

Low Antigenicity of the Polysaccharide Region of *Helicobacter pylori* Lipopolysaccharides Derived from Tumors of Patients with Gastric Cancer

SHIN-ICHI YOKOTA,¹ KEN-ICHI AMANO,^{2*} SHYUNJI HAYASHI,³ AND NOBUHIRO FUJII³

Sumitomo Pharmaceuticals Research Center, Konohana-ku, Osaka 554,¹ Central Research Laboratory, Akita University School of Medicine, Akita 010,² and Department of Microbiology, Sapporo Medical University, Sapporo 060,³ Japan

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We have examined the antibody response to *Helicobacter pylori* lipopolysaccharide (LPS) during natural infection in humans. The sera of over 70% of *H. pylori*-infected individuals were found to contain immunoglobulin G antibodies against the LPS fractions isolated from smooth strains of *H. pylori* but not against those derived from rough strains, as determined by enzyme-linked immunosorbent assay. These results taken together with the immunoblot data indicated that the polysaccharide region of *H. pylori* LPS is antigenic in humans. However, the antigenicity of the polysaccharide varied, depending on the strain. We found that smooth *H. pylori* strains isolated from the tumors of patients with gastric cancer showed significantly lower antigenicity than smooth strains derived from patients with chronic gastritis and gastric and duodenal ulcers. The results suggest that the levels of antigenicity of the polysaccharide region of *H. pylori* LPS in humans correlate with the nature of the gastroduodenal diseases and that they allow a particular distinction to be made between gastric cancer and other gastroduodenal diseases, especially chronic gastritis.

Helicobacter pylori is an emerging candidate for the cause of chronic gastritis and peptic ulcers (11, 12, 14). Furthermore, *H. pylori* infection is thought to be one of the causative factors of gastric cancer (6, 15). Recently, extensive structural and immunological studies of *H. pylori* lipopolysaccharide (LPS) have been carried out. The O polysaccharide region of *H. pylori* LPS is a major antigenic determinant (13), as are those of other typical bacterial LPSs. Interestingly, many *H. pylori* strains have an O polysaccharide containing epitopes that mimic the structures of the Lewis antigens, as shown by chemical (3, 4) and immunological (2, 16, 17) studies. The Lewis antigens, which are made up of fucosylated lactosamine structures, are known to be tumor antigens on cancer cells and blood group antigens and granulocyte marker antigens on normal cells. Hence, the immunological response to the Lewis antigen-containing O polysaccharides was considered to play a role in the pathogenicity of *H. pylori* through the establishment of an autoimmune response (2). We are interested in the antigenicity of *H. pylori* LPS during natural infection in humans. In the present study, we show that the antigenicity of *H. pylori* LPS varies from strain to strain. We discuss the relationship between LPS antigenicity and disease.

MATERIALS AND METHODS

Bacterial strains. Clinical strains of *H. pylori* were isolated from the biopsy specimens of lesions obtained from patients with chronic gastritis (strains CG1 to -19), gastric ulcers (strains GU1 to -18), duodenal ulcers (strains DU1 to -9), and gastric cancer (from tumorous and nontumorous tissues, strains CA1 to -10 and CAIN to -10N, respectively) in the Sapporo Medical University Hospital (Sapporo, Japan). *H. pylori* 43504-r was a spontaneous rough mutant derived from ATCC 43504, which was purchased from the American Type Culture Collection (Rockville, Md.). After three to five laboratory subcultures, these bacteria were grown on brain heart infusion agar plates supplemented with 10% (vol/vol) horse

blood at 37°C for 5 days in microaerophilic conditions by using the Gas Pack system (BBL, Cockeysville, Md.) without a catalyst. The organisms were collected, washed with phosphate-buffered saline three times, and lyophilized.

Human sera. The sera of 25 patients with chronic gastritis (14 sera), gastric ulcers (4 sera), duodenal ulcers (4 sera), and gastric cancer (3 sera) were collected from the Sapporo Medical University Hospital and Akita University Hospital (Akita, Japan). The sera were collected independently of the isolation of *H. pylori* strains used in this study. The sera of 83 healthy adult volunteers were obtained from Akita University Hospital. The status of *H. pylori* infection of each individual was monitored by the *H. pylori* antibody determiner enzyme immunoassay kit (high-molecular-weight cell-associated protein [HM-CAP] kit) (Kyowa Medics, Tokyo, Japan).

