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Increased prevalence of seropositivity for non-gastric *Helicobacter* species in patients with autoimmune liver disease

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Various *Helicobacter* species have been isolated from the stomach, intestinal tract and liver of a variety of mammalian and some avian species, and *Helicobacter* DNA has been detected in human bile and liver samples. An immunoblot assay was established to analyse serum antibody responses to non-gastric *Helicobacter* species in patients with autoimmune liver diseases, in comparison with healthy individuals. Sera from 36 patients with primary sclerosing cholangitis (PSC), 21 with primary biliary cirrhosis, 19 with autoimmune chronic hepatitis and 80 blood donors were analysed by immunoblot, using cell-surface proteins from *Helicobacter pullorum*, *Helicobacter bilis* and *Helicobacter hepaticus* as antigens. Prior to testing, sera were cross-absorbed with a whole-cell lysate of *Helicobacter pylori*. Antibody reactivity to various proteins of these three *Helicobacter* species was measured by densitometric scanning and results were processed by computer software to estimate antigenic specificity. Results were also compared with antibody response to *H. pylori*. For *H. pullorum*, reactivity to at least two of the proteins with molecular masses of 48, 45, 37, 20 and 16 kDa, for *H. hepaticus*, reactivity to the 76, 30 and 21 kDa proteins and for *H. bilis*, reactivity to the 22 and 20 kDa proteins, seemed to have high specificity. Positive immunoblot results with sera from patients with PSC to antigens of *H. pullorum*, *H. bilis* and *H. hepaticus* were found in 38, 22 and 25 % of cases, respectively, and from patients with other autoimmune liver diseases, in 30, 22 and 22 % of cases, respectively. Prevalence of serum antibodies to non-gastric *Helicobacter* species was significantly higher in patients with autoimmune chronic liver diseases than in healthy blood donors ($P < 0.001$). Increased antibody levels to enterohepatic *Helicobacter* species raise questions concerning an infectious role of these emerging bacterial pathogens in human autoimmune liver diseases.

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INTRODUCTION

Genetic, environmental and microbial factors are believed to play a role in the development of chronic liver diseases of presumed autoimmune aetiology, such as primary sclerosing cholangitis (PSC) and primary biliary cirrhosis (PBC). Unknown environmental factors or infectious agents could trigger an immunological reaction and an inflammatory response that result in the destruction of intra- and extra-hepatic bile ducts (Lee & Kaplan, 1995; Sutton & Neuberger, 2002). No causative microbial agent has been defined clearly for humans. Normal intestinal microflora or previously unrecognized intestinal pathogens might contribute to the

development of disease in susceptible hosts. More recently, DNA of different *Helicobacter* species was detected in human bile and gall-bladder samples, suggesting that these organisms may colonize or translocate to the biliary tract of humans and may be associated with hepatobiliary diseases (Fox *et al.*, 1998, 2001; Leong & Sung, 2002; Ljungh & Wadström, 2002).

Isolation of a *Helicobacter* species from a human liver was reported recently (de Magalhaes Queiroz & Santos, 2001). We observed that PSC and PBC patients were positive for *Helicobacter* spp. in liver biopsies by PCR and DNA sequence analysis and visualized *Helicobacter* in Kupffer cells by immunohistochemistry (Nilsson *et al.*, 2000a; Wadström *et al.*, 2001). Similar findings were reported for patients with cholangio- and hepatocellular carcinoma (Nilsson *et al.*,

Abbreviations: EIA, enzyme immunoassay; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis.

2001). Furthermore, a significant difference in antibody response was found in patients with chronic liver disease compared to blood donors (Ananieva *et al.*, 2002).

Helicobacter and human heat-shock protein 70 (Hsp70) share cross-reactive epitopes, suggesting a possible auto-immune role in disease pathogenesis (Ward *et al.*, 1996). High antibody reactivity to these proteins was also found in sera of alcoholic patients with chronic liver disease (Nilsson *et al.*, 2000b).

