Antibody Response of Patients with Helicobacter pylori-Related Gastric Adenocarcinoma: Significance of Anti-CagA Antibodies

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The aim of this study was to search for a specific antibody pattern in sera from patients suffering from Helicobacter pylori-related gastric adenocarcinoma (GAC). The serological response of 22 patients suffering from GAC, 31 patients with gastroduodenal ulcer, and 39 asymptomatic subjects was analyzed using immunoblotting performed with three H. pylori strains: strain ATCC 43579; strain B110, isolated from a patient with ulcers; and strain B225, isolated from a patient with GAC. In addition, the latex agglutination test Pyloriset Dry was used to analyze ambiguous sera. H. pylori seropositivity was 75% in the GAC group, 61.3% in the ulcer group, and 56.4% in the asymptomatic group. Anti-CagA antibodies were found more often in the GAC group (48.8%) and in the ulcer group (47.3%) than in the asymptomatic group (21.2%). These percentages depended on the strain used as an antigen: in the GAC group, the anti-CagA frequencies were 93.3, 40, and 13.3% with strains B225, B110, and ATCC 43579, respectively. Thus the presence of anti-CagA antibodies was increased in patients suffering from H. pylori-related GAC, in particular when the CagA antigen was from a GAC strain. These data suggest the existence of a CagA protein specifically expressed by H. pylori strains isolated from GAC patients.

Gastric adenocarcinoma (GAC) is the second-most-common cancer in the world, with an estimated incidence of 700,000 new cases a year (32). Epidemiological studies suggest that Helicobacter pylori-related gastritis, together with environmental and genetic factors, plays a role in the initiation of GAC (24). H. pylori is a gram-negative, spiral shaped, flagellated microaerophilic bacterium that was identified in the early 1980s (46). It colonizes the stomach of about 50% of all humans and is responsible for the majority of chronic gastritis and peptic ulcer cases (4, 7, 18). A history of H. pylori infection has been found in 50 to 90% of patients with GAC (11, 50). Since 1991, H. pylori has been classified as a group 1 carcinogen, and the relative risk of cancer has been estimated to be 3.6 times higher in patients with H. pylori infection than in noninfected patients (16, 20, 33, 41). Nevertheless, H. pylori infection is more widespread than gastric cancer in Japan, where the prevalence of gastric cancer is high; 0.04% of Japanese subjects were seropositive for H. pylori infection in 1997 and 1998. The first group included 20 patients (9 men and 11 women) with GAC (11, 50). Since 1991, H. pylori has been classified as a group 1 carcinogen, and the relative risk of cancer has been estimated to be 3.6 times higher in patients with H. pylori infection than in noninfected patients (16, 20, 33, 41). Nevertheless, H. pylori infection is more widespread than gastric cancer in Japan, where the prevalence of gastric cancer is high; 0.04% of Japanese subjects who are seropositive for H. pylori suffer from gastric carcinoma (1). Recently, the involvement of H. pylori infection in gastric carcinogenesis has been confirmed in a Mongolian gerbil experimental model (21, 47). Thus, Koch’s postulates for H. pylori as a cause of GAC seem to be fulfilled (45).

These results led to research of the specific determinants of both host and bacterium that predispose H. pylori-infected individuals to GAC. Two virulence factors have been found more frequently in H. pylori strains isolated from patients with ulcers or cancer than in strains isolated from patients with gastritis (5, 36, 44, 49). The first one is the vacuolating cytotoxin (VacA); the second one is the cytotoxin-associated antigen (CagA) that reflects the presence of the CagA pathogenicity island, including about 30 genes of unknown function (9).

We previously showed that a specific antibody response pattern is found in the sera from patients suffering from H. pylori-associated peptic ulcer (3). The aim of this study was to extend these data and to search for specific antibody patterns in sera from H. pylori patients suffering from GAC; subjects suffering from peptic ulcer and asymptomatic subjects served as controls. To investigate antibody patterns, immunoblot assays were carried out with three H. pylori strains: one strain isolated from a patient with GAC, one strain isolated from a patient with duodenal ulcers, and the reference strain, ATCC 43579.

