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Etiologic Role of *Helicobacter pylori* in the Pathogenesis of Histological Chronic Gastritis

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=Abstract=**The aims of this study were 1) to investigate the prevalence rate of *H.pylori*-associated chronic gastritis in patients with non-ulcer dyspepsia (NUD), 2) to observe the effect of eradication of *H.pylori* on the improvement in histological findings of chronic gastritis, 3) to detect and measure the specific antibody to *H.pylori* in patients' sera with ELISA, and 4) finally to study the immune response of serum IgG against *H.pylori* according to the severity of histological gastritis with immunoblot. Out of total 77 patients, 54 had *H.pylori* in their gastric antral mucosa, 48 had moderate-to-severe chronic gastritis, and the remaining 6 had normal-to-mild. In contrast, out of 23 patients without *H.pylori*, 17 had normal-to-mild chronic gastritis, and only 6 had moderate-to-severe. After treatment with colloidal bismuth subcitrate (CBS) alone or CBS and amoxicillin, *H.pylori* cleared up in 61.5% and 83.3% of the patients, respectively. In the placebo group, however, not a single case lost *H.pylori*. When *H.pylori* was successfully eradicated, improvement in histological gastritis was found in 88.8%, while it was 30% when not eradicated ($P<0.002$) in repeated biopsies. The detection rate of anti-*H.pylori* by ELISA in patients with *H.pylori* was significantly higher (90.7%) than in patients without *H.pylori* (21.7%) ($P<0.001$). Moreover, the levels of IgG antibody in patients with *H.pylori* were significantly different between the patients with normal-to-mild (median:0.52) and those with moderate-to-severe gastritis (median:1.28) ($P<0.005$). Major protein bands of acid glycine extract of *H.pylori* on SDS-PAGE were 62 kD, 33 kD, 25 kD, 20 kD, and 14 kD, while protein bands reacting with patient's sera were 62 kD, 33 kD, and 14 kD. Sera from patients with *H.pylori* and severe gastritis commonly reacted with 62 kD, 33 kD, and 14 kD antigen. However, sera from patients with *H.pylori* and normal-to-mild gastritis showed a faint band with only 62 kD.**

In conclusion, it was strongly suggested that *H.pylori* play a causative role in the pathogenesis of histological chronic gastritis. ELISA in this study using acid glycine extract of *H.pylori* may be useful in the diagnosis of *H.pylori*-associated histologically moderate-to-severe gastritis. In some cases, *H.pylori* was detected from normal gastric mucosa, where negligible antigen-antibody reaction between serum IgG and *H.pylori* was identified by immunoblot.

Key Words: *Helicobacter pylori, Histological chronic gastritis, Acid glycine extract antigen, ELISA, Immunoblot, Negligible antigen-antibody reaction.*

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INTRODUCTION

Since Warren (1983) reported that S-shaped bacilli resembling *Campylobacter jejuni* were found in the epithelial layer of patients with chronic active gastritis, showing histologically granulocyte infiltration, evidence that this organism—named *Helicobacter pylori* (*H.pylori*, previously *Campylobacter pylori*)—is a pathogen, and not simply a saprophyte harmlessly colonizing the gastric pyloric antrum, has been accumulated. Contrary to early reports, it does not seem that *H.pylori* is the etiologic agent for peptic ulcer disease. However, it is generally accepted that there is a relationship between chronic active gastritis with increased polymorphonuclear leucocytes (PMN) in the gastric mucosa and *H.pylori*. There are examples of animal studies which produced gastritis in germ free piglets, which had been inoculated with *H.pylori* (Lambert *et al.*, 1987), and examples of human experiments in which chronic active gastritis was induced by inoculation with *H.pylori* in normal healthy subjects (Marshall *et al.*, 1985; Morris and Nicholson, 1987). However, there are some points to resolve before concluding that *H.pylori* is the etiologic agent for gastritis. They are: the studies of natural history of gastritis, the relationship between the damage of gastric mucosa by various foods and gastritis, and the importance of gastritis as the cause of upper gastrointestinal symptoms such as epigastric hunger pain.

Referring to the fact that the infection rate of *H.pylori* increases with age (Graham *et al.*, 1987) and the known age-related increase in prevalence of chronic gastritis, it is natural for *H.pylori*, which is found at the gastric antrum, to be considered the etiologic agent for B-type gastritis. However, in order to prove that *H.pylori*, is the etiologic agent for B-type gastritis, one must satisfy Koch's third and fourth postulates. The third postulate states that after a susceptible animal is inoculated, it must produce the same disease. The fourth postulate states that the same bacteria must be discovered from the diseased animal after inoculation. To satisfy the above postulates, there are two reports of human experiments (Marshall *et al.*, 1985; Morris and Nicholson, 1987) as well as an example of an animal study with gnotobiotic piglets inoculated with *H.pylori* isolated from humans which produced gastritis (Lambert *et al.*, 1987). However, there were limitations in these experiments due to the small number of subjects and ethical problems encountered in the human experiments. The only susceptible animal other than a human is a gnotobiotic piglet. Also, from the gastric mucosa of the monkey, dog, cat, and mouse, bacteria similar in shape to *H.pylori* are found. This hinders progress in the animal studies (Palmer, 1954; Vial and Orrego, 1960; Lockard and Boler, 1970; Baskerville and Newell, 1988).

Therefore, after medical treatment that can eradicate *H.pylori*, if one can observe an improvement of gastritis by histology and subjective symptoms, this may be considered indirect proof that *H.pylori* is the etiologic agent for chronic gastritis and, furthermore, it may be possible to find clinical relevance.

