Cross-Reactivity between Immune Responses to Helicobacter bilis and Helicobacter pylori in a Population in Thailand at High Risk of Developing Cholangiocarcinoma

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Received 16 April 2008/Returned for modification 15 May 2008/Accepted 24 June 2008

Helicobacter bilis DNA has been detected in human tissue and is a candidate for etiologic investigations on the causes of hepatic and biliary tract diseases, but reliable serologic tests need to be developed in order to pursue such investigations. The scope of this study was to assess the specificity of two assays for H. bilis immune response allowing for H. pylori, and their cross-reactivity in a population in Thailand at high risk for cholangiocarcinoma. Plasma samples from 92 Thai volunteers were independently tested in two laboratories (Massachusetts Institute of Technology [MIT] and Lund). MIT performed three analyses of H. pylori and H. bilis based either on (i) outer membrane protein (OMP) with no preabsorption or on antigens derived from whole-cell sonicate before (ii) or after (iii) preabsorption with H. pylori sonicate protein. Lund used cell surface proteins from H. pylori and H. bilis as antigens. Testing for H. bilis was preabsorbed with a whole-cell lysate of H. pylori. More than 80% of the samples were positive for H. pylori in both laboratories. As tested by MIT, 58.7% (95% confidence interval, 47.9 to 68.9%) were positive for H. bilis by OMP and 44.5% (34.1 to 55.3%) were positive for H. bilis sonicate protein, but only 15.2% (8.6 to 24.2%) remained positive after preabsorption with H. pylori sonicate protein. Lund found 34.5% of the samples positive for H. bilis (22.0 to 41.0%), which was statistically compatible with all three MIT results. Serologic responses to OMPs of the two bacteria coincided in 66 and 45% of the samples in the MIT and Lund assays, respectively. We found high cross-reactivity between the immune responses to H. pylori and H. bilis antigens. More-specific H. bilis antigens need to be isolated to develop serologic tests suitable for epidemiological studies.

An increasing number of Helicobacter species (other than Helicobacter pylori) that colonize the enterohepatic tract of animals and humans have been identified in recent years (8). Some cause hepatitis and hepatocellular carcinoma in animal models (10) and bacteremia in both immunocompromised and immunocompetent hosts (19, 24). The documented role of H. pylori in developing duodenal and gastric ulcer disease and stomach cancer in humans (22) justifies the hypothesis that some of these bacteria may have a role in human enterohepatic diseases.

The presence of H. bilis DNA, one of the eight species identified in humans, was first described in the bile and gallbladder tissue of Chilean patients affected by cholecystitis in a region where the incidence of gallbladder cancer was high (9). The DNA of enteric species, including H. bilis, H. hepaticus, and H. pullorum, has been identified by PCR in neoplastic and normal tissues of cancer patients (29), in pediatric liver tissue (45), and in various chronic hepatic disorders, including malignancy and intra- and extrahepatic biliary diseases (2, 6, 20, 23, 37). In particular, H. bilis DNA was more common in Thai and Japanese patients affected by malignancies of the biliary tract than in patients affected by nonmalignant disorders (23).

Because H. bilis has been associated with chronic hepatitis (11, 12), inflammatory bowel disease (3, 13, 40), and cholecystitis (25) in mouse models, potential H. bilis infection in humans appears to be suitable for epidemiological investigations.

Epidemiological studies require, however, the availability of noninvasive tests to compare large numbers of cases and suitable controls.

In the context of a long-term prospective investigation on cholangiocarcinoma in rural Thailand (42), we assessed whether individuals from the resident population expressed detectable plasma immunoglobulin G (IgG) to H. bilis antigens that can be discriminated from antibody responses to H. pylori infection. To establish reliable biomarkers based on minimally invasive tests is a necessary prerequisite to the development of epidemiological studies on the etiological relevance of different Helicobacter species.

