Original Articles

Coexistence of *Helicobacter pylori* spiral and coccoid forms in experimental mice

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Subject headings *Helicobacter pylori*, antibodies; ELISA; spiral form; coccoid form; mouse

Abstract

AIM To infect mice with *Helicobacter pylori* and detect immune response against two form of *H. pylori*.

METHODS An isolate of *H. pylori* obtained from a patient with gastric cancer was used to infect mice. Fifty mice were divided into eight groups. Two groups served as negative control without any inoculation and internal negative control with 0.5M NaHCO3 and brain heart infusion (HBI), respectively. Mice in each experimental group were first inoculated with 0.5M NaHCO3 and then *H. pylori* suspension for 3 times at a 2-day interval. Mice from controls and infectious groups were sacrificed at a weekly interval postinfection. Gastric samples were trimmed, inoculated onto chocolate blood agar and then incujbated in microaerophilic atmosphere at 37iæ for 14 days. Sera were examined for immunoglobulins against *H. pylori* spiral and coccoid antigens by ELISA.

RESULTS After inoculation *H. pylori* was isolated in one mouse from one week postinfection. No *H. pylori* was detected in control mice. However, urease test was positive in 50% (5/10) control mice, 70% (7/10) mice inoculated with NaHCO3 and BHI and 77% (23/30) mice infected with *H. pylori*. The systemic immune responses of the mice to *H. pylori* strain were determined by ELISA. The mice showed immune responses to both *H. pylori* spiral and coccoid antigens one week after infection with *H. pylori*. The peak mean absorbances of antibodies against spiral and coccoid forms were four weeks postinfection which showed 6 and 18 times higher than that of negative control group respectively ($P<0.01$).

CONCLUSION Spiral and coccoid forms of *H. pylori* coexist in experimental mice studied.

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Received 1998-11-09

INTRODUCTION

Helicobacter pylori colonizes stomach of human being and causes gastritis and peptic ulcer[1]. It has been reported that this organism exists in two forms, spiral form and coccoid form[2,3]. Many investigations are being performed on whether coccoid form is degenerative or viable. Hua and Ho[3] reported that similar to the exponential cultures, ageing coccoid form produces alkaline phosphatase, acid phosphatase, leucin arylamidase and naphthol-AS-β1-phosphophdrolase and remains genetically unchanged suggesting that it is highly likely to be viable. It was found that specialized attachment sites such as the "adhesion pedestal", "cup-like indentation" and "abutting adhesion" were seen in the interaction between coccoids and epithelial cells. These adherence patterns were similar to those observed with spiral form in gastric biopsy specimens in vivo, suggesting coccoid could be a differentiated infective form of *H. pylori*[4]. Therefore, this form was suspected to play a critical role in the transmission of *H. pylori* and could be one of the causes of recrudescence of *H. pylori* infection after antibiotic treatment. In this study we investigated mouse immune response against *H. pylori* after oral infection with the bacterium and demonstrated coexistence of spiral and coccoid forms of *H. pylori* in mouse.

MATERIALS AND METHODS

Animals

Female BALB/c mice weighing about 25g were obtained from the Laboratory Animal Center, National University of Singapore. Mice were 5 weeks old when they were sent to laboratory and maintained for one week to allow them to adapt to the new environment. Mice were fed with a

commercial rodent diet and provided with sterile water.

Bacterial strain

An isolate of *H. pylori* H132 obtained from a patient with gastric cancer was used for this study. Strain H132 was isolated on chocolate blood agar base No.2 medium with 5% horse blood at 6 days of incubation of biopsy at 37℃ under microaerophilic environment. The bacterium was inoculated into brain heart infusion (BHI) broth supplemented with 10% horse serum and 0.4% yeast extract in a flask at 37℃ for 2 days. The brother culture was centrifuged at 4000×g for 20min. The supernatant was discarded and fresh BHI broth supplemented with 10% horse serum and 0.4% yeast extract was added to the pellet. The suspension was mixed gently. The inoculum was incubated at 37℃ for another 2 days. The concentration of spiral form was determined by spread plate method and bacterial counting chamber. In this experiment the concentration of *H. pylori* spiral form was about 1-5×10^8CFU/ml.

