Reactivities of Lewis Antigen Monoclonal Antibodies with the Lipopolysaccharides of Helicobacter pylori Strains Isolated from Patients with Gastroesophageal Diseases in Japan

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We have examined the reactivity of monoclonal antibodies (MAbs) specific for Lewis antigens (Leα, Leβ, Leγ, and Leδ) with Helicobacter pylori lipopolysaccharides (LPS) by immunoblot analysis and enzyme-linked immunosorbent assay (ELISA). Sixty-eight strains of H. pylori were isolated from patients with chronic gastritis, gastric and duodenal ulcers, and gastric cancer in Japan. The cells were treated with protease K, and the resulting fractions were used as a source of LPS for the immunoassays. In the immunoblot analysis, 28 isolates (41%) and 29 isolates (42%) reacted with anti-Leα and anti-Leβ MAbs, respectively, while 4 isolates (6%) and 7 isolates (10%) reacted with anti-Leγ and anti-Leδ MAbs. On the other hand, in ELISA, the number of isolates that reacted with anti-Leα MAbs fell significantly to 21 isolates (30%) but the number of isolates that reacted with the other anti-Lewis antigen MAbs remained relatively unchanged. These data show that the immunoblotting technique is more sensitive than the ELISA technique for the detection of immunocomplexes of anti-Leα MAbs and components of H. pylori LPS. Furthermore, human serum was found to react with the synthetic Lewis antigens regardless of the status of the individual's H. pylori infection. This means that humans may naturally possess antibodies against Lewis antigens in the absence of H. pylori infection.

The microaerophilic bacterium Helicobacter pylori has been suggested to be a causative agent of chronic gastritis, gastric and duodenal ulcers, and gastric carcinoma (17). Despite its importance as a human pathogen, the characterization of the surface structures of this bacterium is still incomplete. Especially, knowledge about the biological significance and structure of H. pylori lipopolysaccharide (LPS), one of the key components of the outer membrane of the bacterium, is limited. Recently, Moutiala et al. (16) reported that H. pylori LPS was less toxic than the LPS from Enterobacteriaceae when tested for mitogenicity, pyrogenicity, and toxicity. Perez-Perez et al. (19) also reported low biological activity of H. pylori LPS in mediation of macrophage activation. Aspinall et al. (5–7) determined the structures of the LPS O polysaccharides of three strains of H. pylori and found them to be the same as the Lewis x (Leα) and Lewis y (Leβ) determinants of the human cell surface glycoconjugates. More recently, Appelmelk et al. (3) suggested that the mimicry of Lewis antigens by this organism might explain the autoimmune mechanism of H. pylori-associated type B gastritis.

In this study, we have compared the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns of H. pylori LPS isolated from patients with chronic gastritis, gastric and duodenal ulcers, and gastric carcinoma in Japan and examined the reactivity of the LPS fractions with monoclonal antibodies (MAbs) specific for Lewis and other mammalian carbohydrate antibodies by immunoblotting and enzyme-linked immunosorbent assay (ELISA). Furthermore, we have examined the reactivity of H. pylori LPS with sera obtained from humans infected or not infected with H. pylori.

MATERIALS AND METHODS

Bacterial strains and cultivation. H. pylori 43504-r was a spontaneous rough mutant derived from ATCC 43504, which was purchased from the American Type Culture Collection (Rockville, Md.). Clinical strains of H. pylori were isolated from lesion biopsy specimens obtained from patients with chronic gastritis (strains CG1 to CG19), gastric ulcers (GU1 to GU18), duodenal ulcers (DU1 to DU9), and gastric cancer (tumor and nontumor sites; CA1 to CA10 and CA1N to CA12N, respectively) in the Sapporo Medical University Hospital (Sapporo, Japan). After three to five laboratory subcultures, these bacteria were grown on brain heart infusion agar plates supplemented with 10% horse blood at 37°C for 1 h. For H. pylori LPS, the whole cells were treated with proteinase K (PK) (Merck, Darmstadt, Germany) as described previously (1, 25), dissolved in 0.1 M NaCl, and the LPS was purified by DEAE-cellulose chromatography and dialysis (26).

