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Th immune response induced by *H pylori* vaccine with chitosan as adjuvant and its relation to immune protection

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Abstract

AIM: To study the immunological protective effect of *H pylori* vaccine with chitosan as an adjuvant and its mechanism.

METHODS: Female BALB/c mice were randomly divided into seven groups and orally immunized respectively with PBS, chitosan solution, chitosan particles, *H pylori* antigen, *H pylori* antigen plus cholera toxin (CT), *H pylori* antigen plus chitosan solution, *H pylori* antigen plus chitosan particles once a week for four weeks. Four weeks after the last immunization, the mice were challenged twice by alive *H pylori* (1×10^9 CFU/mL) and sacrificed. Part of the gastric mucosa was embedded in paraffin, cut into sections and assayed with Giemsa staining. Part of the gastric mucosa was used to quantitatively culture *H pylori*. ELISA was used to detect cytokine level in gastric mucosa and anti- *H pylori* IgG1, IgG2a levels in serum.

RESULTS: In the groups with chitosan as an adjuvant, immunological protection was achieved in 60% mice, which was significantly higher than in groups with *H pylori* antigen alone and without *H pylori* antigen ($P < 0.05$ or 0.001). Before challenge, the level of IFN and IL-12 in gastric mucosa was significantly higher in the groups with chitosan as an adjuvant than in the control group and the group without adjuvant ($P < 0.05$ or 0.005). After challenge, the level of IFN and IL-12 was significantly higher in the groups with adjuvant than in the groups without adjuvant and antigen ($P < 0.05$ or 0.001). Before challenge, the level of IL-2 in gastric mucosa was not different among different groups. After challenge the level of IL-2 was significantly higher in the groups with adjuvant than in the control group ($P < 0.05$ or 0.001). Before challenge, the level of IL-10 in gastric mucosa was significantly higher in the groups with chitosan as an adjuvant than in other groups without adjuvant ($P < 0.05$ or 0.01). After challenge, the level of IL-10 was not different among different groups. Before challenge, the level of IL-4 in gastric mucosa was significantly higher in the groups with chitosan as an adjuvant than in other groups without adjuvant ($P < 0.05$). After challenge, the level of IL-4 was significantly higher in the groups with chitosan particles as an adjuvant than in the group with CT as an adjuvant ($P < 0.05$), and in the group with chitosan solution as an adjuvant, the level of IL-4 was significantly higher than that in control group, non-adjuvant group and the groups with CT ($P < 0.05$ or 0.001). The ratio of anti- *H pylori* IgG2a/IgG1 in serum was significantly lower in the groups with chitosan as an adjuvant than in the groups with CT as an adjuvant or without adjuvant ($P < 0.01$).

CONCLUSION: *H pylori* vaccine with chitosan as an adjuvant can protect against *H pylori* infection and induce both Th1 and Th2 type immune response.

Key words: *H pylori*; Chitosan; Vaccine; Adjuvant; Th immune response

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INTRODUCTION

Colonization of *H pylori* in the stomach is associated with the risk of developing different gastroduodenal diseases including chronic gastritis, peptic ulcer, mucosa-associated lymphoid tissue lymphoma and gastric cancer. The current treatment against *H pylori* infection is a combination therapy with two different antibiotics plus a proton-pump inhibitor with or without colloidal bismuth, which can eradicate the bacteria in most cases. However, this therapy has some major drawbacks, including high cost and development of antibiotic resistance. So vaccination

would be a suitable alternative or complement to antibiotic treatment to eradicate the bacteria. A large number of animal experiments have shown that immunization with *H pylori* antigen in combination with certain adjuvants can prevent and even eliminate *H pylori* infection[1-3]. At present, the effective adjuvants are cholera toxin (CT) and *Escherichia coli* heat-labile toxin (LT), but they cannot be used in humans due to their toxicity and side effects. There is no effective and non-toxic adjuvant for humans. Chitosan is a deacetylated product of chitin, which is non-toxic, non-irritable, non-antigenic, bioadhesive, biocompatible and biodegradable[4]. Some studies showed that chitosan as an immune adjuvant could effectively enhance the immune response of local mucosa[5-7] and antigen presentation[8]. But there is no report about chitosan as an adjuvant for *H pylori* vaccine. In the present study, mice were vaccinated with a bacterial whole-cell sonicate of *H pylori* plus chitosan and then challenged by *H pylori*, in order to delineate its effect and possible mechanisms against *H pylori* infection.