SDS-PAGE and immunoblotting. LPS fractions were prepared by proteinase K (Merck, Darmstadt, Germany) treatment. Briefly, the lyophilized cells were dissolved in the sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (9) at a concentration of 2 mg/ml and incubated at 100°C for 10 min. Then 200 µl of a solution of proteinase K (2.5 mg/ml) per ml was added, and the mixture was incubated at 37°C overnight followed by incubation at 65°C for 2 h. The LPS fraction was applied to a 12.5% (wt/vol) polyacrylamide gel slab and electrophoresed. The LPS profile on the gel was developed by silver staining according to the method of Hitchcock and Brown (7). Immunoblotting was accomplished by the method described previously (1). After the LPS fraction was transferred from the gel to a GVHP filter (Nihon Millipore, Yonezawa, Japan), the filter was incubated with human sera (1:100 dilution) as the first antibody. A horseradish peroxidase-conjugated goat anti-human immunoglobulin G (IgG) antibody (Dako, Copenhagen, Denmark) and 3,3'-diaminobenzidine (Nakarai Tesque, Kyoto, Japan) were used as the second antibody and the substrate, respectively.

Enzyme-linked immunosorbent assay (ELISA). The proteinase K-treated cells described above were used as a source of LPS fractions. *Salmonella minnesota* R595 (Re mutant) LPS was purchased from Sigma (St. Louis, Mo.). The LPS fraction was diluted 1:50 or dissolved at a concentration of 10 µg/ml with 50 mM sodium carbonate buffer (pH 9.6). Then the diluent was dispensed in a 96-well polyvinyl microtiter plate (Becton Dickinson, Oxnard, Calif.) and incubated at 4°C overnight. The plate was blocked with 1% (wt/vol) bovine serum albumin by incubation at 37°C for 2 h. Human serum was diluted 1:3,000 with phosphate-buffered saline containing 0.05% Tween 20 and dispensed to the microplate. Horseradish peroxidase-conjugated goat F(ab')₂ anti-human IgG antibodies (BioSource International, Camarillo, Calif.) and TMB peroxidase substrate system (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) were used as the second antibody and the substrate, respectively. Absorbance at 450 nm (*A*₄₅₀) was measured after the reaction was terminated with 1 M phosphoric acid. Binding activity was expressed as the mean *A*₄₅₀ value of triplicate measurements.

Statistical analysis. The statistical significance of the differences between mean values of binding activity in ELISA was carried out by the Student *t* test.

* Corresponding author. Mailing address: Central Research Laboratory, Akita University School of Medicine, 1-1-1, Hondo, Akita 010, Japan. Phone: 81-188-33-1166, ext. 3151. Fax: 81-188-37-4398. E-mail: amanocr1@med.akita-u.ac.jp.

