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G. GOŚCINIAK [1], A. PRZONDO-MORDARSKA [1], E. PONIEWIERKA [2], B. IWAŃCZAK [3], A. BILSKA [1]

# RELATIONSHIP BETWEEN ANTIBODY TO CYTOTOXIN AND *HELICOBACTER PYLORI* INFECTION

[1] Departments of Microbiology, [2] Gastroenterology and [3] Pediatrics, University of Medicine, Wrocław, Poland

Broth culture supernatants from 14 (34%) out of the 41 *H. pylori* strains tested, induced vacuolization in Intestine 407 cells in titers ranging from 1:2 to 1:64. 20% of *H. pylori* strains isolated from children and 42% of strains isolated from adults expressed vacuolating activity.
Serum antibody to cytotoxin produced by *H. pylori* was detected with a neutralization assay. Anticytotoxic antibodies were present in all sera from patients infected with cytotoxic *H. pylori* strains. The toxin-neutralizing activity of sera from individuals infected with *H. pylori* suggests that the cytotoxin is produced *in vivo*.

K e y w o r d s: *H. pylori, vacuolating cytotoxin, antibody to cytotoxin.*

## INTRODUCTION

*Helicobacter pylori* (*H. pylori*) is a recently recognized bacterial pathogen associated with diverse pathologies of varying severity, such as chronic gastritis, peptic ulceration, mucosa-associated lymphoid tissue lymphoma, and gastric cancer.

One of the major distinguishing features between *H. pylori* strains, which may help to explain the different disease outcomes in infected individuals, is the expression of a vacuolating toxin (1). In 1988, Leunk *et al.* (2) first described a cytotoxic protein in the extracellular medium of various strains of *H. pylori*. This protein is able to induce the formation of vacuoles in the cytoplasm of eucariotic cells cultured *in vitro*. The vacA gene is present in all *H. pylori* strains but its product, a 87 kD vacuolating toxin, is expressed by only 40—60 per cent of strains (3, 4). The role of this toxin in the pathogenesis of *H. pylori*-associated disease remains controversial, but similar vacuolating cytopathic effects have been reported in patients (5, 6).

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Atherton *et al.* (7) demonstrated that vacA genes are mosaic, consisting of any one of three signal sequences (s1a, s1b, s2) and one of two middle-region alleles (m1, m2). Importantly, it was shown that this diversity is related to function in that s1/m1 genotypes express increased cytotoxin activity *in vitro*, and are associated with more peptic ulceration, whereas s2/m2 genotypes are non-cytotoxic (8, 9).

Most cytotoxic strains also produce a highly immunogenic protein called CagA. About 10% of *H. pylori* isolates harbouring cagA are not cytotoxic, and a similar proportion of cytotoxic strains does not carry cagA (10—12). The role of CagA in pathogenesis is still obscure.

Serum antibody to *H. pylori* is detectable in individuals infected with the bacterium (4, 13—16). Measurement of specific antibody has a diagnostic value for the detection of infection by *H. pylori*. The aim of this work was to determine the production frequency of vacuolating cytotoxin by strains of *H. pylori* isolated from patients with gastritis and investigate the presence of cytotoxin neutralizing antibodies in the sera of these patients.

## MATERIAL AND METHODS

Forty one strains of *H. pylori* were isolated from the gastric mucosa of 15 children and 26 adult patients with gastritis hospitalized at the Departments of Gastroenterology and Pediatrics, University of Medicine, Wrocław, Poland. These strains were identified as *H. pylori* by Gram strain morphology and by positive urease, oxidase, and catalase tests. The strains were maintained frozen at −70°C in Brucella broth containing 15% glycerol.