MATERIALS AND METHODS

Reagents and bacterial strains

Chitosan and 88.5% deacetylated chitosan powder were purchased from Qisheng Biological Products Limited Company, Shanghai. Campylobacter agar base and Brucella broth were purchased from Shanghai Reagent Providing and Research Centre for Diarrhea Disease Control, China. ELISA kits for IL-2, IFN, IL-12, IL-4 and IL-10 were purchased from Bender MedSystem (USA). Sheep-anti-mouse IgG1 and IgG2a peroxidase conjugate was purchased from Zymed-Laboratories INC (USA). *H pylori* Sydney strain 1 (SS1) was kindly provided by *H pylori* Strain Pool, China.

Animals

Female BALB/c mice, 6-8 wk of age and 22.5 g of mean weight, were purchased from Animal Centre of China Academy of Sciences, Shanghai (licensing number: SCXK (HU) 2002-0010). The mice were housed in a specific pathogen-free environment with free access to food and water.

Culture of H pylori

The Sydney strain of *H pylori* was used throughout the experiments. *H pylori* was grown in Campylobacter agar base containing 7.5% sheep blood under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) at 37℃ for 2-3 d, and harvested from the agar plates by eluting with broth culture. Bacterial density was detected at 660 nm. One OD was 10^8/mL.

Preparation of H pylori antigen

After cultured for 2-3 d, the Sydney strain of *H pylori* was eluted with phosphate buffer saline (PBS), and centrifuged at 1000 × *g* for 10 min. The pellet was washed three times with PBS, and cells were disrupted by sonication. After centrifugation at 8000 × *g* for 30 min at 4℃, supernatant was collected and stored at -85℃ until use. Protein concentration was determined.

Preparation of chitosan particles

Ddeacetylated (88.5%) chitosan powders were suspended in saline to the final concentration of 10 mg/mL and sonicated at 80 HZ output power two times for 5 min each with a sonicator, at 1 min intervals. Following a light centrifugation (50 r/min, 10 min), small particles in the supernatants were removed, filtered through a 400/2800 stainless steel mesh, and further centrifuged to collect the particles at 1400 r/min for 10 min as previously described[9].

Preparation of chitosan solution

Deacetylated (88.5%) chitosan stock solution was prepared at 3% (w/w) in 0.8% (v/v) acetic acid 0.9% (w/v) saline and heated at 37℃ to dissolve[10].

Vaccination and challenge of mice

BALB/c mice were randomly divided into 7 groups: (1) control (PBS alone), 15 mice; (2) chitosan solution alone, 12 mice; (3) chitosan particles alone, 13 mice; (4) *H pylori* antigen alone, 15 mice; (5) *H pylori* antigen plus chitosan solution, 15 mice; (6) *H pylori* antigen plus chitosan particles, 15 mice; (7) *H pylori* antigen plus CT, 12 mice.

BALB/c mice were orally immunized with *H pylori* antigen (1.2 mg/mouse), chitosan particles (500 μg/mouse), CT (5 μg/mouse), and 0.5% chitosan solution, once a week for four weeks. Chitosan particles were whipped into a stable emulsion by sonication with a sonifier at 20 HZ before immunization[9]. Four weeks after the last immunization, the mice were challenged twice by alive *H pylori* (1×10^9/mL, 0.5 mL/mouse).

Before challeng, five mice were randomly collected respectively from the control group, *H pylori* antigen group, *H pylori* antigen plus chitosan solution group and *H pylori* antigen plus chitosan particles group and killed. Samples were collected for further use. The other mice were killed 4 wk after the last challenge. Blood was collected by removing eyeballs immediately before the mice were killed. The stomach was isolated for histology, examination of *H pylori*, and determination of cytokine level. The stomach was washed in sterile 0.8% NaCl and cut longitudinally into two pieces. One was used for quantitative culture of *H pylori*, while the other was used for histology and determination of cytokine expression.