Since the report of Warren (1983), there are numerous articles which show the association between *H.pylori* and chronic active gastritis with increased PMN in the epithelial layer. However, others report that *H.pylori* is also found in histologically normal mucosa (Malfertheiner and Bode, 1987; Humphreys *et al.*, 1988; Burnie *et al.*, 1988; Loffeld *et al.*, 1988; Jung *et al.*, 1988). These conflicting results may indicate that there are different virulences for different strains of *H.pylori* or a diversity in the human host immune response against *H.pylori*.

Bacteria that are similar to *H.pylori* found in gastric mucosa of mammals such as cats and dogs do not cause any inflammatory response and exist as commensal (Lockard and Boler, 1970; Vial and Orrego, 1960). However, in the gastric mucosa of the rhesus monkey, *H.pylori*-like bacteria are associated with severe infiltration of lymphocytes and histiocytes. It is known that the immune response against *H.pylori*-like bacteria is diverse in different animal hosts (Baskervile and Newell, 1988).

However, so far there has been no systematic study concerning *H.pylori* found in the histologically normal human gastric mucosa. Therefore, even though *H.pylori* is detected from the spectrum

of histologically normal gastric mucosa to severe gastritis, the difference may originate from either a different virulence of strain or diverse host immune response against *H.pylori*. Under the above assumption, by using the immunoblotting technique, observing the different antigen-antibody reactions between the normal histologic group and the gastritis group is worthwhile to see if *H.pylori* plays a role in the pathogenesis of gastritis.

If we rely only on the stain or culture to confirm the presence of *H.pylori* in the gastric mucosa, it is possible to get false test results since *H.pylori* are scattered as patches on the gastric mucosa. Therefore, if *H.pylori* can elicit a human host response by specific antibody, serologic assay such as ELISA may be able to overcome the above problem.

To clarify the role of *H.pylori* as the pathogen in chronic gastritis and using only patients with nonulcer dyspepsia (NUD) who did not have macroscopic peptic ulcers on endoscopy, the authors investigated the infection rate of *H.pylori* and the frequency of histological gastritis, and then obsereved the histological changes of the gastric mucosa and attending symptoms after eradication of *H.pylori* with medication.

Also, in order to identify the significance of the presence of *H.pylori* in the normal gastric mucosa, the authors measured the specific antibody against *H.pylori* by the ELISA method and proceeded with an immunoblot test according to the severity of the histology from the gastric antral mucosa of patients with *H.pylori* infection.

MATERIALS AND METHODS

1. Subjects

Patients who presented with upper gastrointestinal symptoms such as epigastric pain underwent endoscopy and were found to have no abnormal apperance such as peptic ulcers or polyps. On abdominal ultrasound, there were no gallstones or pancreatitis, and the physical examination and serum chemistry were all normal in 77 patients who were defined as NUD patients. We excluded patients who, within one week of endoscopy, had used steroids, non-steroidal antiinflammatory drugs, H_2-blockers, or any antibiotics. Also, any patients with a chronic wasting disease, a previous history of gastrectomy, renal failure, pregnancy, or lactation were excluded.

2. Drug administration and follow-up study

Twelve patients who were positive for *H.pylori* were given bismuth compound, colloidal bismuth subcitrarte (CBS), and amoxicillin, 13 received only CBS and 13 others a placebo. CBS and the placebo were given in 2 tablets 30 minutes before breakfast and dinner (CBS dose 480mg). Amoxicillin was given 500mg t.i.d., and a repeat endoscopy was done within 48 hours after 4 weeks of therapy by the same endoscopist.

3. Processing of the biopsy specimens

From each patient within 5cm of pylorus, 4 biopsy specimens were taken, and for serologic tests, blood samples were drawn and serum stored at -20℃. Two of the biopsies were touch-printed on slides, Gram stained, fixed with formalin, and then stained with hematoxylin-eosin. The other 2 biopsies were ground on sterile petri dishes and inoculated over a chocolate agar.

To prevent cross-contamination from other patients after each biopsy was performed, the biopsy forceps were immersed in glutaraldehyde, brushed with isopropyl alcohol, and washed under tap water.

4. Bacterial culture

For the culture medium, chocolate agar, which contained Skirrow formula (vancomycin, trimethoprim, polymyxin B), amphotericin B, and Isovitalex (BBL) was used. To keep microaerobic atmosphere and high humidity, it was incubated for 3 to 4 days in CO_2 incubator at 37℃.

Using the above culture method, as soon as typical clear, colorless, waterdrop-shaped colonies were found, they were Gram stained. Once typical curved Gram negative bacilli were observed, they were subcultured, and after 5 days biochemical identification was carried out with catalase, oxidase, and urease. If catalase, oxidase, and urease tests were all positive, bacterial colonies were amplified and prepared as an antigen for the

ELISA test.

Different bacteria that were used in the inhibition assay to evaluate the specificity of ELISA such as *Campylobacter fetus* (ATCC 27374), *Campylobacter jejuni* (ATCC 33291), and *Escherichia coli* (ATCC 12809) were supplied by the National Institute of Health in Korea.

5. Determination of the histological severity of gastritis

The severity of gastritis was determined by a slightly modified method from Karttunen *et al.* (1978).

When polymorphonuclear leucocytes (PMN) were observed, the following key was used: grade 0: no PMN were seen; grade 1: 1 or 2 PMN were observed at the lamina propria between the foveolar epithelium; grade 2: PMN were scattered around the lamina propria; grade 3: PMN were grouped and filled the lamina propria.

When PMN infiltrated through the epithelium (exocytosis), the following key was used: grade 0: no PMN were seen; grade 1: 1 PMN was found rarely within the epithelal pit (PMN that penetrated the antral mucosa were usually found between the epithelial layers); grade 2: on the average 1 PMN was found within the epithelial pit; grade 3: grouped PMN that penetrated through the epithelial layer formed a microabscess.

