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Genetics

Molecular characterization of the 128-kDa immunodominant antigen of *Helicobacter pylori* associated with cytotoxicity and duodenal ulcer

(vacuolating cytotoxin/peptic ulcer/gastritis/nucleotide sequence/ELISA)

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ABSTRACT **_Helicobacter pylori_ has been associated with gastritis, peptic ulcer, and gastric adenocarcinoma. We report the nucleotide sequence and expression of an immunodominant antigen of _H. pylori_ and the immune response to the antigen during disease. The antigen, named CagA (cytotoxin-associated gene A), is a hydrophilic, surface-exposed protein of 128 kDa produced by most clinical isolates. The size of the _cagA_ gene and its protein varies in different strains by a mechanism that involves duplication of regions within the gene. Clinical isolates that do not produce the antigen do not have the gene and are unable to produce an active vacuolating cytotoxin. An ELISA to detect the immune response against a recombinant fragment of this protein detects 75.3% of patients with gastroduodenal diseases and 100% of patients with duodenal ulcer ($P < 0.0005$), suggesting that only bacteria harboring this protein are associated with disease.**

Helicobacter pylori is a curved, microaerophilic, Gram-negative bacterium that was isolated in 1983 from stomach biopsy specimens of patients with chronic gastritis (1). Originally named *Campylobacter pylori*, it has been recognized to be part of a separate genus, *Helicobacter* (2). The bacterium colonizes the human gastric mucosa and the infection can persist for decades. During the last few years, the presence of the bacterium has been found to be associated with chronic gastritis type B, a condition that may remain asymptomatic in most of the infected persons but that increases considerably the risk of peptic ulcer and gastric adenocarcinoma. Recent studies strongly suggest that *H. pylori* infection may be either a cause or a cofactor of type B gastritis, peptic ulcer, and gastric tumors (3–5). *H. pylori* is believed to be transmitted orally (6), and the risk of infection increases with age (7, 8) and crowding (9, 10). In developed countries, the presence of antibodies against *H. pylori* antigens increases from <20% in people 30 years old to >50% in 60-year-olds (11, 12), while in developing countries >80% of the population is infected by the age of 20 (8).

The virulence factors of *H. pylori* are still poorly understood. The factors that have been identified so far are (*i*) the flagella, which are probably necessary to move across the mucous layer (13); (*ii*) the bacterial urease, which is necessary to neutralize the acidic environment of the stomach and allow the initial colonization (14, 15); and (*iii*) a high molecular weight cytotoxic protein, formed by monomers of 87 kDa, that causes formation of vacuoles in eukaryotic epithelial cells, produced by most *H. pylori* strains associated with disease (16, 17). Another protein, whose size has been estimated to be 120, 128, or 130 kDa by various authors (18–21), has been described in the culture supernatant of many cytotoxic strains of *H. pylori*, suggesting that it plays a role in pathogenesis. This protein is very immunogenic in humans infected with *H. pylori*, since specific antibodies are detected in sera of virtually all infected individuals (22). Here we describe the molecular cloning and sequence determination of the 128-kDa protein.¶ We show that the gene coding for this protein varies in size in different clinical isolates and is present only in cytotoxin-producing strains.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, Phage and Media. The *H. pylori* strains producing the vacuolating cytotoxin were G10, G27, G29, G32, G33, G39, G56, G65, G105, and G113A. The noncytotoxic strains were G12, G21, G25, G47, G50, and G204. They were isolated from endoscopy biopsy specimens at the Grosseto Hospital (Tuscany, Italy). The strain CCUG 17874 (cytotoxin-positive) was obtained from the Culture Collection of the University of Gotheborg. The noncytotoxic strains Pylo 2U+ (urease-positive) and Pylo 2U− (urease-negative) were obtained from F. Megraud (Centre Hospitalier, Bordeaux, France).