All strains used in this study were investigated for the production of vacuolating cytotoxin activity. Examined were also control reference strains of *H. pylori* (NCTC 11634 and ACTC 49503 which were previously reported as cytotoxin producing ($Tox^+$) and T × 30 A, previously reported as non-cytotoxin producing ($Tox^-$) (16). For the cytotoxin production assay, *H. pylori* strains were thawed and streaked onto 7% horse blood Columbia agar. Brucella broth (Difco) supplemented with 5% fetal calf serum, 1% vitox (Oxoid) was inoculated and incubated at 37°C in a 10% $CO_2$, 5% $O_2$ and 85% $N_2$ atmosphere during 48 hours on a shaker. After centrifugation at 15.000 rpm for 15 min at 4°C, cell free supernatants were stored at −70°C.

### *Cytotoxin assay for cell vacuolation*

Intestine 407 cells (Human embryonic intestine cells) were grown in Optimem medium (Gibcko), supplemented with fetal calf serum 6%, 2 mM glutamine, 50 μg gentamicin/ml, and 60 μg/ml anti-PPLO reagent (Gibco). Ninety-six well culture plates were seeded with a suspension of 1.0 ml of freshly trypsinised cells at a final concentration of $5 \times 10^3$ cells per well, and incubated at 37°C for 18—24 hours in a 5% $CO_2$ atmosphere.

Cell-free culture broth supernatant was diluted 1:2 to 1:128 and 0.1 ml of each dilution was added to samples of tissue culture medium. These were incubated for 24 h at 37°C in an atmosphere containing 5% $CO_2$. Intracellular vacuolation in >50% of the Intestine 407 cells in a well indicated the presence of the cytotoxin.

### *Neutralization of H. pylori cytotoxin activity*

Antibody specific for the cytotoxin was detected by neutralization 41 serum samples were obtained from patients with proven *H. pylori* infection. The complement in test sera was inactivated by heating at 56°C for 30 min and then diluted 1:4 in tissue culture medium. Equal volumes of diluted sera and cytotoxic preparations from the homologous strains or reference isolate (*H. pylori* ACTC 49503) were incubated for 1 h at 37°C in 5% $CO_2$ and then added to Intestine 407 cells. Following overnight incubation at 37°C, the wells were observed by phase-contrast microscopy for cytotoxic effects. The results were considered positive if vacuolization of the 407 Intestine cells was inhibited. Wells containing cytotoxin preparations without serum served as controls for Intestine 407 cell vacuolation. In addition, two neutralizing and two non-neutralizing control sera included in each assay.

## RESULTS

We tested 41 clinical *H. pylori* isolates and three control strains for the production of vacuolizing cytotoxins. $Tox^+$ control strains, NCTC 11634 and ACTC 49503, produced vacuolization of Intestine 407 cells in a titer of 1:64, whereas broth culture from the $Tox^-$ strain T × 30 A did not cause vacuolization in any dilution tested. Of the 41 *H. pylori* strains tested, 14 (34%) expressed vacuolating cytotoxic activity in titers ranging from 1:2 to 1:64 (*Table 1*). Out of the total number of 15 strains isolated from children 3 (20%) produced vacuolating cytotoxin, whereas 11 (42%) of strains isolated from 26 adults had vacuolating cytotoxic activity.

The next step of investigation was to determine whether infection with cytotoxin producing strains induced the production of neutralizing antibodies in serum (*Table 2*). We found that serum from all 14 patients (100%) infected with vacuolating cytotoxin producing *H. pylori* strains contained anticytotoxin antibodies. These sera neutralized vacuolating activity in broth culture filtrates of cytotoxic *H. pylori*. Of the twenty seven sera collected from patients infected with non-cytoxic. *H. pylori,* five (18.5%) neutralized vacuolating activity in cytotoxin produced from reference strains.

*Table 1.* The production frequency of vacuolating cytotoxin by strains of *H. pylori.*

| Group of patients | No (%) of strains producing vacuolating cytotoxin |
|---|---|
| Children 15 | 3 (20%) |
| Adults 26 | 11 (42%) |
| Total 41 | 14 (34%) |

*Fig. 1* compares cytotoxin activity when the presence of neutralizing antibodies in serum. In both children and adults, the presence of antibody to cytotoxin in sera was more frequently observed than the presence of vacuolating cytotoxic strains. That is: 34% of patients were infected with vacuolating cytotoxic strains, whereas 46% of all patients produced anti-cytotoxin antibody.