When lymphocytes were found, the following key was used: grade 0: lymphocytes were rarely found in the lamina propria; grade 1: a few lymphocytes were found in the lamina propria; grade 2: many lymphocytes were present in the lamina propria but there was no widening of the lamina propria; grade 3: lamina propria was extended and filled with lymphocytes and also plasma cells.

The overall severity of gastritis was graded as follows: grade 0: the total of the PMN grade and PMN infiltration grade was 0 or 1 and the lymphocyte infiltration grade was less than 1; grade 1: PMN grade and PMN infiltration grade was 0 or 1 and lymphocyte infiltration grade was greater than 2; grade 2: the sum of the PMN grade and PMN infiltration grade was 2 or 3; grade 3: the sum of the PMN grade and PMN infiltration grade was greater than 4.

6. Preparation of the antigen

The method used was slightly modified from Goodwin *et al.* (1987). After biochemical identification, the amplified bacterial colonies were scraped with a loop into sterile distilled water to make a thick rich medium and then centrifuged twice for more than 5 minutes each at 4℃ and 4000 g. Then they were washed with sterile distilled water. A rich medium was made with 2.5 ml of glycine buffer added per 0.1 g of bacterial liquid to 0.2 M glycine hydrochloride (pH 2.2) (Sigma Chemical). This medium was stirred for 15 minutes at 25℃ using a magnetic spin bar, then centrifuged for 15 min. at 4℃ at 11,000 g (Beckman J2-21M/E). The supernatant was collected and neutralized with 0.1 N NaOH addition to a pH of 7.2-7.4 using a pH meter. This neutralized supernatant within the sterile distilled water was dialysed for 24 hours at 4℃ using a dialysis membrane (maximum filtered molecular size: 12,000,Union Carbide).

Protein was ultrafiltrated using a vacuum pump through immersible-CX ultrafilter (Millipore, Bedford, MA), and its concentration was quantified by the Lowry method(1958).

7. Enzyme linked immunosorbent assay (ELISA)

According to the checkerboard titration method, it was decided that the optimal antigen concentration would be 6 ug/ml, serum to 1:100 and the conjugate concentration to 1:1000 dilution.

After concentrated antigen was diluted to 6 ug/ml by 0.5 M carbonate buffer (pH 9.6), 200 ul was distributed to each well of polyvinyl chloride, microtiter, and flat-bottomed plates (Dynatech Lab.) and fixed for 24 hours at 4℃. This antigen attached plate was transferred to 25℃, and each well was washed 3 times with 0.3% PBS-Tween 20.

After the serum was diluted to 1:100 by serum diluent (PBS-Tween 20 with 5 mg/ml of bovine gammaglobulin, 1 mg/ml of gelatin), 100 ul was distributed to each well of the microtiter plate in triplicate and fixed for 90 minutes at 25℃.

Each well was washed 3 times with 0.3% PBS-Tween 20.

Peroxidase-labeled goat antibody to human IgG (Sigma Chemical, A8775) was diluted to 1:1000

with diluent (PBS with bovine serum albumin 20 ug/ml, gelatin 1.0 mg/ml), and 100ul was distributed to each well of the plate and was fixed for 90 minutes at 25℃.

Each well was washed 3 times with 0.3% PBS-Tween 20,then with PBS alone twice.

For color reaction substrate 0-phenylenediamine (Sigma Chemical, P-9029) was dissoved in a solution of 0.012% H_2O_2 to phosphate citrate buffer (pH 5.0), and 100 ul was distributed to each well and reacted for 14 minutes at 25℃.

Color reaction was stopped with 25 ul of 4 M/L H_2SO_4 and the absorbance value of the plate was determined using ELISA reader (Dynatech Lab., MR 700) at 492 nm. For each plate, a blank well was used as a baseline measurement.

Two types of control serum were located on each plate. Sera from 10 patients with very high titers of antibody were pooled and used as positive control serum. Negative control sera from 8 patients were diluted to 1:100 and placed on each plate, and the readings of these wells had to be less than 0.1 absorbance value for the plate to be accepted.

8. Inhibition assay

In order to evaluate the specificity of ELISA, 8 sera with high antibody titers were pooled and divided into 5 aliquots. One was used as the control serum, while the remaining 4 were incubated with 20 ug of acid glycine extracts from *H. pylori*, *C. fetus*, *C. jejuni*, and *E. coli* for 30 minutes at 37℃. Then, 200 ul of serum was diluted from 1:10 to 1:160 and triplicated on the microtiter plate, and ELISA was carried out as described above.

9. SDS-PAGE(sodium dodecyl sulfate-polyacrylamide gel electrophoresis)

A slight modification of the discontinuous buffer procedure by Laemmli(1970) was used. For sample buffer, acid glycine extract from *H.pylori* was added to 50 mM Tris-hydrochloride (pH 6.8), 5% betamercaptoethanol (v/v), 2% SDS, 10% glycerol, and 0.01 % bromphenol blue, and after boiling for 5 minutes at 100℃, it was centrifuged for 5 minutes at 15,000 rpm.

Adjusting the concentration of the stacking gel and the separating gel of the polyacrylamide to 5% and 11%, respectivey, it was cooled to 10℃, and electrophoresis was carried out using a Midget electrophoresis unit (LKB, 2050) for 40 minutes at 40 mA. After electrophoresis, the gel was silver-stained. For measuring molecular weight, a low molecular weight proteins electrophoresis calibration kit (Pharmacia, Sweden) was used as a standard protein.