Escherichia coli strains DH10B (BRL), TG1 and K-12 ΔH1 Δ*trp* (23) and Y1088, Y1089, and Y1090 (24) have been described. pBluescript SK(+) and SK(−) (Stratagene) were used as cloning vectors. The pEx34a, -b, and -c plasmids for the expression of MS2 fusion proteins have been described (23). The phage vector used for the expression library was from the λgt11 cloning kit (BRL). *E. coli* strains were cultured in LB medium (24). *H. pylori* strains were plated onto selective media (5% horse blood in Columbia agar base with Dent's or Skirrow's antibiotic supplement and 0.2% cyclodextrin) or in *Brucella* broth liquid medium containing 5% fetal bovine serum (6) or 0.2% cyclodextrin (25).

Growth of *H. pylori* and DNA Isolation. *H. pylori* strains were cultured in solid or liquid media for 3 days at 37°C, either in a microaerophilic atmosphere [Oxoid (Basingstoke, U.K.) or Becton Dickinson gas-pack generators] or in an incubator with air plus 5% CO_2 (26). Bacteria were harvested, suspended with lysozyme at 100 μg/ml in 0.1 M NaCl/10 mM Tris·HCl/1 mM EDTA, pH 8, and incubated at room temperature for 5 min. SDS was added (1%, wt/vol) and the mixture was heated at 65°C. Proteinase K was added (25 μg/ml) before incubation at 50°C for 2 hr. DNA was purified by CsCl gradient centrifugation with ethidium bromide, pre-

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¶The nucleotide sequence reported in this paper has been deposited in the GenBank data base (accession nos. X70038 and X70039).

cipitated with 77% (vol/vol) ethanol, and recovered with a sealed glass capillary (24).

Construction and Screening of a λgt11 Expression Library. To generate the library, we used genomic DNA from the CCUG 17874 strain partially digested with the restriction enzymes *Hae* III and *Alu* I. After fractionation in a 0.8% agarose gel, the DNA between 0.6 and 8 kb was eluted by using a Costar Spin-X (0.22-μm) microcentrifuge filter. The products from each digestion were combined and used to construct the expression library with the λgt11 cloning kit (BRL) and the Gigapack II Gold packaging kit (Stratagene). The library, which contained 0.8–1 × 10^6 recombinant phages, was amplified in *E. coli* Y1088, yielding 150 ml of lysate with 10^9 phages per ml, 85% of which were recombinant and had an average insert size of 900 bp. Immunological screening was done with the Protoblot system (Promega).

Plasmid Libraries. Attempts to make complete genomic libraries of partially digested chromosomal DNA by using standard vectors that accommodate large DNA inserts, such as EMBL4 or λDASH (24), encountered the difficulties described by many authors in cloning *H. pylori* DNA and failed to give libraries containing the entire *H. pylori* genome (26). In particular, the gene of the 128-kDa antigen could not be detected in any of the several libraries constructed in these vectors. Therefore, the DNA fragments of interest were cloned in pBluescript SK(+) by using genomic DNA from strains CCUG 17874 and G39 digested with *Hin*dIII. DNA ligation, electroporation of *E. coli* DH10B, screening, and amplification were performed in accord with ref. 27. Libraries of *Hin*dIII fragments ranging from 70,000 to 85,000 colonies with a background ≤ 10% were obtained.

DNA Sequencing. DNA sequencing was performed with Sequenase 2.0 (United States Biochemical) and DNA fragments (Fig. 2*A*) subcloned in pBluescript KS(+). Each strand was sequenced at least three times. The region between nt 1533 and 2289, for which a DNA clone was not available, was amplified by PCR and sequenced by using asymmetric PCR and direct sequencing of amplified products (*fmol* system; Promega). The overlap of this region was confirmed by one- and two-side anchored PCR (28, 29): an external universal anchor (5′-GCAAGCTTATCGATGTCGACTCGAGCT-3′/5′-GACTCGAGTCGACATCGA-3′) containing a protruding 5′ *Hin*dIII sequence and the recognition sites of *Cla* I, *Sal* I, and *Xho* I was ligated to primer-extended DNA and amplified. A second round of PCR with nested primers was then used to obtain fragments for cloning and sequencing. Sequence data were assembled and analyzed with the GCG package (Genetics Computer Group, Madison, WI) running on a VAX 3900 under the VMS operating system. The GenBank and EMBL data bases were examined with the EMBL VAXcluster computer.