Animal experimental design

Fifty mice were included in this experiment. They were divided into eight groups. Two groups with ten mice each. One of these 2 groups served as negative control without any inoculation while the second group of 10 mice was inoculated with 0.3ml of 5 m $NaHCO_3$ and 0.3mL BHI serving as internal negative control. The remaining 30 mice were divided into six groups of 5 mice each. Mice in each experimental group were first inoculated with 0.3ml 0.5M $NaHCO_3$. An hour following that, 0.3ml of *H. pylori* suspension was administered with a gastric gavage. The procedure was repected 3 times at 2-day interval for these 30 mice.

Two mice from the controls and five mice from one infection group were sacrificed at weekly interval postinfection. Before being sacrificed, the mice were fasted for one day with free access to water. The mice were sacrificed by cervical dislocation. Stomachs were dissected for microbiological analyses. Five hundreds microliters of blood samples were taken from the heart of sacrificed mice for immune response studies.

Microbiological analyses

Gastric samples were examined within one hour. Samples of antrum were trimmed and inoculated on chocolate blood agars with antibiotics (vancomycin 6g/L, nalidixic acid 5 g/L, amphotericin 6 g/L and trimethoprin 10g/L) and without antibiotics. Plates were incubated in microaerophilic atmosphere at 37℃ for 14 days. Typical colonies were indentified by standard methods[5]. Blood of mice was collected from heart and centrifuged at 4000×g for ten minutes. Sera were removed from clot and stored at -20℃. Sera were examined for immunoglobulins against *H. pylori* by ELISA.

ELISA

Antigens of spiral and coccoid form of *H. pylori* were prepared by acid glycine extraction according to a modification method of Goodwin *et al*[6] as described by Vijayakumari *et al*[4]. Protein concentration was determined by the modified Lowry protein assay and the antigens were stored in 1ml aliquots at -20℃ until use.

The stock antigen solution was diluted in carbonate buffer (90% 0.5M Na_2CO_3 and 10% 0.5M $NaHCO_3$, pH 9.6). Aliquot of 200μL of the diluted antigen preparation was added to each well of a microtitre plate (Nunc) to give 1μg of antigen per well. Plates were left for 24 hours at 4℃. Excess antigen was removed and each were was replaced by 300μl of serum diluent (0.02% timerosal, 0.05% Tween 20 and 1g/L gelatin in PBS) and kept at 4℃ for at least 24 hours before used.

The sera to be tested were diluted 1:100 with serum diluent (0.02% thimerosal, 0.05% Tween 20 and 1g/L gelatin in PBS). A 100μl aliquot of each diluted test serum was added to each of the three wells of the microtitre plate. Plates were incubated at room temperature for 90 minutes. The plates were then washed three times with PBS containing 0.02% thimerosal and 0.05% Tween 20.

The second antibody was horse radish peroxidas labelled goat anti-mouse immunoglobulins (Dako) which react with all mouse IgG subclasses, IgA and IgM. It was diluted to 1:4000 with PBs containing 0.02% thimerosal, 0.02% BSA and 0.1% gelatin. A 100μl of the diluted secondary antibody was added to each well of the plate that was subsequently incubated at room temperature for another 90 minutes. It was then washed three times with PBS containing 0.02% thimerosal and 0.05% Tween 20 followed by washing two times with PBS containing 0.02% thimerosal only. A 100μl of substrate containing 40mg of o-phenylenediamine dihydrochloride (OPD, Sigma) and 40μl of 30% hydrogen peroxide (Merck) in 100ml phosphate citrate buffer (0.1M $C_6H_8O_7H_2O$ and 0.2M $Na_2HPO_4H_2O$) at pH 5.0 was added to each well. Plate was left at room temperature in the dark for 15 minutes. A-50μl of 2.5M H_2SO_4 was added to each well to stop reaction. The optical density (OD) of the reaction mixture was read immediately at wavelength of 490nm and 620nm reference filter using an ELISA

reader (Ceres 900 Bio-Ted).

