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Serum antibody response against *Helicobacter pylori* NCTC 11637 smooth- and rough-lipopolysaccharide phenotypes in patients with *H. pylori*-related gastropathy

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Summary The antigenicity of the *Helicobacter pylori* lipopolysaccharide (LPS) molecule during the course of natural *H. pylori* infection in humans was investigated. The IgG and IgA responses against smooth (S)- and rough (R)-form LPS were evaluated in *H. pylori* positive patients with chronic gastritis (CG, $n = 30$) and duodenal ulcer disease (DU, $n = 16$), and in 15 *H. pylori*-negative dyspeptic subjects. The results demonstrated that anti *H. pylori* LPS IgG and IgA antibody levels were significantly enhanced in both groups of *H. pylori*-positive patients compared with *H. pylori*-negative subjects, thus confirming that *H. pylori* LPS is part of the immunogenic antigen profile of the bacterium. In addition, a marked response against R-LPS, which significantly correlated with that observed against S-LPS, was found for both IgG and IgA, thus indicating that core oligosaccharide plays a powerful immunogenic role. Since the *O*-side chain of LPS from *H. pylori* NCTC 11637 contains epitopes which mimic Lewis x (Le^x) antigens, the presence of antibodies to monomeric, trimeric, and polymeric Le^x was also investigated. Antibodies against polymeric Le^x were detected in two patients suffering from chronic atrophic gastritis and active chronic gastritis, respectively.

INTRODUCTION

In the last decade, a broad spectrum of clinical manifestations aetiologically associated with *Helicobacter pylori*-infection has been well documented, and this bacterium is now recognized as a major causative agent of gastroduodenal disease.[1,2] However, our understanding of the pathogenic mechanisms underlying the acute onset of the *H. pylori*-induced gastric inflammatory process is incomplete,[3] and its development leading to chronic gastritis, gastric atrophy and ulcer disease, warrants further investigation. It is known that infection of the gastric mucosa by *H. pylori*, aided by its capability to withstand a virtually hostile environment, results in long-lasting colonization and persistence of this bacterium in close proximity to the epithelial surface of the stomach.[4,5] As a consequence, the continuous shedding of antigenic material elicits both a local and systemic humoral immune response.[6–9] Several lines of evidence have suggested that in *H. pylori*-related gastropathy, immune-mediated phenomena may play a crucial pathogenic role.[10–12] In particular, it has been demonstrated that the *O*-polysaccharide chain of the LPS molecule from diverse

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H. pylori strains contains epitopes which mimic the structure of Le^x and/or Le^y antigens.[13–16] These epitopes, in turn, may elicit the production of antibodies cross-reactive with antigenic determinants of the gastric tissue, thus seemingly mediating local damage.[10,12,17–19] Therefore, a major focus of investigation has been the humoral immune response against *H. pylori* LPS and the contribution of the *O*-polysaccharide chain to the immunogenicity of the *H. pylori* LPS molecule.[18–20]

In the present study, two *H. pylori* LPS phenotypes isolated from *H. pylori* NCTC 11637 smooth (S) and rough (R) bacteria, namely S- and R-form LPS, were used to define the contribution of the *O* chain and core oligosaccharide structures in the development of the antibody response in patients suffering from *H. pylori*-related chronic gastritis (CG) and duodenal ulcer (DU) disease. A further aim of this investigation was to determine the presence of antibodies directed against Le^x antigens in these patients. The results show that, besides antibodies to S-form LPS, a strong humoral IgG and IgA response against R-form LPS was demonstrated in sera from patients with CG and DU, which clearly indicates the immunogenicity of *H. pylori*-LPS core structures. On the other hand, antibodies against synthetic polymeric Le^x were found only in 2 of 30 patients affected by CG, whereas no anti-Le^x antibody response was found in 16 patients suffering from DU disease.