Protein Preparation and ELISA. Protein extracts were obtained from supernatants of *H. pylori* cells that had been treated with 6 M guanidine in phosphate-buffered saline at room temperature for 60 min. Fusion proteins purified by electroelution (30) or by ion-exchange chromatography were used to immunize rabbits and to coat microtiter plates for ELISAs. Sera from people with normal mucosa, from blood donors, and from patients were obtained from A. Ponzetto (Ospedale Le Molinette, Turin, Italy). Clinical diagnosis was based on histology of gastric biopsy samples. Vacuolating activity was tested on HeLa cells (31).

RESULTS

The 128-kDa Protein Is Immunodominant and Associated with Cytotoxicity. When Western blots of *H. pylori* guanidine extracts were probed with sera from patients with gastroduodenal disease, a 128-kDa protein that was a minor component in the Coomassie blue-stained gel (Fig. 1*A*, lane 1) was strongly recognized by all sera tested (e.g., lane 2). The 128-kDa protein was electroeluted (Fig. 1*A*, lane 3) and used to raise a mouse serum that in a Western blot recognized only this protein (lane 4). This serum was then used to detect by Western blotting the 128-kDa protein in extracts of the *H. pylori* strains described in *Materials and Methods*. Remarkably, we found that the antigen was present in all 10 strains that had vacuolizing activity on HeLa cells but was absent in the 8 strains that did not have such activity (Fig. 1*B*). The size of the protein varied slightly among the strains (Fig. 1*B*, lanes 1–3). To describe these properties, the gene coding for this antigen was named cytotoxin-associated gene A (*cagA*). The CagA antigen was not detected by Western blotting in *Campylobacter jejuni, Helicobacter mustelae, E. coli*, and *Bordetella pertussis* (data not shown).

Structure of *cagA*. Approximately 10^6 clones of the λgt11 expression library were screened with the mouse serum specific for the CagA antigen and with a pool of sera from patients with gastroduodenal diseases. The mouse serum detected positive clones at a frequency of 3 × 10^{-3}. Sequence analysis of 8 clones revealed that they all partially overlapped with clone A1 (Fig. 2). The pool of human sera identified many clones containing different regions of the *cagA* gene, including clones 57/D, 64/4, and 24 (Fig. 2) and several clones overlapping clone A1. None of these clones contained the entire *cagA* gene; therefore, the nucleotide sequence of the entire region was determined by using the clones derived from the λgt11 library; clone B1 (Fig. 2), isolated from the *Hin*dIII plasmid library; and fragment 007, obtained by PCR of the chromosomal DNA (Fig. 2). Computer analysis of the 5925-nt sequence (Fig. 3) revealed a long open reading frame, nt 535–3975, that was in frame with the fusion proteins deriving from the λgt11 clones 64/4, 24, A1, and A17 (Fig. 2). Clone 57/D contained an open reading frame only in the 3′