*Table 2.* Presence of neutralizing antibodies in sera of patients infected by cytotoxic ($Tox^+$) and noncytotoxic ($Tox^-$) strains of *H. pylor.*

| Strains of *H. pylori* | | Neutralizing antibodies in sera | |
|---|---|---|---|
| | | + | − |
| $Tox^+$ | 14 | 14(100%) | — |
| $Tox^-$ | 27 | 5(18.5%) | 22(81.5%) |

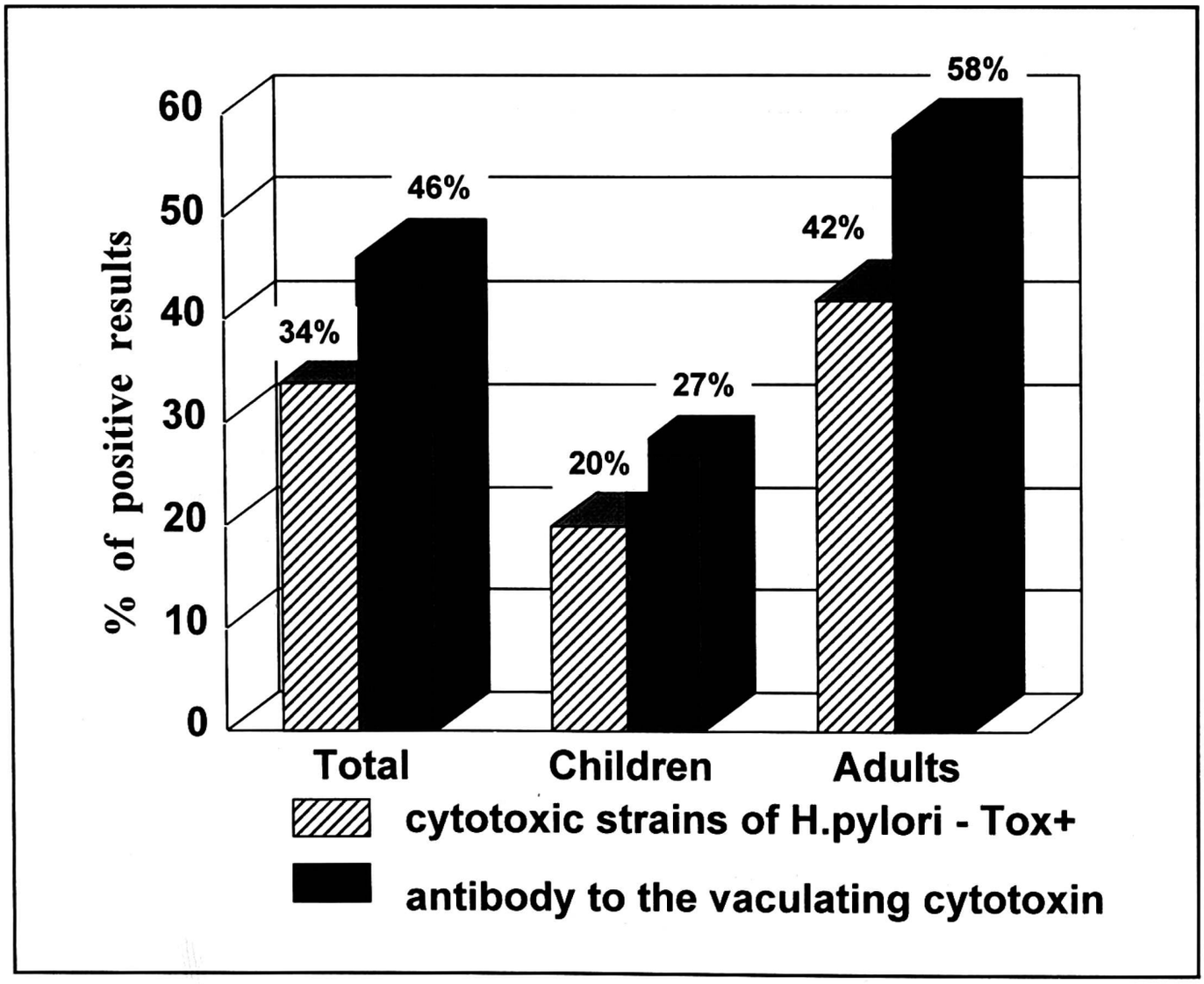


*Fig. 1.* Comparison of cytotoxic activity of *H. pylori* strains with the presence of neutralizing antibody in sera.

## DISCUSSION

In the first stage of the investigation we examined the production frequency of vacuolating cytotoxin by *H. pylori* strains isolated from patients with gastritis. 41 strains were investigated; 15 from children, 26 from adults. Vacuolating cytotoxin activity was evident in 34% of *H. pylori* isolates. According to some authors, 50—60% of *H. pylori* strains express cytotoxic activity in tissue culture supernatants which induce vacuolating of cells *in vitro* (3, 4, 17, 18). These reports relate to strains isolated from adult patients with gastritis, as well as from patients with paptic ulcer disease. The percentage of strains which displayed cytotoxin activity was lower if only patients with gastritis were examined. In the present study we investigated patients with gastritis only.

An Italian investigation analyzed the difference in the production of vacuolating cytotoxin by *H. pylori* between the strains isolated from patients with peptic ulceration and those isolated from patients with chronic gastritis (19). The investigators found that cytotoxin producing *H. pylori* strains were present more frequently in patients with peptic ulceration (66.6%) than in patients with chronic gastritis only (30%). Also, Gossenes *et al.* (16) reported that number of strains producing vacuolating cytotoxin was significantly higher in those isolated from patients with peptic ulceration (48%) than in those isolated from patients with gastritis (24.7%). Other authors confirm that cytotoxin-producing strains appear to be associated more frequently with ulceration (18, 20). Consequently, the results of