MATERIALS AND METHODS

Bacterial strain and culture conditions

H. pylori NCTC 11637 was obtained from the National Collection of Type Culture (London, UK). This *H. pylori* reference strain expresses major virulence determinants such as a 128 kDa immunodominant antigen, CagA, and a potent cytotoxin, VacA, both of which are involved in the most severe expressions of *H. pylori*-related gastropathy.[3,21] *H. pylori* LPS can undergo phase variations from S- to R-LPS.[22,23] For extraction of R-form LPS. *H. pylori* NCTC 11637 was grown on blood agar composed of trypticase soy agar (Difco, Detroit, MI, USA) enriched with 6% horse blood and supplemented with 10% IsoVitaleX (BBL Microbiology Systems, Cockeysville, MD, USA) in an atmosphere of 5% O_2, 10% CO_2, and 85% N_2 at 37°C for 48 h. Bacteria were harvested in sterile distilled water, centrifuged at 5000 *g* at 4°C for 30 min, washed twice, and the bacterial pellets were freeze-dried.[22] Although in vitro passages of *H. pylori* strains on solid medium result in loss of *O*-side chains, when *H. pylori* is grown in a fluid medium this loss is reversed and production of S-form LPS is stabilized.[23–25] Therefore, for extraction of S-form LPS, *H. pylori* NCTC 11637 was grown in a liquid medium of brain-heart infusion (Oxoid, Basingstoke, UK) and 2% fetal calf serum (Oxoid).[24]

Purified antigens

S- and R-form LPS from *H. pylori* NCTC 11637 were isolated as previously described by hot phenol-water extraction and subsequent enzymatic treatment with RNAse A, DNAse II and proteinase K.[22] The purity of LPS preparations was confirmed by routine sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of preparations[22] followed by silver staining.[26] All LPS preparations were converted to their triethylammonium salts by electrodialysis to increase solubility.[27]

Synthetic monomeric and trimeric Le^x antigens were purchased as neoglycoconjugates from Isosep AB (Tullinge, Sweden). The oligosaccharides in the neoglycoconjugates were prepared from human milk. *p*-Aminophenylethyl and acethylphenylenediamine acted as spacers between the oligosaccharides and human serum albumin. As polymeric Le^x antigen, *Schistosoma mansoni* circulating cathodic antigen (CCA), a glycoprotein containing polymeric (n = 25) Le^x, which had been prepared as described previously,[28] was kindly provided by B.J. Appelmelk (Vrije Universiteit, Amsterdam, The Netherlands).

Patient sera

Sixty-one patients (33 males, 28 females; median age 48 years, range 24–68 years) who underwent upper gastrointestinal endoscopy in the Scientific Institute for Digestive Diseases (Castellana Grotte, Italy) because of dyspeptic complaints were enrolled in this study. The status of *H. pylori*-infection was defined by a positive urease CP-test, histological detection of this microorganism in biopsy specimens by Warthin-Starry staining, and serological determination of IgG levels performed by a commercially available ELISA test (Helori-test IgG, Eurospital, Trieste, Italy). Sera were stored at –80°C over a period of 6 months in small aliquots and extensive thawing and freezing of the samples was avoided during the study. The presence of antibodies against *H. pylori* NCTC 11637 S- and R-form LPS, as well as against different purified Le^x antigen preparations, was assessed by an enzyme-linked immunosorbent assay (ELISA).

ELISA

For ELISA, phosphate-buffered saline (PBS) pH 7.4 was used as coating buffer. PBS containing 1% (w/v) bovine serum albumin (PBS-1% BSA) (Sigma Chemical Co., St Louis, MO, USA), PBS-1% BSA-0.05% (v/v) Tween-20, and PBS-0.05% Tween-20 were used as blocking, diluent, and washing buffers, respectively. Each step was performed at 25°C.