Assessment of bacterial load in stomach

The bacterial load in the stomach was determined by quantitatively culture of *H pylori* and improved Giemsa staining, when both of them negative was difined as *H pylori* negative and when anyone of them positive was difined as *H pylori* positive. For assessment of *H pylori* colonization, weighed stomachs were homogenized in 0.3 mL of Brucella broth, 1/4 and 1/8 serial dilutions were spread over the surfaces of serum plates containing 10 mg of vancomycin, 2500 IU of polymyxin and 5 mg of trimethoprim per liter. The plates were incubated for 3-7 d. Colonies were counted to determine the CFU per gram of stomach tissue. In Giemsa staining, the colonization was

Table 1 Rates of immune protection of *H pylori* vaccine with different kinds of adjuvant

Groups	*n*	*H pylori* positive *n* (%)	Rates of immune protection (%)
(1) Control	10	10 (100)	0
(2) Chi-solution	12	12 (100)	0
(3) Chi-particles	13	13 (100)	0
(4) *Hp* antigen	10	10 (100)	0
(5) *Hp* antigen + CT	12	5 (41.67)	58.33[d]
(6) *Hp* antigen + chi-solution	10	4 (40)	60[a,b]
(7) *Hp* antigen + chi-particles	10	4 (40)	60[a,b]
$P < 0.001$			

[a]$P < 0.05$ *vs* (1) and (4) groups; [b]$P < 0.01$ *vs* (2) and (3) groups; [d]$P < 0.01$ *vs* (1)-(4) groups; chi = chitoson.

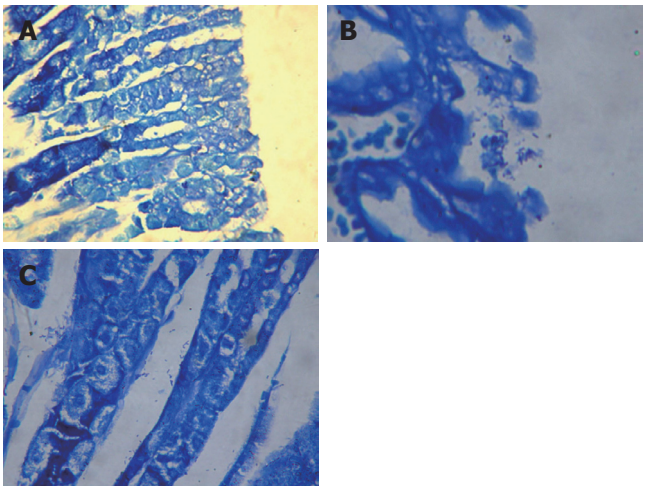

Figure 1 *H pylori* stained with Giemsa in gastric tissue of mice. No *H pylori* found in *H pylori* antigen + chi-particles group (**A**) , and lots of *H pylori* found on surface of gastric mucosa (**B**) and in gastric foveola (**C**) of control group (Giemsa dyeing × 400).

assessed by semiquantitative analysis of *H pylori* in gastric mucosa (nil = 0; 1-2 call/crypt = 1; 3-10 call/crypt = 2; 11-20 call/crypt = 3; > 21 call/crypt = 4)[11].

Determination of cytokines in gastric mucosa by ELISA

After weighed, the gastric mucosa was homogenized in 1.3 mL PBS and the homogenates were centrifuged at 3000 × *g* at 4℃ for 20 min. Supernatant was harvested and diluted at 1:2. For quantification of IL-2, IFN, IL-12, IL-4 and IL-10 in the supernatants, commercial enzyme-linked immunosorbent assay (ELISA) systems were used. The limit of detection was 3 pg/mL for IL-4, 22 pg/mL for IL-10, and 6 pg/mL for IL-12, 8 pg/mL for IL-2, and 8 pg/mL for IFN. The results were represented as pg/mg wet weight of gastric mucosa.

