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Original Article

Immunological response of recombinant H. pylori multi-epitope vaccine with different vaccination strategies

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Abstract: Objective: To investigate different protective effects of recombinant H. pylori multi-epitope antigen (rIB) with cholera toxin subunit B (rCTB) as the intramolecular/extramolecular adjuvant though different immunization routes in a Helicobacter pylori infected mouse model. Methods: By using rCTB as the intramolecular/extramolecular adjuvant of rIB, BALB/c mice were immunized through oral administration or intramuscular injection, on day 0, 14, 28. Every 14 days, ELISA was used to detect serum specific IgG and IgA titers after immunization. After the last immunization, H. pylori SS1 challenge was performed, and urease test, Gram staining after smearing of mouse gastric tissue, PCR, pathology and immunohistochemistry were used to evaluate preventive effect of the recombinant protein vaccine. Results: After immunization three times, intramolecular injection could induce high titers of serum specific IgG antibody, and the antibody titer in rIB group, rCTB+rIB and rBIB group was 2000, 5000 and 7500, respectively ($P < 0.05$). Specific IgA antibody was only detected in rBIB oral administration group. The immune protection rate in rBIB oral administration group was significantly higher than that in rBIB intramolecular injection group (33.3% vs. 83%), indicating significant difference. Conclusion: rCTB has good intramolecular/extramolecular immune adjuvant effects, and its intramolecular immune adjuvant effect is better. Both intramolecular injection and oral administration of rBIB have immune protective effect against H. pylori challenge, and oral administration of rBIB exerts better immune protective effect.

Keywords: Helicobacter pylori, recombinant vaccine, cholera toxin subunit B, adjuvant, immunological response

Introduction

Helicobacter pylori (H. pylori) is an important pathogenic bacterium discovered by Australian scholars Warren and Marshall in 1982. A large amount of research on molecular biology, epidemiology and related diseases have confirmed that H. pylori is an important pathogenic factor of gastritis, peptic ulcer, gastric precancerous lesion, gastric cancer, gastric MALT lymphoma, etc. Helicobacter pylori is the pathogenic bacterium with the highest infection rate in the world, and half of the population in the world is infected by Helicobacter pylori [1]. In recent years, the overuse of antibiotics results in increasingly prominent problems, including low H. pylori eradication rate, reduced drug choice after failure of eradication and high recurrence rate. Therefore, the development of safe and effective vaccine becomes particularly important [2-5].

UreB is the urease activity unit, which is relatively conservative. It has very strong antigenicity and is the key for bacterial colonization in stomach. UreI is a H. pylori urea channel protein. Mollenhauer et al. have found that UreI gene deletion mutants of H. pylori cannot colonize in stomach. Cholera toxin B (CTB) is a nontoxic receptor binding subunit produced by Vibrio cholerae. CTB can be used as a stable carrier of some exogenous polypeptide to increase the immunogenicity of the epitope of a fused antigen so that the human body can produce a relatively strong immune response to increase the immune protection [6-9].

In earlier stage, genetic engineering recombinant technology was used to construct rCT. UreI and UreB were used as core antigen components to construct rIB, and rBIB was constructed by using rCTB as the intramolecular adjuvant. It's a basic study on vaccine components

Table 1. Treatments in different vaccination group

Groups (n = 6)		0 D	14 D	28 D
Bland group (PBS) (0.2 ml/20 g)		-	-	-
Intramuscular injection (2.5 mg/kg)	rCTB	2.5 mg/kg	2.5 mg/kg	2.5 mg/kg
	rIB	2.5 mg/kg	2.5 mg/kg	2.5 mg/kg
	rCTB+rIB	2.5 mg/kg	2.5 mg/kg	2.5 mg/kg
	rBIB	2.5 mg/kg	2.5 mg/kg	2.5 mg/kg
Oral group (10 mg/kg)	rCTB	10 mg/kg	10 mg/kg	10 mg/kg
	rIB	10 mg/kg	10 mg/kg	10 mg/kg
	rBIB	10 mg/kg	10 mg/kg	10 mg/kg
	rCTB+rIB	10 mg/kg	10 mg/kg	10 mg/kg

Note: "-" does not contain the components in the mixed solution.