MATERIALS AND METHODS

Plasma samples of 92 subjects from a cohort of 24,000 volunteers recruited in northeastern Thailand between 1990 and 2001 were retrieved from the cohort
bioBank. At recruitment, each volunteer signed informed consent and donated a blood sample that was divided into several aliquots and stored at −20°C (42). The 92 subjects were selected to represent the cohort: 32 were men, the mean age of the group was 55.0 ± 10.0 years (range, 20 to 70 years), and 19.6% were positive for Opistorcha veverini, a liver fluke endemic in this population. Infection with hepatitis B and C viruses was not assessed in this set of subjects. However, the prevalences of participants positive for hepatitis B surface antigen and core and surface antigens to hepatitis C in a random sample tested at baseline were 10 and 4%, respectively (unpublished data).

The 92 vials of plasma were thawed, split into two aliquots (0.2 to 0.4 ml), and shipped in dry ice to the two laboratories at the Massachusetts Institute of Technology (MIT) in Cambridge, MA, and the University of Lund (Lund) in Sweden. The laboratories conducted the analyses independently and were blinded with respect to any characteristics of the participants.

Laboratory assays at MIT: bacterial strains and culture conditions. The MIT laboratory performed two analyses for antibody titers to H. pylori and H. bilis, one based on outer membrane proteins (OMPs) with no preabsorption and the second based on antigens derived by whole-cell sonication (51). In the second assay, comparisons were made between IgG levels before and after preabsorption with H. pylori sonicate protein. To prepare OMPs, H. pylori and H. bilis were cultured in brucella broth containing 5% fetal bovine serum for 24 h under microaerobic conditions. After 3 washes in phosphate-buffered saline (PBS) and examination for bacterial contaminants using Gram staining and phase microscopy, the pellet was resuspended in 4 ml of 1% N-octyl-β-D-glucopyranoside (Sigma, St. Louis, MO) for 30 min at room temperature. Insoluble material was removed by ultracentrifugation at 100,000 × g for 1 h. After dialysis against PBS for 24 h at 4°C, supernatant protein concentration was measured by the Lowry technique (Sigma). For sonication, bacterial pellets from broth cultures were resuspended in sterile PBS and sonicated on ice (Artrek Sonic Dismembranator; Artrek Systems, Framingdale, NY). Sonication was for four cycles of 30 s on and 30 s off at a duty cycle of 50% and with power applied slowly to 60 W. After sonication, the mixture was examined by phase-contrast microscopy to confirm the absence of intact bacteria, followed by determination of the protein concentration as described above. OMP antigens for the first analysis consisted of OMP extracts harvested from clinical isolates of H. pylori (Hp1018, Hp1010, NQ366, NQ1725, and NQ1708) mixed in equal amounts based on protein concentration. The ATCC type strain of H. bilis (ATCC 51630) was used. Sonicate antigens for the second analysis were prepared from four of the five previously listed clinical isolates of H. pylori (Hp1018, Hp1010, NQ366, and NQ1708), also mixed in equal amounts based on protein analysis. H. pylori sonicate was prepared using the same ATCC type strain as described above.

For all assays, a checkerboard titration of reagents was performed to identify the optimal enzyme-linked immunosorbent assay (ELISA) conditions. Sera from eight confirmed H. pylori-positive (four from Louisiana and four from Colombia) and five confirmed negative patients (two from Louisiana and three from Colombia) were used as controls (based on PCR, culture, and histology). Serum concentration to H. pylori was defined as ELISA values that exceeded the mean plus three times the standard deviation of the ELISA optical density of sera from five confirmed negative H. pylori seca. Serum concentration to H. bilis could not be proven and was based on ELISA values generated from the samples from the five confirmed H. pylori-negative patients.

MIT laboratory, first analysis. For plasma IgG measurement, 96-well Immulon II plates (ThermoLab Systems, Franklin, MA) were coated with 100 μl per well of 1-μg/ml concentrations of H. pylori or H. bilis OMP in carbonate buffer (pH 9.6) overnight at 4°C. Sera were diluted 1:1,000 and incubated on wells for 1 h at 37°C. The biotinylated secondary antibody was goat anti-human IgG (Southern Biotechnology Associates, Birmingham, AL), which was used at a 1:10,000 dilution for 1 h at 37°C. Incubation with extravidin peroxidase for 30 min at 37°C (Sigma) was followed by a 30-min incubation with ABTS [2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)] diaminonium salt substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD) for color development at room temperature. Absorbance (optical density) at 405/562 nm was recorded by an ELISA plate reader (Dynatech MR7000; Dynatech Laboratories, Inc., Chantilly, VA).