Ninety-six flat-bottom well polyvinylchloride plates (Falcon MicroTest III, Becton Dickinson, Oxnard, CA, USA) were incubated overnight with the different antigen preparations (100 ng of antigen/100 μl of PBS/well). The coated plates were washed 3 times with PBS-0.05% Tween-20 and then blocked with PBS-1% BSA for 1 h. After 2 washings, the plates were incubated with diluted sera in PBS-1% BSA-0.05% Tween-20 (1:100) for 1 h. Subsequently, plates were washed and, unless otherwise stated, 100 μl/well of horseradish peroxidase (HRP)-conjugated affinity purified goat antibodies to human IgG or IgA (Sigma), diluted 1:5,000, were added and then incubated for 1 h. After 3 washings, 100 μl of freshly prepared solution of *o*-phenylendiamine dihydrochloride (0.5 mg/ml) and hydrogen peroxide (0.015%) in citrate-phosphate buffer (pH 5) was added. At the end of a 30 min incubation period in the dark, the colorimetric reaction was stopped by adding 50 μl/well of 1 M sulfuric acid and the absorbance was read at 492 nm with a Multiskan plate reader (Labsystem, Helsinki, Finland). All samples were analyzed in duplicate. The blank value, estimated in duplicate on each plate, was obtained using 100 μl of diluent buffer instead of serum. Moreover, non-specific binding of each serum sample, determined on PBS-1% BSA coated wells, was subtracted. Results are expressed as optical density (OD) units. Sera were assessed on the same day and the same batches of reagents were used.

The ELISAs to detect antibodies against monomeric or trimeric Lewis^x antigens were performed as described above except that a mouse monoclonal antibody (CB10, IgM isotype, produced previously[17]) which binds to monomeric and trimeric form of Le^x, was used as a positive control and HRP-conjugated goat anti-mouse IgM (Jackson Immunoresearch Laboratories, Avondale, PA, USA) was utilized as secondary antibody. This positive control gave an OD of 2.950 and 2.810 on binding to trimeric and monomeric Le^x, respectively.

Statistics

Statistical analysis was performed using 2-tailed Mann-Whitney U-test, Wilcoxon signed rank test and Spearman rank correlation as appropriate. P values < 0.05 were taken as statistically significant.

RESULTS

Patient selection

Forty-six out of 61 dyspeptic patients enrolled for this study were infected by *H. pylori*: 30 (17 males, 13 females) were affected by CG and 16 (10 males, 6 females) suffered from DU. The remaining 15 patients (6 males, 9 females) were *H. pylori*-negative and were considered as controls in this study.

Antibody response to *H. pylori* NCTC 11637 S- and R-form LPS

IgG isotype response

The anti S- form LPS IgG response was significantly higher in patients with CG (median OD 1.938; 25/75 percentiles 0.331/2.829; P < 0.0001, Mann-Whitney U-

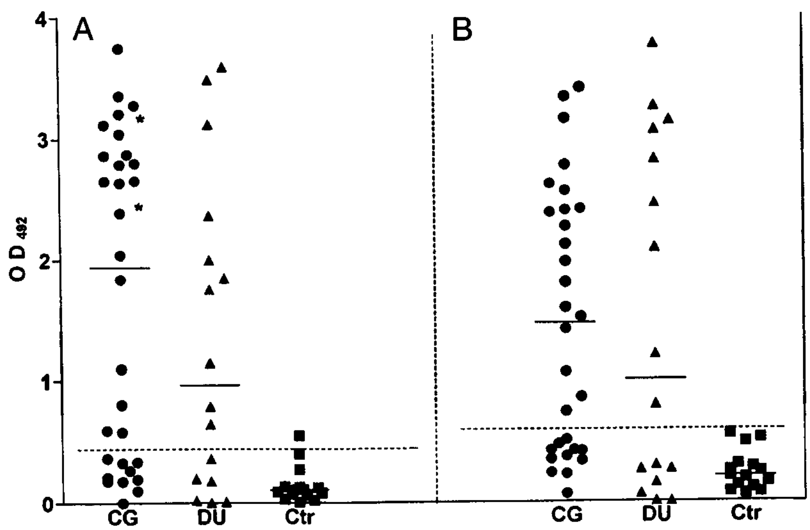

Fig. 1 IgG levels against *H. pylori* NCTC 11637 S-form LPS (**A**) and R-form LPS (**B**) in sera from *H. pylori*-infected patients with chronic gastritis [CG, n = 30 (filled circles)], duodenal ulcer [DU, n = 16 (filled triangles)], and from *H. pylori*-negative controls [Ctr, n = 15 (filled squares)]. Horizontal bars indicate the median values. The dotted lines represent the cut-off points (2 SD above the mean of control group). Sera from patients indicated with an asterisk showed a marked reactivity with polymeric Lewis^x antigen. (A) CG versus Ctr, P < 0.0001; DU versus Ctr, P = 0.0068. (B) CG versus Ctr, P < 0.0001; DU versus Ctr, P = 0.042; two-tailed Mann-Whitney U-test.

