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# Detection of *Campylobacter pyloridis* in Patients with Antrum Gastritis and Peptic Ulcers by Culture, Complement Fixation Test, and Immunoblot

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**The association of *Campylobacter pyloridis* with antrum gastritis and peptic ulcers was described. We investigated antral biopsies from 180 patients who underwent gastroscopy. By culture or Gram stain or both, we found overall 98 (54%) of them to be positive for *C. pyloridis*. In the various groups the following percentages were found to be positive: normal antral mucosa 3% ($n$ = 30); moderate superficial antrum gastritis, 49% ($n$ = 83); severe superficial antrum gastritis, 86% ($n$ = 44); duodenal ulcer, 83% ($n$ = 54); and gastric ulcer, 72% ($n$ = 18). A serological screening that used a complement fixation test yielded the following results: highest rates of positive complement fixation titers were seen in patients with severe gastritis and those with duodenal ulcers, both with 79%; the lowest incidence was in a group of 20 blood donors, with 5%. Positive complement fixation titers in gastritis patients also correlated well with characteristic patterns on immunoglobulin G and A immunoblots, while there was no specific reactivity observed on immunoglobulin M immunoblots.**

*Campylobacter*-like organisms that have recently been named *Campylobacter pyloridis* (9, 17) have become an interesting object in the discussion of the etiology of gastritis and peptic ulcer. Marshall and Warren (10, 20) were the first to describe the association of the presence of *Campylobacter*-like organisms in the antral mucosa with histological evidence of antrum gastritis as well as with peptic ulcers, especially duodenal ulcers. A causative relationship, however, has not yet been established.

In this study, we looked for *C. pyloridis* in antral biopsies from 180 patients and correlated it with the presence of peptic ulcers and antrum gastritis. Furthermore, we looked at the humoral immune response by means of a complement fixation (CF) test. For a more detailed analysis the immunoblot technique was used, which also enabled us to differentiate among immunoglobulin G (IgG), IgA, and IgM immune responses.

## MATERIALS AND METHODS

**Patients.** A total of 180 patients who had been referred to the Department of Medicine and who underwent upper gastrointestinal endoscopy for various reasons were finally evaluated. In 104 of these patients blood samples were taken at the time of endoscopy.

**Biopsies.** Biopsies were taken from the antral mucosa as far away as possible from mucosal lesions, placed into thioglycolate medium, and cultured within 3 h of collection. In most cases, parallel specimens from the antrum were placed in Formalin, sent to the pathology laboratory, and routinely processed for histology.

**Microscopy and culture.** Each biopsy was first rubbed across the surface of a blood agar plate (blood agar base no. 2 [Oxoid Ltd., Basingstoke, U.K.]; CM 271, containing 7% sheep blood and Skirrows selective supplement [Oxoid SR 69]) and then across a microscope slide. The blood agar plates were then incubated at 37°C in a microaerophilic atmosphere (produced by a Campy Pak disposable hydrogen-plus-carbon dioxide generator envelope [BBL Microbiology Systems, Cockeysville, Md.] in an anaerobic jar without catalyst) for 4 to 5 days. A Gram stain was performed on the biopsy smears. Organisms were classified as *C. pyloridis* on the basis of their typical appearance as Gram-negative rods or, if cultured, their typical colony morphology, as well as their strongly positive reactions for oxidase, catalase, and urease.

**CF test.** A thick suspension of *C. pyloridis* organisms harvested from a 4-day-old culture was sonicated, using a Sonifier B-12 (Branson Sonic Power Co., Danbury, Conn.), three times for 30 s and used as antigen. Sheep erythrocytes, guinea pig complement, and rabbit amboceptor were all obtained from Behringwerke, Marburg, Federal Republic of Germany. The CF test was performed following standard procedures.

**SDS-PAGE.** A washed pellet of a 4-day-old culture of a *C. pyloridis* strain, containing approximately 400 μg of protein (as determined according to the method of Lowry et al. [7]) was suspended in 300 μl of sodium dodecyl sulfate (SDS) sample buffer (0.06 M Tris hydrochloride, pH 6.8, containing 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromphenol blue). The mixture was then heated for 5 min in a boiling-water bath and centrifuged at 10,000 × $g$ for 2 min at room temperature. The solubilized supernatant of the sample was then subjected to SDS-polyacrylamide gel electrophoresis (PAGE) at a constant current of 40 mA for 4 h, using a 0.15-cm-thick slab gel of 12% acrylamide as running gel and 3.5% acrylamide as stacking gel in a discontinuous buffer system as described by Laemmli (5).