Table 2. Anti-SS1 IgG antibody titer after immunization with different sample ($\bar{x} \pm s$, n = 6)

Groups	Serum IgG titers					
	Intramuscular injection			Oral group		
	14 D	28 D	42 D	14 D	28 D	42 D
Bland group (PBS)	0	0	0	0	0	0
CTB	0	0	0	0	0	0
IB	0	1200 ± 400	2000 ± 0	100 ± 0	120 ± 51	200 ± 0
IB+CTB	50 ± 0	1200 ± 900	5000 ± 0	100 ± 0	150 ± 50	200 ± 100
BIB	100 ± 0	2800 ± 900	7500 ± 3763	100 ± 0	200 ± 0	300 ± 100

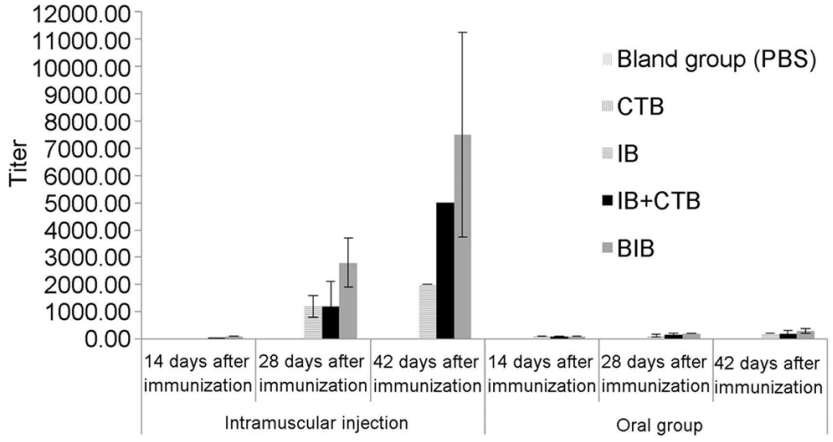

Figure 1. Anti-SS1 IgG titers after immunization with different antigens ($\bar{x} \pm s$, n = 6).

and vaccine immune routes, and we evaluated the induced immune response and immune protective effect differences through different combinations and different immunization routes by using recombinant H. pylori multi-epitope antigen (rIB) and using cholera toxin subunit B as the adjuvant molecule, so as to provide experimental evidence for the research of H. pylori vaccine.

Materials and methods

Strain, reagents and animals

H. pylori Strain SS1 (Sydney strain 1), to which animal model had adapted, was purchased from Hebei Medical University. rBIB, rIB and rCTB were all prepared by our project group. SPF level BALB/c mice of 6 weeks old (males:females = 1:1) were provided by Huaxi Experimental Animal Center of Sichuan University.

BALB/c mice grouping and immunization

BALB/c mice were randomly divided into nine groups and each group included six mice. rIB, rCTB, rBIB antigens were diluted into 1 mg/ml. All animals received fasting and water deprivation for 12 h before intramuscular injection or oral administration. Then, according to the immunization treatment requirements in **Table 1**,

Table 3. Anti-SS1 IgA titers after immunization with different antigens ($\bar{x} \pm s$, n = 6)

Groups	Serum IgA titers					
	Intramuscular injection			Oral group		
	14 D	28 D	42 D	14 D	28 D	42 D
Bland group (PBS)	0	0	0	0	0	0
CTB	0	0	0	0	0	0
IB	0	0	0	0	0	0
IB+CTB	0	0	0	0	0	0
BIB	0	0	0	100 ± 0	120 ± 51	120 ± 0

in intramuscular injection immunization groups (IM groups), mice underwent intramuscular injection immunization from left and right legs; in oral administration immunization groups (oral groups), mice received 0.2 ml by intragastric administration. After initial immunization, booster immunization once was performed on day 14 and 28, respectively, and the dose and method were the same as the initial immunization.

H. pylori challenge

14 days after the last immunization, 6 mice in each group were all challenged by freshly cultured H. pylori. Before the challenge, fasting and water deprivation lasted for 12 h. Each mouse received gastric gavage of H. pylori bacterial liquid 200 μl with about 1×10^8 cFu of bacterial bodies by oral route; the infection challenge was repeated two more times by once every other day. Four hours after each challenge, food and water supplies were restored.

Serum specific IgG and IgA antibodies detection

After mice were immunized, samples were collected once every 14 days. Indirect ELISA assay was used to detect serum specific IgG and IgA.

