Spectrotypic analysis of antibodies to Helicobacter pylori in patients with antral gastritis and duodenal ulcer

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Abstract

Aims—To investigate the anti Helicobacter pylori (H pylori) spectrotype associated with (a) antral gastritis and duodenal ulcer; (b) the H pylori eradicating treatment.

Methods—Spectrotypic analysis was performed by isoelectric focusing and reverse blotting (IEFRB) in a cross sectional study on sera from 70 patients with antral gastritis and duodenal ulcer. In addition, a longitudinal study was performed on 40 of these patients (20 with antral gastritis and 20 with duodenal ulcer) who underwent eradicating treatment.

Results—The cross sectional study showed that the oligoclonal spectrotype was present in 74% of antral gastritis patients and in 85% of duodenal ulcer patients. In only a minority of subjects (23% with antral gastritis and 3% with duodenal ulcer) was a polyclonal spectrotype observed. The longitudinal study showed a reduction in the intensity of the spectrotypic bands in 5/10 antral gastritis patients with eradicated H pylori as opposed to only 2/10 patients without eradication. A reduction was also observed in 6/11 eradicated 0/9 non-eradicated patients with duodenal ulcer. Collectively, a reduction in the spectrotype was observed in 11/21 patients (52%) who—independently of the disease—underwent H pylori eradication, as opposed to 2/19 of the non-responder patients (10-5%). The polyclonal spectrotype was found exclusively in four patients with antral gastritis, all belonging to the group without eradication of H pylori after eradicating treatment.

Conclusions—The anti H pylori oligoclonal spectrotype is the most common pattern observed in patients with antral gastritis and duodenal ulcer. After H pylori eradicating treatment the spectrotype does not change qualitatively, but the polyclonal pattern seems to be predictive of a poor response to eradication.

Keywords: Helicobacter pylori, antibody, spectrotype, antral gastritis, duodenal ulcer.

Colonisation of gastric mucosa by Helicobacter pylori (H pylori) is considered to be the cause of chronic gastritis,1 a strong risk factor for peptic ulcer disease2 and gastric cancer.3 Several clinical trials have shown that bacterial eradication by antibiotic treatment dramatically reduces the recurrences of peptic ulcer4 and has even been able to induce a regression of a primary low grade B cell gastric lymphoma.5 The crucial role of H pylori in the development of these serious gastric diseases is also documented by the scientific efforts to develop a specific vaccine.6 Serological assays to identify specific anti H pylori antibodies are useful tools in diagnosing and monitoring the disease activity without the need for invasive methods.7 Of particular interest was the use of anti H pylori specific isoelectric focusing and reverse blotting (IEFRB) in the definition of the humoral immune response in patients with gastric cancer.8 IEFRB is a technique capable of resolving antibodies at the level of single clone products9 and therefore it provides a good indication of the activity of B cell clones specifically engaged in the humoral immune response. The spectrotypic profile can be mono-, oligo-, or polyclonal, depending on the number of B cell clones (one, few, or several) actively secreting antibodies. A monoclonal pattern is characterised by one band cluster (from three to five bands, expressing a microclonal heterogeneity),10 an oligoclonal pattern by a limited number of band clusters, and finally, a polyclonal response is defined by several band clusters or by diffuse smears through the gel, due to superimposition of the sera spectrum.11 The type of response observed presumably depends on many factors, including the nature of the antigen, the repertoire of the host Ig genes, and host antigen processing.12 Consequently, spectrotype is an individual characteristic, a sort of “immune fingerprinting” directly related to both specific antigen and host factors, that develops during the vaccination13 and does not change during the course of a chronic disease.14 It has been shown that the anti H pylori oligoclonal spectrotype is observed with higher frequency in patients with gastric cancer.9

The aim of this paper was to study by IEFRB the sera from patients with other H pylori associated chronic diseases, such as antral gastritis and duodenal ulcer, and to monitor, at spectrotypic level, the reduction of specific anti H pylori antibodies commonly observed by quantitative assays, such as enzyme linked immunosorbent assay (ELISA), after successful H pylori eradication treatment.
Methods

PATIENTS

Cross sectional study

Seventy consecutive patients with upper gastrointestinal symptoms referred for upper gastrointestinal endoscopy and found to have histologically confirmed *H pylori* positive antral gastritis (n = 35; M/F: 21/14; age range 21–62 years, mean age 45 years) or duodenal ulcer (n = 35; M/F: 18/17; age range 18–64 years, mean age 46 years), and positive for anti *H pylori* IgG in ELISA were enrolled in the study.