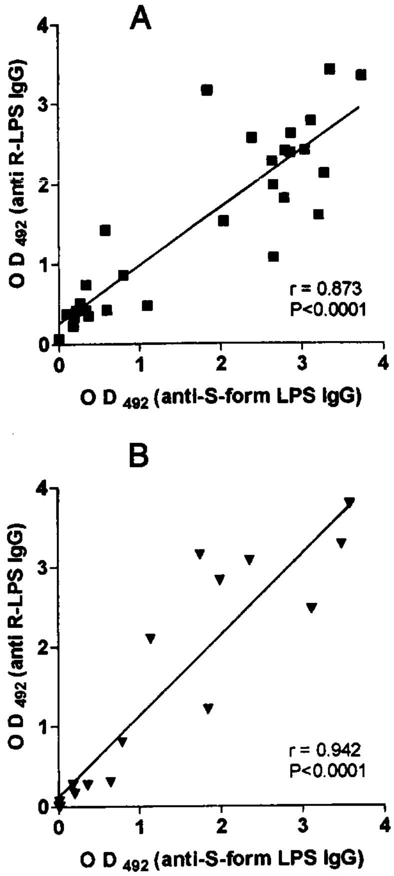

Fig. 2 Correlation between IgG antibodies against *H. pylori* NCTC 11637 S- form (x axis) and R-form (y axis) LPS in sera from *H. pylori*-infected patients with chronic gastritis (**A**) and duodenal ulcer (**B**).

test) and DU (0.964; 0.189/2.179; P = 0.0068) when compared with *H. pylori*-negative controls (0.107; 0.066/0.131). No statistically significant difference was found between CG and DU patients (P = 0.387; Fig. 1A). Similarly, the IgG response against R-form LPS was significantly enhanced in CG (1.471; 0.424/2.394; $P <$ 0.0001) and DU (1.004; 0.212/2.947; $P = 0.042$) patients compared with controls (0.208; 0.121/0.296) (Fig. 1B).

As shown in Figure 2, a significant positive correlation between the anti-S- and anti-R-form LPS IgG response was observed in both CG and DU patients ($r = 0.873$; $P <$ 0.0001, and $r = 0.942$; $P < 0.0001$, respectively, Spearman r test).

IgA isotype response

With respect to the IgA response, in both CG (0.05; 0.028/0.089; $P < 0.0001$) and DU (0.038; 0.022/0.101; $P =$ 0.0003) patients, higher anti S-form LPS antibody levels were detected when compared with controls (0.01; 0.003/0.013), whereas no significant difference was observed between CG and DU patients ($P = 0.62$; Fig.3A). Likewise, anti R-form LPS IgA production was significantly enhanced in CG (0.081; 0.03/0.223; P = 0.043) and DU (0.111; 0.03/0.177; $P = 0.041$) patients, the values of controls were 0.035; 0.024/0.06 (Fig. 3B). The difference between the IgA response in CG and DU patients was not significant ($P = 0.954$). Also, a positive correlation was demonstrated between anti-S- and anti-R-form LPS IgA antibodies in both CG and DU patients (r = 0.859; P < 0.0001 and r = 0.722; P = 0.0016, respectively; Fig. 4).