Determination of H pylori-specific antibodies in serum

H pylori-specific antibodies (IgG1 and IgG2) in serum were detected by indirect ELISA. Each well of microtiter plates was coated with 100 μL of *H pylori* antigen solution at the concentration of 20 μg/mL in 0.01 mol/L sodium carbonate-bicarbonate buffer (pH 9.6) overnight at 4℃. After suction, 200 μL of 0.1% BSA solution in PBS-Tween-20 was added to each well and further incubated for 1 h at 37℃. After washed three times with PBS-Tween-20, 100 μL of diluted (1/100) serum samples was added to wells and incubated for 1 h at 37℃. After washed three times with PBS-Tween-20, 100 mL of sheep-anti-mouse IgG1 and IgG2a peroxidase conjugate was added to wells and incubated further for 1 h at 37℃. After washed three times with PBS-Tween-20, 100 μL of o-phenylendiamine solution containing 0.01% H_2O_2 was added to wells and incubated for 30 min at room temperature. The reaction was stopped by the addition of 50 μL of 2 mol/L sulfuric acid, and color development was measured by a plate reader at 492 nm. The results were represented as A value of samples/A value of control.

Table 2 *H pylori* colonization score in gastric mucosa

Groups	*n*	*H pylori* colonization score			
		0	1	2	3
(1) Control	10	0	2	4	4
(2) Chi-solution[d]	12	1	8	2	1
(3) Chi-particles[d]	13	0	10	1	2
(4) *Hp* antigen[c]	10	1	5	2	2
(5) *Hp* antigen + CT[a,d]	12	7	2	1	2
(6) *Hp* antigen + chi-solution[b]	10	6	4	0	0
(7) *Hp* antigen + chi-particles[b]	10	7	3	0	0
$H = 57.181$, $P < 0.001$					

[b]$P < 0.01$ *vs* (1)-(4) groups; [a]$P < 0.05$ *vs* (4) group; [c]$P < 0.05$ *vs* (1) group; [d]$P < 0.01$ *vs* (1) group; chi = chitoson.

Statistical analysis

Differences in the protection rate against *H pylori* infection were analyzed by Fisher's exact test. Differences in *H pylori*-specific antibody and cytokine level in gastric mucosa among experimental groups were detected for statistical significance by analysis of variance or Student's *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

Rates of immune protection against H pylori infection

Significant difference was found in the rates of immune protection of vaccines with different adjuvants against *H pylori* infection ($P < 0.001$), and the protection rates were significantly higher in the groups with adjuvant than in the groups without adjuvant and antigen ($P < 0.05$ or 0.001, Table 1).

H pylori colonization score in gastric mucosa

Significant difference was observed in density of *H pylori* colonization among different groups ($P < 0.001$), and the density of *H pylori* colonization was significantly lower in the groups with adjuvant than in the groups without adjuvant or antigen ($P < 0.05$ or 0.001, Table 2, Figure 1).

CFU/g of H pylori in gastric mucosa

Significant difference was found in *H pylori* colonization among different groups, as indicated by the number of CFU/g of *H pylori* in gastric mucosa ($P = 0.001$). The number of CFU/g of *H pylori* was significantly lower in the groups with chitosan as an adjuvant than in other

Table 3 Density of *H pylori* colonization in gastric mucosa

Groups	*n*	Median of *H pylori* colony number (CFU/g)
(1) Control	10	4.74×10^5
(2) Chi-solution	12	0.73×10^5
(3) Chi-particles	13	1.83×10^5
(4) *Hp* antigen	10	0.48×10^5
(5) *Hp* antigen + CT	12	0
(6) *Hp* antigen + chi-solution	10	0
(7) *Hp* antigen + chi-particles	10	0
$H = 27.43$, $P = 0.001$		

chi = chitoson.

groups without adjuvant ($P < 0.05$ or 0.01). There was no significant difference in *H pylori* colonization among the groups with CT as an adjuvant or without adjuvant ($P > 0.05$, Table 3).