**Immunoblot.** Protein transfer to nitrocellulose was performed according to a modification (J. Heesemann, submitted for publication) of a vacuum-blotting procedure described by Peferoen et al. (14). Briefly, one sheet of nitrocellulose (BA 85; Schleicher & Schuell, Dassel, Federal

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TABLE 1. Correlation of detection of *C. pyloridis* in antral biopsies with pathohistology of the antrum and peptic ulcers[a]

| Diagnosis | *C. pyloridis* grading | | | | No. positive/ total (%) |
|---|---|---|---|---|---|
| | Negative | + | ++ | +++ | |
| Histological | | | | | |
| Normal antral mucosa | 29 | 1 | | | 1/30 (3) |
| Moderate antrum gastritis | 42 | 20 | 13 | 8 | 41/38 (49) |
| Severe antrum gastritis | 6 | 17 | 10 | 11 | 38/44 (86) |
| Endoscopic | | | | | |
| Duodenal ulcer | 9 | 15 | 13 | 17 | 45/54 (83) |
| Gastric ulcer | 5 | 7 | 1 | 5 | 13/18 (72) |

[a] For quantitative grading of *C. pyloridis*, see text (Results).

Republic of Germany) and two sheets of Whatman 3M paper, all soaked in blotting buffer (0.025 M Tris, 0.193 M glycine, 20% methanol, pH 8.3) as described by Towbin et al. (19), and ten sheets of dry Whatman 3M paper were applied to each side of the gel in that order. This "sandwich" was then subjected to a vacuum gel dryer (model 224; Bio-Rad, Munich, Federal Republic of Germany) for 1 h with initial heating for 10 min.

After removing the Whatman 3M paper, the nitrocellulose-gel sandwich was inserted into a blocking buffer (0.01 M $Na_2HPO_4/NaH_2PO_4$, 0.15 M NaCl, pH 7.5, 0.5% Tween 20, 0.1% sodium azide). The liberated gel was stained with Coomassie blue (2). After the blocking procedure, the nitrocellulose was cut into approximately 3-mm-wide strips. Each strip was incubated with 5 ml of patient serum diluted 1:100 in blocking buffer overnight with gentle shaking at room temperature. The strips were subsequently washed three times for 10 min in blocking buffer and then incubated for 3 h at room temperature with alkaline phosphatase rabbit anti-human IgG (H plus L chains) (dianova GmbH, Hamburg, Federal Republic of Germany), anti-human IgA (α-chain specific) alkaline phosphatase conjugate (goat; Sigma Chemical Co., St. Louis, Mo.), or alkaline phosphatase goat anti-human IgM (dianova), each diluted 1:1,000 in blocking buffer. The nitrocellulose strips were then washed twice in blocking buffer and once in 0.15 M Veronal (Winthrop Laboratories, New York, N.Y.)-acetate buffer, pH 9.6, prior to the enzyme reaction.

Stock solutions for the alkaline phosphatase reaction that was performed as described by Blake et al. (1) were prepared as follows: 5-bromo-1-chloroindoxyl phosphate (5 mg/ml in dimethylformamide) (U.S. Biochemicals Corp., Cleveland, Ohio), Nitro Blue Tetrazolium (1 mg/ml in Veronal-acetate buffer [U.S. Biochemicals Corp.]), and 2 M $MgCl_2$. The strips were then incubated for 10 min at room temperature in freshly made substrate buffer prepared from the stock solutions consisting of 20 μl of $MgCl_2$, 1 ml of the 0.1% Nitro Blue Tetrazolium, 0.1 ml of the indoxyl phosphate, and 9 ml of the Veronal-acetate buffer. The enzyme reaction was terminated by washing the strips with plain tap water.

## RESULTS

**Correlation of the presence of *C. pyloridis* with antrum histology and peptic ulcer disease.** *C. pyloridis* was grown from 78 (43%) of the 180 patients' biopsies and was seen on 91 (51%) of the Gram-stained smears. In total, 98 (54%) patients were positive for *C. pyloridis* on culture or Gram stain or both.

The 180 patients evaluated included only patients who showed either gastric or duodenal ulcer on endoscopy or who showed one of the following histological pictures: normal antrum mucosa, moderate or severe superficial antrum gastritis as designated by the pathologist. Other diagnoses, e.g., neoplastic changes or atrophic gastritis, were not included in this study.