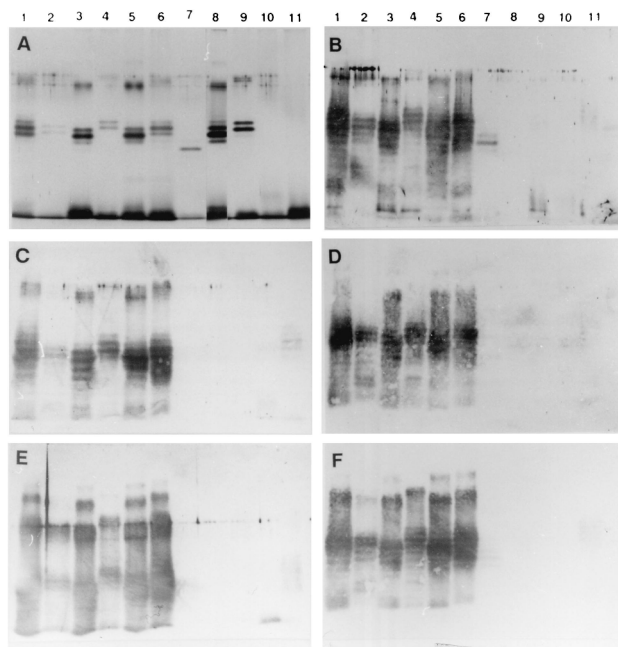


FIG. 1. SDS-PAGE analysis of LPS fractions derived from *H. pylori* CG7 (lanes 1), CG9 (lanes 2), GU2 (lanes 3), GU10 (lanes 4), DU1 (lanes 5), DU2 (lanes 6), CA2 (lanes 7), CA6 (lanes 8), CA8 (lanes 9), 43504-r (lanes 10), and CG10 (lanes 11). Shown are a silver-stained SDS-polyacrylamide gel (A) and similar gels immunoblotted with sera from patients with chronic gastritis (B), gastric ulcers (C), duodenal ulcers (D), and gastric cancer (E) and from a healthy adult infected with *H. pylori* (F). Samples corresponded to 3.4 μ g of dry cells per lane.

RESULTS

Antibody response to *H. pylori* LPS. We chose 11 strains of *H. pylori* for a detailed characterization. The LPS profiles on silver-stained SDS-polyacrylamide gels are shown in Fig. 1A. Among them, 43504-r and CG10 were rough strains and thus did not display polysaccharide chains. All the other strains were smooth strains, and their polysaccharide chains appeared as a series of ladder bands on the silver-stained gels.

First we examined the status of *H. pylori* infection of each individual with the conventional serum diagnosis kit HM-CAP. The sera of 24 of 25 patients and 21 of 83 healthy volunteers were found to be positive for *H. pylori* infection. The antibody response to LPS was determined by ELISA with the LPS fraction isolated from proteinase K-treated cells as the immobilized antigen. From the results of ELISA at various dilutions of sera, 3,000-fold dilution was found to be suitable for detecting the titer of antibody specific to *H. pylori* LPS and eliminating nonspecific binding (data not shown). More than 70% of the sera of *H. pylori*-infected individuals contained IgG against *H. pylori* LPSs derived from the smooth strains of the CG, GU, and DU series (Fig. 2). Among the other sera (about 30% of the sera with *H. pylori* infection), significant binding to the LPS was observed in a few sera at lower dilutions, such as 100-fold. However, the specific binding to the LPS was not observed even at lower dilutions in most of the negative sera.

We found a significantly low titer of IgG against the LPS of smooth strains derived from the tumors of gastric cancer (CA series). Also, almost none of the sera showed significant levels of IgG against rough strains (43504-r and CG10) or against the *S. minnesota* Re mutant, a severely defective enterobacterial LPS mutant strain (8) used as a control to measure the titer of nonspecific antibody to lipid A. The specificity of sera of *H. pylori*-infected individuals was assessed by immunoblot analysis