Cytokine level in gastric mucosa

Before challenge, there was no significant difference in IL-2 level among different groups. But there was significant difference in levels of IFN, IL-12, IL-10 and IL-4 among different groups ($P < 0.05$ or 0.005), which were significantly higher in the groups with chitosan as an adjuvant than in other groups without adjuvant ($P < 0.005$, Table 4). After challenge, there was significant difference in levels of IFN, IL-12, IL-2 and IL-4 among different groups. The level of IL-2 was significantly higher in the groups with chitosan solution as an adjuvant than in the groups without antigen ($P < 0.05$ or 0.001). Moreover, the level of IL-2 was significantly higher in the groups with chitosan particles and *H pylori* antigen alone than in the control group and the group with chitosan solution alone ($P < 0.05$). The level of IFN and IL-12 was significantly higher in the groups with adjuvant than in the groups without antigen or adjuvant ($P < 0.05$ or 0.001). The level of IL-4 was significantly higher in the groups with chitosan particles than in the groups with CT ($P < 0.05$). Moreover, the level of IL-4 in the groups with chitosan solution as adjuvant was significantly higher than in other groups with chitosan solution alone, *H pylori* antigen alone and CT as adjuvant ($P < 0.05$). There was no significant difference in the level of IL-10 among different groups ($P > 0.05$, Table 5).

There was significant difference in the levels of IL-2, IFN, IL-12, IL-10 and IL-4 before and after challenge by alive *H pylori*. The levels of IL-2, IFN and IL-12 were significantly higher in the groups with *H pylori* antigen after challenge than those before challenge ($P < 0.05$). The levels of IL-10 were significantly lower in the groups with adjuvant after than before challenge ($P < 0.05$). The level of IL-4 was significantly lower in the groups with chitosan particles as adjuvant after challenge than before challenge ($P < 0.05$). The level of cytokines before and after challenge was not significantly different in the other group ($P > 0.05$, Figure 2).

Level of anti-Hp IgG2a, IgG1 and ratio of IgG2a/IgG1 in serum

After challenge, there was significant difference in the levels of anti-Hp IgG2a, IgG1 and ratio of IgG2a/IgG1 in serum among different groups ($P < 0.001$). The level of anti-Hp IgG2a was significantly higher in the groups with chitosan particles than in other groups with *H pylori* antigen alone or chitosan alone and the control group ($P < 0.05$ or 0.001). The level of anti-Hp IgG2a was significantly higher in the groups with CT or chitosan solution than in the control group ($P < 0.05$). The level of anti-Hp IgG1 was significantly higher in the groups with adjuvant than in other groups with chitosan alone and in the control group ($P < 0.05$). Moreover, the level of anti-Hp IgG1 was significantly higher in the groups with *H pylori* antigen than in the control group ($P < 0.05$). The ratio of IgG2a/IgG1 was significantly lower in the groups with chitosan than in the groups with CT, *H pylori* antigen alone and in the control group ($P < 0.01$, Table 6).

DISCUSSION

All effective vaccines need a suitable antigen-presenting system that depends on adjuvant or vehicle[12]. *H pylori* antigen alone cannot induce protective immune response. Antigen-presenting system can introduce exogenous antigen into cells, and can enhance the immune response to antigen and even change the type of immune response. Many studies showed that chitosan can effectively promote local immune response and enhance antigen presentation[5-8]. In this study, we evaluated a vaccine delivery system with chitosan, the rate of immune protection of vaccine with chitosan as adjuvant against *H pylori* infection was 60%, which was significantly higher than with *H pylori* antigen alone or chitosan alone, indicating that chitosan can be used as a mucosa adjuvant of *H pylori* instead of CT.

$CD4^+$ helper T cells (Th) in mice can be divided into Th1 and Th2 subtypes. Th1 cells can synthesize and secrete IL-2, IL-12, IFN-γ, take part in cell-mediated immune response and promote the production of IgG2a by B cells. Th2 cells can also secrete cytokines such as L-4, IL-5, and IL-10, help B cells produce antibody, take part in humoral immune response and promote the production of antibodies such as IgG1, IgE and IgA. Negative feedback exists in the two types. Th can regulate immune response. Recently, different Th response types induced by *H pylori* vaccine and their effects in immune response are the main point in the mechanism of *H pylori* vaccine, indicating that the balance of Th1 and Th2 response is involved in the protection mechanism of *H pylori* vaccine. In natural *H pylori* infection, the presence of Th1 is the primary immune response. Th1-mediated cell immunity cannot protect against *H pylori* infection and is related to the severity of *H pylori* infection[13-15]. At the same time, $CD4^+$ Th2 secreting IL-4 and IL-10 is depressed, thus IgA secreted by B cells is reduced, leading to persistent *H pylori* infection. After immunization, the type of immune response has changed from Th1 to Th2. Hatzifoti *et al*[16] reported that immunization of mice with DNA vaccine encoding urease B genes could up-regulate the expression of Th2 cytokine IL-10. Mohammadi *et al*[17] found that stimulating immune response to Th2 could reduce the number of *H pylori* and the intensity of inflammation of gastric mucosa, indicating that if the type of immune response