Four antral biopsies were taken: two for histological diagnosis (haematoxylin and eosin (H and E) and Giemsa stains) and *H pylori* status assessment, one for culture and one for the urease test (CP-TEST). \(^{15}\)

Histology and culture were done without knowledge of the *H pylori* status.

The histological appearances were graded according to Whitehead \(^{14}\) as modified by Marshall. \(^{17}\) *H pylori* colonisation was determined by positive Giemsa staining and CP-TEST or culture or both. \(^{15}\)

Eleven age and sex matched subjects (M/F: 8/3; age range 24–61 years, mean age 44 years) with upper gastrointestinal symptoms, enrolled during the same period, not colonised by *H pylori* and histologically normal, were used as a control group.

Longitudinal study

Twenty out of 35 patients with antral gastritis and 20 out of 35 patients with duodenal ulcer, all histologically confirmed *H pylori* positive, were followed up for 12 months after stopping eradication treatment. Treatment was based on a two week course of colloidal bismuth subcitrate 120 mg four times daily plus tetracycline 500 mg three times daily. Sera from these subjects were studied by IEFBRB just before beginning treatment and then 12 months after the end of the treatment course.

ELISA test

Specific anti *H pylori* IgG was measured by a previously validated ELISA technique with a sensitivity and a specificity of 94%. \(^{16,18}\) Goat anti-human peroxidase labelled IgG conjugates were used and the antigen was a crude sonicate of *H pylori* strain B1, kindly provided by John Holton (Department of Microbiology, Middlesex School of Medicine, London, UK).

*H pylori* conjugation

A sonicate of *H pylori* strain B1 was labelled with horseradish peroxidase (HRP) using a preactivated and stable reagent (Immuno Pure Activated Peroxidase, Pierce). Briefly, 300 µg of *H pylori* (diluted in 0.02 M Tris, 0.14 M NaCl, pH 7.4) was dialysed extensively against conjugation buffer (1 M NaHCO₃, pH 9.5–9.6, 0.9% NaCl). Then, 1 mg of preactivated HRP was mixed with the dialysed *H pylori* and incubated overnight at 4°C. Quench buffer (0.2 M glycine, 30 µl) was added and allowed to react for two hours at room temperature.

Finally, 350 µl of stabilising solution (1% bovine serum albumin in distilled water) was added and the mixture dialysed extensively against dialysis buffer (50 mM sodium phosphate, pH 6.8; 0.9% NaCl; 0.2% thimerosal). The same protocol was used for the conjugation of affinity chromatography purified anti *H pylori* F(ab')₂ with HRP.

ISOELECTRIC FOCUSING AND REVERSE BLOTTING (IEFBRB)

Isoelectric focusing was performed in agarose gel (0.5 mm, 1% agarose, 10% sorbitol, and 3% ampholines, pH 3.5–9.5; Pharmacia-LKB Biotechnologies), as previously reported. \(^{19,20}\) A 20 µl aliquot of serum, diluted 1:4 in 0.9% NaCl, was applied to paper sample strips near the anode of a Multiphor apparatus (Pharmacia-LKB). Proteins were allowed to migrate for three hours, while voltage level was increased progressively from 300 to 1200 V. Sample paper strips were removed after one hour. At the end of the run, IEF-separated proteins were blotted for 90 minutes onto a nitrocellulose sheet premoistened with distilled water and laid on the agarose gel, covered with Whatman 3MM dry paper and paper towelling. The resulting layered materials were pressed under a 1 kg weight. Non-specific binding was blocked by immersing the nitrocellulose sheet in 5% non-fat dry milk in phosphate buffered saline containing 0.1% Tween-20 (PBS-Tw) for one hour at room temperature. Thereafter, the membrane was incubated with 1-4 µg/ml of purified HRP-conjugated *H pylori* strain B1 for one hour at room temperature and then overnight at 4°C. Alternatively, the membrane was incubated with 6 µg/ml of unconjugated purified *H pylori* strain B1 overnight at 4°C, followed by an incubation with 2 µg/ml of affinity chromatography purified, HRP conjugated, anti-*H pylori* F(ab')₂, overnight at 4°C. The nitrocellulose sheet was then washed 10 times in PBSTw at room temperature. Specific anti *H pylori* immunoglobulin was detected using an "enhanced chemiluminescence (ECL) system kit" according to the manufacturer’s instructions (Amersham International). After incubation with detection reagents, the nitrocellulose sheet was exposed to Kodak x-Omat AR film (Eastman Kodak) at room temperature. The exposure time varied from one minute to one hour depending on the amount of target protein on the blot.