MIT laboratory, second and third analyses. The ELISA conditions were identical to the first analysis except that sonicate proteins were used as antibody coating. To preabsorb plasma with H. pylori antigens to minimize cross-reactive IgG to H. bilis, the amount of sonicate protein used was standardized by adjusting the concentration to achieve and optical density of 0.5 at a wavelength of 540 nm when mixed with 1 ml of plasma diluted to 1:1,000. Tubes were placed on a mixing platform for 1 h at room temperature and then overnight at 4°C. A paired sample was incubated with PBS instead of protein and subjected to the same incubation conditions. Nonabsorbed and preabsorbed samples were then plated against H. pylori or H. bilis sonicate antigens for the ELISA. The data generated on serial days of assay were normalized by including a set of the same three samples on every plate and adjusting the values for interday variability.

Lund laboratory. (i) Bacterial strains and culture conditions. A reference strain of H. pylori (17874 obtained from the Cell Culture Collection, University of Gothenburg, Gothenburg, Sweden [CCUG]) and an H. bilis strain (CCUG 38995) were cultured on brucella blood agar (Becton Dickinson, Franklin Lakes, NJ) supplemented with 5% horse blood, 10% inactivated horse serum, 1% IsoVitalex (Becton Dickinson), and 1% hemin (ICN, Aurora, OH) for 3 to 4 days at 37°C in a microaerobic atmosphere (Anoxomat; MART Microbiology, Lichtenvooorde, The Netherlands). Cells of the two species were harvested, washed twice in PBS (pH 7.2; 0.02 M sodium phosphate, 0.13 M NaCl) and used for subsequent protein extraction.

(ii) Extraction of CSPs. An acid glycine extraction of cell surface proteins (CSPs) of the two Helicobacter species was performed as follows. Washed cells were resuspended in 0.2 M glycine hydrochloride (pH 2.2; 4 g of cells/100 ml) supplemented with protease inhibitors (Complete; Roche Diagnostics, Mannheim, Germany) and stirred magnetically for 15 min at 20°C. Cells were removed by centrifugation at 12,000 × g for 15 min at 8°C, and the supernatants were neutralized with NaOH and dialyzed for 18 h at 8°C against PBS. Protein was quantified by the Bradford method using the Bio-Rad protein assay (Bio-Rad, Richmond, CA) and bovine serum albumin as a standard. Protein extracts were divided into aliquots and kept frozen at −22°C until use.

(iii) Serology. To remove potentially cross-reacting antibodies between H. pylori and the enteric H. bilis, a preabsorption step was performed. Plasma samples were tested for antibodies to antigens of H. pylori by enzyme immunoassay (ELISA) and immunoblotting were absorbed with a whole-cell lysate of H. pylori (Hp10018 serum was added for 16 h at 8°C, washed with PBS plus 0.05% Tween [pH 7.2]), and blocked for 1.5 h at 22°C with 2% bovine serum albumin in PBS-T. Samples, diluted 1/800, were applied and incubated for 1 h at 37°C. As a reference standard, human gamma globulin (Pharmacia & Upjohn, Stockholm, Sweden) was used. A pool of 10 sera, determined to be negative by ELISA for H. pylori and H. bilis, was included as a negative control, and a polyclonal rabbit antiserum to the two Helicobacter species was applied as a positive control. After a washing step, an alkaline phosphatase-conjugated anti-human IgG (diluted 1/30,000; Sigma, St. Louis, MO) or an anti-rabbit IgG antibody (diluted 1/500; DacoCytomation AS, Glostrup, Denmark) was used as a secondary antibody, and the plates were again incubated for 1 h at 37°C. Antibody absorbances were visualized by adding a substrate buffer containing 1 mg of p-nitrophenyl phosphate (Sigma) per ml in a diethanolamine buffer (pH 9.8). The absorbance was measured in a spectrophotometer at 405 nm when mixed with 1 ml of plasma diluted 1:1,000. Tubes were placed on a mixing platform for 1 h at room temperature and then overnight at 4°C. A paired sample was incubated with PBS instead of protein and subjected to the same incubation conditions. Nonabsorbed and preabsorbed samples were then
TABLE 1. MIT laboratory: within-subject association between _H. pylori_ and _H. bilis_ immune response