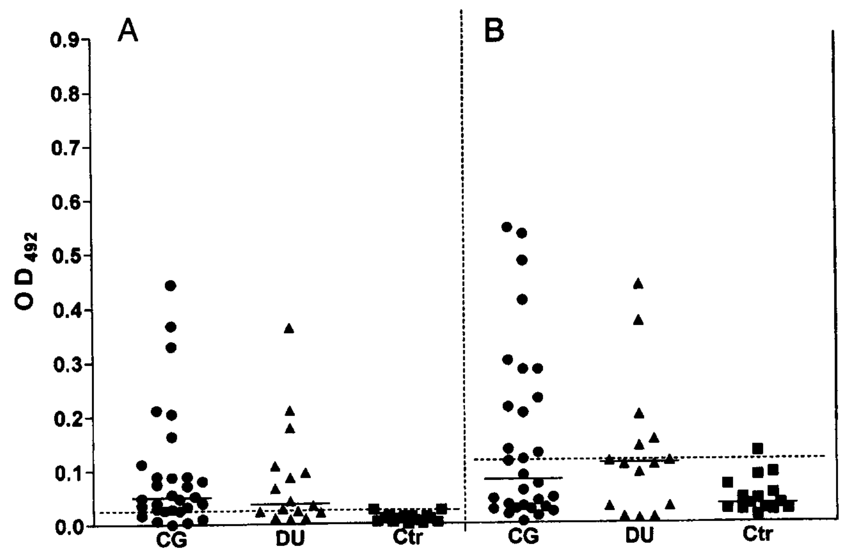

Fig. 3 IgA levels against *H. pylori* NCTC 11637 S- form LPS (A) and R-form LPS (B) in sera from *H. pylori*-infected patients with chronic gastritis [CG, $n = 30$ (filled circles)], duodenal ulcer [DU, $n = 16$ (filled triangles)], and from *H. pylori*-negative controls [Ctr, $n = 15$ (filled squares)]. Horizontal bars indicate the median values.The dotted lines represent the cut-off points (2 SD above the mean of control group). (CG versus Ctr, $P < 0.0001$; DU versus Ctr, $P = 0.0003$. (B) CG versus Ctr, $P < 0.043$; DU versus Ctr, $P = 0.041$.

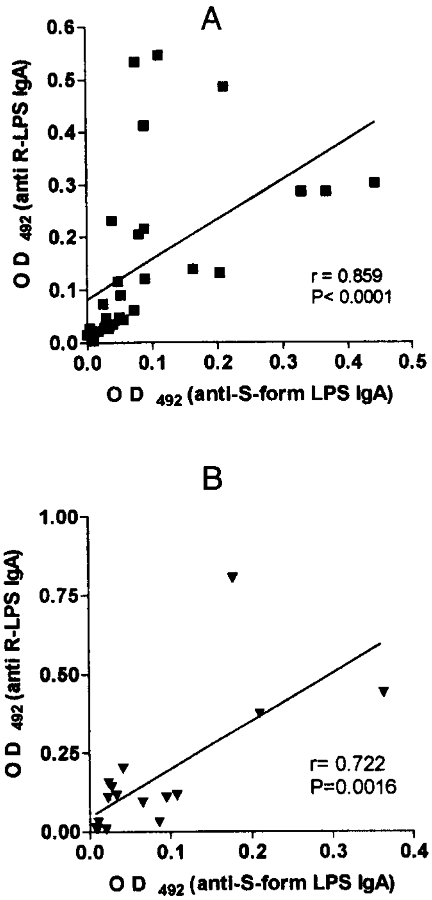

Fig. 4 Correlation between IgA antibodies against *H. pylori* NCTC 11637 S-form (x axis) and R-form (y axis) LPS in sera from *H. pylori*-infected patients with chronic gastritis (A) and duodenal ulcer (B).