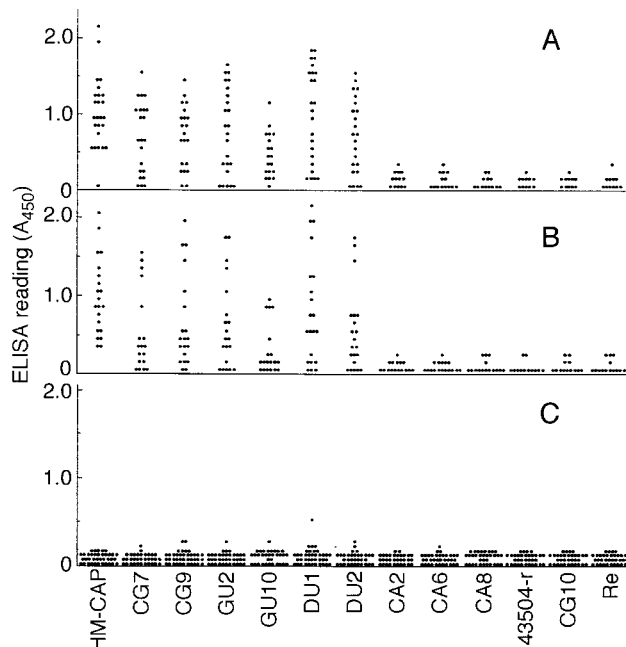


FIG. 2. Titers in human sera of IgG to various *H. pylori* LPSs as determined by ELISA. Sera were obtained from patients with gastroduodenal diseases (A), healthy individuals with *H. pylori* infections (B), and healthy individuals without *H. pylori* infections (C). Human sera were diluted 3,000-fold and applied to a microplate coated with *H. pylori* LPS, and the bound IgG was measured. Serum titer is expressed as the mean value of the ELISA readings (A_{450}) of triplicate measurements. HM-CAP was from the HM-CAP ELISA kit. Re, LPS from *S. minnesota* R595 (Re mutant).

of proteinase K-treated cells. The sera stained the high-molecular-weight regions of the blots with a characteristic ladder or smear banding pattern in the smooth strains of the CG, GU, and DU series (Fig. 1B to F). The results indicated that the sera of many of the *H. pylori*-infected individuals contained IgG specific for the polysaccharide region, but not for the core oligosaccharide-lipid A region, of clinical isolates of *H. pylori* LPS, with the exception of the LPS of isolates from gastric cancer patients (CA series). The sera of the noninfected individuals contained few antibodies to any of the *H. pylori* LPS (Fig. 2C and data not shown).

Comparison of antigenicities of LPSs of *H. pylori* strains derived from various sources. The antigenicities of LPSs from 69 *H. pylori* strains were analyzed by using 10 randomly selected high-titer human sera as probes (Fig. 3). The LPS of *H. pylori* showed varying antigenicities, depending on the strain. Rough strains showed low antigenicity regardless of their clinical sources. Eight of nine smooth strains derived from tumors of gastric cancer patients showed low antigenicities. The low antigenicities of strains derived from these tumors was a significant difference from those of strains isolated from other gastroduodenal diseases, especially chronic gastritis. Furthermore, we compared the antigenicities of paired strains derived from the tumorous and nontumorous tissues of individual gastric cancer patients (Fig. 4). In 2 out of 10 cases, isolated strains derived from nontumorous tissues were highly antigenic whereas strains derived from tumors showed low antigenicities.

DISCUSSION

In this study, we have demonstrated that the polysaccharide region of *H. pylori* LPS is a potent immunogen in humans during natural infections. The LPSs from the smooth strains,

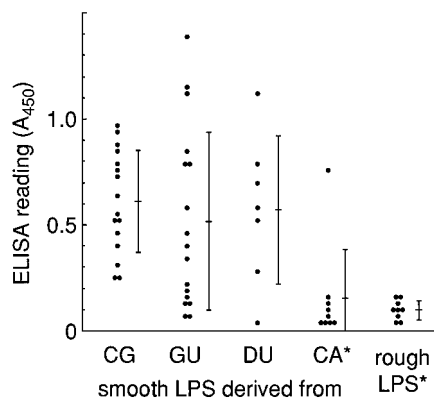


FIG. 3. Reactivity of IgG in human sera to *H. pylori* LPSs isolated from various sources in ELISA. The clinical sources of the smooth strains were patients with chronic gastritis (CG), gastric ulcers (GU), duodenal ulcers (DU), and gastric cancer (CA). Seven rough strains were derived from CG (three strains), GU (one strain), DU (two strains), and CA (one strain). The LPS fraction was used as the coated antigen. Human serum (diluted 1:3,000) was applied to the LPS plate, and the bound IgG was measured. Ten human sera with high titers of IgG to *H. pylori* LPS were randomly selected for this study. The reactivity to each LPS of *H. pylori* strains is expressed as the mean value of ELISA readings of 10 human sera. The groups marked with asterisks were significantly different from the others at the $P < 0.05$ level.

which carry the O polysaccharide chains, could be classified into two groups, high antigenicity and low antigenicity. We focused our attention on the relationship between the antigenicity and the nature of the clinical sources of *H. pylori*. The antigenicities of the *H. pylori* strains isolated from patients with gastric tumors were significantly lower than those of strains isolated from patients with other gastroduodenal diseases. The low level of antigenicity may be related to the progression of the disease. This may be particularly relevant to gastric cancer compared to, for instance, chronic gastritis. It should be emphasized, however, that the low antigenicity was not due to the lack of the O polysaccharide, because the low-antigenicity strains expressed normal levels of polysaccharide chains.