The aim of the present study was to analyse immune responses to cell-surface proteins of *Helicobacter pullorum*, *Helicobacter bilis* and *Helicobacter hepaticus*, in order to explore the possible role of these related emerging pathogens in human autoimmune liver diseases.

METHODS

Patient samples. Serum samples from patients (aged 31–88 years) with PSC ($n = 36$), PBC ($n = 21$) or autoimmune hepatitis ($n = 19$) were analysed. Sera were collected from a prospective series of patients investigated at the Department of Medicine, University Hospital of Malmö, for suspected chronic liver disease due to elevated concentrations of liver enzymes and/or clinical symptoms of >6 months duration. Patients with PBC were all women and all had a cholestatic biochemical profile with a positive test for anti-mitochondrial antibodies, elevated plasma concentrations of IgM and a liver biopsy that was compatible with the diagnosis. Among the patients with PSC, 15 were women. The diagnosis of PSC was verified in each case by endoscopic retrograde cholangiography. Among the patients with autoimmune chronic hepatitis, 16 were women. These patients all had anti-nuclear antibodies and smooth muscle antibodies, as well as increased plasma concentrations of IgG and liver biopsies that were compatible with the diagnosis. All patient sera were negative for hepatitis C virus (RIBA3) and hepatitis B surface antigen (ELISA) (Verbaan *et al.*, 1998). Blood donor serum samples ($n = 80$), collected randomly at the blood-transfusion unit at the University Hospital of Malmö, were included to represent a healthy population without any gastrointestinal or liver diseases for comparison of antibody reactivity to antigens of *Helicobacter* species. All serum samples were kept frozen at $-20\text{ }^{\circ}\text{C}$ until they were analysed.

Bacterial strains and antigen preparations. A human isolate of *H. pullorum* (strain CCUG 33838; Cell Culture Collection, University of Gothenburg), *H. bilis* strain CCUG 38995^T and *H. hepaticus* strain CCUG 33637^T (murine isolates) were cultured on Brucella blood agar as described previously (Kornilovs'ka *et al.*, 2002) for 4 days at $37\text{ }^{\circ}\text{C}$ in a microaerobic atmosphere (Anoxomat; MART Microbiology). *Helicobacter pylori* strain CCUG 17874^T was cultured (low passage; fewer than four times) on GAB-CAMP agar (Soltesz *et al.*, 1992) without antibiotics for 3 days at $37\text{ }^{\circ}\text{C}$ in a microaerobic atmosphere (Anoxomat; MART Microbiology).

Acid/glycine extraction of cell-surface proteins of *H. bilis*, *H. hepaticus* and *H. pylori* was performed as described previously (Lelwala-Guruge *et al.*, 1992). This method did not efficiently release cell-surface proteins from *H. pullorum*; therefore, a water-washing procedure was used for this bacterium (Kornilovs'ka *et al.*, 2002). Protein concentrations of the extracts were determined by the Bradford method, using the Bio-Rad protein assay and BSA (Bio-Rad) as standard.

SDS-PAGE and immunoblot assay. SDS-PAGE was performed under reducing conditions (Laemmli, 1970) by using Criterion cell electrophoresis equipment (Bio-Rad). Extracted *Helicobacter* proteins

were separated in a 10–20% gradient gel with a 5% stacking gel (Criterion pre-cast gels; Bio-Rad) (Kornilovs'ka *et al.*, 2002). Separated *Helicobacter* proteins were transferred electrophoretically to a polyvinylidene difluoride membrane as reported previously (Nilsson *et al.*, 1997). For each immunoblot assay, a polyclonal rabbit antiserum to each of the four *Helicobacter* species was included as calibrator (Kornilovs'ka *et al.*, 2002). Protein bands obtained by immunoblot (after the absorption step described below) were scanned by using a GS-710 Imaging densitometer and compared and processed by Quantity One software (both from Bio-Rad). For *H. pullorum*, reactivity to at least two of the proteins with molecular masses of 48, 45, 37, 20 and 16 kDa, for *H. hepaticus*, reactivity to the 76, 30 and 21 kDa proteins and for *H. bilis*, reactivity to the 20 and 22 kDa proteins, seemed to have high specificity. Criteria for an *H. pylori*-positive immunoblot were followed as described previously (Nilsson *et al.*, 1997).