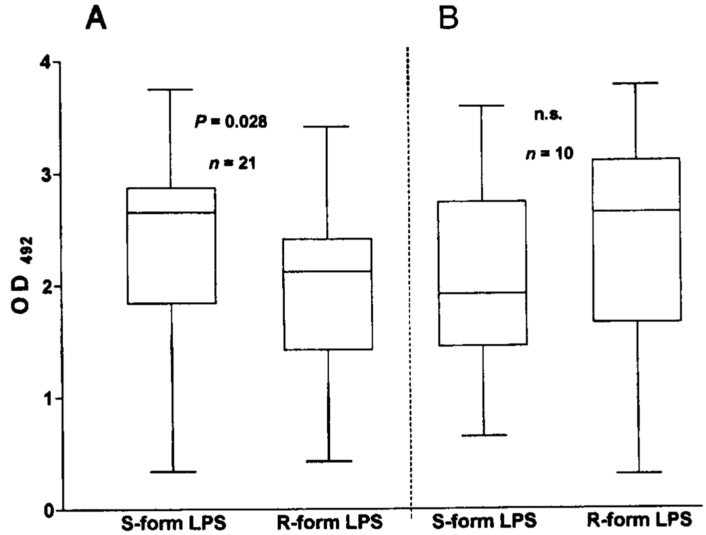

Fig. 5 Comparison between anti-*H. pylori* NCTC 11637 S-form and R-form LPS IgG response in sera from *H. pylori*-infected patients with anti S-form and/or anti R-LPS antibody levels exceeding 2 SD above the mean of controls (cut-off point). The data are depicted as box-plot diagrams, with the box encompassing the range of values from the 25 percentile (lower bar) to the 75 percentile (upper bar). The horizontal line within the box represents the median, and the vertical lines above and below the box signify the maximum and minimum values. (**A**) Chronic gastritis ($n = 21$); (**B**) duodenal ulcer ($n = 10$).

Analysis of difference between anti-S- and anti-R-form LPS antibody response

In order to assess whether any difference regarding the degree of anti-S- and anti-R-form LPS antibody response could be demonstrated in patients who showed antibody reactivity against LPS from *H. pylori* NCTC 11637, patients were subdivided into 2 groups: those with anti-S- and/or anti-R-form LPS antibody levels exceeding 2 standard deviations (SD) above the mean of controls, arbitrarily assumed as the cut-off, were classified as anti-*H. pylori* LPS-positive; whereas those with values less than the cut-off were regarded as anti-*H. pylori* LPS-negative. The comparison of paired data between anti-S- and anti-R-form LPS antibody levels in positive patients was performed using the Wilcoxon signed rank test.

With respect to the IgG response, the cut-off values were 0.446 and 0.570 for anti-S- and anti-R-form LPS, respectively. Twenty-one CG patients (18 CG patients had both anti-S- and R-form LPS antibodies, 1 exhibited only anti-S- and 2 only anti-R-form LPS antibodies) and 10 DU patients (9 DU patients had both anti-S- and anti-R-form LPS, 1 displayed only anti-S-form LPS antibodies) were positive.

Regarding the IgA anti-S- and R-form LPS response, the cut-off values were 0.026 and 0.121, respectively. Sera from 23 CG patients (12 CG patients had both anti-S- and anti-R-form LPS antibodies, 11 showed only anti-S-form LPS antibodies) and 11 DU patients (5 DU patients had both anti-S- and anti-R-form LPS antibodies, 5 exhibited only anti-S-, and 1 only anti-R-form LPS antibodies) were positive.

Among those patients positive for anti-*H. pylori* LPS antibodies, subjects suffering from CG showed an IgG response against anti-S-form LPS (2.652; 1.837/2.872) significantly higher than that directed against R-form LPS (2.124; 1.420/2.414; $P = 0.028$; Fig. 5A). On the other hand, in DU patients more elevated anti-R-form LPS IgG levels were found, although the difference with anti-S-form LPS IgG levels was not statistically significant ($P = 0.37$; Fig. 5B). Comparison between the anti-S- and anti-R-form LPS IgA response showed that, in both CG and DU individuals, the values of IgA against R-form LPS were more elevated than that observed against S-form LPS, but the difference was statistically significant only in the case of DU patients ($P = 0.007$; Fig. 6).

Anti-Le^x antibody response

The presence of serum antibodies in patients against purified monomeric, trimeric and polymeric Le^x antigens was also evaluated. Anti-polymeric Le^x IgG antibodies were detected in 2 patients endoscopically diagnosed as suffering from chronic atrophic gastritis (OD 0.434; cut-off = 0.254) and active CG without signs of atrophy (OD 0.794), respectively. Sera of these patients are marked with an asterisk in Figure 1. No reaction of human sera with monomeric or trimeric Le^x antigen was detected.

