Pathogenicity and immune prophylaxis of cag pathogenicity island gene knockout homogenic mutants

Huan-Jian Lin, Jing Xue, Yang Bai, Ji-De Wang, Ya-Li Zhang, Dian-Yuan Zhou

H pylori

INTRODUCTION
Helicobacter pylori (H pylori) infected over half of human adults in the world, especially in Asian-Pacific countries. Only a small portion of patients display clinical symptoms such as peptic ulcer and gastric cancer[1-14]. The difference between the infection and the outcomes is determined by both bacterial factors and host responses. Among the bacterial factors, cag pathogenicity island (PAI) had been studied widely, while the immune response to the infection is the overwhelming host factor that affects the outcome of infection. In respect of the bacterial factors, cytotoxin associated gene A (cag A) protein is usually considered as a toxic marker of the bacterium. A related gene cluster, PAI encodes several proteins with the similar biological activities. To define the relationship between cagPAI and the pathogenicity of H pylori, three pairs of H pylori harboured cagPAI and their isogenic PAI knockout mutants were utilized. Their roles in the binding and apoptosis inducing activities as well as the immune protection were compared in vivo and in vitro.

MATERIALS AND METHODS

Methods
H pylori culture and H pylori sonicate (SON) preparation were performed by standard procedures There was no difference between the groups of wildtype and knockout bacteria in the rate of growing (Figure 1).

Flow cytometry was used to detect adhesion of H pylori binding to gastric epithelial cells H pylori strain (ATCC26695) was marked by 10 mL/L PKH-26 and washed three times. The liquid after the third washing was used in the control groups, then bound to gastric epithelial cells (KatoIII) (bacteria/cells = 300:1), and incubated at 37 °C for 60 min. After washed, the sample was detected by FACS.

Figure 1 Equivalent growth of wildtype and knockout strains after 2 d culture.

Table 1 cagA positive wildtype bacteria and their cagA knockout homogenic mutants

| H pylori          | cagPAI     |
|-------------------|------------|
| LC11              | + wildtype |
| AH244             | knockout cagPAI knockout of LC11 |
| 84183             | + wildtype |
| 2-1               | knockout cagPAI knockout of 84183 |
| 26695             | + wildtype |
| 8-1               | knockout cagPAI knockout of 26695 |

CONCLUSION: The role of cagPAI in the pathogenicity and prophylaxis of H pylori infection remains to be cleared.

REFERENCES
Lin HJ, Xue J, Bai Y, Wang JD, Zhang YL, Zhou DY. Pathogenicty and immune prophylaxis of cag pathogenicity island gene knockout homogenic mutants. World J Gastroenterol 2004; 10(22): 3289-3291.
http://www.wjgnet.com/1007-9327/10/3289.asp

INTRODUCTION: The role of cagPAI in the pathogenicity of H pylori infection as well as the stimulation of the proliferation rate of growing (Figure 1).
ELISA was used to detect apoptosis of gastric epithelial cells induced by H pylori. The basic mechanism was to detect the release of DNA combined histone. According to the recommended procedures, cracked Kato-III cells with anti-histone monoclonal antibody were incubated and color was developed after adding substrate. Absorbency at the 405 nm level was measured in the enzyme-tagging instrument. Then, the apoptotic index (AI) was calculated in comparison with control group.

Mice were divided into 4 groups, five mice each group, except for the natural death. PBS Group: 200 µL PBS/one mouse was fed once via mouth as negative control; CT Group: CT (10 µg) 200 µL/per mouse was fed once via mouth as adjuvant control Group; CagPAI positive Group: ultrasonic smashed H pylori 26695 (100 µL)and CT (10 µg) was fed at a time via mouth; CagPAI negative Group: ultrasonic smashed H pylori 8-1 (100 µL) and CT (10 µg) were fed once via mouth.

**Immunity procedures**

Before immunization and inoculation of H pylori, the mice were prohibited from water and food for over 12 h. Thirty min before immunization, the mouse was fed with 200 µL (0.01 mol/L) NaHCO3 solution to neutralize gastric acid. The experimental group and the control group were inoculated with antigens and adjuvants, immunized on d 0, 7, and 14. They were allowed to drink water 1 h after immunization. Each group was attacked with H pylori 106 CFU once every other day. 3 wk after the latest immunization. The mice were killed 8 wk after the latest attack.

**Evaluation of bacterial implantation**

The spleen and stomach were removed immediately after the mouse neck was broke. The spleen was kept in the axenic cell culture media temporarily and dealt with it for 4 h. The stomach was cut along the greater curvature, washed with axenic fluid, then some tissues were sampled for rapid urea enzyme test and histological examination. Histological examination was graded semi-quantitatively according to the standard as follows: 0 point: no H pylori; 1 point: 1-2 H pylori in some gastric pits; 2 points: 3-10 H pylori in majority of gastric pits and 3 points: over 10 H pylori in majority of gastric pits.