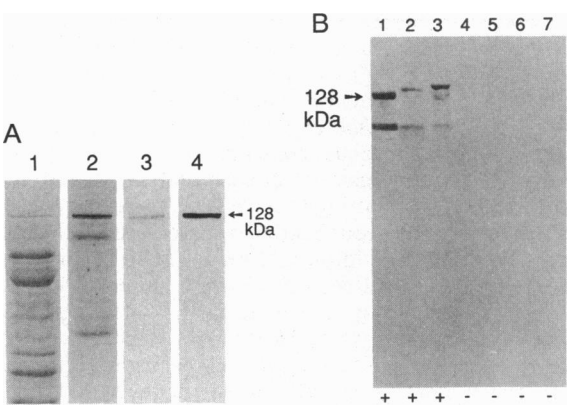

FIG. 1. (*A*) Lane 1: SDS/polyacrylamide gel showing a guanidine extract of *H. pylori* CCUG 17874, stained with Coomassie blue. Lane 2: Western blot of the extract shown in lane 1, probed with serum of a patient with duodenal ulcer; similar patterns were obtained with sera from all patients with gastroduodenal diseases. Lane 3: SDS/polyacrylamide gel showing the purified 128-kDa antigen stained with Coomassie blue. Lane 4: Western blot of the extract shown in lane 1, probed with a mouse serum raised against the purified protein shown in lane 3. (*B*) Western blot showing the expression and size variability of the CagA antigen. Representative cytotoxin-positive strains (CCUG 17874, G33, and G39 in lanes 1–3, respectively) and cytotoxin-negative *H. pylori* strains (G50, G21, Pylo 2U+, and Pylo 2U− in lanes 4–7, respectively), were tested with the mouse antiserum specific for the 128-kDa antigen. Presence (+) or absence (−) of vacuolizing activity is indicated below each lane. Arrow indicates the 128-kDa CagA antigen. A degradation product is present in the three positive strains. The remaining cytotoxin-positive strains tested (see *Materials and Methods*) showed an immunoreactive band with a molecular mass similar to, or slightly higher than, that found in strain G39. The remaining cytotoxin-negative strains were also negative for the CagA antigen.

end of the cloned fragment and therefore could not make a gene fusion with the β-galactosidase gene of λgt11. The presence of an immunoreactive protein in the λgt11 clone 57/D could be explained only by the presence of an endogenous promoter driving the expression of a nonfused protein. This hypothesis was proven by subcloning in both directions the insert 57/4 into pBluescript and showing that an immunoreactive protein was obtained in both cases. Conclusive evidence that the gene indeed coded for CagA was obtained by subcloning the inserts A17 and 64/4 in the pEx34B plasmid vectors to obtain fusion proteins that were purified and used to immunize rabbits. The sera obtained recognized specifically the 128-kDa band in cytotoxic *H. pylori* strains, giving a pattern identical to that shown in Fig. 1*A*, lane 4 (data not shown).

The *cagA* gene codes for a putative 1147-aa protein of 128,012.73 Da and pI 9.72. The basic properties of the purified protein were confirmed by two-dimensional gel electrophoresis. The codon usage and the G+C content (37%) of the gene were similar to those of other *H. pylori* genes (13, 26). A putative ribosome binding site, AGGAG, was identified 5 bp upstream from the proposed ATG start codon. Computer search for promoter sequences of the region upstream from the ATG start codon identified sequences resembling either −10 or −35 regions; however, a region with good consensus to an *E. coli* promoter or resembling published *H. pylori* promoter sequences was not found. Primer extension analysis of purified *H. pylori* RNA showed two transcription start sites, 104 and 214 bp upstream from the ATG start codon (data not shown). Canonical promoters could not be identified upstream from either transcription start. The expression of a portion of CagA by clone 57/D suggests that *E. coli* also recognizes a promoter in this region, but it is not clear whether *E. coli* recognizes the same promoters as *H. pylori* or whether the *H. pylori* DNA, which is A+T-rich, provides *E. coli* with regions that may act as promoters. A Rho-independent terminator was identified downstream from the stop codon (Fig. 3). CagA is very hydrophilic and does not show leader peptide or transmembrane sequences. The most hydrophilic region, aa 600–900, includes the repetition of EFKNGKNKDFSK and EPYIA and the presence of a stretch of six asparagines (Fig. 3).