RESULTS

After inoculation *H. pylori* was isolated in only one mouse from one week postinfection. The isolate was identified by spiral morphology, Gram negative, urease positive and naphthol-AS-B1-phosphohydrolase, leucine arylamidase and alkaline and acid phosphatase in API ZYM test. No *H. pylori* was detected in control mice and mice inoculated with $NaHCO_3$ and BHI. However, urease test was positive in 50% (5/10) control mice, 70% (7/10) mice inoculated with $NaHCO_3$ and BHI and 77% (23/30) mice infected with *H. pylori*.

In macroscopic findings, no visible gastric erosion or ulceration was seen in either *H. pylori*-infected mice or control mice.

The systemic immune responses of the mice to *H. pylori* strain were determined by ELISA. The distribution of OD values and the mean OD values of antibodies against *H. pylori* spiral antigens in different groups were shown in Figure 1. The sera of negative control group or the group inoculated with BHI gave very low absorbance except one mouse in negative control group with an OD of 0.616. The mice showed immune responses to *H. pylori* spiral antigens one week after infection with *H. pylori*. Two weeks postinfection, the mean OD value was doubled than that of negative control group (P<0.05). The peak mean absorbance was four weeks postinfection which showed six times higher than that of negative control group (P<0.01). However, mouse serum antibodies against *H. pylori* spiral antigens decreased gradually 5 weeks postinfection.

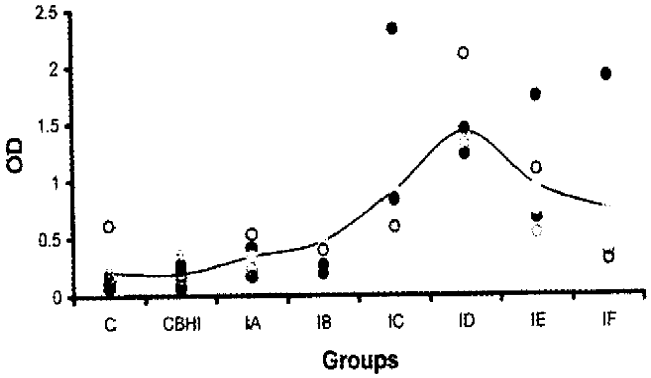

Figure 1 Distribution of Mouse antibodies against *H. pylori* spiral antigens.

C: negative control. CBHI: mice inoculated with $NaHCO_3$ and BHI. IA: one week postinfection. IB: two weeks postinfection. IC: three weeks postinfection. ID: four weeks postinfection. IE: five week postifinfection. IF: six weeks postinfection. The line represents mean OD value.

The mouse antibodies against *H. pylori* coccoid antigens were also detected. The distribution of OD values and the mean OD values of mouse serum antibodies against *H. pylori* coccoid antigens in different groups were shown in Figure 2. One week postinfection, the antibodies against coccoid antigens could be detected (P < 0.05) and were almost 4 times higher than that of control. Four weeks postinfection, mouse serum antibodies against *H. pylori* coccoid antigens in infection group were about 18 times higher than that of control group (P <0.01). The mouse serum antibodies against *H. pylori* coccoid antigens decreased gradually 5 weeks postinfection.

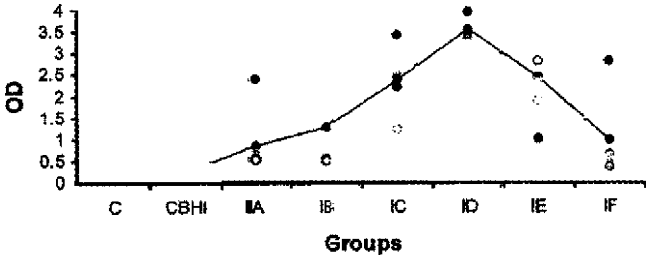

Figure 2 Distribution of OD of mouse antibodies against *H. pylori* coccoid antigens.