Immunoabsorption experiments. To remove potentially cross-reacting antibodies between *H. pylori* and the other three *Helicobacter* species, a whole-cell lysate of *H. pylori* (CCUG 17874^T) was used. Ten microlitres of serum was added to 1 ml sonicated cells (A_{540} , 1.5) and incubated for 16 h at $8\text{ }^{\circ}\text{C}$ under constant shaking. Cells were removed by centrifugation at 12 000 g for 15 min and supernatants were used for the immunoblot assay. As a control for complete absorption of *H. pylori* antibodies, an *H. pylori* enzyme immunoassay (EIA) was performed as described previously (Ananieva *et al.*, 2002).

Polyclonal rabbit antisera to the *Helicobacter* species (Kornilovs'ka *et al.*, 2002) were used for extended absorption experiments. In addition, cross-reactivity was analysed by a dot-blot assay. Antisera were absorbed step-wise as described above, with whole cells of: (i) *H. pylori* + *Escherichia coli* (ATCC 25922); (ii) *H. pylori* + *E. coli* + *Lactobacillus paracasei* (Ljungh *et al.*, 2002); and (iii) *H. pylori* + *E. coli* + *L. paracasei* + *Campylobacter jejuni* (ATCC 33560^T). Membrane strips were coated with antigens of the above bacteria and with antigens of '*Flexispira rappini*' (CCUG 23435), *Helicobacter canis* (CCUG 33835), *H. pullorum*, *H. bilis* and *H. hepaticus* (10 µg per dot) and probed with the polyclonal non-absorbed and absorbed rabbit antisera (diluted 1 : 200). Dots were visualized by using the same buffer as described for the immunoblot assay. After an initial absorption step with *H. pylori* antigens only, any additional absorption steps did not affect the test results with patient sera.

Statistics. Statistical analysis included descriptive statistics for each group; significant differences between the patient groups and healthy blood donors were calculated by the non-parametric F-test.

RESULTS

To illustrate differences in antibody levels before and after absorption, a densitogram of three scanned immunoblot strips that contained separated proteins of the non-gastric *Helicobacter* species and was probed with a patient serum (strongly reactive before absorption) was prepared (Fig. 1). Each peak corresponds to one band on the immunoblot strip and height corresponds to its density. With this patient's serum, almost all reactivity to *H. bilis* disappeared, whereas the serum seems to contain antibodies specific to *H. hepaticus*.

In the dot-blot assay with antisera raised against antigens of *H. pullorum*, *H. bilis* and *H. hepaticus*, a high degree of cross-reactivity was demonstrated, which was reduced significantly by using the preabsorption step. Reactivity to proteins of '*F. rappini*' remained and a weak response to proteins of