Diversity of *cagA* Is Generated by Internal Duplications. To find out the mechanism of size heterogeneity of the CagA proteins in different strains, we analyzed the structure of one of the strains with a larger CagA protein (G39) by Southern blotting, PCR, and DNA sequencing. The results showed that the *cagA* genes of G39 and CCUG 17874 were identical in size until position 3406, where the G39 strain was found to contain an insertion of 204 bp, made by two identical repeats of 102 bp. Each repeat was found to contain sequences deriving from the duplication of three segments of DNA (sequences D1, D2, and D3 in Fig. 2) coming from the same region of *cagA* and connected by small linker sequences. The region where the insertion occurred and the insertion itself are represented in Fig. 2.

***cagA* Is Absent in Noncytotoxic Strains.** To investigate why the CagA antigen was absent in the noncytotoxic strains, DNA from two of them (G50 and G21) was digested with *Eco*RI, *Hin*dIII, and *Hae* III for Southern blotting. Two probes internal to *cagA*, nt 520–1840 and 2850–4331 (Fig. 4), recognized bands in strains CCUG 17874 and G39. The bands varied in size in the two strains, in agreement with the gene diversity reported in Fig. 2. However, neither probe hybridized with the G50 and G21 DNA.

Presence of Serum Antibodies Against the CagA Antigen Correlates with Gastroduodenal Diseases. To study the quantitative antibody response to the CagA antigen, we purified to homogeneity the fusion protein produced by the A17 fragment subcloned in pEx34 and used this antigen to coat microtiter plates for an ELISA. In this assay, the patients with gastroduodenal pathologies had an average ELISA titer that was significantly higher than that found in randomly selected blood donors and people with normal gastric mucosa (Fig. 5). To evaluate whether the antibody titer correlated with a particular gastroduodenal disease, the sera from patients with known histological diagnosis were tested in the ELISA. Patients with duodenal ulcer had an average antibody titer significantly higher than that for all the other diseases (Fig. 5). Altogether, the ELISA was found to be able to predict 75.3% of the patients with any gastroduodenal disease and 100% of the patients with duodenal ulcer.

DISCUSSION

H. pylori culture supernatants have been shown by different authors to contain an antigen of 120, 128, or 130 kDa that is recognized by sera of patients infected with *H. pylori* (18–21).