our investigation are similar to those reported in other publications. It is important to note that our data is refer to *H. pylori* strains derived from both adults and children. In our study, 42% of broth culture filtrates displaying vacuolating cytotoxin activity were derived from adults and only 20% from children. The results obtained suggest that the frequency of infection with $Tox^+$ *H. pylori* strains increases with the age of patients.

In this investigation, the titers of cytotoxic activity in broth culture filtrates were usually low: dilutions ranged from 1:2 to 1:64. As in our study use was made of unconcentrated broth culture filtrates, the titers were lower than those reported in literature (3). The concentrated culture supernatants from clinical *H. pylori* isolates investigated by Cover (3) produced vacuolization in titers ranging from 1:10 to 1:160. Glupczynski (21) showed that unconcentrated broth culture supernatant from *H. pylori* strains induced vacuolation in Intestine 407 cells in titers from 1:8 to 1:20.

In the next stage of the investigation we examined the presence of antibody to the cytotoxin, using a neutralization assay. Neutralizing antibodies to the cytotoxin were found in 100% of sera taken from patients infected with toxigenic *H. pylori* strains. 18.5% of patients infected with nontoxigenic strains

had antibodies with neutralized control cytotoxin activity. This might be explained by the loss of ability to produce cytotoxin in *in-vitro* culture, an insensitive assay for the cytotoxin, or by coinfection with both toxigenic and nontoxigenic isolates (22).

To sum up, neutralization of cytotoxin by the sera of individuals infected with *H. pylori* suggests that the cytotoxin is produced *in vivo*. We also found the neutralization test to be more sensitive than the vacuolization fest for the detection of infection by cytotoxin producing *H. pylori* strains.

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Author's address: G. Gościniak, Department of Microbiology University of Medicine, 4 Chałubińskiego Str., 50-368 Wrocław, Poland.