Evaluation for colonization of Helicobacter pylori in gastric tissue

On day 28 after the last challenge, the mice were sacrificed, and stomach tissue in the mice was sampled under sterile condition. Along the greater curvature the stomach was longitudinally cut open and along the lesser curvature the stomach was cut into two halves. One half was placed into 4% neutral formalin for fixation so as to prepare for HE staining and immunohistochemistry, and another half was further longitudinally divided into three equal parts, which would be used for urease test, smearing and Gram stain, PCR detection, respectively.

Results

Detection of serum specific IgG antibody

In rIB oral group, rBIB IM group, rBIB oral group, rIB+rCTB IM group and rIB+rCTB oral group, low level of serum anti-H. pylori specific IgG antibody could be detected from day 14 after the mice were immunized, while in rIB IM group no specific IgG antibody was detected. On day 28 after immunization, the titers of every IM group and every oral group increased to some degree, but there was significant difference ($P = 0.000$) in serum IgG antibody titer between IM groups and oral groups. On day 42 after immunization, titer in each IM group was significantly higher than the second immunization, while the difference between different IM groups was significant; specific IgG antibody titers were in order by rBIB IM group > rIB+rCTB IM group > rIB IM group. On day 42 after immunization, the titer in each oral group increased, but there was no significant difference between different oral groups, detailed in **Table 2**; **Figure 1**.

Detection of serum specific IgA antibody

After oral immunization three times by using rCTB as intramolecular adjuvant plus using rIB protein (i.e. rBIB), serum specific IgA antibody could be detected ($P = 0.000$) and the titer was up to 1:120. Meanwhile, serum IgA antibody could not be detected in other groups. The results are shown in **Table 3**.

Analysis for colonization of Helicobacter pylori in gastric tissue

On day 28 after the challenge, four methods, i.e., PCR, urease test, immunohistochemistry and Gram staining, were used to detect H. pylori colonization in gastric tissue of the mice. If two methods of the four were positive, positive H. pylori infection was judged. The results are shown in **Table 4**.

Table 4. Result of protection rates of different groups

	Groups	Positive rate (number of positive/number of test, n = 6)				Infection rate (%)	Protection rate (%)
		PCR	RUT	Immunohistochemistry	Gram stain		
Bland group (PBS)		6/6	6/6	6/6	6/6	100	0
Intramuscular injection	CTB	6/6	6/6	6/6	6/6	100	0
	IB	6/6	6/6	6/6	3/6	100	0
	CTB+IB	6/6	6/6	6/6	6/6	100	0
	BIB	4/6	4/6	4/6	4/6	66.7	33.3
Oral group	rCTB	6/6	6/6	6/6	6/6	100	0
	rIB	5/6	6/6	6/6	6/6	100	0
	rCTB+rIB	6/6	6/6	6/6	6/6	100	0
	rBIB	1/6	1/6	3/6	1/6	16.7	83.3

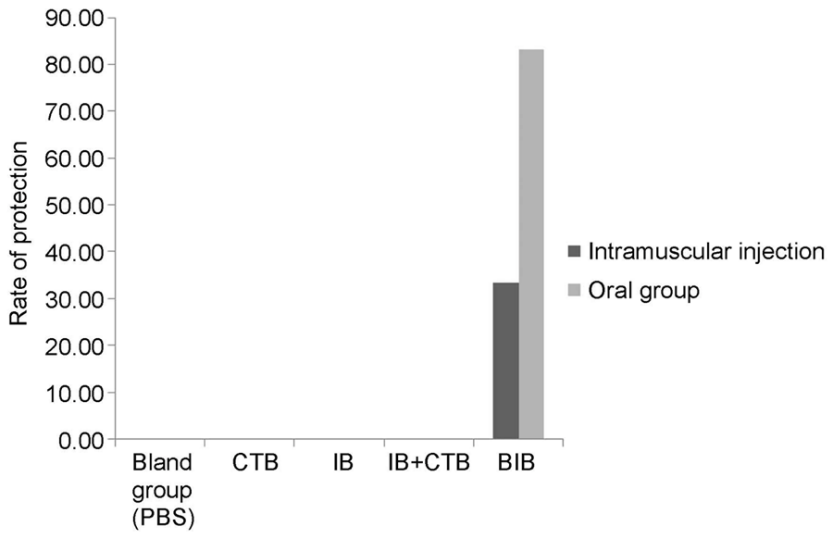

Figure 2. Result of protection rates of different groups.