Patients with Gastroduodenal Diseases in Japan

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In this study, we have compared the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) patterns of H. pylori LPS isolated from patients with chronic gastritis, gastric and duodenal ulcers, and gastric carcinoma in Japan and examined the reactivity of the LPS fractions with monoclonal antibodies (MAbs) specific for Lewis and other mammalian carbohydrate antibodies by immunoblotting and enzyme-linked immunosorbent assay (ELISA). Furthermore, we have examined the reactivity of H. pylori LPS with sera obtained from humans infected or not infected with H. pylori.

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MAbs against Lewis antigens. The specificities of MAbs against Lewis antigens are listed in Table 1. Clones 73-30 and H18A were purchased from Seikagaku Kogyo (Tokyo, Japan); clones BG-7 (P12), BG-8 (F3), and BG-6 (T218) were purchased from Signet Laboratories (Dedham, Mass.); and clones MAB2108 (7E) and MAB2102 (2.5E) were purchased from Chemicon (Temecula, Calif.).

Synthetic Lewis oligosaccharide conjugates. The synthetic Lewis antigens were conjugated with either human serum albumin (HSA) purchased from Iosep AB (Tullinge, Sweden) or a biotinylated polymer probe (BP) purchased from Seikagaku Kogyo (Tokyo, Japan). Human sera. The sera of 25 patients with chronic gastritis, gastric ulcers, duodenal ulcers, and gastric cancer and the sera of 83 healthy adult volunteers were obtained from the hospitals of Sapporo Medical University and Akita University School of Medicine (Akita, Japan). The status of H. pylori infection of each individual was monitored with an enzyme immunoassay kit with Determiner H. pylori antibody (HM-CAP) (Kyowa Medics, Tokyo, Japan) and a biotinylated polymer probe (BP) purchased from Seikagaku Kogyo.

ELISA. For HSA-conjugated Lewis antigens, the HSA conjugates were dissolved in PBS at a concentration of 10 μg/ml and were dispensed into a Microtiter III flexible assay plate (Becton Dickinson, Oxford, Calif.). After incubation at 4°C overnight, the plate was blocked with 1% HSA. For BP-conjugated Lewis antigens, Streptavidine-Plus (ProZyme, San Leandro, Calif.) at a concentration of 5 μg/ml was dispensed into a microplate. The BP conjugates, at a concentration of 2 μg/ml, were dispensed to the streptavidin-coated plates and incubated at 37°C for 1 h. For H. pylori LPS, the whole cells were treated with protease K (PK) (Merck, Darmstadt, Germany) as described previously (1, 25), dissolved in 0.1 M NaCl, and the LPS was purified by DEAE-cellulose chromatography and dialysis (26).
at a concentration of 10 μg/ml with 50 mM sodium carbonate buffer (pH 9.6), and dispensed into a microplate. After incubation at 4°C overnight, the plate was blocked with 1% HSA. MAbs against Lewis antigens and human sera were diluted with PBS containing 0.05% Tween 20 and 3% HSA at appropriate concentrations and used as primary antibodies. Horseradish peroxidase-conjugated goat anti-murine immunoglobulin antibody (BioSource International, Camarillo, Calif.) or anti-human immunoglobulin G (IgG) antibody (BioSource International) was used as a secondary antibody, and the tetramethyl benzidine peroxidase substrate system was used to reveal the immune complexes (Kirkegaard & Perry Laboratories, Gaithersburg, Md.). Absorbance at 450 nm was measured after termination of the reaction with 1 M phosphoric acid.

**SDS-PAGE and immunoblotting.** SDS-PAGE and immunoblotting were performed as described previously (1, 2, 25). Before applying samples to gel, bacterial whole cells were treated with PK as described above. The PK-treated samples (LPS fractions) were applied to 12.5% (wt/vol) gel and electrophoresed. The gel was stained with silver according to the method of Hitchcock and Brown (12) for detecting the LPS profile. Immunoblotting was accomplished by a method described previously (1, 25). After transfer from the gel to a polyvinylidene difluoride (GVHD) filter (Nihon Millipore, Yonezawa, Japan), the filter was incubated with human serum (1:100 dilution) as the primary antibody. Horseradish peroxidase-conjugated goat anti-murine immunoglobulin antibody (BioSource International) was used as the secondary antibody.