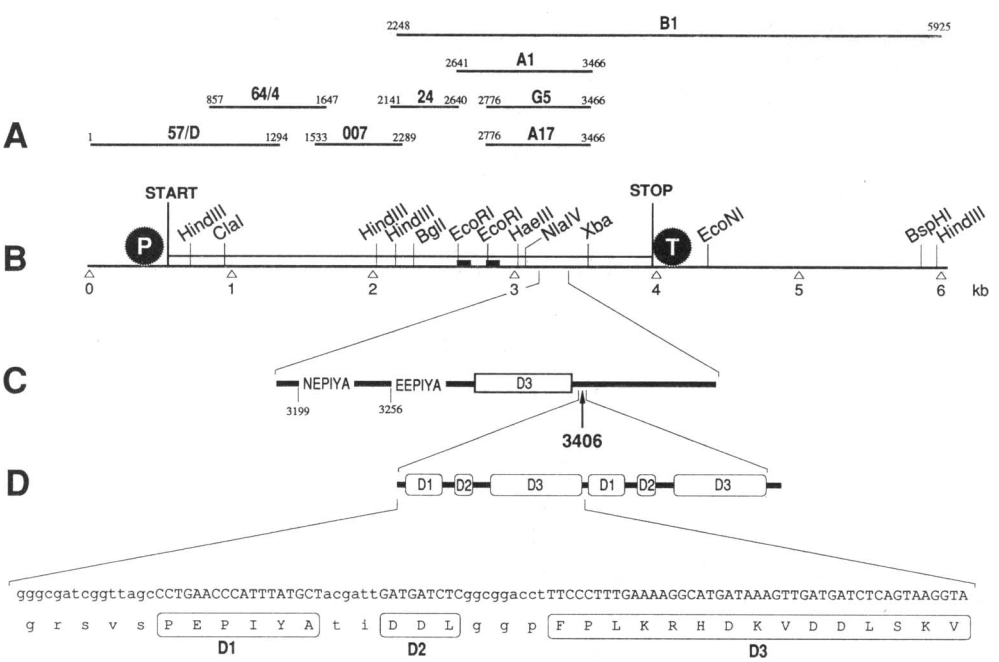

FIG. 2. (*A*) Clones used to identify and sequence the *cagA* gene. Clones A1, 64/4, G5, A17, 24 and 57/D were obtained from the λgt11 library. Clone B1 was from a plasmid library of *Hin*dIII fragments; 007 was obtained by PCR. (*B*) Map of the *cagA* gene. P, promoter; T, terminator. (*C*) Position of insertion of the repeated sequence of G39. (*D*) Nucleotide and amino acid sequence of one of the repeated sequences found in strain G39. Uppercase letters indicate the sequences D1, D2, and D3 duplicated from *cagA*; lowercase letters indicate the linkers nucleotides and amino acids.