By using Fisher exact test for analysis, the results showed that the protection rate against H. pylori challenge in rBIB oral group was higher by 50% than rBIB IM group, and there were significant differences in H. pylori challenge infection rate and protection rate against H. pylori challenge between rBIB oral group and other groups. (P = 0.000). See **Figure 2**.

Immunohistochemical results

On day 28 after infection challenge of H. pylori, immunohistochemistry showed that blank control group (PBS group), rCTB IM group, rIB IM group, rCTB+rIB IM group and rCTB oral group, rIB oral group, rCTB+rIB oral group all had cluster of H. pylori colonization in gastric tissue samples; in only 4 of 6 BALB/c mice in rBIB IM group, dispersedly distributed H. pylori were detected; in only 3 of 6 BALB/c mice in rBIB oral group, H. pylori colonization was detected. See **Figure 3**.

HE staining

On day 28 after infection challenge of H. pylori, pathological HE staining results showed that in non-immunized control group, rCTB IM group, rIB IM group, rCTB+rIB IM group and rCTB oral group, rIB oral group, rCTB+rIB oral group, rBIB IM group, majority of the samples exhibited: necrosis, shedding, inflammatory cell infiltration and other pathological changes; in rBIB oral group, HE staining of the gastric tissues rarely exhibited obvious lesion. See **Figure 4**.

Pathological comprehensive score of each immunization group

Referring to Arlin B. Rogers scoring criteria [10], each of inflammation, hemorrhage, edema, H. pylori bacterial body colonization, necrosis, atrophy was scored into 1, 2, 3, 4 points according to the lesion severity, and the total points were 4 (theoretical total of 6 items was 24 points); in one group, the accumulative total score of all mice with 6 items was 24 × 6 = 144 points, and in each group, the actual pathologi-