**RESULTS**

**SDS-PAGE patterns of LPS from *H. pylori.*** The molecular sizes and microheterogeneity of LPS fractions from 69 strains of *H. pylori* that had been treated with PK were compared on SDS-PAGE gels after silver staining (Fig. 1). The LPS of most strains showed some ladder bands, resembling those of the smooth-type LPS of enterobacteria, in the high-molecular-weight area of the gel and one or two bands, characteristic of rough-type LPS, in the low-molecular-weight area. However, LPS from strains CG5, CG10, CG12, CA1, and CA7 showed only one band in the fast-migration zone of the gel. Although the LPSs from strains CA1N and DU8 also showed single bands, the migration rates of these bands were somewhat retarded compared to those of the LPS bands described above. LPS from strain 43504-r showed some bands in the low-molecular-weight area but no ladder bands. LPS from strain CA3N had only ladder bands. Furthermore, strains GU4, GU6, and DU7 exhibited either low amounts of LPS or no LPS at all. The different numbers of bands and the altered migration rates of the major bands in all of these LPS fractions indicated that the LPSs from different strains of *H. pylori* are structurally distinct.

**Specificity of anti-Lewis antigen MAbs for synthetic Lewis antigens.** Figure 2 shows that the Lewis antigen MAbs recognized the synthetic Lewis antigens, thus confirming the specificity of these MAb preparations. Two anti-Leₐ MAbs (73-30 and BG-7) reacted with only 2 Leₐ substrates (Leₐ-HSA and

**TABLE 1. MAbs against Lewis antigens used in this study**

<table>
<thead>
<tr>
<th>MAb</th>
<th>Specificity</th>
<th>Isotype</th>
<th>Source</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>73-30</td>
<td>Leₐ</td>
<td>Murine IgM</td>
<td>Seikagaku Kogyo</td>
<td>26</td>
</tr>
<tr>
<td>BG-7 (P12)</td>
<td>Leₐ</td>
<td>Murine IgM</td>
<td>Signet Lab</td>
<td>20, 21, 24</td>
</tr>
<tr>
<td>H18A</td>
<td>Le₉</td>
<td>Murine IgG3</td>
<td>Seikagaku Kogyo</td>
<td>10</td>
</tr>
<tr>
<td>H18A</td>
<td>Le₉</td>
<td>Murine IgM</td>
<td>Signet Lab</td>
<td>20, 21, 24</td>
</tr>
<tr>
<td>BG-8 (F3)</td>
<td>Le₇</td>
<td>Murine IgG1</td>
<td>Chemicon</td>
<td>22, 23</td>
</tr>
<tr>
<td>MAB2102 (2.25LE)</td>
<td>Le₇</td>
<td>Murine IgG1</td>
<td>Signet Lab</td>
<td>20, 21, 24</td>
</tr>
<tr>
<td>MAB2102 (2.25LE)</td>
<td>Le₇</td>
<td>Murine IgG1</td>
<td>Chemicon</td>
<td>22, 23</td>
</tr>
<tr>
<td>BG-6 (T218)</td>
<td>Le₇</td>
<td>Murine IgM</td>
<td>Signet Lab</td>
<td>20, 21, 24</td>
</tr>
</tbody>
</table>

**FIG. 1.** SDS-PAGE profile of PK-treated whole cells of *H. pylori* strains stained with silver (1, 2). The strains were isolated from lesion biopsy specimens from patients with chronic gastritis (CG1 to CG19), gastric ulcers (GU1 to GU18), duodenal ulcers (DU1 to DU9), and gastric cancer (tumor and nontumor sites; CA1 to CA10 and CA1N to CA12N, respectively). AT, *H. pylori* 43504-r.
Tri-Le^x^-HSA). Two anti-Le^b^ MAbs (H18A and BG-8) mainly reacted with the Le^b^ substrate, but the H18A MAb also weakly reacted with Le^o^ substrates. Two anti-Le^o^ MAbs (BG-5 and MAB2108) reacted with Le^-^-HSA, but only BG-5 reacted with Le^-^-BP. Two anti-Le^y^ MAbs (BG-6 and MAB2102) reacted with Le^-^-HSA and Le^-^-BP; however, MAB2102 also reacted with Le^-^-HSA but not with Le^-^-BP. Based on the reactivities of MAB2102 and MAB2108 with two Le^o^ substrates, these MAbs may be recognizing the number of sugar residues of Le^-^-HSA (four saccharides) and Le^-^-BP (three saccharides). In the low-dilution range (1/100), BG-5 and BG-6 weakly cross-reacted with other substrates.