Table 4 Levels of IL-2, IFN-γ, IL-12, IL-10 and IL-4 in gastric mucosa before challenge (mean ± SD)

Groups	*n*	IL-2	IFN-γ	IL-12	IL-10	IL-4
(1) Control	5	19.9 ± 12.6	28.6 ± 10.2	118.9 ± 46.2	67.2 ± 32.5	4.19 ± 2.95
(2) *Hp* antigen	5	20.6 ± 3.5	33.6 ± 13.7	165.3 ± 47.4	104.3 ± 19.1	6.49 ± 2.61
(3) *Hp* antigen chi-solution	5	28.6 ± 9.3	58.5 ± 12.2[b]	283.5 ± 93.7[c,d]	255.3 ± 131.8[c,d]	14.70 ± 8.73[a]
(4) *Hp* antigen+ chi-particles	5	27.6 ± 13.9	59.8 ± 15.2[b]	283.4 ± 99.6[c,d]	237.1 ± 98.3[c,d]	14.48 ± 6.84[a]
		F = 0.739	F = 7.948	F = 6.083	F = 6.228	F = 4.189
		P = 0.544	P = 0.002	P = 0.006	P = 0.005	P = 0.023

[a]P < 0.05 *vs* (1) and (2) groups; [b]P < 0.01 *vs* (1) and (2) groups; [c]P < 0.05 *vs* (2) group; [d]P < 0.01 *vs* (1) group; chi = chitoson.

Table 5 Levels of IL-2, IFN-γ, IL-12, IL-10 and IL-4 in gastric mucosa after challenge (mean ± SD)

Groups	*n*	IL-2	IFN-γ	IL-12	IL-10	IL-4
(1) Control	10	34.3 ± 11.8	48.7 ± 27.8	233.7 ± 125.7	86.4 ± 38.2	3.87 ± 1.99
(2) Chi-solution	11	31.8 ± 27.4	56.8 ± 26.1	308.5 ± 178.7	100.7 ± 52.6	4.47 ± 1.89
(3) Chi-particles	10	51.1 ± 42.6	58.8 ± 28.1	311.3 ± 129.6	88.4 ± 51.7	7.21 ± 4.02[g]
(4) *Hp* antigen	9	97.9 ± 64.2[a]	59.4 ± 15.0	319.4 ± 136.3	89.3 ± 29.01	4.89 ± 3.15
(5) *Hp* antigen + CT	10	80.9 ± 60.2[c]	93.6 ± 23.5[e,f]	487.0 ± 289.3[e,f]	81.8 ± 49.5	3.67 ± 1.76
(6) *Hp* antigen + chi-solution	10	124.3 ± 75.2[b]	107.5 ± 42.0[d]	525.6 ± 112.2[d]	108.5 ± 39.0	8.78 ± 4.96[h,i]
(7) *Hp* antigen + chi-particles	10	88.6 ± 57.0[a]	105.9 ± 48.1[d]	554.0 ± 164.4[d]	93.1 ± 39.2	6.59 ± 1.38[k]
		F = 3.370	F = 6.346	F = 5.448	F = 0.745	F = 3.214
		P = 0.002	P < 0.001	P < 0.001	P > 0.05	P = 0.003

[a]P < 0.05 *vs* (1) and (2) groups; [b]P < 0.001 *vs* (1)-(3) groups; [c]P < 0.05 *vs* (1) group; [d]P < 0.01 *vs* (1)-(4) groups; [e]P < 0.05 *vs* (2)-(4) groups; [f]P < 0.01 *vs* (1) group; [g]P < 0.05 *vs* (1), (2) and (5) groups, [h]P < 0.01 *vs* (1), (2) and (5) groups; [i]P < 0.05 *vs* (4) group; [k]P < 0.05 *vs* (5) group; chi = chitoson.