C: negative control. CBHI: mice inoculated with $NaHCO_3$ and BHI. IA: one week postinfection. IB: two weeks postinfection. IC: three weeks postinfection. ID: four weeks postinfection. IE: five weeks postinfection. IF: six weeks postinfection. The line represents mean OD value.

DISCUSSION

Anumber of animal models have been developed to provide information on pathogenesis, immunity and therapy for *H. pylori* infection. These include models in nonhuman primates[7,8], gontobiotic and conventional piglets[9,10]. However, there were contradictory reports in murine model study. Karita et al[11] detected colonization of *H. pylori* in the germfree athymic and euthymic mice up to 10 weeks after inoculation, but observed temporary colonization in conventional euthymic mice. Cantorna and Balish[12] tried in vain to colonize clinical strains of *H. pylori* in the alimentary tract of germfree rodents. Marchetti *et al*[13] were able to detect colonization of bacterium and gastric pathology in specific-pathogen-free mice up to 8 weeks following challenge with fresh clinical isolates whereas a laboratory strain failed to establish infection. Watanabe *et al*[14] successfully demonstrated the long-term infection with *H. pylori* induces adenocarcinoma in Mongolian

gerbils.

In the present study, attempts were made to colonized specific-pathogen-free mice with a fresh *H. pylori* isolate. *H. pylori* was isolated in one mouse. Culture of this fastidious micro-organism is always a challenge with regard to the proper conditions and interfering contaminants which may inhibit the growth of *H. pylori.* This may be the reason that in this study only one *H. pylori* strain was isolated from one mouse. Urease test was reported to be a highly effective detection method especially in the absence of other microflora as in human stomach or germfree animals[15,16]. However, this method of detection could be ineffective when used in conventional mice due to the presence of other urease-producing microflora which could lead to false positive results[17]. In this study, urease test was found positive in the gastric specimens of 50% (5/10) control mice, 70% (7/10) mice inoculated with $NaHCO_3$ and BHI and 77% (23/30) mice infected with *H. pylori*. The indiscrimination of urease test results among different experimental groups of mice made it difficult to determine whether positive urease tests were caused by *H. pylori* colonization or contaminating urease producers. The result is in agreement with report of Xia *et al*[17] that urease test may not be a suitable method of detection in conventional or specific-pathogen-free mice model.

The serum immune response of mice against *H. pylori* spiral and coccoid antigens was investigated. One week postinfection, the mouse antibodies against *H. pylori* increased. The peak mean OD values of antibodies were four weeks postinfection. Coincidently, the profile of antibodies against coccoid antigens was similar. It was shown that four weeks postinfection, antibodies against coccoid antigens increased much higher than that of spiral antigens. Could there be more coccoid form than spiral form in the stomach of mice where the environment is not so favourable to the growth of *H. pylori*? Bhatia *et al*[18] observed the effect of the presence of Lactobacillus acidophilus or its metabolites on inhibition of *H. pylori* growth in *in vitro* culture. The presence of significant number of *Lactobacillus* in gastrointestinal tract of mice might affect *H. pylori* and promote it to convert to coccoid form. The detection of antibodies against *H. pylori* spiral and coccoid antigens is consistent with the observation in patients with gastroduodenal disease that both forms coexist in stomach and could involve in the outcomes of different gastric disorders[19]. It was reported that after antibiotic treatment for *H. pylori* infection, if failure happened the majority of patients were recrudescence[20], i.e. the patients re-infected with same strains of *H. pylori.* This relapse occurred between 5-50 months after treatment, while 4 weeks after treatment those patients showed eradication of *H. pylori* based on micribiological methods. One reason could be that two forms of *H. pylori* coexist in stomach. When the environmental condition is unfavourable to *H. pylori*, most of the cells might convert to coccoid form which is difficult to be detected by commonly used microbiological methods.

In this study we demonstrated coexistence of spiral and coccoid forms of *H. pylori* in experimental mice. This factor should be considered in clinical management since coccoid form might be viable and pathogenic as suggested by some investigators[3,4].

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