**Spleen cell proliferation text**

Spleen tissue was triturated into homogenates at asepsis environment. The spleen were counted and implanted in the 96 well plate (2×10^5 cells/well) containing 500 ng/mL PMA and 10 ng/mlonominycin RPMI1640 (including cow blood serum, mycillin, Hepes ect), H pylori ultrasonic smashed antigen was added into them in terms of 2.5, 5, 10 mg/mL, cultured for 24 h, then H'-thymidine was added, and cultured for 8 h, then the cell were collected and degraded. Each control group was compared with the blank control group in the count, and the proliferation quociety was calculated. Differential proliferation quociety equals each group’s proliferation quociety/PBS group’s proliferation quociety ×100%.

**Statistical analysis**

Data and their variance were analyzed using EXCEL software. P<0.05 was considered statistically significant.

**RESULTS**

**Adherence of CagPAI and H pylori**

CagPAI positive culture and mutant strains showed a different bacteria/cell ratio, and were, marked with PKH26, then they were incubated with stomach cell Kato-III, the adherence ability was measured with flow cytometry positive cells and mean fluorescence intensity (MFI) were calculated. No difference was found between wild strain and mutant strain. The result was displayed in Figure 2.

**Figure 2** Binding of cag PAI wildtype H pylori and their mutant to gastric epithelial cell Kato-III.

**Apoptosis-induced ability of cagPAI and H pylori**

CagPAI and mutant strain were cultured with gastric epithelia cells at the ratio 300:1 for 24 h. The cells were degraded, the cell apoptosis was detected by ELISA. We found that the apoptosis-induced ability of 26 695 was slightly higher than that of 8-1 strain, and no difference was found in other couples. The result is displayed in Figure 3.

**Figure 3** Apoptosis of gastric epithelial cells induced by positive cagPAI and knockout H pylori strains (determined by ELISA). ^P<0.05 vs the control.

**Immune prevention to the cagPAI and H pylori**

The protection rate of groups PBS, CT, 26695 and 8-1 was 0%, 0%, 40%, 60% (Table 2). CagPAI positive strain ATCC26695 and mutant strain 8-1 companied with mucous membrane adjuvant cholera toxin prevented H pylori from growing in the stomach of mice. There was no difference between the CagPAI positive strain and CagPAI negative strain. We found that full-protect rate of the two H pylori immune group was only 20% or so. It was found that immune was apparently decrease in semi-quantitative counting. No obvious difference was found between groups, and CT group had no prevention effect. The result is displayed in Figure 4.

**Table 2** Ultrasonic smashed substance’s protection against mice H pylori (result of urease test)

| Immune project | Urease test (+)/number of animals (piece) | Protecting ratio (%) |
|----------------|--------------------------------------|---------------------|
| PBS            | 5/5                                  | 0                   |
| CT             | 5/5                                  | 0                   |
| 26695 SON+CT   | 3/5                                  | 40*                 |
| 8-1 SON+CT     | 2/5                                  | 60                  |

*P<0.05, vs control groups.
SS1 strain which was used to infect animals was cag (+) strain, but we found that there was no difference in preventive effect between cagPAI (+) strain and mutant strain. Although there was no statistical difference, mutant strain had a higher protective trend. It showed that the immune prevention was not caused by protein coded by cagPAI. We do not support regarding cagPAI gene product is immune antigen of *H pylori* bacterin.

Cellular immunity, especially CD4+ T cellular immunity is a very important mechanism, which can help B cells produce sIgA. We selected the spleen cell proliferation test as the target in immune. Because B cells could not live in *vitro*, the proliferation cells were mainly T cells. Both cagPAI (+) and cagPAI (-) strain were stimulated to proliferate T cells, indicating that some special T cells clones are existed in spleen cells. The number of cagPAI mutant strains is more than that of cagPAI (+) strains, agreeing with the histology.

The effect of cagPAI on *H pylori* immune and pathogenesis is very complex and needs further study.

### DISCUSSION

CagA is cytotoxin correlate protein, which is one of *H pylori* nosogenetic factors. The PAI is regarded as one of the signs of *H pylori* infection. Early researches found that this positive sign was higher in peptic ulcer and gastric carcinoma than in gastritis. For example, foreign researchers found that anti-CagPAI in serum was positive in 93% of the gastric carcinoma patients, it is different in Asian-Pacific area, the positive ratio of the bacterial strain was much higher. A large number of reports approved that the pathogenicity of CagA (+) was stronger than that of CagA (-), inducing the production of IL-8 and GRO-α, EATN78 could produce and activated related nucleic transcription factors. Few reports about comparison of wildtype strain isogenic mutant strain *in vitro* were available. We studied three pairs of cagPAI wildtype strain and cagPAI gene-knockout mutant strain constructed by Dr. Bery. No difference was found between the growing speed and activity of the two strains.

Therer were many gene produces concerned with *H pylori* adherence, including NLBH, porins, BabA etc[4,9]. Our study showed that there was no difference in adherence to the epithelia of stomach between cagPAI (+) strain and cagPAI (-) strain.

