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Immune Responses to Bile-Tolerant *Helicobacter* Species in Patients with Chronic Liver Diseases, a Randomized Population Group, and Healthy Blood Donors

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Bile-tolerant *Helicobacter* species such as *Helicobacter pullorum*, *Helicobacter bilis*, and *Helicobacter hepaticus* are associated with hepatic disorders in animals and may be involved in the pathogenesis of chronic liver diseases (CLD) in humans. Antibody responses to cell surface proteins of *H. pullorum*, *H. bilis*, and *H. hepaticus* in serum samples from patients with CLD, a randomized population group, and healthy blood donors were evaluated by using enzyme linked immunosorbent assay (ELISA). The results were compared with the antibody responses to *Helicobacter pylori*. For analysis of a possible cross-reactivity between bile-tolerant *Helicobacter* species and *H. pylori*, sera from a subpopulation of each group were absorbed with a whole-cell extract of *H. pylori* and retested by ELISA. Results before absorption showed that the mean value of the ELISA units for *H. pullorum* was significantly higher in patients with CLD than in healthy blood donors (*P = 0.01*). Antibody reactivity to cell surface protein of *H. hepaticus* was also significantly higher in the CLD patients than in the healthy blood donors and the population group (*P = 0.005* and *P = 0.002*, respectively). Following the absorption, antibody responses to *H. pullorum* decreased significantly in all three groups (*P = 0.0001* for CLD patients, *P = 0.0005* for the population group, and *P < 0.0001* for the blood donors), indicating that cross-reactivity between *H. pylori* and other *Helicobacter* spp. occurs. The antibody responses to *H. hepaticus* and *H. bilis* in CLD patients remained high following absorption experiments compared to ELISA results before absorption. The significance of this finding requires further investigations.

During the last two decades, research on the *Helicobacter* genus has focused on *Helicobacter pylori*-associated diseases such as chronic gastritis, peptic ulceration, gastric cancer, and mucosa-associated lymphoid tissue lymphoma (7, 17, 18, 20, 23, 30, 31, 38). Recently, other spiral-shaped bacteria belonging to the *Helicobacter* genus have been identified in the intestinal tracts and livers of humans, other mammals, and birds. These microorganisms have been reported to be associated with gastroenteritis, hepatitis, and other diseases in humans and animal species (1, 4, 10, 34).

*Helicobacter pullorum* can be transmitted in the feces of asymptomatic poultry and was first isolated from the livers and intestinal contents of laying hens with vibriotic hepatitis (2, 5, 36). In humans, *H. pullorum* was detected by PCR from the bile of patients with chronic cholecystitis (12). Two cases of human enteritis associated with *H. pullorum*, one of them in an immunocompromised patient, have also been reported (6, 36, 37).

*Helicobacter bilis* was first identified in inbred mice with chronic hepatitis (14). By using sequencing of PCR-amplified 16S rRNA gene fragments, DNA from *H. bilis* was also detected in the gall bladders of five out of eight Chileans with chronic cholecystitis (12). However, culture and isolation of *H. bilis* were unsuccessful in that study.

In 1992, pathologists at the National Cancer Institute reported that *Helicobacter hepaticus* could be isolated from A/JCr mice suffering from hepatocellular carcinoma (11, 42). Neither chemicals nor a virus induced the tumor, but *H. hepaticus* was cultured regularly from murine liver suspensions, specifically, from the extracellular space of the hepatic canalici.

A number of patients infected with hepatic viruses develop cirrhosis and hepatocellular carcinoma. The risk factors currently recognized cannot fully explain the pathogenesis of this process. Therefore, a bacterial coinfection, particularly of *Helicobacter* spp., could be involved in further morphological changes following the viral damage of the liver. Bile-tolerant *Helicobacter* spp. have been reported to produce a cytotoxic distending toxin, which causes progressive cell enlargement and eventual cell death in eukaryotic cell lines (43, 44). In addition, it is now evident that in primates certain *Helicobacter* species induce liver, bile tract, and pancreatic diseases (13). Several bile-tolerant *Helicobacter* species cause bile duct and liver diseases in animals and humans (6, 12, 26). The significance of these *Helicobacter* spp. in human disease and the true prevalence in the general population remain to be determined.