DISCUSSION

In research on the putative pathogenic mechanisms underlying the pathologies aetiologically related to *H. pylori*-infection, great interest has focused on *H. pylori* LPS as a virulence determinant of the bacterium.[29–33] In fact, it has been demonstrated that *H. pylori* LPS is endowed with a low biological activity structurally related to a unique acylation and phosphorylation pattern of the lipid A moiety.[22,31,32,34,35] Such a biological feature, which has been suggested to be a possible phylogenetically selective advantage allowing the bacterium to persist in the host and to escape from its defence response,[23,31,33] might also represent a low grade, long-lasting noxious stimulus possibly involved in the chronicization of *H. pylori*-associated inflammatory reaction.[32,36] The putative pathogenic role of *H. pylori* LPS molecule has been further emphasized because of molecular mimicry between the *O*-polysaccharide chain and the blood group Le^x and/or Le^y antigens.[13–15] Moreover, certain monoclonal antibodies obtained from mice immunized with *H. pylori* also recognize Le^x or Le^y antigens[37] and *H. pylori* infection in humans elicits the production of antibodies cross-reacting with the gastric mucosa.[10,17–19] It has been suggested that auto-antibodies against the above mentioned Le antigens, cross-reacting with structurally related antigenic determinants present on the gastric epithelial surface, may trigger autoimmune phenomena.[10,33] The possible involvement of these auto-antibodies in the progression of chronic gastritis to atrophy has been suggested.[10–12,19]

The present work was undertaken to clarify the relative contributions of the different regions within *H. pylori* LPS to the antigenicity of this molecule. Our results demonstrate that *H. pylori* LPS is an important component of the immunogenic antigen profile of the bacterium since IgG as well as IgA responses can be detected in *H. pylori*-positive individuals suffering from CG and DU, but not in *H. pylori*-negative dyspeptic patients. Furthermore, a positive correlation was found in both groups of *H. pylori*-infected patients when IgG and IgA antibody responses against S- and R-form LPS were

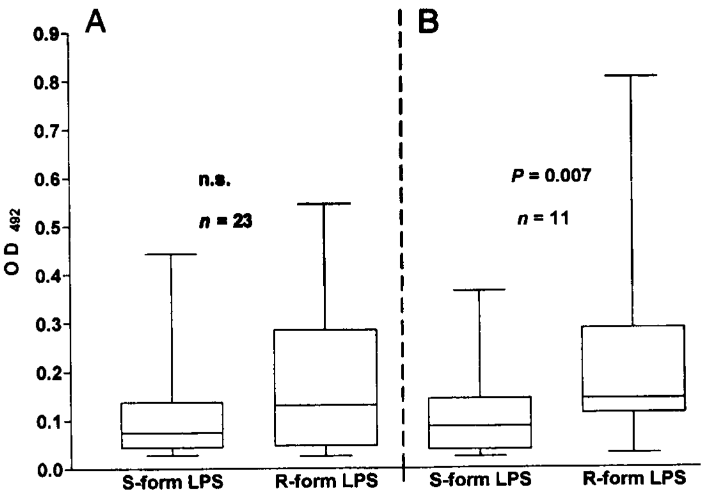

Fig. 6 Comparison between anti-*H. pylori* NCTC 11637 S-form and R-form LPS IgA response in sera from *H. pylori*-infected patients with anti S-form and/or anti R-form LPS antibody levels exceeding 2 SD above the mean of controls (cut-off point). The data are depicted as box-plot diagrams, with the box encompassing the range of values from the 25 percentile (lower bar) to the 75 percentile (upper bar). The horizontal line within the box represents the median, and the vertical lines above and below the box signify the maximum and minimum values. (**A**) Chronic gastritis (n = 23); (**B**) duodenal ulcer (n = 11).

compared. The most straightforward explanation for this finding is that, besides the well-known antigenic role of the *O*-polysaccharide chain in inducing antibodies during natural infection by *H. pylori* in humans, the core oligosaccharide contains immunogenic determinants as well. The evidence that the core oligosaccharide region within *H. pylori* behaves as an immunostimulant is also supported by the in vitro demonstration of TNF-α release and membrane tissue factor induction as a consequence of peripheral blood mononuclear cell exposure to *H. pylori* R-form LPS.[32,38]