DENSIITOMETRIC ANALYSIS

The spectrotype obtained from the sera of the patients followed up in the longitudinal study was measured by densitometric analysis on an enhanced laser densitometer (LKB 2222-020 UltraScan XL). The absorbance values were determined for each band at the two different times. Based on differences observed in triplicate measurements on identical samples, which averaged 20% (data not shown), changes of intensity greater than 60% were considered significant. \(^{21}\)
Spectrotypic analysis of Helicobacter pylori antibodies

### Table 1: Spectrotypic analysis in patients with antral gastritis and duodenal ulcer

<table>
<thead>
<tr>
<th>Patients</th>
<th>Spectrotype</th>
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<tr>
<td></td>
<td>Negative</td>
<td>Monoclonal</td>
<td>Oligoclonal</td>
<td>Polyclonal</td>
</tr>
<tr>
<td>Antral gastritis</td>
<td>35</td>
<td>1(3%)</td>
<td>-</td>
<td>26(74%)</td>
</tr>
<tr>
<td>Duodenal ulcer</td>
<td>35</td>
<td>2(6%)</td>
<td>-</td>
<td>30(85%)</td>
</tr>
<tr>
<td>Control group</td>
<td>11</td>
<td>1(100%)</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>

Figure 1 Different kinds of anti H pylori antibody spectrotype in patients with antral gastritis (lanes a, b, c) or duodenal ulcer (lanes d, e, f). Lane g refers to a normal control. Isoelectric point (pI) of bands is reported on the y axis. All the observed patterns are reported, as follows. Lane a: monoclonal, diffusely distributed over a wide pH range; lane b: monoclonal, mainly clustered in neutral and alkaline areas; lane c: oligoclonal, diffusely distributed over a wide pH range; lane d: oligoclonal, mainly clustered in the neutral area; lane e: oligoclonal, mainly clustered in acidic and neutral areas; lane f: monoclonal; lane g: negative.

STATISTICAL ANALYSIS
The analysis of the difference in the prevalence of oligoclonal spectrotype between patients with antral gastritis and duodenal ulcer and of the difference in percentage of reduction/disappearance of the anti H pylori spectrotype bands after antibiotic therapy was performed by χ² analysis.

Results
CROSS SECTIONAL STUDY
Twenty six out of 35 antral gastritis patients (74%) showed an oligoclonal spectrotype, whereas eight showed a polyclonal spectrotype. One patient was totally negative. Thirty out of 35 duodenal ulcer patients (85%) showed an oligoclonal, two a monoclonal, and one a polyclonal spectrotype. Two patients were negative. None of the negative controls showed a positive spectrotype (table 1; fig 1).

These data show that the oligoclonal spectrotype is the most frequent anti H pylori spec-

<table>
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<th>Table 2: Longitudinal spectrotypic analysis in 40 patients with antral gastritis and duodenal ulcer, divided according to the clinical outcome after eradication treatment</th>
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<tbody>
<tr>
<td>Patients</td>
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<tr>
<td></td>
</tr>
<tr>
<td>Antral gastritis</td>
</tr>
<tr>
<td>Duodenal ulcer</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

U/I = Unmodified/Increased; R/D = Reduced/Disappeared

* p<0.05 v R/D, H pylori not eradicated; † p<0.001 v R/D, H pylori not eradicated; ‡ p<0.005 v R/D, H pylori not eradicated.

LONGITUDINAL STUDY
IEFRB was performed before and 12 months after treatment to eradicate H pylori in 20 patients with antral gastritis and 20 with duodenal ulcer. The treatment was successful in 10 antral gastritis patients and in 11 duodenal ulcer patients.

The spectrotypic characteristics before and 12 months after the treatment did not change qualitatively, irrespective of the disease and the treatment. Quantitative variations in the intensity or in the number of bands were observed instead in relation to the treatment; these variations could be best appreciated by laser densitometry (fig 2).

H pylori was eradicated in 10 patients with antral gastritis, all showing an oligoclonal spectrotype. In five of them a reduction or disappearance in the intensity or number of bands of the spectrotype was observed, whereas in only two out of the 10 non-eradicated patients was such a reduction detectable (p<0.05) (table 2).