10. Immunoblotting

After electrophoresis, the gel and nitrocellulose membrane (LKB 2005-106, 0.20um) were fixed for 15 minutes to blotting buffer (Towbin transfer buffer: 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3). Then it was cooled to 4℃ in an electrophoretic transfer unit (LKB 2051 Midget Multiblot) containing blotting buffer, and transfer was carried out for 4 hours using 70 volts.

After transfer, the standard molecular weight protein portion was cut, and the molecular weight was determined by amido-black 10 B protein stain method. In order to avoid nonspecific binding of the nitrocellulose membrane, blocking was carried out for 1 hour at 40℃ in blocking buffer(3% (w/v) BSA/PBS).

After washing with PBS for 10 minutes, the sample serum which contained the first antibody was diluted to 1:100 with blocking buffer and reacted with the nitrocellulose membrane for 2 hours at room temperature.

After washing with PBS for 10 minutes, peroxidase-labeled goat antibody to human IgG (Sigma Chemical, A8419) was diluted to 1:1000 in blocking buffer and reacted with the nitrocellulose membrane for 1 hour at room temperature. After reaction, it was washed with PBS for 10 minutes.

For the developer 0.06% 3-3'-diaminobenzidine (Sigma Chemical) was added to 10ml of 50 mM Tris (pH 7.6), 0.003% H_2O_2 was added, and then this was incubated for 3 minutes at room temperature. The reaction was then stopped with PBS washing, dried at room temperature, and the result was observed.

11. Statistical analysis

For statistical analysis, a Minitab (version 5.1.1)

program was used. Student t-test, paired t-test, Chi-square test, z-test, and a Mann Whitney test was done, and when the P value was less than 0.05, it was considered to be statistically significant.

RESULTS

1. Infection rate of *H.pylori* and prevalence of histological gastritis

Of the 77 NUD patients, *H.pylori* was detected in 54 (70%) but not in 23 patients (30%). Out of the 54 patients with *H.pylori*, 48 (88.8%) had grade 2 or 3 histological gastritis, but only 6 (26%) out of 23 patients without *H.pylori* had grade 3 gastritis. There were more frequent cases of moderate-to-severe histological gastritis in patients with *H.pylori* than those without *H.pylori* ($P<0.005$ by Chi-square test) (Table 1).

Table 1. Grade of histological gastritis according to the presence and absence of *H.pylori*

	Grade of Histological Gastritis	
	Grade 0-1	Grade 2-3
H.pylori(−) (n=23)	17	6
H.pylori(+) (n=54)	6	48*

* $p<0.005$ by Chi-square test

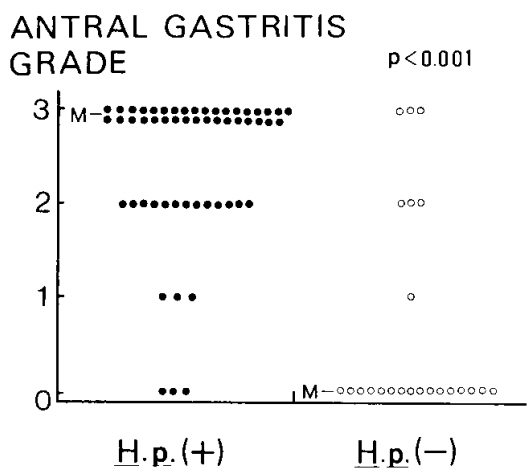

Fig. 1. Comparison of histological findings of antral mucosa in patients with and without *Helicobacter pylori* in the antral mucosa M = median

2. Comparison of inflammatory cell infiltrate according to presence of *H.pylori*

In comparing the overall severity of gastritis, it was significantly higher in the *H.pylori* positive group than in the negative group ($P<0.001$ by Mann Whitney test)(Fig. 1).

3. ELISA

a) Inhibition assay

The effect of different acid-glycine extracts on the titer of *H.pylori* antibody in a highly positive serum is shown in Fig. 2. *H.pylori* acid-glycine extract adsorbed the antibody in the positive control serum, thus confirming the presence of specific antibody against *H.pylori*.

However, *C.fetus* acid-glycine extract also decreased the absorbance of positive control serum, suggesting a degree of cross reactivity between antibodies to *H.pylori* and extracts from other bacteria.

b) SDS-PAGE profile of *H.pylori* acid-glycine extract

The SDS-PAGE profile of acid-glycine extract of *H.pylori* is presented in Fig. 3. Major protein bands were 62 kD, 33 kD, 25 kD, 20 kD, and 14 kD.

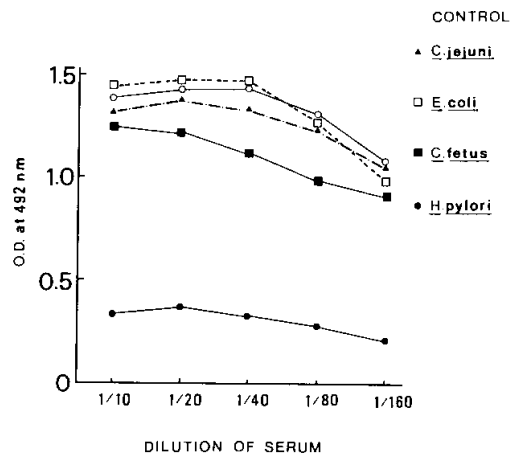

Fig. 2. Inhibition assay. The effect of adsorption by acid-glycine extracts of four different bacteria on optical densities of serum containing antibodies to *Helicobacter pylori*