CagPAI (+) strain could cause apoptosis and proliferation in stomach epithelia[10]. However, it was proved by ELISA in our research that *in vitro* cagPAI was not concerned with apoptosis-induced ability, which did agree with the result of Le’Negrate. They found that cagE in cagPAI participated in the process of Fas-induced *H pylori* causing stomach epithelial apoptosis due to different the strain. we chose two strains in three pairs of strains, so the result was probably accurate.

In the immune prevention of *H pylori* infection, it was reported that cagA could prevent infection of the cagPAI (+). In our research, SS1 strain which was used to infect animals was cag (+) strain, but we found that there was no difference in preventive effect between cagPAI (+) strain and mutant strain. Although there was no statistical difference, mutant strain had a higher protective trend. It showed that the immune prevention was not caused by protein coded by cagPAI. We do not support regarding cagPAI gene product is immune antigen of *H pylori* bacterin.

Cellular immunity, especially CD4+ T cellular immunity is a very important mechanism, which can help B cells produce sIgA. We selected the spleen cell proliferation test as the target in immune. Because B cells could not live in *vitro*, the proliferation cells were mainly T cells. Both cagPAI (+) and cagPAI (-) strain were stimulated to proliferate T cells, indicating that some special T cells clones are existed in spleen cells. The number of cagPAI mutant strains is more than that of cagPAI (+) strains, agreeing with the histology.

The effect of cagPAI on *H pylori* immune and pathogenesis is very complex and needs further study.

### REFERENCES

1. Bai Y, Zhang YL, Wang JD, Zhang ZS, Zhou DY. Construction of the non-resistant attenuated Salmonella typhimurium strain expressing *Helicobacter pylori* catalase. *Diyi Junyi Daxue Xuebao* 2003; 23: 101-105
2. Vandenplas Y. *Helicobacter pylori* infection. *World J Gastroenterol* 2000; 6: 20-31
3. Blaser MJ. Hypothesis: the changing relationships of *Helicobacter pylori* and humans: implications for health and disease. *J Infect Dis* 1999; 179: 1523-1530
4. Covacci A, Censi S, Bugnoli M, Petracca R, Burroni D, Macchia G, Massone A, Papini E, Xiang Z, Figura N, Rappuoli R. Molecular characterization of the 128-kDa immunodominant antigen of *Helicobacter pylori* associated with cytotoxicity and duodenal ulcer. *Proc Natl Acad Sci U S A* 1993; 90: 5791-5795
5. Blaser MJ, Perez-Perez GI, Kleanthous H, Cover TL, Peek RM, Chyou PH, Stemmermann GN, Nomura A. Infection with *Helicobacter pylori* strains possessing CagA is associated with an increased risk of developing adenocarcinoma of the stomach. *Cancer Res* 1995; 55: 2111-2115
6. Odenbreit S, Puls J, Sedlamayer B, Gerland E, Fischer W, Haas R. Translocation of *Helicobacter pylori* CagA into gastric epithelial cells by type IV secretion. *Science* 2000; 287: 1497-1500
7. Bai Y, Dan HL, Wang JD, Zhang ZS, Odenbreit S, Zhou DY, Zhang YL. Cloning, expression, purification and identification of immunogenic region of four *Helicobacter pylori* adhesin genes in AlpA gene. *Prog Biochem Biophys* 2002; 29: 922-926
8. Bai Y, Zhang YL, Chen Y, Wang JD, Zhou DY. Study of Immunogenicity and safety and adherence of conserved region of four *Helicobacter pylori* adhesin *in vitro*. *Prog Biochem Biophys* 2003; 30: 422-426
9. Bai Y, Zhang YL, Wang JD, Zhang ZS, Zhou DY. Cloning and immunogenicity of conserved region of adhesin gene of *Helicobacter pylori*. *Zhonghua Yi Xue Za Zhi* 2003; 83: 726-739
10. Bai Y, Wang JD, Zhang ZS, Zhang YL. Construction of the Attenuated Salmonella typhimurium strain expressing *Helicobacter pylori* conserved region of adhesin antigen. *Chin J Biotech* 2003; 19: 77-82
11. Bai Y, Chang SH, Wang JD, Chen Y, Zhang ZS, Zhang YL. Construction of the E.coli clone expressing adhesin BabA of *Helicobacter pylori* and evaluation of the adherence activity of BabA. *Diyi Junyi Daxue Xuebao* 2003; 23: 293-295
12. Bai Y, Zhang YL, Wang JD, Lin HJ, Zhang ZS, Zhou DY. Conservative region of the genes encoding four adhesins of *Helicobacter pylori* cloning, sequence analysis and biological information analysis. *Diyi Junyi Daxue Xuebao* 2002; 22: 869-871
13. Rudnicka W, Covacci A, Wadstrom T, Chmiela M. A recombinant fragment of *Helicobacter pylori* CagA affects proliferation of human cells. *J Physiol Pharmacol* 1998; 49: 111-119
14. Wang RX, Zhang LY, Yin DL, Muison RA, Shi Y. Protein kinase C regulates fas (CD95/APO-1) expression. *J Immunol* 1998; 161: 2201-2207

Edited by Wang XL and Zhang JZ. Proofread by Xu FM