Our study raises a number of questions. Why do strains isolated from gastric tumors express a low-immunogenic LPS? One possibility is that low-antigenicity LPS is tumorigenic. This hypothesis suggests that tumorigenic strains of *H. pylori* may

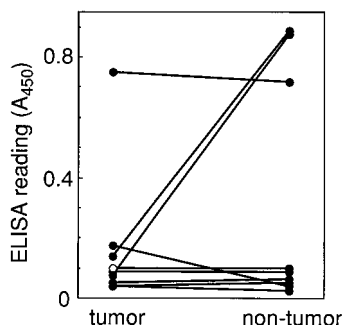


FIG. 4. Comparison of the LPS antigenicities of paired *H. pylori* strains derived from tumorous and nontumorous tissues of the same patient as measured by ELISA. The experimental procedures were the same as those described in the legend to Fig. 3. Ten randomly selected human sera (diluted 1:3,000) with high titers of IgG to *H. pylori* LPS were applied to a microplate coated with the LPS fraction, and the bound IgG was measured. The reactivity to the LPS of each *H. pylori* strain is expressed as the mean value of ELISA readings of 10 human sera. Symbols: closed circle, smooth strain; open circle, rough strain.

exist. However, there is no data in the literature in support of the tumorigenicities of individual *H. pylori* strains. Another possibility is that *H. pylori* cells carrying a low-antigenicity LPS are selected for during infection of the host. Changes in the structure of the cell surface antigens during infection would allow *H. pylori* to escape from the host immune system. This would be applicable to gastric cancer, which is regarded as the later stage of a series of inflammatory reactions (5). The gastric cancer patients would have been exposed to the inflammatory stimuli caused by *H. pylori* infection longer than the patients with other gastroduodenal diseases. In any case, it will be necessary to conduct longitudinal studies of the antigenic changes of *H. pylori* cells during the course of infection. Our finding that two strains isolated from the tumors of patients have low antigenicities compared to those of strains isolated from the nontumorous tissues of the same patient (Fig. 4) deserves further investigation. It would be interesting to know, for example, whether or not these strains are derived from the same *H. pylori* organism.

Another question is what structures of *H. pylori* LPS represent the antigenic determinants. Data from immunoblotting (Fig. 1B to F) indicates that the determinants are located on the polysaccharide region of the LPS preparation. Recently, Lewis antigen structures were identified as being part of the polysaccharides of many *H. pylori* LPSs. Appelmek et al. suggested that the Lewis antigen, especially Lewis^x, structure is an antigenic epitope in mammals, including humans (2), and Wirth et al. indicated that the distribution of Lewis^x and Lewis^y structures was related to a gene, *cagA*, associated with pathogenicity. However, we have evidence to show that the distributions of antigenic determinants and of Lewis antigens in *H. pylori* LPSs may not be so directly related. In fact, some strains from gastric tumors, which show low antigenicities, expressed Lewis antigens and some highly antigenic LPSs were found not to contain Lewis antigens (1a, 18). At least some antigenic determinants for humans with natural infections seem to be immunologically distinct from Lewis structures, although we do not question the fact that the determinants located on the polysaccharide chains contain the Lewis antigens.

H. pylori is recognized as a category 1 (definite) human carcinogen by the World Health Organization and the International Agency for Research on Cancer (10). However, there is little experimental evidence showing cancer development induced by *H. pylori* infection. The observations in the present paper suggest that *H. pylori* strains isolated from tumors are different from those from other sources. Further study of this phenomenon should provide useful information concerning *H. pylori* pathogenicity with respect to gastric cancer. Moreover, the method described here could eventually be applied to the screening of individuals who are at the highest risk of developing gastric cancer induced by *H. pylori* infection.