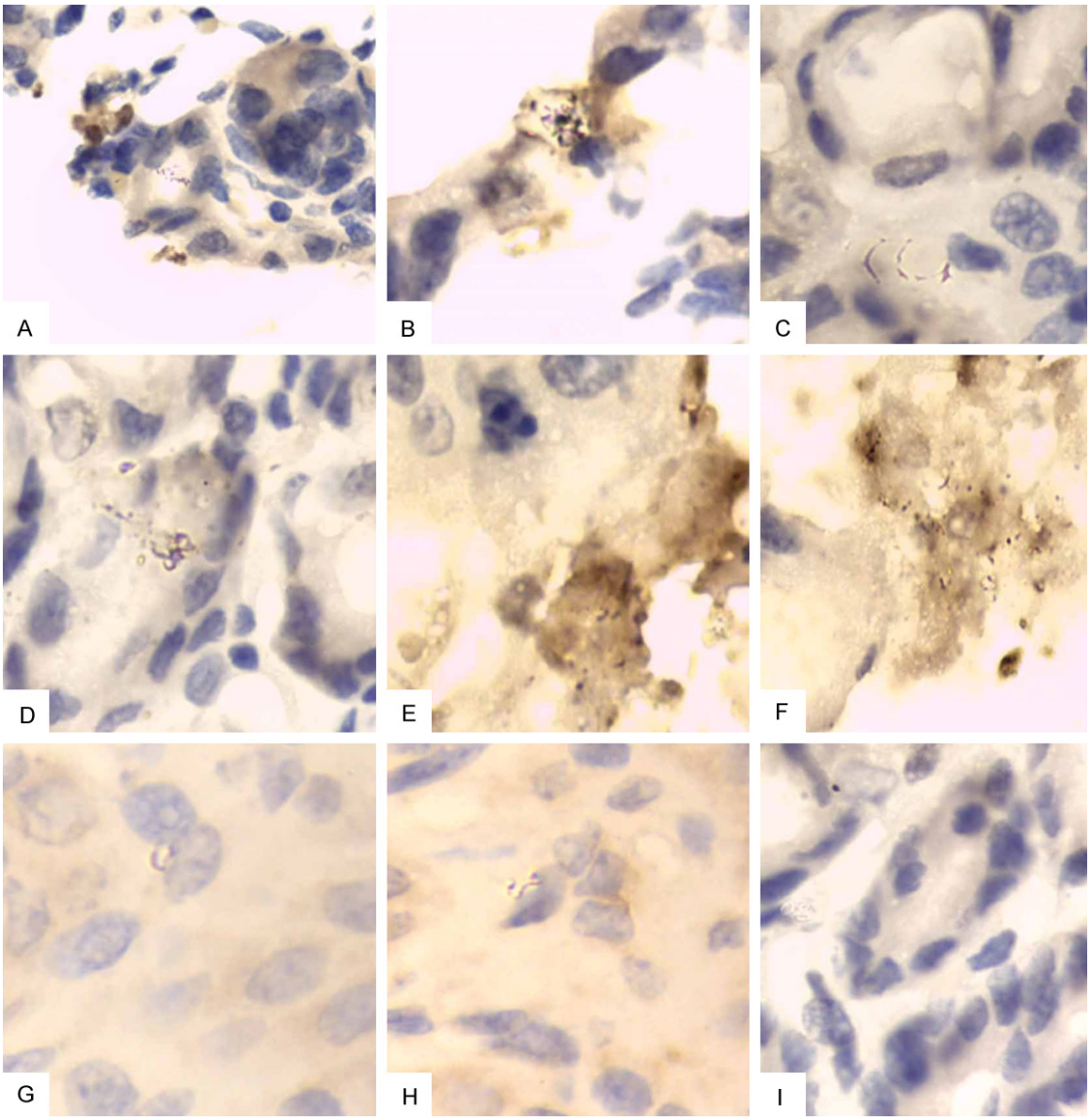

Figure 3. Mouse gastric tissue (immunohistochemistry, × 1000). A: Blank control group; B: rCTB IM group; C: rCTB oral group; D: rIB IM group; E: rIB oral group; F: rIB+rCTB IM group; G: rIB+rCTB oral group; H: rBIB IM group; I: rBIB oral group.

cal accumulative score was divided by theoretical total score (144 points) and was multiplied by 100%, and then the gastric pathological injury degree (%) was obtained. The 1-gastric pathological injury degree (%) was the gastric pathological injury decline degree (%).

Pathological score statistics showed that after the mice received infection challenge of H. pylori, the gastric pathological injury degree in rBIB oral group was the lowest (only 14.6%), and was lower by 64.6% than blank group; compared with oral adjuvant group, the gastric pathological injury decline degree in rBIB oral group increased by 47.9%; compared with rBIB IM group, the gastric pathological injury decline degree in rBIB oral group increased by 27.1%; compared with rIB recombinant group and rCTB+rIB group, the gastric pathological injury decline degree in rBIB oral group increased by 47.9% and 45.8%, respectively, and there were significantly differences between the groups (P = 0.000). The results are shown in **Table 5**.

Pathological score statistics showed that after challenge of H. pylori, the gastric pathological