**Reactivity of anti-Lewis antigen MAbs with H. pylori LPS.**

The specificity of anti-Lewis antigen MAbs for H. pylori LPS was tested by carrying out immunoblotting and ELISA of PK-treated whole cells. On the basis of the reactivities of the anti-Lewis antigen MAbs to the synthetic antigens, we decided that all MAbs except MAB2102 were suitable for the analysis of H. pylori LPS. Figure 3 shows the reactivities of anti-Lewis antigen MAbs to 12 H. pylori LPS fractions subjected to immunoblot analysis. LPS from 43504-r and CG7 did not react with any MAbs. However, the LPS from these latter strains showed O polysaccharide-containing LPS on silver-stained PAGE gels. LPS from GU9 and CA4 reacted with only anti-Le^-^-MAbs, and LPS from CG9 and GU10 reacted with anti-Le^o^ and anti-Le^b^ MAbs, respectively. On the other hand, LPS from DU1 and DU2 reacted with anti-Le^o^ and anti-Le^b^ MAbs, and LPS from GU2 and CA6 reacted with anti-Le^o^ and anti-Le^b^ MAbs. Furthermore, LPS from CG4 and CA2 reacted with anti-Le^-^- and anti-Le^o^- MAbs. Generally, the reactivity of anti-Le^-^- MAbs with H. pylori LPS was weak in comparison to that of anti-Le^-^- MAbs. Table 2 summarizes the data from immunoblot analysis and ELISA concerning the reactivities of these MAbs to all the H. pylori LPSs under study. On immunoblots, 28 (41%) and 29 isolates (42%) reacted with anti-Le^-^- and anti-Le^-^- MAbs, respectively, while only 4 (6%) and 7 isolates (10%) reacted with anti-Le^-^- and anti-Le^-^- MAbs, respectively. In ELISA, the isolates recognized by anti-Le^-^- MAbs decreased significantly to 21 isolates (30%) but the numbers of isolates recognized by the other anti-Lewis antigen MAbs scarcely changed compared to the numbers obtained by immunoblot analysis. We classified these LPSs into nine groups according to their reactivities with anti-Lewis antigen MAbs. The nonre-
active type A was further divided into two types, A(S) and A(R), according to the presence and absence of O-polysacch-
side moieties, respectively, as deduced from silver-stained SDS-PAGE gels. H. pylori LPSs were mainly classified into
types A(S), A(R), B, C, and F on the basis of the immunoblot analysis and into types A(S), A(R), B, and C on the basis of
the ELISA. If the numbers of strains detected by immunoblotting and ELISA are compared, H. pylori strains with LPSs of
type A(S) were detected at a higher frequency by ELISA, unlike H. pylori strains with LPSs of types B and C. These data suggested
that immunoblotting was more sensitive than ELISA for the detection of Lewis antigens by anti-Lewis antigen MAbs in the
LPS fractions of H. pylori, especially for the detection of Le x-related antigens.

Reactivity of human sera against synthetic Lewis antigens.
We analyzed the reactivity of sera from patients with chronic gastritis, gastric ulcers, duodenal ulcers, and gastric cancer and
the sera from H. pylori-infected individuals and uninfected controls with five kinds of synthetic Lewis antigens (Fig. 4). All
the human sera recognized these antigens individually, and the immunological reactions were independent of the status of
H. pylori infection. The results suggest that the presence of antibodies against Lewis antigens in human serum is unrelated to
infection by H. pylori.

FIG. 4. Reactivities of human sera, infected or not infected with H. pylori,
with synthetic Lewis antigens, as assayed by ELISA. 1, Le x-HSA; 2, Tri-Le x-HSA;
3, Le y-HSA; 4, Le a-HSA; 5, Le b-HSA. Human sera were diluted to 1:100. ELISA
readings (A 0.450) were expressed as antibody titers. The bars indicate the means
and standard deviations.