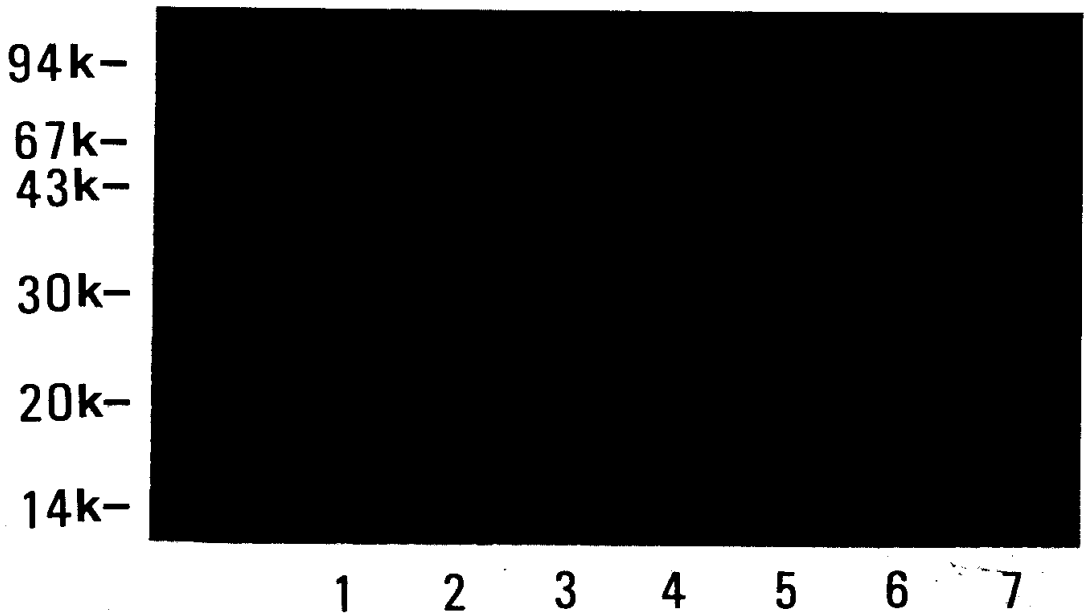

Fig. 3. SDS-PAGE of seven *Helicobacter pylori* isolated from different people
1 = Reference strain of *Helicobacter pylori*

c) ELISA according to the presence of *H.pylori*

The result of ELISA according to the presence of *H.pylori* is shown in Fig. 4. Optical density of the serum was measured at 492 nm, if 0.4 was the cut-off value, sensitivity was 91%, and specificity 78%.

d) Comparison of ELISA titer according to severity of histological gastritis

When *H.pylori* was not detected and gastric mucosal histology showed normal or grade 1 gastritis, the median ELISA value was put as 0.00. But when *H.pylori* was detected and the histology showed more than grade 2 gastritis, the median ELISA value 1.28. There was a highly significant statistical difference between these two groups ($P<0.001$ by Mann Whitney test) (Table 2).

Even if *H.pylori* was detected, when mucosal histology showed normal or mild gastritis of grade 1, the median ELISA value was 0.52, but when the histology was more than moderate gastritis (grade 2 or 3), the median ELISA value was 1.28. There was a highly significant statistical difference between these two groups also ($P<0.005$ by Mann Whitney test).

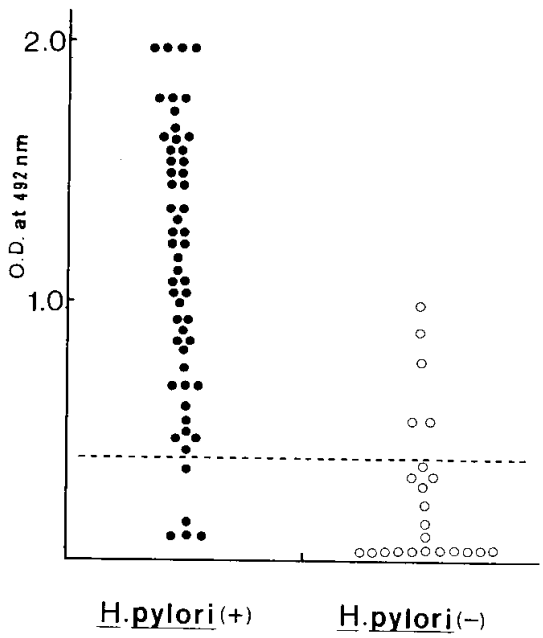

Fig. 4. Serum IgG antibody to acid glycine extract of *Helicobacter pylori*. Left, optical densities of sera of patients with *H.pylori* ; right, those of sera of patients without *H.pylori*

Table 2. Level of IgG antibody to *H.pylori* by ELISA according to histological grading and to the presence of *H.pylori* in the tissue

	Grade of Histological Gastritis	
	Grade 0-1	Grade 2-3
H.pylori (−)	0.00* (n=17)	0.41 (n=6)
H.pylori (+)	0.52** (n=6)	1.28*** (n=48)

* vs ** p>0.05 by Mann Whitney test
* vs *** p<0.001 by Mann Whitney test
** vs *** p<0.005 by Mann Whitney test
* median value (absorbance at 492 nm)

Based on the above results, we could find that the serum level of antibody against *H.pylori* measured by ELISA reflected the severity of histological gastritis associated with *H.pylori* rather than just the presence of *H.pylori*.