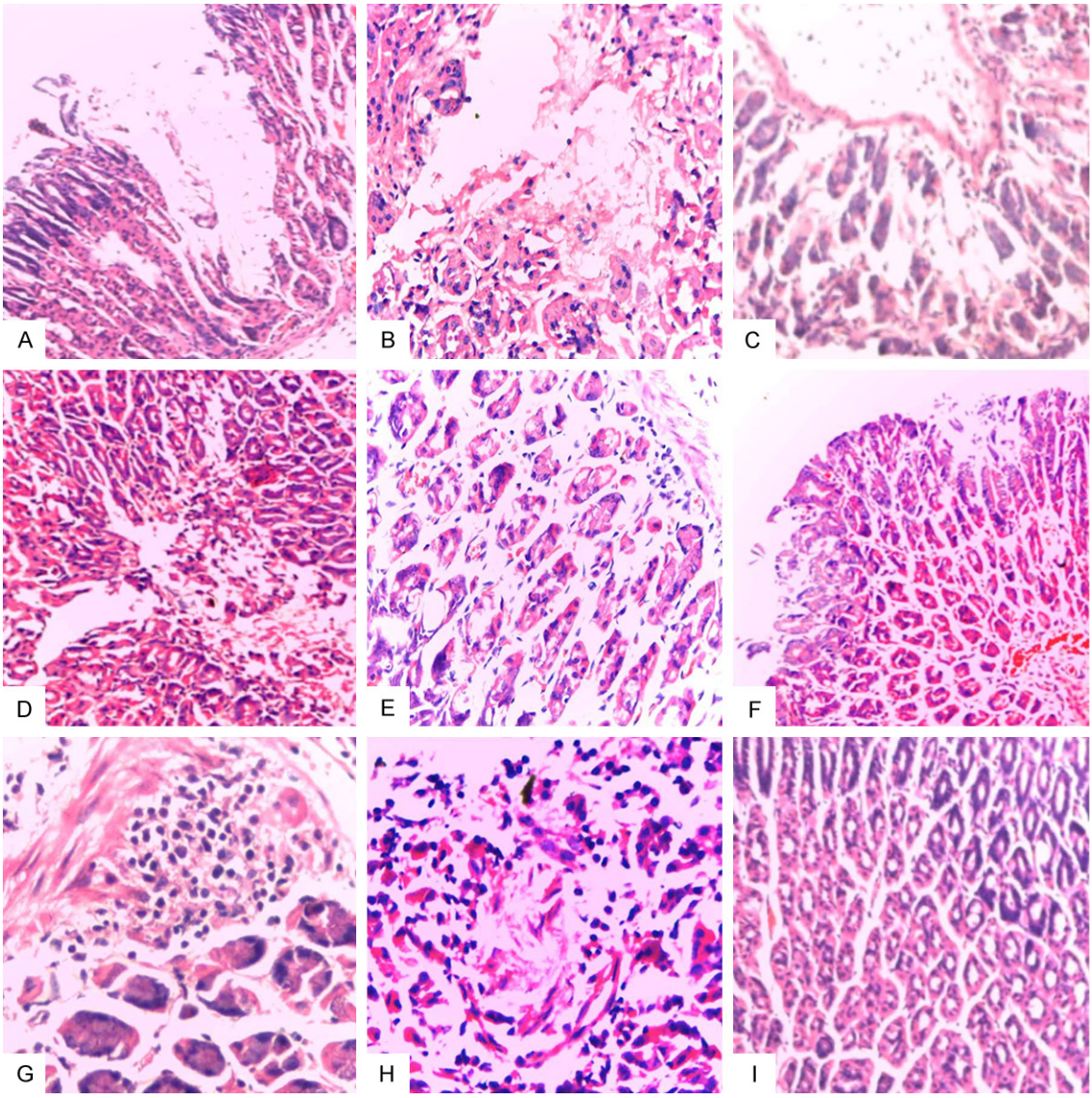

Figure 4. Mouse gastric tissue (HE, × 400). A: Bland control group: within gastric intrinsic membrane, glands reduced by 2/3, and severe atrophy existed; B: rCTB IM group: on the gastric surface, there were epithelial focal necrosis with inflammatory cell infiltration; C: rCTB oral group: mild shedding, necrosis with inflammatory cell infiltration; D: rIB IM group: epithelial hemorrhagic necrosis on the surface; E: rIB oral group: obvious inflammatory cell infiltration within the lamina propria; F: rIB+rCTB IM group: superficial ulcer with bleeding; G: rIB+rCTB oral group: lymphocytic infiltration in deep part of the lamina propria; H: rBIB IM group: focal necrosis; I: rBIB oral group: normal.

injury degree in rBIB oral group was the lowest (only 14.6%) and was lower by 54.6% than non-treated control group (blank control) group; compared with rBIB IM group, the gastric pathological injury degree in oral rBIB group decreased by 27.1%; compared with oral adjuvant group, the gastric pathological injury decline degree in oral rBIB group increased by 47.9%; compared with rCTB adjuvant group, rIB recombinant group and rCTB+rIB group, the gastric pathological injury decline degree in oral rBIB group was higher by 47.9%, 47.9% and 45.8%, respectively. There was significantly difference between the groups (P = 0.000). The results are shown in **Figure 5**.

