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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 vibrionic 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 canaliculi.

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 cytolethal 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.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. *H. pullorum* strain CCUG 33838 (Culture Collection, University of Gothenburg, Gothenburg, Sweden) (human isolate), *H. bilis* murine strain CCUG 38995, and *H. hepaticus* murine 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 Biomedical Inc., Irvine, Calif.) and grown for 3 days (*H. pullorum* and *H. bilis*) or 5 days (*H. hepaticus*) under microaerobic conditions (3% H_2, 10% CO_2, 5% O_2, and 82% N_2) 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 acid glycine 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 8°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 used. Harvested cells of *H. pylori* (strain 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 Homogenizor U 2000B; Braun, Melsungen, Germany). To 1 ml of sonicated cells in PBS (A_{540} of 1.5), 10 μl of serum was added and incubated for 1 hour 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 for serology. As 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 (to ≤25) (21) in all three groups (6.7 for CLD patients, 11.5 for the population group, and 3.2 for blood donors).

TABLE 1. Antibody responses to cell surface proteins of *Helicobacter* spp. in CLD patients, a randomized population group, and blood donors, obtained by ELISA before absorption

Species	ELISA result (mean RAA value ± SD)[a] for:		
	Patients with CLD (n = 29)	Population group (n = 189)	Blood donors (n = 100)
H. pullorum	35.1 ± 23.3[b]	37.2 ± 15.0	26.9 ± 12.2[b]
H. bilis	23.4 ± 18.3	29.9 ± 17.8	24.2 ± 14.4
H. hepaticus	28.2 ± 23.5[c,d]	17.8 ± 16.1[c]	18.0 ± 14.3[d]
H. pylori	24.2 ± 17.2[e]	68.0 ± 31.1[e,f]	37.4 ± 29.6[f]

[a] Differences between mean values in the three groups were calculated by using the *t* test.
[b] $P = 0.01$.
[c] $P = 0.002$.
[d] $P = 0.005$.
[e] $P < 0.0001$.
[f] $P < 0.0001$.

Subjects. The subjects in the three groups analyzed in this study had similar socioeconomic status and represented urban citizens of Estonia.

(i) Patients. Serum samples from 29 patients with various CLD (23 males and 6 females; mean age, 44.4 ± 10.9 years) were analyzed. They represented patients studied at the Department of Gastroenterology, University Hospital of Tartu. The diagnoses were based on disease history, biochemical and immunological findings, and liver histology (9). Increased levels of liver enzymes in sera and/or clinical symptoms lasting more than 6 months confirmed the chronicity of the disease. Twenty patients had hepatic cirrhosis, seven had chronic hepatitis, one had autoimmune hepatitis, and one had chronic alcoholic hepatitis.

(ii) Population group. A total of 200 subjects formed a representative sample of the adult population from the southern Estonian town of Karksi-Nuia. These subjects were randomly selected from 1,461 persons (637 males and 824 females; median age, 42.3 years) who had previously participated in and donated blood for a large seroepidemiological study of several immunologically mediated diseases (40, 41). Of these 200 subjects, samples from 189 (82 males and 107 females; mean age, 41.9 ± 16.2 years) were analyzed in the present study, since 11 serum samples were used up.

(iii) Blood donors. Serum samples from blood donors (36 males and 64 females; mean age, 37.7 ± 11.4 years) were randomly collected at the Blood Centre of the University Hospital of Tartu; all tested negative for hepatitis B and C viruses. All serum samples were kept frozen at −20°C until analyzed.

Statistical analysis. Differences between mean values of the ELISA results (RAA) in the three groups were calculated using the *t* test. Differences were recognized as statistically significant at a *P* value of ≤0.05. Association between age and ELISA results for bile-tolerant *Helicobacter* spp. and association between ELISA results for *H. pylori* and for bile-tolerant *Helicobacter* species were calculated using regression analysis in the SAS system. The differences between ELISA results before and after absorption within the subpopulation of samples for each group were compared using Wilcoxon's rank sum test for paired data.

RESULTS

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

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

[a] Differences between mean values in the three groups were calculated by using the t test.
[b] The mean RAA value for the three *Helicobacter* species was highest in the population group, with a P value of < 0.001.

H. pylori ($P = 0.003$ and $P = 0.001$, respectively, for *H. bilis*, and $P = 0.006$ and $P < 0.0001$, respectively, for *H. hepaticus*). A positive association between age and the antibody response to *H. pullorum* was seen in patients with CLD ($P = 0.03$), and a positive association between age and the antibody response to *H. bilis* was seen in the healthy blood donors ($P = 0.002$).

The antibody response to *H. pylori* in the population group was significantly higher than for the CLD patients and the blood donors ($P < 0.0001$ for the CLD patients and $P < 0.0001$ for the blood donors). A high prevalence (87%) of antibodies to *H. pylori* in this population sample was established previously (23, 41), and similar results were obtained in the present study.

ELISA results after absorption. Cross-reactivity between *H. pylori* and the other *Helicobacter* spp. was analyzed using 29 sera from CLD patients, 30 sera from the population group, and 30 blood donor sera. The population and blood donor samples were selected at random. The outcome of the ELISA results following the absorption experiment is presented in Tables 2 and 3. The mean RAA values for *H. pullorum* decreased significantly in all three groups after absorption ($P = 0.0001$ for patients, $P = 0.0005$ for the population group, and $P < 0.0001$ for blood donors). The antibody response to *H. bilis* following absorption decreased significantly in the blood donors only ($P = 0.02$).

In both the population and blood donor groups, the mean RAA value for *H. hepaticus* increased significantly ($P < 0.0001$ 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).

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

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)	ELISA result (mean RAA value ± SD)[a] for:					
	H. pullorum		*H. bilis*		*H. hepaticus*	
	Before absorption	After absorption	Before absorption	After absorption	Before absorption	After absorption
Patients (29)	35.2 ± 23.4[b]	17.1 ± 7.7[b]	23.4 ± 18.3	19.7 ± 10.2	29.3 ± 24.2	29.3 ± 11.5
Population group (30)	40.5 ± 10.6[c]	31.8 ± 15.1[c]	29.8 ± 18.5	26.8 ± 10.6	22.6 ± 19.3[d]	38.3 ± 9.5[d]
Blood donors (30)	31.4 ± 10.8[e]	10.5 ± 6.6[e]	21.7 ± 10.7[f]	16.8 ± 12.3[f]	16.9 ± 12.9[g]	28.2 ± 12.9[g]

[a] 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.
[b] $P = 0.0001$.
[c] $P = 0.0005$.
[d] $P < 0.0001$.
[e] $P < 0.0001$.
[f] $P = 0.02$.
[g] $P = 0.0001$.

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 of 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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