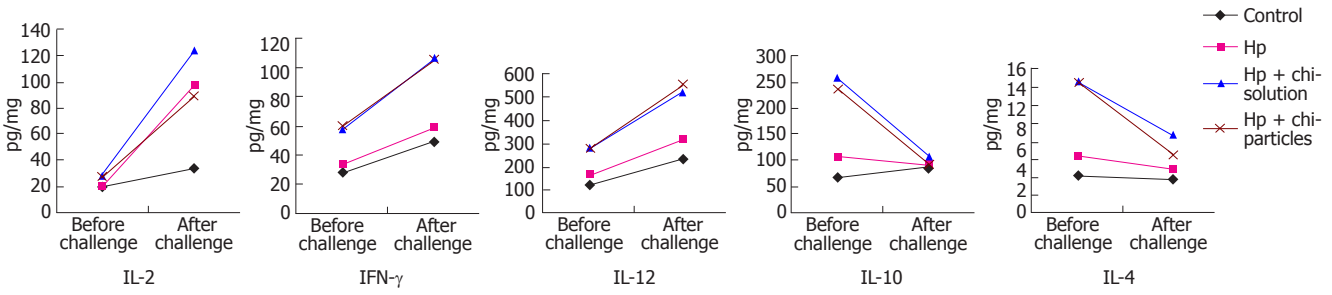

Figure 2 Levels of IL-2, IFN-γ, IL-12, IL-10 and IL-4 in gastric mucosa before and after challenge in different groups.

Table 6 Levels of anti-HpIgG2a, IgG1 and ratio of IgG2a/IgG1 in serum after challenge (mean ± SD)

Groups	*N*	IgG2a	IgG1	IgG2a/IgG1
(1) Control	10	4.44 ± 2.09	2.77 ± 1.18	2.13 ± 1.24
(2) Chi-solution	11	6.28 ± 3.62	3.96 ± 2.32	1.64 ± 0.85
(3) Chi-particles	10	5.56 ± 2.27	4.57 ± 2.09	1.43 ± 0.37[g,h]
(4) *Hp* antigen	9	9.21 ± 6.80[a]	6.43 ± 4.88[a]	2.356 ± 1
(5) *Hp* antigen + CT	10	11.22 ± 5.52[a]	7.71 ± 5.11[d,e]	2.19 ± 0.86
(6) *Hp* antigen + chi-solution	10	11.48 ± 5.55[a]	9.04 ± 5.35[b]	1.06 ± 0.4[f]
(7) *Hp* antigen + chi-particles	10	16.13 ± 10.20[b]	8.02 ± 5.29[c,d]	1 ± 0.37[f]
		F = 4.572	F = 10.308	F = 3.780
		P < 0.001	P < 0.001	P < 0.001

[a]P < 0.05 *vs* (1) group; [b]P < 0.01 *vs* (1)-(3) groups; [c]P < 0.05 *vs* (2) and (3) groups, [d]P < 0.01 *vs* (1) group; [e]P < 0.05 *vs* (2) group; [f]P < 0.01 *vs* (1), (4) and (5) groups; [g]P < 0.05 *vs* (5) group; [h]P < 0.01 *vs* (4) group.

induced by immunization has changed from Th1 to Th2, *H pylori* colonization in gastric mucosa can be inhibited by producing Th2 cytokines such as IL-4. Saldinger *et al*[18] immunized *H. felis* -infected mice with oral rUreB and CT, and found that infected mice were cured 3 wk after the 4th immunization. they also found that immunization could lead to the proliferation of $CD4^+$ T cells in the spleen of mice accompanying gradual decrease in IFN-γ and increase in IL-4, indicating that immunization of mice with rUreB and CT could induce gradual Th2 immune