In the evaluation *C. pyloridis* organisms were graded quantitatively according to Marshall and Warren (10) as follows: negative, no typical organisms seen; +, spiral bacteria rarely seen; ++, scattered bacteria seen in most high-power fields; +++, numerous bacteria observed in most high-power fields. Those cases where *C. pyloridis* was cultured but not seen on the Gram film of the biopsy were graded as +. These results are summarized in Table 1. In viewing these data, it has to be taken into account that some patients are represented twice in the table because they showed histological evidence of gastritis and at the same time presented with a peptic ulcer at endoscopy. If these patients with peptic ulcers are excluded from the gastritis groups, the *C. pyloridis*-positive yield is reduced for patients with moderate gastritis from 49 to 40% and for those with severe gastritis from 86 to 77%. Thus, the highest yield for *C. pyloridis* is found in patients with duodenal ulcer (83%).

All patients with duodenal ulcer where antrum histology was available showed some degree of antrum gastritis; the corresponding data are shown in Table 2. These results show that almost all (18 of 19) duodenal ulcer patients with severe antrum gastritis were positive for *C. pyloridis*.

**CF test.** Results of the CF test were expressed as titers. CF titers below 5 were valued as negative, while those equal to or higher than 5 were taken to be reactive. Details of these test results are shown in Table 3. Basically, the same groups of patients have been listed as in Table 1. However, each group has been subdivided into two groups on the basis of whether they were positive or not for *C. pyloridis* on culture or Gram stain or both.

Furthermore, a group of 20 blood donors was also included of which only 1 was reactive in the CF test and only at a titer of 5. Patients with severe gastritis and those with duodenal ulcer had the highest incidence of CF reactivity (Table 3).

**SDS-PAGE and immunoblot.** Figure 1 shows on the left-hand side next to the marker protein bands the Coomassie blue stain of an SDS-polyacrylamide gel of a whole-cell lysate of the *C. pyloridis* strain that served as antigen for both the immunoblot and the CF test. The major protein bands of this strain as well as of some other *C. pyloridis* strains we tested (data not shown) had calculated molecular weights of 79,000, 72,000, 63,000, 60,000, 55,000, 30,000, 26,000, and 24,000.

Figure 1 also shows some IgG and IgA immunoblots from three patients with antrum gastritis and from three patients with normal antral mucosa. The corresponding CF titers are listed below the blot strips. IgM blots are not shown because

TABLE 2. Correlation of detection of *C. pyloridis* in duodenal ulcer patients with grade of antrum gastritis[a]

| Diagnosis | *C. pyloridis* grading | | | | No. positive/ total (%) |
|---|---|---|---|---|---|
| | Negative | + | ++ | +++ | |
| Duodenal ulcer with moderate antrum gastritis | 7 | 6 | 4 | 4 | 14/21 (67) |
| Duodenal ulcer with severe antrum gastritis | 1 | 6 | 5 | 7 | 18/19 (95) |

[a] For quantitative grading of *C. pyloridis*, see text (Results).

TABLE 3. Antibodies to *C. pyloridis* in different groups of patients with *C. pyloridis* present or absent on Gram stain or culture or both and, as a control, in a group of 20 blood donors[a]

| Diagnosis | *C. pyloridis* | No. with CF titers of: | | | | | | No. positive/total (%) |
|---|---|---|---|---|---|---|---|---|
| | | <5 | 5 | 10 | 20 | 40 | 80 | |
| Histological | | | | | | | | |
| Normal antral mucosa | Present | 1 | | | | | | 0/1 (0) |
| | Absent | 8 | 3 | 1 | | | | 4/12 (33) |
| Moderate antrum gastritis | Present | 6 | 5 | 7 | 2 | 1 | | 15/21 (71) |
| | Absent | 9 | 3 | 1 | 1 | | | 5/14 (36) |
| Severe antrum gastritis | Present | 4 | 4 | 6 | 2 | | 1 | 13/17 (76) |
| | Absent | | 1 | | | 1 | 1 | 3/3 (100) |
| Endoscopic | | | | | | | | |
| Duodenal ulcer | Present | 4 | 7 | 6 | 2 | | | 15/19 (79) |
| | Absent | | | | | | | |
| Gastric ulcer | Present | 5 | 5 | | 3 | 1 | | 9/14 (64) |
| | Absent | 5 | 1 | | | | | 1/6 (17) |
| Blood donors | | 19 | 1 | | | | | 1/20 (5) |

[a] CF titers below 5 were considered to be negative.

they all only revealed a single band of about 60 kilodaltons (kDa).