As to the possible correlation between antibody production against S- and/or R-form LPS and the nature of *H. pylori*-dependent gastric pathology, the anti-S-form LPS IgG response was of a significantly greater order of magnitude than that directed against R-form LPS in CG patients. However, IgG levels appear not to be related to the severity of the gastric inflammatory process (data not shown). In contrast, a marked trend to prevalence of an anti-R-form LPS IgG response occurs in *H. pylori*-patients affected by DU disease. With reference to the IgA response, predominantly anti-R-form LPS antibody levels were detected in CG patients and the tendency was even more pronounced in individuals suffering from DU. From a pathogenic standpoint, the question arises as to whether an enhanced IgG antibody response against S-form LPS in patients suffering from CG may be involved in the acute onset and, thereafter, gastric mucosal damage as a consequence of auto-immune events and/or immune complex-mediated inflammation. On the other hand, a more pronounced anti-R-form LPS IgG production may follow the unmasking of core structures possibly due to conformational changes of LPS molecule in the course of a prolonged *H. pylori* infection which may result in an ulcerative process. With respect to the anti-R-form LPS IgA response in both CG and DU patients, our data support the evidence that core oligosaccharide structures are able to elicit the production of this class of immunoglobulins. It would be of pathogenic interest to evaluate whether the pattern of antibody responses at mucosal site is the same as that observed in sera from patients with *H. pylori*-related gastropathy or, alternatively, whether the IgA secretory production is more predominant within the gastric habitat.

As to the pathogenesis of *H. pylori*-dependent gastropathy, previous studies have suggested a role for anti-Le^x antibodies.[10,11,19] Consequently, a further aim of this study was to assess whether, in *H. pylori*-infected patients, Le^x determinants contained in the *O*-side chain may contribute to the antigenicity of S-form LPS. In previous reports, the presence of such (auto)antibodies was mainly investigated by evaluating the ability of sera from *H. pylori*-positive patients to inhibit the binding of anti-Le^x monoclonal antibodies to the corresponding antigenic epitopes present on *H. pylori* LPS molecule.[10,18] In this respect, a significant correlation between the degree of reactivity against *H. pylori* S-form LPS and the ability of sera to inhibit the binding of anti-Le^x monoclonal antibody CB10 to *H. pylori* was also reported.[10] However, a bias in interpreting data obtained with this methodological approach is represented by the possibility that the reported inhibition could be due to serum antibodies directed to determinants close to Le^x epitopes. These antibodies, even though not specifically recognizing Le^x antigens, could prevent the binding of anti-Le^x monoclonal antibodies because of sterical hindrance. Thus, in this study, we used monomeric, trimeric and polymeric Le^x neoglycoconjugates as coating antigen in a direct immunoenzymatic assay to determine the presence of anti-Le^x antibodies in patient sera.

Our results show that, in spite of high levels of antibodies against S-form LPS, anti-Le^x antibodies were found in two CG patients when polymeric, but not monomeric or trimeric Le^x molecules were used as coating antigen. In contrast to the limited distribution of these antibodies in the patients investigated in this study, Amano and coworkers have recently detected by ELISA the presence of antibodies against different purified Lewis antigens (i.e. monomeric Le^x, trimeric Le^x, Le^y, Le^a and Le^b) in sera from *H. pylori*-patients affected by chronic gastritis, gastric ulcer, duodenal ulcer, gastric cancer as well as from *H. pylori*-infected healthy subjects and non-infected controls.[39] Since no differences between the groups studied were found, these authors suggested that the presence of antibodies against Le antigen in human sera is unrelated to infection by *H. pylori*.[39] On the other hand, Appelmelk et al reported a preferential reaction of human anti-*H. pylori* antibodies with polymeric rather than trimeric or monomeric Le^x and Le^y antigens.[10] Collectively, these results support the view that only polymeric Le^x antigens would be recognized in vitro by such antibodies, seemingly because of the importance of epitope structural conformation in a polymer reflecting the actual situation of Le^x which is expressed as a polymer within the *O*-chain of the *H. pylori* LPS molecule. In conclusion, our findings demonstrate a marked serum immune response of both the IgG and IgA isotypes against *H. pylori* S- and R-form LPS in patients with CG and DU associated with *H. pylori* infection. However, their reciprocal role in the pathogenesis of *H. pylori*-dependent disease and involvement in auto-immune phenomena requires further investigation.

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