4. Negative conversion of *H.pylori* after medication and accompanying change of histological gastritis and symptoms

Thirty-eight out of 77 patients who had undergone endoscopy, follow-up gastroscopy with biopsy was possible after 4 weeks of administering medicine. As for age, severity of gastritis, and epigastric pain, there was no significant difference among the 3 groups, but in the male-to-female ratio in the placebo group, the female was a little higher (Table 3).

a) Negative conversion of *H.pylori* after medication

From the CBS and amoxicillin combination therapy group, *H.pylori* was eradicated in 10 out of 12 (83.3%) ; from the CBS mono-therapy group, it was eradicated in 8 out of 13 (61.5%), while none were eradicatd from the placebo group. There was a significant difference of negative conversion rate between the CBS and amoxicillin combination therapy group versus the placebo, and the CBS monotherpy group versus the placebo group (P<0.001 by Z-test, each). The negative conversion rate was higher in combination therapy group compared to CBS mono-therapy group but was not statistically significant (P>0.05 by Z-test) (Table 4, Fig. 5).

b) Change of histological gastritis and epigastric pain according to medication

The severity of histological gastritis and epigastric pain improved in accordance with the negative conversion of *H.pylori* after medication. There was a significant difference between the combination therapy group and placebo group and between the CBS mono-therapy and placebo group. However, there was no statistical significant difference

Table 3. Characteristics of patients randomly assigned to receive medication

	CBS + Amoxicillin	CBS	Placebo	Statistical Significance
Patients (n)	12	13	13	NS
Age/Mean (SE)	40.2 (3.2)	38.4 (4.3)	37.6 (2.5)	NS
Sex (M:F)	6:6	6:7	4:9	p<0.05
Gastritis of grade 2 or 3	12	11	13	NS
Epigastric pain	11	12	12	NS

* NS: not significant

Table 4. Negative conversion rates of *H.pylori* and associated effects

	CBS + Amoxicillin (n=12)	CBS (n=13)	Placebo (n=13)
Negative conversion rate of *H.pylori*	10/12 (83.3%)	8/13 (61.5%)	0/13 (0%)
Improvement of histological gastritis	11/12 (91.6%)	9/13 (69.2%)	2/13 (15.4%)
Improvement of epigastric pain	9/11 (81.8%)	8/12 (66.6%)	3/12 (25.0%)

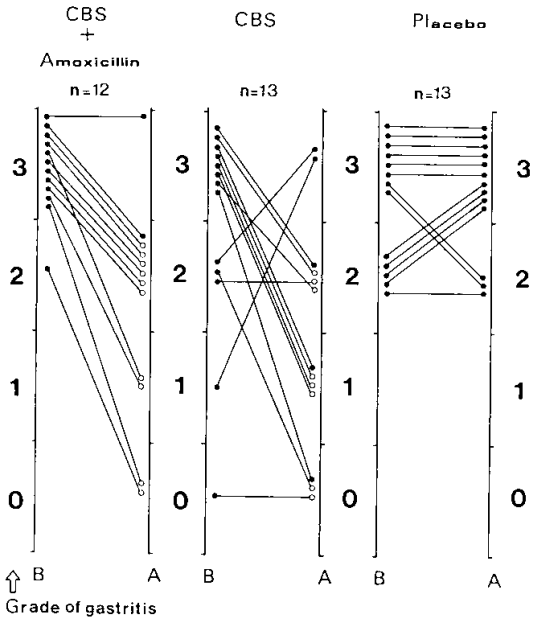

Fig. 5. Grades of histological gastritis before and after treatment
● = *H.pylori* (+), ○ = *H.pylori* (−)
B = before, A = after

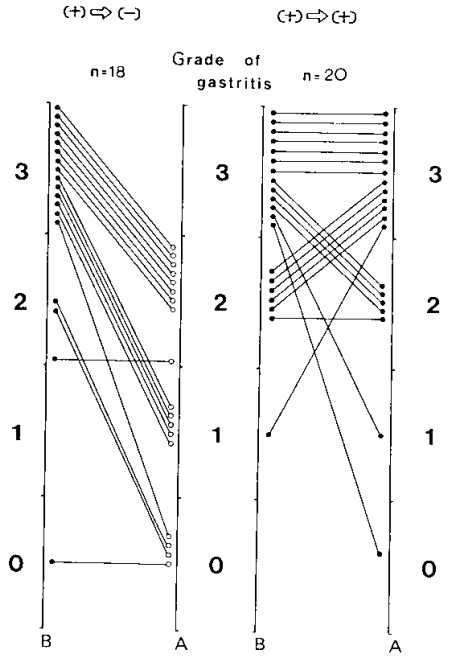

Fig. 6. Grades of histological gastritis before and after treatment in *Helicobacter pylori* eradicated and persistent groups.
● = *H.pylori* (+), ○ = *H.pylori* (−)
B = before, A = after

between the combination therapy group and the CBS mono-therapy group (Table 4, Fig. 5).

5. Improvement of histological gastritis and symptoms according to negative conversion of *H.pylori*

Comparing the 18 patients who turned negative for *H.pylori* after either combination or mono-therapy and the 20 patients who were still positive for *H.pylori* despite the medical therapy or with placebo, 16 of the former group (88.8%) and only 6 of the latter group (30%) showed improvement in histological gastritis. This was statistically significant ($P<0.002$ by Z-test). Also, epigastric pain improved in 12 out of the former (66.6%) patients who became negative versus only 6 out of the latter who were persistently positive (30%). There was a significant difference between both study groups ($P<0.002$ by Z-test) (Table 5, Fig. 6).