TABLE 2. Reactivity of H. pylori LPS with anti-Lewis antigen MAbs

<table>
<thead>
<tr>
<th>LPS type</th>
<th>No. of strains (%) detected by ELISA</th>
<th>Immunoblotting</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>A(S)</td>
<td>8 (12)</td>
<td>15 (22)</td>
<td></td>
</tr>
<tr>
<td>A(R)</td>
<td>10 (14)</td>
<td>10 (14)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>17 (25)</td>
<td>12 (17)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>15 (22)</td>
<td>12 (17)</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>1 (1)</td>
<td>3 (4)</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>2 (3)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>9 (13)</td>
<td>8 (12)</td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>2 (3)</td>
<td>1 (1)</td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>4 (6)</td>
<td>7 (10)</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>1 (1)</td>
<td>1 (1)</td>
<td></td>
</tr>
</tbody>
</table>

In the immunoblotting, all MAbs against Lewis x, y, a, and b antigens, except MAB2102 (anti-Le b), were used as primary antibodies at a dilution of 1:250. In the
ELISA, 73-30 (1:50 dilution) and BG7 (1:20) were used as anti-Le x MAbs, H18A (1:100) and BG8 (1:50) were used as anti-Le y MAbs, BG5 (1:20) and MAB2108 were
used (1:100) as anti-Le a MAbs, and BG6 (1:20) was used as the anti-Le b MAb.

DISCUSSION
Recently, Aspinall et al. reported that the O-polysaccharides of LPSs from the H. pylori strains NCTC11637 (originally the same strain as ATCC 43504) and MO19 are structurally similar
to the Lewis x and y blood group antigens, respectively (5–7). LPS from H. pylori strain P466 contains structurally similar
portions of both the Lewis x and the Lewis y antigens (5). These antigens are also present in human mucosa (14). Fur-
thermore, Simoons-Smit et al. proposed that the typing of H. pylori would be possible by testing the reactivity of anti-
Lewis antigen MAbs with these LPSs, and Wirth et al. supported their proposal.

In this paper, we report the reactivities of several kinds of MAbs against Lewis antigens with the LPSs of 69 strains of H. pylori isolated from patients with gastroduodenal diseases by
immunoblotting and ELISA. Based on the results, H. pylori LPS was classified into nine groups, without any apparent
correlation with the status of the diseases. Specifically, we were able to demonstrate the extensive reactivities of anti-Le x
and anti-Le y MAbs with H. pylori LPS compared to those of anti-Le a and anti-Le b MAbs, confirming results obtained previously
by Appelmelk et al. (3), Simoons-Smit et al. (22), and Wirth et al. (24). They reported that over 80% of H. pylori isolates
contained Le x and/or Le y antigens, while our results showed that only 70 and 60% of H. pylori isolates reacted with anti-Le x
and/or anti-Le y MAbs, respectively, as assayed by immunoblotting and ELISA. This discrepancy may originate from differ-
ences in the assay techniques for determining antibody titers or in the way the bacteria were cultivated.

Previously, the microbial expression of mammalian carbohydrate antigens was reported. Campylobacter jejuni LPS was shown to possess epitopes made up of glycosphingolipids of the
ganglioside group (4). Furthermore, Streptococcus bovis expresses sialyl-Le x (11), and ova and adult worms of Schistosoma
mansoni express Le x as an antigen (13). The mimicry was thought to contribute to the ability of the bacteria to escape the
host immune response. Indeed, H. pylori LPS was shown previously to have low biological activities with respect to endo-
toxicity (15, 16), neutrophil activation (18), and regulation of adhesion molecule expression (8). These low activities might
be related to not only the fatty acid composition of lipid A but also the mimicry of the host carbohydrate antigens, namely, the
Lewis antigens. On the other hand, Lewis antigen expression by H. pylori has possibly led to autoimmune diseases against host
cells expressing similar antigens (3).

Recently, we measured the reactivity of H. pylori-infected
human serum with *H. pylori* LPS (25). The results indicated that the polysaccharide regions of *H. pylori* are immunogenic in humans. The results also indicated that the polysaccharide regions of *H. pylori* strains isolated from tumors of gastric cancer patients showed significantly lower immunogenicity compared to those isolated from gastroduodenal disease patients. Based on these results and the data presented in this study, we conclude that the antigenicity of *H. pylori* LPS in humans is independent of the Lewis antigen content of the LPS. It will be important to better define the structure of the polysaccharide region of *H. pylori* LPS that reacts with *H. pylori*-infected human serum, and indeed, work in this direction is already in progress.

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