response , thus eliminating *H. felis*. However, some studies showed that Th1 and Th2 response together is better than Th2 response only in preventing *H pylori* infection[19]. Gottweln *et al* reported that the two kinds of *H pylori* vaccine with complete Freund's or aluminum as adjuvant, which induce Th1 and Th2 immune response respectively, could induce protective immune response *in vivo* in mice, indicating that Th1 and Th2 immune response have the effect of immune protection. Eisenberg *et al*[21] immunized neonatal and adult mice with *H pylori* antigen and complete or incomplete Freund's adjuvant and found that the number of T cells producing Th1 cytokines like IFN-γ, IL-2 and Th2 cytokines like IL-4 IL-5 increased, have the effect of immune protection. Sommer *et al*[22] used CpG oligonucleotide as adjuvant to induce Th1 immune response, and found that it could not protect against *H pylori* infection but lead to more serious gastritis. Thus in the protective immune response to *H pylori*, Th1 and Th2 are needed. It must have a balance between Th1 and Th2 to achieve immune protection and to prevent tissue from damaging by serious inflammation[23].

In our study, before challenge by alive *H pylori*, the levels of IFN, IL-12, IL-4 and IL-10 were significantly higher in the groups with chitosan than in other groups without adjuvant, indicating that in the early stage of immune, they induce immune response to both Th1 and Th2. But 4 wk after challenge the levels of IL-2, IFN and IL-12 were significantly higher in the groups with adjuvant than in groups without adjuvant or in control group, indicating that after challenge they could promote the production of Th1 cytokines. The levels of IL-4 and IL-10 were significantly lower after challenge than before challenge. Chen *et al*[24] found that in the early stage of *H pylori* challenge (5 wk), the level of Th2 cytokines was significantly lower, even undetectable. Yu *et al*[25] found that after oral immunization with *H pylori* antigen plus LT, Th1 and Th2 immune response are induced in the early and advanced stage respectively, indicating that oral immunization can induce Th1 as well as Th2 immune response, which is in accordance to our study. In our study, after *H pylori* challenge the level of IL-4 was significantly higher in the group with chitosan particles as adjuvant than in the group with CT as adjuvant, and the level of IL-4 was significantly higher in the group with chitosan solution as adjuvant than in the groups with CT as adjuvant, chitosan solution alone and *H pylori* antigen along, indicating that *H pylori* vaccine with chitosan is better than that with CT in inducing Th2 cytokines especially IL-4. In addition, after *H pylori* challenge the levels of anti-Hp IgG2a and IgG1 were significantly higher in the groups with adjuvant than in the control group. IgG2a and IgG1 were induced by Th1 and Th2 immune response respectively, indicating *H pylori* vaccine can up-regulate Th1 and Th2 immune response. But the ratio of IgG2a/IgG1 in serum was significantly lower in the groups with chitosan as adjuvant than in other groups with CT as adjuvant or *H pylori* antigen alone, indicating that chitosan as an adjuvant can reverse the inhibition of Th2 induced by *H pylori* infection and return to the balance between Th1 and Th2, thus contributing to the immune protection against *H pylori* infection.

Chitosan can regulate immune response. Studies showed that chitosan as a mucosa adjuvant of the vaccine against meningococci and Bordetella bronchiseptica could successfully induce protective immune response[26,27]. Seferian *et al*[28] inoculated BALB/c mice with chitosan plus β- human chorionic gonadotropin, and found that the mixed immune response to IgG1, IgG2a and IgG2b antibodies could be observed in the groups with chitosan emulsion as adjuvant. Bivas-Benita *et al*[29] immunized mice with oral Toxoplasma gondii GRA1 protein and DNA vaccine-loaded chitosan particles, and successfully induced specialized anti- GRA1 IgG1 and IgG2a, indicating that it can enhance immune respons to Th1 and Th2. McNeela *et al*[30] found that immunization with chitosan plus diphtheria toxin could induce both systemic and local specific humoral immune response. At the same time, it could enhance immune response to Th1 and Th2.

In conclusion, *H pylori* vaccine with chitosan as adjuvant could induce Th1 and Th2 immune response, and partially reverse the inhibition of Th2 induced by *H pylori* infection and recover the balance between Th1 and Th2. As an adjuvant of *H pylori* vaccine, chitosan is better than CT in immune protection against *H pylori* infection.

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