The 110-kDa protein band was reactive on the IgG blot in six of eight tested sera from gastritis patients, but in none of the 12 tested sera from patients with normal antral mucosa. On the IgA blot this protein band also showed reactivity with 6 of 8 sera from gastritis patients and also 1 of the 12 sera from patients with normal histology.

Similarly, a 22-kDa band appeared on seven of eight IgG blots from the gastritis patients and in none of the IgG blots from patients with normal antrum mucosa. On the IgA blots, however, this protein band did not show reactivity with any of the sera tested.

## DISCUSSION

The study presented here shows a strong correlation between superficial gastritis of the antrum and the occurrence of *C. pyloridis* on the antral mucosa as evidenced by microscopy or culture of the organism or both. The correlation was more pronounced with severe gastritis (86%) than

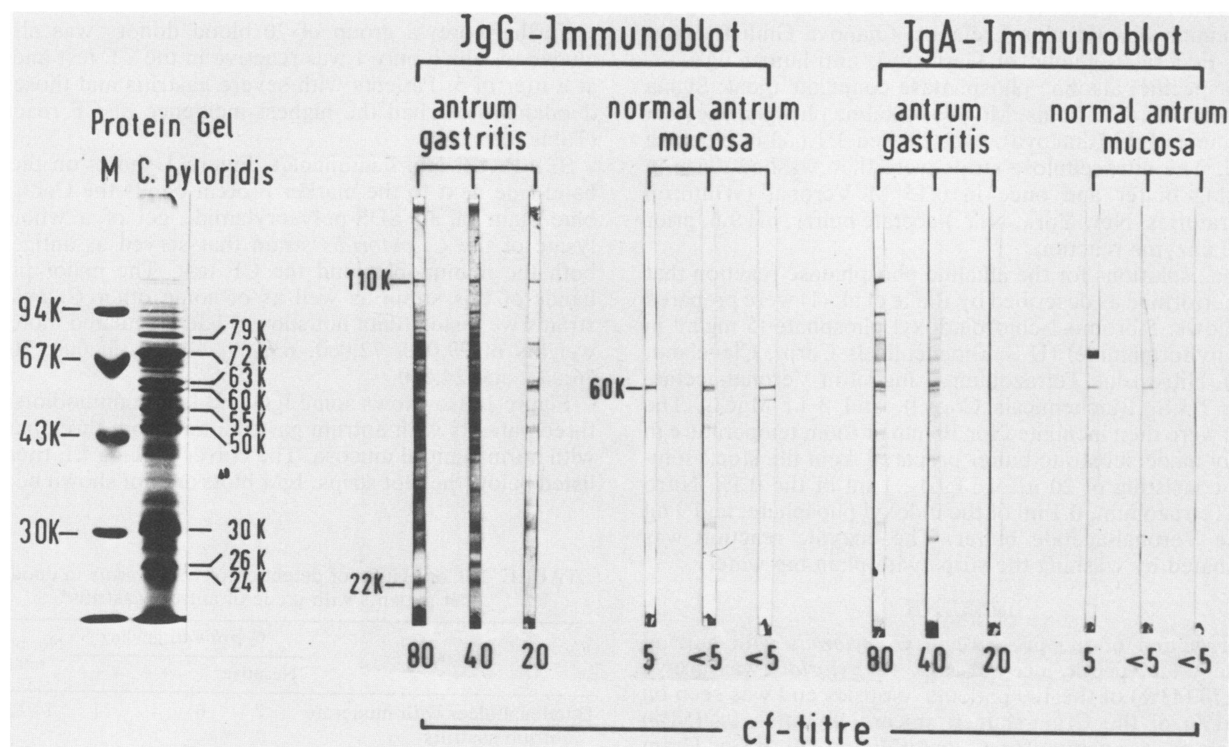


FIG. 1. SDS-PAGE of a whole-cell lysate of *C. pyloridis* with marker proteins (M) on the left. Below the IgG and IgA immunoblots developed with sera from either three patients with antrum gastritis or three patients with normal antrum mucosa, the corresponding CF titers are listed.

with moderate gastritis (49%). Isolation of *C. pyloridis* also correlated strongly with a diagnosis of duodenal ulcer (83%) and somewhat less with gastric ulcer (72%). The correlation was most pronounced in patients who suffered from a duodenal ulcer and, at the same time, showed severe superficial gastritis of the antrum (95%). In all patients with duodenal ulcer where histology of the antrum was available, either moderate ($n = 21$) or severe ($n = 19$) superficial gastritis of the antrum was present. On the other hand, only 1 of 30 patients with a normal histology of the antrum showed evidence for the presence of *C. pyloridis*. Similar rates of isolation of *C. pyloridis* from the antral mucosa of patients with superficial antrum gastritis or peptic ulcers or both have also been reported by other authors (3, 6, 10, 11, 15).