Of particular interest is the observation that in none of the four patients with a polyclonal spectrotype was H pylori eradicated.

H pylori was eradicated in 11 patients with duodenal ulcer, all showing an oligoclonal spectrotype. In six of them a reduction in the spectrotype was detectable, whereas in none of the nine non-eradicated patients was a reduction in spectrotype observed (p<0.001) (table 2).

Collectively, a reduction or disappearance of the spectrotype was observed only in patients with the oligoclonal variety, and in particular in 11/21 patients (52%) who, independently of the disease, underwent H pylori eradication, as opposed to 2/19 of the non-responder patients (10-5%) (p<0.005). Some representative examples are given in fig 2, which shows densitometry performed on sera from four patients, two with and two without modifications in spectrotypic band intensity.

As described in Methods, the recognition of iso-electrofocused specific antibodies was performed by a direct link with HRP-H pylori antigen or with unconjugated H pylori antigen, followed by the reaction with affinity purified, HRP conjugated anti H pylori F(ab')² antibodies, in order to exclude possible epitope masking due to conjugation procedures. No significant differences, however, were observed in the spectrotypes obtained with the two methods (data not shown).

Discussion
Several papers in the last few years have established the strong association between H pyl-
ori and antral gastritis, peptic ulcer, and, more recently, gastric cancer and lymphoma.1-3,22 The study of the immune response to *H. pylori* provided more insights not only into the pathogenesis of these diseases but also into the diagnosis, making it possible to reduce the number of endoscopies by about 25-30%.24,25 Furthermore, the analysis of antibody response to *H. pylori* has allowed indirect monitoring of possible bacterial eradication after antibiotic treatment.26-28

Spectrotypic analysis, as performed by IEF-FRB, allows the antibody response to be indirectly analysed at the level of a single secreting B cell clone.

Recently, in the course of a multicentre Italian study, an oligoclonal spectrotype was identified in 69% of patients with gastric cancer, irrespective of the histological type.6 We applied spectrotypic analysis to the study of patients with antral gastritis and duodenal ulcer, in the majority of whom, irrespective of the disease, we found an oligoclonal spectrotype. Thus the same oligoclonal spectrotype that has been reported to be associated with gastric cancer is also frequently present in patients with antral gastritis and duodenal ulcer, indicating that oligoclonality, more than being just a marker of disease, seems to be a characteristic of the humoral response to *H. pylori*.

In addition, we studied some of the patients who underwent eradication treatment to follow possible variations in the spectrotype induced by the treatment. Fifty percent of the patients with antral gastritis in whom *H. pylori* was eradicated had a reduction or disappearance of the spectrotype, whereas this occurred in only two patients out of 10 where *H. pylori* was not eradicated, showing a partial reduction of the spectrotype. The result is even more evident in patients with duodenal ulcer: 67% of the patients with *H. pylori* eradication versus none of the patients without eradication showed a reduction in the spectrotype. This is in line with the clinical and bacteriological response to treatment and with the behaviour of the quantitative antibody response to *H. pylori*, as assessed by ELISA, indicating that when this pathogen is eradicated the humoral immune response tends to extinguish, with a progressive reduction of the antibodies produced by different B cell clones. It should be emphasised that,
among the 20 longitudinally studied patients with antral gastritis, four had a polyclonal spectrotype and in none of these was H pylori eradicated. This is indirect evidence that most of the specific antibodies in the H pylori infected patients do not seem to be protective and do not help bacterial clearance during antibiotic treatment. The oligoclonal spectrotype may be interpreted as an expression of a limited recruitment of actively secreting specific B cell clones. This pattern might be related to the strict localisation of the infection to the gastric mucosa and to the reduced recirculation of the specifically responding lymphoid cells. On the other hand, polyclonal spectrotype, far from being the expression of more protective antibody response, seems to be related to a deeper bacterial colonisation of the gastric mucosa, such as to induce a larger diffusion of bacterial products and a response to a broader range of antigens, but more difficult to eradicate.

In conclusion, the oligoclonal spectrotype seems to be the hallmark of the humoral response to H pylori, not only in gastric cancer but even more so in other H pylori associated chronic diseases such as antral gastritis and duodenal ulcer. Spectrotypic analysis of the sera of these patients has allowed us to identify—among the positive sera found on ELISA—the polyclonal pattern as being a possible predictive marker of unresponsiveness to the anti H pylori treatment. The results are a further indication for the poor protective value of anti H pylori circulating antibodies.