Table 5. Effects of negative conversion of *H.pylori* on histological gastritis and epigastric pain compared with those of *H.pylori* persistent group

	Negative Conversion of *H.pylori* (n=18)	Persistence of *H.pylori* (n=20)	Statistical Significance
Improvement of histological gastritis	16/18 (88.8%)	6/20 (30%)	$p<0.002$*
Improvement of epigastric pain	12/18 (66.6%)	6/20 (30%)	$p<0.02$**

*, ** by *z*-test

trol serum. This tells us that there was a cross reactivity between antibodies against *H.pylori* and *C.fetus*. Other investigators also reported cross reactivity between *H.pylori* and other *Campylobacter* species (Goodwin *et al.*, 1987; Newell, 1987). However, it does not seem to be a great problem in performing the ELISA for *H.pylori*, because in the case of *C.jejuni* infection, it produces acute enteritis in humans, the antibody increases within 2 weeks, and the serum levels of IgG and IgM return to normal value within 3 months (Blaser and Duncan, 1984). However, infection with *H.pylori* is chronic and persistent.

To detect serum antibody against *H.pylori*, secondary antibody used in this study was against only human IgG made in goat. The reason for this is that infection with *H.pylori* is chronic, so it was hard to expect serum IgM to rise. Also, other investigators report that it was hard to discriminate the infection status with serum IgM antibody (Goodwin *et al.*, 1987; Perez-Perez *et al.*, 1988).

After carrying out ELISA against *H.pylori*, it was possible to confirm the specificity of this test against *H.pylori* by inhibition assay. However, the sensitivity and specificity was only 91% and 78%, respectively, for two reasons. First, even though there is an *H.pylori* infection, in certain cases there is little or no inflammatory reaction in the gastric mucosa, suggesting that some human hosts do not show any immune response. Therefore, the serum antibody is low. This can be understood from the fact that *H.pylori* can be detected from 6% to 43% even from histologically normal gastric mucosa (Jones *et al.*, 1986; Loffeld *et al.*, 1988; Humphreys *et al.*, 1988). Second, even if the serum level of antibody by ELISA is high due to actual infection with *H.pylori*, the sensitivity of the current methods used to determine *H.pylori* infection—such as Gram stain, hematoxylin-eosin stain, or bacterial culture —is low. Besides, due to patchy distribution of *H. pylori* colonization, the ELISA result could be interpreted as false positive. The first problem can be resolved by using immunoblotting to see the difference of antigen-antibody reaction between the human host and *H.pylori* antigen according to the severity of histological gastritis. However, the second problem seems hard to overcome since there is not yet a gold standard method of detecting *H.pylori*. Major protein bands of SDS-PAGE profile of the acid-glycine extract of *H.pylori* were 62 kD, 33 kD, 25 kD, 20 kD, and 14 kD. This was similar to that reported by Newell (1989) : 61 kD, 56 kD, 31 kD, and 25 kD. However, there was a difference from those reported from Perez-Perez *et al* (1987), 62 kD, 53 kD, 48 kD, and 30 kD. These differences seem to originate from the differences in antigen processing, such as dialysis, ultrafiltration, and other procedures.

Compared to the *H.pylori* negative group with histologically normal-to-mild gastritis, the *H.pylori* positive group with gastritis of grade 2 or 3 had higher optical densities in ELISA. Such findings correspond with those of other studies using different patient groups, peptic ulcer disease (Jung *et al.*, 1988) and different kinds of antigen, that is sonicated bacteria (Perez-Perez *et al.*, 1988). These facts suggest that *H.pylori* elicit an immune response and increase the serum antibody in human beings.

The proportion of females in the medication treatment group was slightly higher than that of the placebo group. However, in *H.pylori* infection and its related problems, there is no known difference according to male to female ratio, so it would not have any effect on the study result. There are differences in the negative conversion of *H.pylori* after medication, from 40% to 90% (McNulty *et al.*, 1986; Marshall *et al.*, 1987; Humphrey *et al.*, 1988; Rauws *et al.*, 1988; Morgan *et al.*, 1988). It was thought to originate from the differences in the type of medicine, duration of medication, administration method, and interval between the end of the treatment and the follow-up gastroscopic biopsy.

The reason we chose epigastric pain as the main criteria to evaluate subjective symptom improvement according to negative conversion of *H.pylori* was based on the result of Chua *et al.* (1989). That is, if we divide the NUD patients according to their symptoms, there are two groups, one with epigastric pain that signals peptic ulcer disease and the other with early satiety, abdominal distension, flatuence, and nausea suggesting gastric dysmotility problems. It was reported that *H.pylori* was detected in 90% of the patients from the former group and only in 19% from the latter group. This im-

plies that epigastric pain was more related with *H.pylori* infection.

Negative conversion rate of *H.pylori*, consequent improvement of histology and subjective symptoms with combination therapy using bismuth compound and amoxicillin was superior to those of mono-therapy using only bismuth compound, although statistically not significant. This is believed to be due to the fact that CBS works only locally over the gastric mucosa compared to amoxicillin which is absorbed into the bloodstream and works systematically. Therefore, when combination therapy was used, the antibiotic effect was additive.

With amoxicillin alone as a single agent, the negative conversion rate and histological improvement was equal to those of CBS alone or in combination with amoxicillin (Rauws *et al.*, 1988). In cases where *H.pylori* was eradicated with medication, the improvement of histological gastritis and subjective symptoms was much higher than the group with persistence of *H.pylori*. This strongly suggests that *H.pylori* is the etiological agent of histological chronic gastritis. However, with antibiotics such as erythromycin, the negative conversion rate was only 6% (McNulyty *et al.*, 1986). This may point to the importance of considering the stability in low pH and the capacity of diffusing into the gastric crypt when choosing antibiotics.

The reason that the 25% of patients experienced relief of their symptoms with only a placebo is that some proportion of the symptoms of NUD may be attributable to psychological factors. However, in the CBS group or CBS with amoxicillin group there was more of a statistically significant difference in the reduction of epigastric pain than in the placebo group, suggesting that *H.pylori* play a bigger role as an etiologic agent in NUD patients with histological gastritis.