On histology the organisms appear to be located within the mucin layer of the antral mucosa, sometimes in close contact with the luminal surface of the epithelial cells of the mucosa. However, in accordance with other authors (3, 18), we could not detect signs of tissue invasion. If this were indeed so, one would hardly expect a humoral immune response in the host.

However, as we could demonstrate by using a CF test with the sonic extract of a *C. pyloridis* strain as antigen, increased CF titers were seen much more often in patients with gastritis in whom *C. pyloridis* was present than in patients without detectable *C. pyloridis*. In addition, only 1 of 20 healthy blood donors showed a positive CF titer. The highest rates of positive CF titers were observed in patients with severe gastritis and those with duodenal ulcer (both with 79%).

To find out more about the immune response to *C. pyloridis*, we made use of the immunoblot method. By this method distinct patterns of immune response to various protein antigens can be differentiated. Furthermore, the patterns gained for IgG, IgM, and IgA can be compared with one another.

Basically, the IgG and IgA blots correlated quite well with the CF titers; i.e., sera with higher CF titers also generally reacted with more protein bands on the blots. The IgA blots, however, revealed a much smaller number of bands than the IgG blots. Although there was no single clear-cut pattern for gastritis patients versus patients with normal antral mucosa on either the IgG or the IgA blots, certain bands (e.g., a band of about 110 kDa) reacted with the majority of sera from gastritis patients while only rarely reacting with sera from patients with normal antrum mucosa.

All sera reacted with a protein of about 60 kDa. This applied also to the IgM blot, whereas practically none of the tested sera reacted with any other protein band on the IgM blot regardless of the CF titer. That almost all sera were positive for the 60-kDa protein is indicative of a reaction that is not specific for *C. pyloridis*.

Rathbone et al. (16), using an enzyme-linked immunoassay, also demonstrated significantly raised IgG and IgA serum antibody titers in a *C. pyloridis*-positive group of patients, while the IgM titers were similar in the bacteria-positive and bacteria-negative groups. Since we are apparently dealing with a chronic infection, this finding is not too surprising. Also, an IgA response is rather characteristic of an infection that is taking place at the mucosal level. Kaldor et al. (4) also demonstrated significantly higher IgG antibody titers to *C. pyloridis* in peptic ulcer patients, using an enzyme-linked assay; however, he did not look for an IgA or IgM response. He did, however, show specificity of the IgG antibodies by absorption procedures, particularly with other *Campylobacter* species.

Similar serological findings were also reported by other authors (3, 8). Some authors also reported higher antibody titers in adults than in children, indicating that the infection is acquired later in life (4, 8).

That not all gastritis patients positive for *C. pyloridis* on culture or microscopy also showed raised CF titers or characteristic bands on the immunoblot might of course be explained by antigenic differences of infecting *C. pyloridis* strains. However, Pearson et al. (13) as well as Megraud et al. (12), when comparing protein profiles of different *C. pyloridis* strains by PAGE, did not find major differences between them. Pearson, who performed PAGE after ultrasonication of whole-cell extracts, found in all the tested strains the same major protein bands with molecular weights of 62,000, 53,000, 50,000, 39,000, 25,000, 21,000, and 14,000. Megraud, using SDS-PAGE, found a somewhat different profile with major protein bands of 74, 64, 58, 43, 21, 17, and 12 kDa. These discrepancies may be explained by differences in the techniques used. *C. pyloridis* strains we looked at by SDS-PAGE analysis also appeared similar and showed major protein bands of 79, 72, 63, 60, 55, 50, 30, 26, and 24 kDa.

However, minor protein bands, which may barely be visible on Coomassie-stained gels, may appear as major bands on immunoblot analysis, e.g., a protein band of about 110 kDa that, as has been mentioned above, was reactive with the majority of sera from gastritis patients but with hardly any of the sera from patients with normal antral mucosa. Thus, antigenic differences between infecting strains may indeed play a role in the immune responses of different patients. Further studies by immunoblot analysis to elucidate this question of antigenic differences are under way.

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