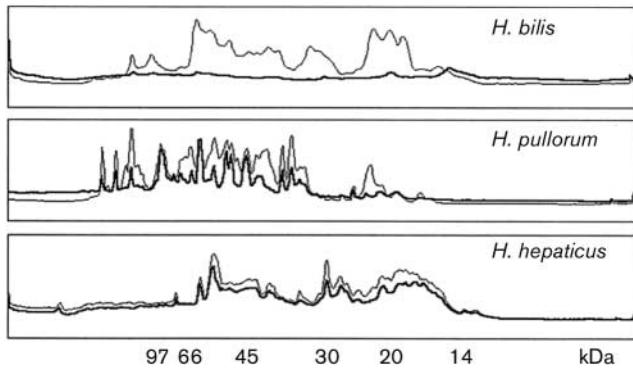


Fig. 1. To illustrate differences in the antibody response before and after absorption, a densitogram of three scanned immunoblot strips was prepared, each containing equal amounts of separated proteins of non-gastric *Helicobacter* species, and probed with a patient serum diluted 1:100 (strongly reactive before absorption). Each peak corresponds to one band on the immunoblot strip and the height corresponds to its density. With this serum, almost all reactivity to *H. bilis* disappeared, whereas this patient's serum seems to contain antibodies specific to *H. hepaticus*. Thin line, non-absorbed serum; thick line, absorbed serum.

H. canis was also noted. Cross-reactivity with proteins of *C. jejuni*, *L. paracasei*, *E. coli*, *H. pullorum*, *H. bilis*, *H. hepaticus* and *H. pylori* disappeared after the absorption step.

None of the preabsorbed blood donor sera reacted with any of the 48, 45, 37, 20 or 16 kDa proteins of *H. pullorum*, whilst one of 80 sera (1%) was positive for the *H. bilis* proteins (20 and 22 kDa). To the *H. hepaticus* proteins (76, 30 and 21 kDa), three of 80 (4%), and to *H. pylori*, 13 of 80 (16%), sera were positive (Table 1).

Sera from PSC patients contained antibodies to at least two of the specific *H. pullorum* proteins in 14 of 36 (38%), to *H. bilis* in eight of 36 (22%) and to *H. hepaticus* in nine of 36 (25%), respectively. Results obtained by immunoblot in the group of PSC patients were statistically significant, compared to the

immunoblot results of donor sera ($P < 0.05$, $P < 0.001$ and $P < 0.001$, respectively). Among sera from patients with autoimmune liver diseases other than PSC, 12 of 40 (30%) were positive by *H. pullorum* immunoblot, nine of 40 (22%) were positive by immunoblot to *H. bilis* and five of 40 (12%) were positive by immunoblot to *H. hepaticus*. These results differed significantly from those of blood donors ($P < 0.001$ for the three species) (Table 1). A positive *H. pylori* immunoblot was found in eight of 36 (22%) of the PSC sera and in 23 of 40 (57%) of sera from patients with other autoimmune disorders (Table 1).

DISCUSSION

Enteric, bile-tolerant *Helicobacter* species, such as *H. pullorum*, *H. bilis* and *H. hepaticus*, are fastidious microaerophilic microbes that are extremely difficult to isolate from stool samples and other clinical specimens. They are probably inhibited by other microbes in complex test samples, as well as by certain bile and intestinal metabolites. In this study, we aimed to develop and optimize antibody-based detection tests, analogous with some other fastidious bacterial pathogens that are difficult to isolate, e.g. *Leptospira*, *Treponema*, *Borrelia*, *Ehrlichia* and various *Rickettsia* species. Furthermore, difficulty in obtaining human liver, gall-bladder and bile samples for PCR diagnosis should stimulate the development of immuno-based assays to detect infections with these new enteric pathogens.

The absorption procedure to remove possible cross-reacting *H. pylori* antibodies should rule out the risk that infection with this pathogen would give a false-positive result in the immunoblot, not excluding cross-reactivity between various enterohepatic *Helicobacter* species. Cross-reactivity to proteins of '*F. rappini*' was observed in the dot-blot assay, most probably resulting from homology between heat-shock proteins of various *Helicobacter* species. However, weak reactivity to *H. canis* was also found; this species has not so far been found in human liver or bile samples. Much less or no cross-reactivity was observed to proteins of *C. jejuni*, *L.*

Table 1. Immunoblot results of sera from patients with autoimmune liver diseases and healthy individuals

Sera were absorbed with sonicated cells of *H. pylori* prior to testing for antibody reactivity to antigens of *H. pullorum*, *H. bilis* and *H. hepaticus*.