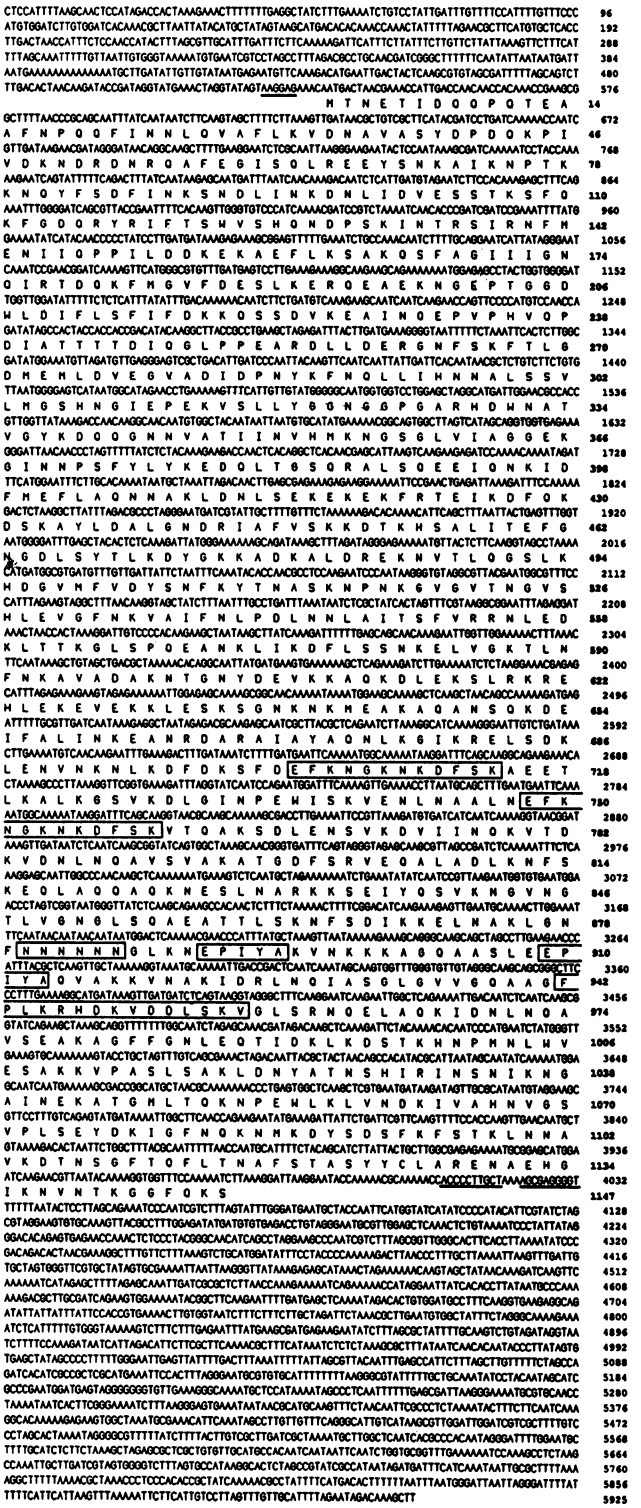

FIG. 3. Nucleotide sequence of the DNA region shown in Fig. 2*A* and amino acid sequence (single-letter code) of the CagA protein. The important features of the gene and protein are marked: the ribosome binding site and the terminator are underlined. Repeated sequences and six consecutive asparagines are boxed.

It was not clear whether the difference in size of the antigen described was due to interlaboratory differences in estimating the size of the same protein, to actual size variability of the same antigen, or to different molecules described by the different laboratories. Until recently, these molecules were often described also as having a vacuolating activity in HeLa cells (21). In this work we have purified the 128-kDa antigen,

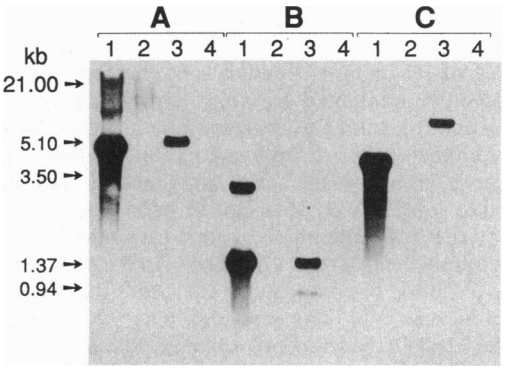

FIG. 4. Southern blot of chromosomal DNA deriving from the cytotoxic strains CCUG 17874 (lanes 1) and G39 (lanes 3) and the noncytotoxic strains G21 (lanes 2) and G50 (lanes 4) cut with *Eco*RI (*A*), *Hin*dIII (*B*), or *Hae*III (*C*). The probe used contained nt 520–1840 of the *cagA* gene. Similar results were obtained with a probe containing nt 2850–4331 (data not shown).

confirmed its high immunogenicity, cloned its gene, and shown that this varies in size in different isolates that are cytotoxic on HeLa cells, while it is absent in the isolates without cytotoxic activity. In agreement with a recent report (16), we have found that the 128-kDa molecule purified by gel chromatography is devoid of cytotoxic activity and that cytotoxicity is associated with fractions containing an 87-kDa protein not crossreacting with the CagA antigen (C. Montecucco, M.B., and R.R., unpublished data). These data indicate that the 128-kDa molecule is not the cytotoxin but is somehow associated with it. The high immunogenicity and the association with cytotoxicity of this molecule suggested that it be named cytotoxin-associated gene A (CagA) antigen.‖

The association between the presence of the *cagA* gene and cytotoxicity may suggest that the product of *cagA* is necessary for the transcription, folding, export, or function of the

‖After our manuscript had been submitted for publication we learned that the same gene had also been studied by M. Blaser and coworkers (32). Jointly we decided to adopt the name CagA.