| Antigen and _H. bilis_ detection result | No. of samples (% positive) with result for _H. pylori_ detection with OMP<sup>a</sup> | % Agreement | Kappa | P |
|----------------------------------------|-------------------------------------------------|-------------|-------|---|
| OMP (no preabsorption)                 |                                                 |             |       |   |
| Positive                               | 49                                              | 57 (66.3)   | 0.243 | 0.003 |
| Negative                               | 26                                              | 12 (28.4)   |       |   |
| Total                                  | 75 (81.5)                                       | 17 (22.6)   |       |   |
| Sonicate                               |                                                 |             |       |   |
| Positive                               | 38                                              | 3 (4.1)     | 56.5  | 0.007 |
| Negative                               | 37                                              | 14 (20.3)   |       |   |
| Total                                  | 75                                              | 17 (22.6)   |       |   |
| Preabsorbed sonicate<sup>b</sup>       |                                                 |             |       |   |
| Positive                               | 14                                              | 0 (0.0)     | 33.7  | 0.0786 |
| Negative                               | 61                                              | 17 (22.6)   |       | 0.03 |
| Total                                  | 75                                              | 17 (22.6)   |       |   |

<sup>a</sup> Percent positive values are indicated in parentheses.

<sup>b</sup> That is, the _H. bilis_ sonicate after preabsorption with the _H. pylori_ sonicate.

The prevalence of _H. pylori_-positive individuals was high in both laboratories: 75 of 92 samples analyzed by MIT (Table 1), yielding a prevalence of 81.5% (95% CI = 72.1 to 88.9%), and 77 of 88 analyzed by Lund (Table 2; four vials did not contain enough material for all assays), yielding a prevalence of 87.5%.

TABLE 2. Lund laboratory: within-subject association between _H. pylori_ and _H. bilis_ immune response

| _H. pylori_ cell surface protein | No. of samples with result for _H. bilis_ cell surface proteins after preabsorption with _H. pylori_ lysate | % Agreement | Kappa | P |
|---------------------------------|----------------------------------------------------------------------------------------------------------|-------------|-------|---|
| Positive                        | 29 (87.5%)                                                                                               | 48 (41.0%)  | 0.243 | 0.003 |
| Negative                        | 0 (0.0%)                                                                                                 | 11 (8.7%)   |       |   |
| Total                           | 29 (87.5%)                                                                                               | 48 (41.0%)  |       |   |

<sup>a</sup> The percentages of samples with a positive response to _H. pylori_ and _H. bilis_ were 87.5% and 22.7%, respectively.

<sup>b</sup> Percent agreement, 45.5; kappa, 0.136; P < 0.005.

<sup>c</sup> There were four missing values for _H. pylori_.
the association between classification can reduce a true odds ratio of 10 (magnitude of 46.7% (OMP). In epidemiological studies, such a level of mis-