Discussion

Our study compared differences of immune responses produced by three combination

Table 5. Results of pathological scores of different groups

	Groups	Pathological scores of gastric in each group (n = 6)						Degree of gastric mucosa injury	Declined degree of injury
		Inflammation	Hemorrhage	Edema	H. pylori	Necrosis	Atrophy		
	PBS	18	18	18	18	24	18	79.2%	20.8%
Intramuscular injection	CTB	18	9	9	24	18	18	66.7%	33.3%
	IB	18	12	9	24	18	12	64.6%	35.4%
	CTB+IB	18	12	6	18	18	18	62.5%	37.5%
	BIB	12	6	6	12	12	12	41.7%	58.3%
Oral group	CTB	18	9	9	24	18	12	62.5%	37.5%
	IB	12	12	12	18	24	12	60.4%	39.6%
	CTB+IB	18	12	9	24	12	12	62.5%	37.5%
	BIB	9	0	6	3	3	0	14.6%	85.4%

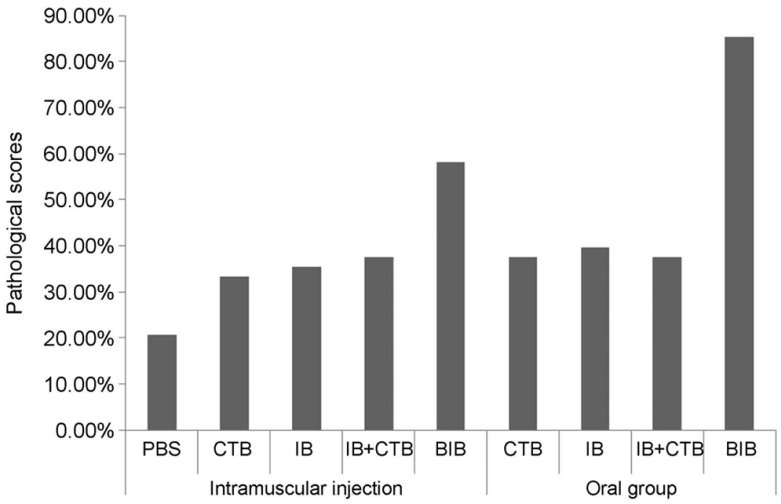

Figure 5. Pathological injury score of the infected stomach in each group of mice.

ways, i.e., rIB, rIB+rCTB and rBIB, with two medication routes, i.e., intramuscular injection and oral administration, and then evaluated immune protective effects generated by different immunizations through challenge of H. pylori. Detection results of serum specific IgG antibody showed: among IM groups, on day 14 after immunization, the titers of specific IgG antibody in rIB+rCTB group and rBIB group were 50 and 100, respectively, and in group with rIB alone, group with rCTB alone and non-immunized control group, specific IgG antibody had not been produced; after immunization twice, rIB and rIB+rCTB group titers were all 1200 as well as rBIB group titer reached 2800; after immunization three times, rIB group antibody titer was 2000, rCTB+rIB group antibody titer was 5000 and rBIB group antibody titer was 7500, significantly higher than other groups. The above results have indicated that as the extramolecular adjuvant and intramolecular adjuvant, while rCTB enhances the humoral immune response, it accelerates the humoral immunity response and the intramolecular adjuvant effect is stronger. Detection results of serum specific IgA antibody showed: every IM group had not produced serum specific IgA antibody; among oral groups, only rBIB oral group with rCTB as intramolecular adjuvant had produced specific IgA antibody significantly higher than other groups, indicating that rCTB can enhance mucosal immunity and the intramolecular adjuvant effect is strong. These results have demonstrated that rBIB containing intramolecular adjuvant is the best vaccine antigen component.

At present most studies believe that specific IgA is the key to clear H. pylori [11, 12], similar to the results of this study. In this study, each of IM groups (rIB, rCTB, rBIB, rCTB+rIB) had produced high level of IgG antibody by induction, but could not produce serum IgA antibody by induction; rBIB oral group not only produced high level of IgG antibody by inducing blood, but also produced specific IgA by induction. In the

experiment for evaluating protective effect, rBIB oral group protection rate was as high as up to 83.3%, and the gastric pathological injury degree was the lowest (only 14.6%), and its pathological injury decreased by 54.6% than non-treated control group (blank control); compared with rBIB IM group, its pathological injury degree decreased by 27.1%. Compared with rCTB adjuvant group, rIB recombinant group and rCTB+rIB group, the pathology injury decline degree in rBIB oral group increased by 47.9%, 47.9%, 45.8%, respectively. rBIB oral immunization had the highest infection protection rate against H. pylori and the highest pathological injury decline rate, demonstrating that oral immunization is the best immunization route of rBIB vaccine. This study has provided solid research data for new H. pylori vaccine components and immune routes and lays the foundation for further evaluating rBIB as a new vaccine.

Disclosure of conflict of interest

None.

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