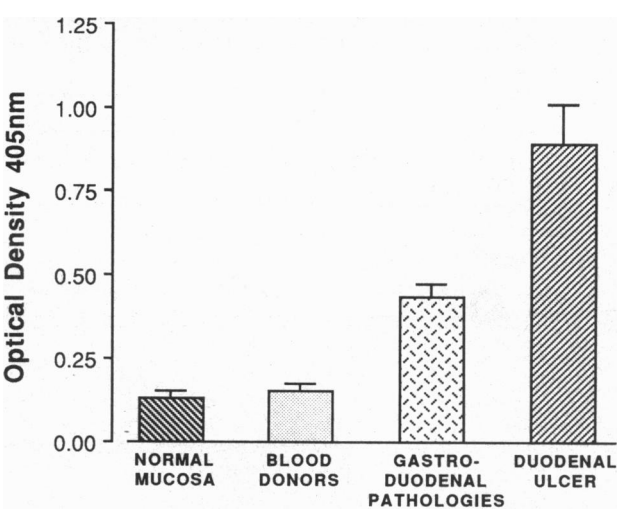

FIG. 5. Average ELISA titers of sera deriving from 6 people with normal mucosa, 37 randomly selected blood donors, 89 patients with gastroduodenal disease, and 20 patients with duodenal ulcer. Error bars indicate the standard error. Percentage of sera with values above the cutoff level was 75.3% in patients with gastroduodenal disease and 100% in patients with duodenal ulcer. Values in patients with gastroduodenal disease and duodenal ulcer were significantly different from those of people with normal mucosa, $P < 0.0005$.

cytotoxin. Indeed, analogies with similar systems (33–35) suggest that a multisubunit protein such as the *H. pylori* cytotoxin (16) is likely to require accessory protein(s) for folding and export. Alternatively, both the cytotoxin and the *cagA* genes may be absent in noncytotoxic strains. This would imply some physical linkage between the two genes.

A peculiar property of the CagA antigen is its size variability. We have found that even among strains isolated within the same city the CagA antigens are heterogeneous in size, suggesting that the *cagA* gene is continuously changing. While the diversity at the genome level of different *H. pylori* is well known (36, 37), so far there is no evidence that this is reflected at the protein level. This suggests that size variability may be peculiar to the CagA antigen and that this may occur under selective pressure. The presence of repeating sequences in antigenic molecules is a finding common to many pathogens such as *Streptococcus, Clostridium difficile, Trichomonas vaginalis, Plasmodium falciparum*, and *Trypanosoma cruzi* (38–42). This mechanism is often used to escape immunity by generating either antigenic diversity or immunodominant nonprotective epitopes that distract the immune system from the protective epitopes. In the case of *H. pylori*, this system duplicates sequences already present in the antigen, without generating antigenic diversity. However, the duplication generates two new peaks of hydrophilicity, rich in prolines, that are predicted to be surface-exposed and very immunogenic. It will be interesting to study whether these repeats give an advantage over the immune system, make a protein that is more functional, or are just neutral and have been propagated because they do not give any disadvantage. The natural competence of transformation of *H. pylori* may suggest that the genome is constantly rearranged by transformation with DNA from dead cells and that each gene undergoes changes by homologous recombination (36, 37). Thus, mutations that give a selective advantage or do not give any disadvantage may be maintained.

The CagA antigen has been described mostly as present in the culture supernatant of strains grown in the presence of calf serum (20). We have grown our bacteria without calf serum, in the presence of cyclodextrin, and found the antigen to be associated with the cell surface. This suggests that the release of the antigen into the supernatant may be due to the action of proteases present in the serum that may cleave either the antigen itself or the complexes that hold the CagA antigen to the bacterial surface. Similar processing activities may release the antigen during *in vivo* growth. The absence of a typical leader peptide sequence suggests the presence of a *sec*-independent export system that is involved in the export of this protein (35).

A feature that makes this antigen very interesting is the high immunogenicity in humans and the correlation of the antibody levels with gastroduodenal disease and with duodenal ulcer in particular. This suggests that only bacteria expressing CagA are associated with duodenal ulcer and therefore that this protein is linked to disease. Recent studies showing that *H. pylori*-positive patients with gastric cancer have significantly increased recognition of the 128-kDa protein relative to infected subjects with non-ulcer dyspepsia confirm this hypothesis (43). Our studies indicate that ELISAs based on recombinant CagA may replace the invasive diagnostic methods that are used today. Finally, the high immunogenicity of CagA suggests that this antigen could be useful for the development of vaccines.

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