| Antigen and H. bilis detection result (MIT) | No. of samples (% positive) with result for H. pylori detection with OMP (Lund)* | % Agreement | Kappa | P       |
|-------------------------------------------|--------------------------------------------------------------------------------|-------------|-------|---------|
|                                            | Positive | Negative | Total |                        |            |            |
| OMP (no preabsorption)                    |                      |          |       |                        |            |            |
| Positive                                  | 20       | 34       | 54 (58.7) | 53.3  | 0.122     | NS c       |
| Negative                                  | 9        | 29       | 38    |                        |            |            |
| Total                                     | 29       | 63       | 92    |                        |            |            |
| Sonicate                                  |                      |          |       |                        |            |            |
| Positive                                  | 20       | 21       | 41 (44.6) | 67.4  | 0.321 <0.001 |
| Negative                                  | 9        | 42       | 51    |                        |            |            |
| Total                                     | 29       | 63       | 92    |                        |            |            |
| Preabsorbed sonicate**                    |                      |          |       |                        |            |            |
| Positive                                  | 7        | 7        | 14 (15.2) | 68.5  | 0.151 0.05  |
| Negative                                  | 22       | 56       | 78    |                        |            |            |
| Total                                     | 29 (31.5)| 63       | 92    |                        |            |            |

* Percent positive values are indicated in parentheses.

** That is, the H. bilis CSPs after preabsorption with H. pylori lysate.

The ability to characterize the infection status of individuals with enterohepatic helicobacters described in the last 20 years would open entirely new perspectives for investigations into the causes of chronic and malignant diseases of the biliary tract in human beings. Cancers of the gallbladder and biliary tract show 10-fold variation worldwide (36), being generally rare but with areas of an exceptionally high incidence in the Andean region in South America, Thailand, Japan, central Europe (36), and northeastern India (28). Established risk factors for the disease such as cholelithiasis, obesity, and parity seem to account for only a small degree of variation, with endemic liver fluke infection in Thailand being the only exception (17). The risk attributed to the parasite, however, could be confounded by enterohepatic Helicobacter infection as a cofactor contributing to biliary carcinogenesis, similar to the role of H. pylori infection in the development of gastric cancer.

Based on molecular evidence that enterohepatic Helicobacter species infect humans (8, 9, 19, 24), it remains unknown how commonly these infections develop and which human populations are at greatest risk. The geographical distribution of H. bilis or other enteric Helicobacter species in humans is unknown but suggests that their prevalence may be low in populations at low risk for nonviral hepatitis and biliary duct malignancies (4, 5, 30, 37, 38, 48) but could contribute to the unexplained increase of extra- and intrahepatic cancer inci-
dence in North America and Europe (18, 39, 44) and the wide geographical variation in the incidence of gallbladder and extrahepatic bile duct cancers observed in other regions (36). Enterohelical Helicobacter infection could also be a factor in the development of cholesterol gallstones and intrahepatic cholelithiasis, as suggested by recent studies in animal models (25–27).

In order to increase the probability of detecting positive subjects, investigators have analyzed patients affected by a variety of disorders of the liver and biliary tract. When serological markers of infection were assessed, more patients were generally found positive compared to blood donors, depending on the specific disease (31). The hepatic disease may, however, modify responses to infection as is well documented for H. pylori and atrophy of the gastric mucosa (15, 33, 41). The nature of a putative association between the enterohelical helicobacters and hepatic diseases in humans will therefore eventually clarified by epidemiological studies that can discriminate subjects harboring active infection from negative subjects, hopefully preceding the diagnosis of clinical disease. It is therefore important to develop inexpensive but accurate serological markers of infection for use in large-scale epidemiological studies. The design of future descriptive investigations of the kind we have presented here will require improved control of the confounding effect of H. pylori infection (16, 34) and the adoption of a highly reproducible diagnostic standard.

Assays based on antigens derived from the population in which the study is performed are more sensitive and specific. A significant limitation to date is that gastric, liver, and lower-bowel samples suitable for PCR and culture have not been commonly available, particularly in high-risk populations. Thus, the interpretation of epidemiological studies such as those presented here must acknowledge the inability to definitively demonstrate H. pylori or H. bilis infection.

ACKNOWLEDGMENTS

The recruitment and the follow-up of the cohort were in part supported by grants from the Buddhist Aid Center of Japan, the Association for International Cancer Research, Scotland, United Kingdom (grant 98–55), and the International Agency for Research on Cancer, Lyon, France. The analyses in the MIT laboratory were supported by NIH grants R01-CA067529 and P30-ES02109. The analyses in the Lund laboratory were supported by an ALF grant from the Lund University Hospital.

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