REFERENCES

- Amano, K., M. Fujita, and T. Suto. 1993. Chemical properties of lipopolysaccharides from spotted fever group rickettsiae and their common antigenicity with lipopolysaccharides from *Proteus* species. *Infect. Immun.* **61**:4350-4355.
- Amano, K., S. Hayashi, T. Kubota, N. Fujii, and S. Yokota. Reactivities of Lewis antigen monoclonal antibodies to the lipopolysaccharides of *Helicobacter pylori* strains isolated from patients with gastroduodenal diseases in Japan. *Clin. Diagn. Lab. Immunol.*, in press.
- Appelmek, B. J., I. Simoons-Smit, R. Negrini, A. P. Moran, G. O. Aspinall, J. G. Forte, T. De Vries, H. Quan, T. Verboom, J. J. Maaskant, P. Ghiara, E. J. Kuipers, E. Bloemena, T. M. Tadema, R. R. Townsend, K. Tyagarajan, J. M. Crothers, Jr., M. A. Monteiro, A. Savio, and J. De Graaff. 1996. Potential role of molecular mimicry between *Helicobacter pylori* lipopolysaccharide and host Lewis blood group antigens in autoimmunity. *Infect. Immun.* **64**:2031-2040.
- Aspinall, G. O., and M. A. Monteiro. 1996. Lipopolysaccharide of the *Helicobacter pylori*.

- cobacter pylori* strains P466 and MO19: structures of the O antigen and core oligosaccharide regions. *Biochemistry* **35**:2498–2504.
4. **Aspinall, G. O., M. A. Monteiro, H. Pang, E. J. Walsh, and A. P. Moran.** 1996. Lipopolysaccharide of the *Helicobacter pylori* type strain NCTC 11637 (ATCC 43504): structure of the O antigen chain and core oligosaccharide regions. *Biochemistry* **35**:2489–2497.
 5. **Correa, P.** 1992. Human gastric carcinogenesis: a multistep and multifactorial process—first American Cancer Society award lecture on cancer epidemiology and prevention. *Cancer Res.* **52**:6735–6740.
 6. **The Eurogast Study Group.** 1993. An international association between *Helicobacter pylori* infection and gastric cancer. *Lancet* **341**:1359–1362.
 7. **Hitchcock, P., and T. M. Brown.** 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *J. Bacteriol.* **154**:269–277.
 8. **Holst, O., and H. Brade.** 1992. Chemical structure of the core region of lipopolysaccharides, p. 135–170. *In* D. C. Morrison and J. L. Ryan (ed.), *Bacterial endotoxic lipopolysaccharides*, vol. I. Molecular biochemistry and cellular biology. CRC Press, Boca Raton, Fla.
 9. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685.
 10. **Logan, R. P. H.** 1994. *Helicobacter pylori* and gastric cancer. *Lancet* **344**:1078–1079.
 11. **Marshall, B. J.** 1983. Unidentified curved bacilli on gastric epithelium in chronic gastritis. *Lancet* **i**:1273–1313.
 12. **Marshall, B. J., and J. R. Warren.** 1984. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* **i**:1311–1315.
 13. **Mills, S. D., L. A. Kurjanczyk, and J. L. Penner.** 1992. Antigenicity of *Helicobacter pylori* lipopolysaccharides. *J. Clin. Microbiol.* **30**:3175–3180.
 14. **National Institutes of Health Consensus Development Panel on Helicobacter pylori in Peptic Ulcer Diseases.** 1994. *Helicobacter pylori* in peptic ulcer diseases. *JAMA* **272**:65–69.
 15. **Parsonnet, J., G. D. Friedmann, D. P. Vandersteen, N. Orentreich, and R. K. Sibley.** 1991. *Helicobacter pylori* infection and the risk of gastric carcinoma. *N. Engl. J. Med.* **325**:1127–1131.
 16. **Sherburne, R., and D. E. Taylor.** 1995. *Helicobacter pylori* expresses a complex surface carbohydrate, Lewis X. *Infect. Immun.* **63**:4564–4568.
 17. **Wirth, H.-P., M. Yang, M. Karita, and M. J. Blaser.** 1996. Expression of the human cell surface glycoconjugates Lewis X and Lewis Y by *Helicobacter pylori* isolates is related to *cagA* status. *Infect. Immun.* **64**:4598–4605.
 18. **Yokota, S., and K. Amano.** Unpublished data.

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