MATERIALS AND METHODS

Patients. Three groups of patients were included in this study. The patients were hospitalized at the University Hospital Center of Brest, France, between 1997 and 1998. The first group included 20 patients (9 men and 11 women) with GAC. The median age was 75.3 years (range, 55 to 95 years) for the men and 73.1 years (range, 53 to 83 years) for the women. Twelve GACs were intestinal-type adenocarcinomas, and eight cancers were diffuse-type adenocarcinomas, according to the Lauren classification (27). The second group included 31 patients (26 men and 5 women) with gastroduodenal ulcers. The median age was 61 years (range, 18 to 80 years) for the men and 72 years (range, 51 to 84 years) for the women. The third group included 39 asymptomatic patients (17 men and 22 women). The median age in this group was 70 years (range, 56 to 87 years) for the men and 76.3 years (range, 63 to 91 years) for the women. No significant demographic differences between the GAC group and the asymptomatic group were present. Serum samples from the 90 patients were collected, aliquoted, and stored frozen at −70°C.

Serological assays. The presence of antibodies to H. pylori in serum was determined using the rapid latex agglutination test Pyloriset Dry (Orion Diagnostica, Foumouze, France) in accordance with the manufacturer’s instructions and a home-made immunoblot assay with saline extracts from three strains: ATCC 43579, B110, and B225 (29). Strain B110 was isolated from a patient with a duodenal ulcer, and strain B225 was isolated from a patient with GAC. The three strains were cagA positive and had the s1 vacA signal sequence (2, 38). They were consequently considered virulent. For the preparation of antigens, H. pylori strains were cultivated on Pylori agar (bioMérieux, Marcy l’Etoile, France).
and incubated at 37°C under microaerobic conditions for 3 to 4 days. Saline extracts corresponding to the water-soluble and surface-exposed antigens were prepared according to a previously described method (3). Briefly, bacterial cells were harvested in sterile 0.15 M NaCl, vortexed for 5 min, and centrifuged (10,000 × g for 10 min at 4°C). The protein concentration of supernatants was determined, and the saline extracts were stored frozen at −70°C. Using a mini-gel apparatus (Bio-Rad, Richmond, Calif.), we carried out sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 50 μg of saline extracts per gel (26).

Molecular mass markers ranging from 14 to 94 kDa (Pharmacia, Uppsala, Sweden) were included on each gel. After migration, proteins were electrotransferred to a nitrocellulose membrane. The membranes were cut into strips and incubated for 1 h at room temperature with serum samples or with monospecific serum (see below) at a 1:100 dilution. They were then incubated for 1 h at room temperature with alkaline phosphatase-conjugated anti-human, anti-rabbit, or anti-mouse immunoglobulin G (Dakopatts, Copenhagen, Denmark). Color reactions were developed with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium (Sigma, St. Quentin Fallavier, France).

To aid identification of the immunoreactive bands detected by the patient’s serum, a set of eight monospecific polyclonal rabbit sera raised to the 20-kDa ferritin-like protein (Felp), the 26- and 35-kDa antigens, the 54-kDa catalase, the 60-kDa HspB, the 76-kDa fumarate reductase, the 87-kDa VacA, and the 125 kDa CagA antigens and two murine monoclonal antibodies raised to the 30-kDa urea and 66-kDa UreB antigens were used. These sera were generously supplied by AVENTIS Pasteur (Marcy l’Etoile, France). Immunoreactive bands were also identified with a calibration curve constructed by plotting the migration distance of the various markers versus their respective molecular masses. Immunoblots were considered to be positive for antibodies to H. pylori when three or more immunoreactive bands were present and when at least one of these three bands corresponded to CagA (125 kDa), HspB (60 kDa), UreB (66 kDa), or UreA (30 kDa) antigens (3). The Pyloriset Dry test was used to analyze ambiguous sera: those sera that were positive by Western blotting with only one immuno blot strain were considered positive with the Pyloriset Dry test also.

The chi-square test was used to compare the frequencies of the immunoreactive bands. Factorial analysis of correspondence was done for every immunoblot series, one series for each of the three H. pylori strains (19, 28, 42). This analysis was used to compare entire immunoblot patterns. The data of the immunoblots were transferred into three two-way tables, one table for each strain. Each table had 90 rows, with 1 row for each serum, and 10 columns, with 1 column for each immunoreactive band. The presence or the absence of each band was encoded as follows: present = 2, absent = 1. Factorial analysis of correspondence (SPAD N software; Cistéa, Saint-Mandé, France) was performed from each table, first considering all sera and then only the seropositive sera.