The major bands in immunoblot using *H.pylori* as an antigen were diverse from 66 kD to 45 kD, down to 31 kD (Perez-Perez and Blaser, 1987), 61 kD, 56 kD, 54 kD, 29 kD, 26 kD (Newell, 1987), and 63 kD, 57 kD, 31 kD (Perez-perez *et al.*, 1988). The reasons for the above diversity were thought to originate from the differences in methodology, such as the kinds of antigen used, or whether antibody against *H.pylori* was from human serum or from immunized animal serum.

However, until now in the immunoblotting study using *H.pylori* as the antigen, the major research concern has been to observe which antibody against *H.pylori* reacts with which portion of the *H.pylori* antigen, not to observe the difference in antigen-antibody reaction according to the severity of gastric mucosal histology.

To test the hypothesis that the reason why in some cases *H.pylori* is detected from normal human gastric mucosa—although, for the most part *H.pylori* causes histological gastritis—is due to the individual difference in the immune response of serum antibody against *H.pylori*, we carried out immunoblotting using acid glycine extract antigen from *H.pylori*. As a result, in cases that were positive for *H.pylori* but histologically normal or mild gastritis of grade 1, there was little or no reaction against *H.pylori* antigen molecule of 33 kD compared to the severe gastritis group of grade 3. When another strain of *H.pylori* antigen was used, the results were identical. Based on the above results, the serum from *H.pylori* positive patient with severe gastritis of grade 3 was immunoblotted against 7 different *H.pylori* antigens. For the control, the serum from *H.pylori* positive patients, but with histologically normal mucosa, was used. It was observed that in the former case there was a strong antigen-antibody reaction against *H.pylori* antigen 62 kD, 33 kD, and 14 kD in molecular weight, but in the control serum, only a faint reaction was observed against only the antigen of 62 kD. This strongly suggests that human host immune response against *H.pylori* is diverse. Actually, even when *H.pylori* is detected, the human host shows a broad spectrum of immune responses, from histologically normal gastric mucosa to severe gastritis. The above results of immunoblot help to explain such a phenomenon.

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=국 문 초 록=

조직학적 만성위염의 병인으로서 *Heliocbacter pylori*의 역할

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조직학적 만성위염의 병인으로서 *H.pylori*의 역할을 밝히기 위해 소화성 궤양과 같은 육안적인 위점막 병변이 없는 non-ulcer dyspepsia (NUD) 환자들을 대상으로 *H.pylori*의 감염률과 조직학적 위염의 빈도를 밝히고 bismuth 제제 단독 혹은 항생제인 amoxicillin과의 병합요법으로 *H.pylori*를 박멸시킨 후 *H.pylori*의 음전에 따른 위점막의 조직학적 소견과 증상의 변화를 관찰하였다. 또한 일부 정상 위점막으로부터도 검출되는 *H.pylori*의 의의를 알아보기 위해 *H.pylori*에 대한 혈청내 항체와 *H.pylori* 사이의 항원 항체반응을 효소면역측정법으로 측정하여 *H.pylori*에 특이한 항체의 존재 유무를 조사하며 *H.pylori*에 감염된 환자의 위 전정부 점막의 조직학적 소견의 경중에 따라 immunoblot 검사를 시행하였다. 실험결과 NUD 환자 77명 중 48명 (62%)에서는 H.pylori와 연관된 조직학적 중등도 내지 중증 위염이 있음이 관찰되었고 *H.pylori*가 존재할 경우 다형핵백혈구와 이의 상피내 침윤, 림프구 등의 염증세포의 침윤도가 유의하게 높았다. *H.pylori*가 검출된 환자군에 bismuth 제제 단독 혹은 amoxicillin과의 병합요법을 4주일간 실시한 경우 각각 61.5%와 83.3%에서 *H.pylori*가 음전되었으나 placebo 투여군에서는 1 예에서도 음전이 없었고 *H.pylori*가 음전된 경우 조직학적 위염과 심와부 동통의 호전이 관찰되었다.

효소면역측정법상 *H.pylori*에 특이한 항체의 존재를 억제검사 (inhibition assay)를 통하여 확인할 수 있었으며 이 항체는 *H.pylori*의 단순한 존재 유무보다는 *H.pylori*와 연관된 조직학적 위염의 정도와 밀접한 관계가 있었다. *H.pylori*의 acid glycine 추출항원을 SDS-PAGE 한 결과 주요 단백항원이 62 kD, 33 kD, 25 kD, 20 kD, 14 kD에서 관찰되었으며 immunoblot 상 *H.pylori*가 검출된 환자의 혈청과 반응하는 항원은 62 kD, 33 kD, 14 kD였다. *H.pylori*가 검출되면서 grade 3의 중증 위염이 있는 경우에는 62 kD, 33 kD, 14 kD 항원에 대하여 공통적으로 강한 항원 항체 반응이 있었으나 *H.pylori*가 검출되더라도 정상조직을 가진 환자의 혈청은 62 kD 항원에 대하여만 미약한 반응이 관찰되었다. 이상의 실험결과는 *H.pylori*가 조직학적 중등도 이상의 만성위염의 병인으로서 원인적 역할을 할 것임을 강력히 시사하며 효소면역측정법은 *H.pylori*에 의한 중등도 이상의 만성위염을 진단하는 데 있어 매우 유용함을 알 수 있었다. 또한 일부 예에서는 *H.pylori*가 조직학상 정상 위점막으로부터도 검출되는데 이 경우에는 immunoblot상 *H.pylori* 항원과 혈청내 IgG 항체 사이에 미약한 단백 분획만을 관찰할 수 있어 *H.pylori*와 인체 사이에 면역반응이 없거나 약하게 일어남을 시사하였다.