The aim of the present study was to determine the antibody responses to cell surface proteins of *H. pullorum*, *H. bilis*, and *H. hepaticus* in three different groups: (i) patients with chronic liver diseases (CLD) of various etiologies, (ii) a randomized population group forming a representative sample of an adult Estonian population, and (iii) healthy blood donors. Results were compared with the antibody responses to *H. pylori*. Cross-reactivity between the bile-tolerant *Helicobacter* spp. and *H. pylori* was evaluated.
MATERIALS AND METHODS

Bacterial strains and culture conditions. H. pullorum strain CCUG 33838 (Culture Collection, University of Gothenburg, Gothenburg, Sweden) (human isolate), H. bilis marine strain CCUG 38995, and H. hepaticus marine strain CCUG 33637 were cultured on brucella blood agar supplemented with 5% horse serum, 5% sheep blood, 1% Isovitalex (Becton Dickinson, Franklin Lakes, N.J.), 0.1% charcoal (Sigma-Aldrich Corp., St. Louis, Mo.), and 1% hemin (ICN Biomedicals Inc., Irvine, Calif.) and grown for 3 days (H. pullorum and H. bilis) or 5 days (H. hepaticus) under microaerobic conditions (3% H2, 10% CO2, 5% O2, and 82% N2) at 37°C. H. pylori strain CCUG 17874 was cultured on GAB-CAMP agar (35) without antibiotics for 3 days at 37°C under microaerobic conditions.

Antigen preparations. Bacterial cells from 10 agar plates of each strain, with confluent bacterial growth, were harvested and washed once in 10 mM phosphate-buffered saline (PBS), pH 7.2. Cell surface proteins of H. bilis, H. hepaticus, and H. pylori were extracted with 0.2 M acetic acid buffer (pH 2.2) as described previously (21). Acid glycine buffer treatment was not efficient in releasing proteins of H. pullorum; instead, water solubilization was found to be an alternative. Harvested cells were washed once in PBS and then resuspended in deionized water (high-pressure liquid chromatography grade) (4 g [wet weight] of cells/100 ml of water). The suspension was stirred magnetically for 10 min at 20°C, and cells were removed by centrifugation at 12,000 × g for 15 min at 8°C. The supernatant was collected and dialyzed for 10 h at 4°C against PBS. The protein concentration was determined using the Bio-Rad (Richmond, United Kingdom) protein assay. The protein profiles of H. pullorum, H. bilis, and H. hepaticus have recently been characterized by proteomic technology (19).

Rabbit antisera to the Helicobacter species. The procedure for immunization of rabbits was recently described (19). In brief, rabbits (Swedish lop-eared) were injected subcutaneously with approximately 1.8 mg of sonicated cell material of H. pullorum strain CCUG 33838, H. bilis strain CCUG 38995, or H. hepaticus strain CCUG 33637, mixed with adjuvant (AdjuPrime Immune Modulator; Pierce, Cheshire, United Kingdom), in six divided doses (days 1, 5, 10, 15, 20, and 25). Three weeks later, the animals were bled and serum was collected.

ELISA. The H. pullorum, H. bilis, and H. hepaticus enzyme-linked immunosorbent assays (ELISAs) were performed as described previously for an H. pylori ELISA (22). In brief, wells (Maxisorp immunoplates; Nunc, Roskilde, Denmark) were coated for 16 h at 8°C with antigen in duplicate (100 μl per well) at a protein concentration of 5 μg per ml. The wells were then blocked for 1.5 h at 22°C with 3% bovine serum albumin in PBS. The plates were washed four times with PBS containing 0.05% Tween 20. Human sera (100 μl per well) were diluted 1:800 and plates incubated for 90 min at 37°C. On each plate a rabbit antiserum to each Helicobacter spp. was included as a positive control (dilution, 1:800; 100 μl per well). Alkaline phosphatase-conjugated anti-human and anti-rabbit immunoglobulin G antibodies (Dako, Glostrup, Denmark) were used as secondary antibodies (dilution, 1:500). Incubation was for 1 h at 37°C. Bound antibodies were visualized by addition of substrate solution containing 1 mg of p-nitrophenylphosphate (Sigma-Aldrich Corp.) per ml in diethanolamine buffer, pH 9.8. The absorbance was measured at 405 nm after 35 min of incubation. It was not possible to establish a reliable cutoff value for the ELISAs with H. pullorum, H. bilis, and H. hepaticus, since no true-positive or -negative human sera were available.

ELISA results are presented as relative antibody activity (RAA). The RAA is the corrected mean absorbance value as a percentage of that of a reference standard (human gamma globulin; Pharmacia & Upjohn, Stockholm, Sweden) (22). The mean RAA values for each Helicobacter spp. were compared for the three studied groups.