**RESULTS**

**Protein profiles of the saline extracts used as antigens in immunoblots.** Saline extracts prepared from the three H. pylori strains included in the study were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a 12% acrylamide gel. They showed qualitative and quantitative differences for numerous proteins (Fig. 1). In particular, the CagA protein was present in a larger amount in strain ATCC 43579 than in strains B225 and B110.

**H. pylori serological status of the 90 patients.** The sera of 31 subjects (34.4%) were negative by both serological tests, the agglutination assay and immunoblotting. The agglutination assay was positive in the sera of 44 (48.9%) of the 90 subjects. Depending on the H. pylori strain used as an antigen in the immunoblot, the number of positive sera ranged from 49 (54.5%) with strain B225, 51 (56.7%) with strain ATCC 43579, to 52 (57.8%) with strain B110. The sera of 42 (46.7%) of the 90 subjects were positive with all three strains; the sera of 11 of 90 subjects (12.2%) were positive with two strains. Among these 53 subjects, the sera of 41 were positive by the agglutination assay. These 53 subjects were considered seropositive. The sera of six subjects (6.7%) were positive with only one strain; of these six subjects, three had sera positive by the agglutination test and were therefore considered seropositive; the three others were considered seronegative.

Thus, 56 of 90 (62.2%) subjects were considered H. pylori seropositive: 15 of the 20 patients belonging to the GAC group (75.0%), 19 of the 31 patients belonging to the ulcer group (61.3%), and 22 of the 39 subjects belonging to the control group (56.4%). The prevalence of seropositive subjects in the three groups was not significantly different. There was no influence of sex on seropositivity: 60.5% of the women and 63.5% of the men were seropositive. H. pylori seropositivity increased with age: 25% of those under 50 and 64% of those over 50 were seropositive. In the GAC group, there was no significant difference in seropositivity between patients with diffuse-type tumors and those with intestinal-type tumors.

**Antibody patterns of the 90 patients.** For each serum sample, the number and nature of the immunoreactive bands observed on the blots varied substantially according to the H. pylori strain used to prepare the antigen. The average number of bands was 5.5, 10.5 and 11.75 with B225, B110, and ATCC 43579, respectively. Figure 2 shows the reactivity patterns of
one negative and one positive serum sample tested by immunoblotting with the three *H. pylori* strains.

We focused our study on the 10 bands which were most often encountered on the blots. These bands, identified by their molecular mass and by reactivity with monospecific antisera, corresponded to eight antigens identified as CagA, VacA, fumarate reductase, UreB, HspB, catalase, UreA, and ferritin-like protein and to two unidentified antigens of 35 and 26 kDa, respectively. For each patient serum sample, the frequencies of each band obtained with each of the three antigenic preparations were established, and the mean frequency of each band was calculated (Fig. 3). Significant differences of mean frequencies between seropositive and seronegative subjects were found for all antibodies. Catalase, HspB, and UreB were the antigens that were most often recognized by sera from seropositive subjects. However, antibody to catalase had a low level of specificity, since it was detected in 83.3% of the seropositive subjects but also in 55% of the seronegative subjects. Antibody to HspB had the best ratio of sensitivity to specificity.

Factorial analysis of correspondence performed with the data of the immunoblots obtained with the three strains confirmed the distinction between the immunoblots of seropositive and seronegative subjects. Data points for the latter were clearly focused on the factorial plane by the negative values of the F1 axis (Fig. 4).

**Antibody patterns of seropositive subjects.** We next compared the antibody patterns of the seropositive subjects, including 15 GAC patients, 19 ulcer subjects, and 22 asymptomatic control subjects, to search for a specific pattern of GAC. Of the 10 antibodies studied, the mean frequencies of only 3 antibodies depend on the clinical origin of the serum. Anti-CagA antibodies were found more often in sera from the GAC patients (48.8%) and the ulcer subjects (47.3%) than in sera from asymptomatic subjects (21.2%) ($P < 0.0001$). Anti-VacA antibodies were more frequent in the asymptomatic control subjects (37.8%) than in the ulcer group (14%) and in the GAC group (20%) ($P < 0.0001$). Anti-ferritin-like protein antibodies were more frequent in the ulcer group (68.8%) than in the GAC group (31%) and in the asymptomatic control group (33.3%) ($P < 0.00001$).