Antigens from:	No. positive sera (%)		
	PSC (n = 36)	Other autoimmune diseases (n = 40)	Blood donors (n = 80)
<i>H. pullorum</i>	14 (38)*	12 (30)*	0 (0)
<i>H. bilis</i>	8 (22)*	9 (22)*	1 (1)
<i>H. hepaticus</i>	9 (25)*	5 (12)*	3 (4)
<i>H. pylori</i>	8 (22)	23 (57)	13 (16)

*Significant difference compared to blood donors ($P < 0.001$).

paracasei and *E. coli*. Immunoblot bands, following cross-absorption of the tested patient samples, could thus be interpreted as specific.

Very few of the blood donors demonstrated seropositivity for antigens from non-gastric *Helicobacter* species, in agreement with a previous EIA study on Estonian blood donor sera (Ananieva *et al.*, 2002).

Antibody reactivity to *H. pullorum* and *H. bilis* was similar with sera from patients with PSC and autoimmune chronic hepatitis, in contrast to the seroreactivity to proteins of *H. hepaticus*, where PSC sera showed a higher antibody response (25 %) than those of autoimmune hepatitis patients (12 %). The significance of this finding requires further studies. The *H. pylori* immunoblot showed that the antibody response among PSC patients was lower than that of autoimmune hepatitis patients, with 22 and 57 % positive tests, respectively. Similar results were obtained by EIA in a previous investigation (Nilsson *et al.*, 2000b), which also included sera from patients with alcoholic liver disease that showed a strong antibody response to *Helicobacter* heat-shock proteins.

To purify highly specific immunogenic proteins as diagnostic antigens for optimized immunoassays for these emerging *Helicobacter* species would be of great advantage. Detection of antibodies to non-gastric, enteric *Helicobacter* species provides, however, only indirect evidence of infection; immunohistochemical staining methods should be developed in parallel, as culture of non-gastric *Helicobacter* species from various clinical specimens continues to be a major problem that limits studies of their prevalence and role in hepatic diseases.

In the first human study published in this field, DNA of *H. bilis* and *H. pullorum* was commonly identified by PCR in Chilean patients with chronic cholecystitis (Fox *et al.*, 1998). The isolation of a *Helicobacter* strain from a human liver (de Magalhaes Queiroz & Santos, 2001) and detection of *Helicobacter* DNA by PCR in bile from patients with bile-duct diseases (Roe *et al.*, 1999; Matsukura *et al.*, 2002) suggest that further systematic studies should be carried out in patients with liver and bile-duct diseases. Interestingly, Avenaud *et al.* (2000) detected *Helicobacter* species in hepatocellular carcinoma and cholangiocarcinoma, and Nilsson *et al.* (2002) detected *Helicobacter* species in patients with pancreatic cancer. These and other gastric bile-sensitive species have been proposed to translocate to the bile tract and liver by using macrophages to carry bacteria from the gut to the mesenteric lymph nodes, to enter the blood stream and seed other sites, e.g. in individuals with poor liver function (Garcia-Tsao, 2001).

Autoimmune diseases are conditions in which the immune system damages normal tissue components of the individual. In patients with autoimmune hepatitis and PBC, significant titres of anti-nuclear and anti-smooth muscle autoantibodies are commonly observed (Alvarez *et al.*, 1999). Chronic viral infections play a crucial role in the initiation and main-

tenance of pathological processes in the liver (Haydon & Neuberger, 2000), but little is known about other microbial pathogens that cause chronic infections, which may trigger an autoimmune response. Animal models to study *Helicobacter* spp. infections should be developed to analyse their relation to autoimmune liver diseases, as well as further attempts to culture microbes from liver specimens.

This study will hopefully stimulate more studies to explore the possible role of enteric bile-tolerant and -sensitive *Helicobacter* species in the pathogenesis of hepatobiliary disease in humans.

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