Absorption experiments. For absorption of potential cross-reactive antibodies, sonicated whole cells of H. pylori (CCUG 17874) were washed once in PBS (pH 7.2) and sonicated in ice at an average power output of 45 W eight times for 60 s each with 30-s intervals (Ultrasonic Homogenizer U 2000B; Braun, Melsungen, Germany). To 1 ml of sonicated cells in PBS (A540 of 1.5), 10 μl of serum was added and incubated for 1 h at 22°C and then for 16 h at 6°C with constant shaking. Cells were removed by centrifugation at 12,000 × g for 10 min, and supernatants were collected. A control of complete absorption of H. pylori antibodies, an H. pylori ELISA with all absorbed sera was performed. After absorption, the mean H. pylori RAA value decreased below the background level (t < 0.05). ELISA results before absorption. The antibody responses to extracted cell surface proteins of H. pullorum, H. bilis, H. hepaticus, and H. pylori from patients with CLD, blood donors, and the population group are presented in Table 1. The mean RAA value for H. pullorum was significantly higher in patients with CLD than in healthy blood donors (P = 0.01). Antibody responses to cell surface proteins of H. hepaticus were also significantly higher in the CLD patients than in the healthy blood donors and the population group (P = 0.005 and P = 0.002, respectively).

In both healthy blood donors and the population group, but not in the CLD patients, antibody responses to H. bilis and H. hepaticus demonstrated an association with seropositivity for...
A positive association between age and the antibody response following absorption decreased significantly (23, 41), and similar results were obtained in the present study. The mean RAA value for patients with CLD was 17.1 ± 7.8, and for the healthy blood donors (P = 0.0001), the antibody response to the blood donors was also evaluated.

Various bile-tolerant microorganisms are often difficult to culture, and liver biopsy sampling is not possible for many patients due to the high risk of bleeding or lack of facilities for this procedure. Thus, serological testing could be an important diagnostic method, since it is easy to perform and standardize. However, antigenic cross-reactivity should be considered, and cross-absorption of patient sera is required prior to testing until specific immunogenic proteins for various Helicobacter species are identified and purified for use in such assays. Since very small amounts of sera are required for ELISA and immunoblot analysis, these methods may also be used for screening of laboratory animals.

The aim of the present study was to analyze the antibody responses to bile-tolerant Helicobacter spp. in patients with CLD in an attempt to find potential associations between these microorganisms and various hepatic diseases. As a comparison, antibody responses in an unselected population group and in blood donors were also evaluated.

It is likely that Helicobacter species have several antigens in common (15, 16, 29); e.g., cross-reactivity between flagellar

### Table 2: Antibody responses to cell surface proteins of Helicobacter spp. in CLD patients, a randomized population group, and blood donors, obtained by ELISA following cross-absorption with H. pylori whole-cell extract

| Species          | Patients with CLD (n = 29) | Population group (n = 30) | Blood donors (n = 30) |
|------------------|----------------------------|---------------------------|-----------------------|
|                  | ELISA result (mean RAA value ± SD)* for: |                          |                       |
|                  | Before absorption | After absorption | Before absorption | After absorption | Before absorption | After absorption |
| H. pullorum      | 17.1 ± 7.8        | 31.8 ± 15.1*          | 10.5 ± 6.6           |
| H. bilis         | 19.7 ± 10.2       | 26.8 ± 10.6*          | 16.7 ± 12.3          |
| H. hepaticus     | 29.3 ± 11.5       | 38.3 ± 9.5*           | 28.2 ± 12.9          |

* Differences between mean values in the three groups were calculated by using the t test.

### Table 3: Antibody responses to cell surface proteins of Helicobacter spp. obtained by ELISA before and after absorption with H. pylori whole-cell extract, in CLD patients, a randomized population group, and healthy blood donors

| Group (n)             | Before absorption | After absorption | Before absorption | After absorption | Before absorption | After absorption |
|-----------------------|-------------------|------------------|-------------------|------------------|-------------------|------------------|
| Patients (29)         | 35.2 ± 23.4*      | 17.1 ± 7.7*      | 23.4 ± 18.3       | 19.7 ± 10.2      | 29.3 ± 24.2       | 29.3 ± 11.5      |
| Population group (30) | 40.5 ± 10.6*      | 31.8 ± 15.1*     | 29.8 ± 18.5       | 26.8 ± 10.6*     | 22.6 ± 19.3*      | 38.3 ± 9.5*      |
| Blood donors (30)     | 31.4 ± 19.8*      | 10.5 ± 6.6*      | 21.7 ± 10.7*      | 16.8 ± 12.3*     | 16.9 ± 12.9*      | 28.2 ± 12.9*     |

* Differences between mean values before and after absorption within the subpopulation of samples for each group were compared by using Wilcoxon’s rank sum test for paired data.

DISCUSSION

There are now at least 23 species in the genus Helicobacter, as well as some putative new species not formally named (10). Thirteen of them colonize the lower intestinal tracts of domestic and laboratory animals, as well as humans. Many of these organisms, which naturally colonize the intestinal crypts, can also colonize the biliary tract of the liver and induce hepatitis in animals, and in some cases they can induce hepatic cancer (26, 33).

The number of recently discovered enterohepatic Helicobacter spp. is growing. The possible pathological implications of these microbes may be important, but little is known about the true prevalence of these pathogens within different population groups.