There was no statistical association between the frequencies of the antibodies and the strain used to prepare the antigen, except for anti-CagA antibodies, which were statistically more frequently detected in the three groups of patients when GAC strain B225 was used as an antigen (Fig. 5). In particular in the GAC group, anti-CagA antibodies were present in 93.3, 40, and 13% of the sera when strains B225, B110, and ATCC 43579 were used as antigens, respectively. Thus, anti-CagA antibodies of subjects suffering from GAC seemed to be more easily detected when immunoblotting was performed with a strain isolated from a patient suffering from the same disease.

Finally, factorial analysis of correspondence restricted to seropositive subjects was realized to analyze entire immuno-
DISCUSSION

In this study, we analyzed the serological responses of patients suffering from *H. pylori*-related GAC. The age and sex distributions of GAC patients studied were representative of our area. The risk that asymptomatic subjects would be affected by *H. pylori* was very low; hence, our control group can be considered free from cancer, despite the fact that no upper gastroduodenal endoscopy was performed.

The seroprevalence of *H. pylori* and its epidemiological characteristics were similar to the data reported by others (13, 14). We found no significant difference in *H. pylori* seroprevalence between GAC patients with diffuse-type tumors and those with intestinal-type tumors, in agreement with some studies but not with others (8, 10, 22, 34). The seroprevalences in the three clinical groups were also similar to the results of other studies (10, 13, 40).

Serology is recognized as one of the most reliable methods for diagnosis of *H. pylori* infection. Therefore, the seropositive patients may be considered *H. pylori* infected, and the seronegative may be considered *H. pylori* free (23, 35, 43). Among the serological methods used to study the antibody response to *H. pylori*, immunoblotting is certainly one of the most powerful (3, 43). This method allows the detection of antibodies and a determination of the specificity of these antibodies. The interpretation of the antibody patterns in *H. pylori*-infected patients has not been well established so far, even if some attempts have already been made (6, 15). Nevertheless, because the antibody response reflects the features of both the infecting strain and the host response, it may be a helpful tool to predict the risk of an *H. pylori*-infected patient developing severe disease. For many years, several studies had sought correlations between antibodies against *H. pylori* and diseases related to this bacterium, using enzyme-linked immunosorbent assay or immunoblot assay, but only one considered all antibodies on immunoblot patterns of GAC patients (25). In our study, we analyzed all antibody patterns by immunoblotting using the factorial analysis of correspondence method. We did not find an antibody pattern specific for GAC. Nevertheless, we found an association between antibodies to ferritin-like protein and ulcer disease (12, 17). To our knowledge, this association has never been reported. In contrast to the majority of the studies, we found that anti-VacA antibodies were more often present in sera from asymptomatic subjects than in sera from the two other patient groups (3, 37, 39, 50). At present, we have no explanation for this finding. As with the majority of the studies, we found an association between anti-CagA antibodies and GAC or gastroduodenal ulcer (11, 25, 37, 39, 48). There was no correlation between the expression of the CagA proteins and their recognition by human serum. Strain ATCC 43579 expresses high CagA protein levels, and strain B225 expresses lower levels. The CagA protein was not observed with strain B110 under our analysis conditions (Fig. 1). However, CagA was more frequently recognized when strain B225 was used as an antigen, a finding which indicates qualitative differences in the CagA proteins.

The most adequate immunoblotting assay would probably be realized with an antigenic extract prepared from the patient strain. However, the infecting strains are not always available and, practically speaking, such an assay would not be feasible. Thus, it may be helpful to define what kind of strain is more relevant in obtaining an antigenic preparation designed for immunoblot assays. Our results suggest that in searching for anti-CagA antibodies in the sera of patients suffering from GAC, the best choice would be a strain isolated from a patient suffering from the same disease. Moreover, the specific recognition of anti-CagA antibodies present in sera from GAC subjects and the CagA antigen of strain B225 could suggest that GAC-associated *H. pylori* strains express a specific type of CagA protein. To confirm these findings, it would be interesting to test more strains isolated from patients in the three clinical groups included in the study and also to analyze and compare the sequence of the cagA genes in the three strains. The structure of the cagA gene of *H. pylori* strain B225 is currently under investigation.

In conclusion, we confirmed the high diversity of the antibody response to *H. pylori* and the strong association between anti-CagA and the presence of GAC. This association is stronger if the CagA protein used as an antigen comes from a strain isolated from a patient with GAC, suggesting the existence of a type of CagA protein more implicated in gastric carcinogenesis.

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