Various bile-tolerant microorganisms are often difficult to culture, and liver biopsy sampling is not possible for many patients due to the high risk of bleeding or lack of facilities for this procedure. Thus, serological testing could be an important diagnostic method, since it is easy to perform and standardize. However, antigenic cross-reactivity should be considered, and cross-absorption of patient sera is required prior to testing until specific immunogenic proteins for various Helicobacter species are identified and purified for use in such assays. Since very small amounts of sera are required for ELISA and immunoblot analysis, these methods may also be used for screening of laboratory animals.

The aim of the present study was to analyze the antibody responses to bile-tolerant Helicobacter spp. in patients with CLD in an attempt to find potential associations between these microorganisms and various hepatic diseases. As a comparison, antibody responses in an unselected population group and in blood donors were also evaluated.

It is likely that Helicobacter species have several antigens in common (15, 16, 29); e.g., cross-reactivity between flagellar

and P = 0.0001, respectively). The mean RAA value for H. hepaticus in the patients with CLD did not change. Compared to the subsamples of CLD patients and blood donors, the antibody responses to the three bile-tolerant Helicobacter species were highest in the population subsample, with P values of <0.001 for all groups for all Helicobacter spp. (Table 2).
proteins of different pathogens was found in a previous study (28). Serological cross-reactivity within species belonging to the genera *Ehrlichia* (39), and *Chlamydia* (24) was reported. It could be speculated that such cross-reactivity also occurs between *Helicobacter* species, based on data from these studies.

In the present study, ELISA results following absorption experiments demonstrated that antibodies to *H. pylori* were completely removed from the analyzed sera, which does not exclude cross-reactivity between bile-tolerant helicobacters. Significant changes in antibody responses to the bile-tolerant species following absorption experiments within the three groups were observed.

The mean RAA value for *H. pullorum* in all three groups analyzed decreased dramatically following absorption, which may be due to cross-reactivity between antigens of *H. pullorum* and *H. pylori*. A similar decrease in the antibody response to *H. bilis* was also observed in the blood donors. The immune responses to *H. hepaticus* and *H. bilis* in patients with CLD remained high following the absorption, indicating that the antibody reactivity was specific to antigens of these two *Helicobacter* spp. or to other helicobacters, not yet identified, that may be involved in the pathogenesis of CLD. We expected to find an increase in the antibody reactivity to *H. hepaticus* in CLD patients, but this was not found, which may be a consequence of suppressed synthesis of proteins, including immunoglobulins, in damaged liver tissue (25). In contrast, the antibody response to *H. hepaticus* in the population group and the blood donors increased significantly following absorption. This finding cannot yet be explained.

Nilsson et al. (29) used immunoblotting to discriminate between antibodies to *H. pylori* and *H. hepaticus*. They found that 39% of patients with CLD were positive for immunoglobulin G antibodies to *H. hepaticus*. After absorption, 30% of patients remained positive, supporting the findings of the present study.

*Helicobacter* DNA was detected in liver tissue from eight patients suffering from primary liver carcinoma in a previous study (3), and it was suggested that *Helicobacter* spp. might be involved in the genesis of primary liver carcinoma. However, the presence of *Helicobacter* species in the livers of those patients might also be a consequence of the tumor process.

Roe et al. (32) detected *Helicobacter* DNA in bile from patients with various bile duct diseases. *Helicobacter* spp., including *H. pylori*, were identified in the liver tissue of patients with primary sclerosing cholangitis and primary biliary cirrhosis by using *Helicobacter* species-specific PCR. Bile and liver samples were also positive by PCR for *Helicobacter* DNA in nearly 50% of patients (27). In another study, 71 and 75% of liver samples from patients with cholangiocarcinoma or hepatocellular carcinoma were PCR positive for *Helicobacter* spp. as determined by using genus-specific primers (26).

Recently, an *H. pylori*-like strain was isolated from the liver of a woman with cirrhosis due to Wilson’s disease (8), which confirms that *Helicobacter* spp. are able to infect the human liver. However, it is not clear whether the organism isolated from this patient was in the infected liver tissue or the bile duct.

In conclusion, a high cross-reactivity between cell surface proteins of bile-tolerant helicobacters and *H. pylori* was found in this study, suggesting that species-specific immunogenic proteins need to be identified and purified for use in enzyme immunoassays. One such protein could be the cytolethal distending toxin of the bile-tolerant *Helicobacter* spp. (43, 44). The antibody responses to *H. hepaticus* and *H. bilis* proteins remained high following absorption in patients with CLD, and these findings should stimulate further investigations to ascertain whether *Helicobacter* spp. might play a role in the pathogenesis of these diseases in humans and other mammals.

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