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Protection Against *Helicobacter pylori* Infection by a Trivalent Fusion Vaccine Based on a Fragment of Urease B-UreB414

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A multivalent fusion vaccine is a promising option for protection against *Helicobacter pylori* infection. In this study, UreB414 was identified as an antigenic fragment of urease B subunit (UreB) and it induced an antibody inhibiting urease activity. Immunization with UreB414 partially protected mice from *H. pylori* infection. Furthermore, a trivalent fusion vaccine was constructed by genetically linking heat shock protein A (HspA), *H. pylori* adhesin A (HpaA), and UreB414, resulting in recombinant HspA-HpaA-UreB414 (rHHU). Its protective effect against *H. pylori* infection was tested in BALB/c mice. Oral administration of rHHU significantly protected mice from *H. pylori* infection, which was associated with *H. pylori*-specific antibody production and Th1/Th2-type immune responses. The results show that a trivalent fusion vaccine efficiently combats *H. pylori* infection, and that an antigenic fragment of the protein can be used instead of the whole protein to construct a multivalent vaccine.

***Keywords*:** *H. pylori*, multivalent vaccine, urease B subunit, *H. pylori* adhesin A, heat shock protein A

Helicobacter pylori is a Gram-negative bacterium that resides extracellularly in the gastric mucosa and infects more than half of the world's population. Although the majority of individuals remain asymptomatic, chronic infection with *H. pylori* is the cause of gastritis, peptic ulcer disease, and raises a risk of gastric cancer (Kusters *et al*., 2006; Correa and Houghton, 2007). Current antibiotic-based triple therapies are impractical for global control due to their high cost and problems with patient compliance and the emergence of antibiotic-resistant strains (Michetti, 1997). Vaccination against *H. pylori* infection could be an effective and economic way to control this pathogen.

The recombinant subunit vaccine is effective and has many advantages, such as definite components, good safety, and suitability for commercial production. Animal experiments have indicated that vaccination with recombinant *H. pylori* subunit antigens, such as urease B subunit (UreB), heat shock protein A (HspA) (Ferrero *et al*., 1995; Li *et al*., 2001), *H. pylori* adhesin A (HpaA) (Sutton *et al*., 2007), and vacuolating toxin A (Marchetti *et al*., 1998), could induce protective immune responses against *H. pylori* infection (Kabir, 2007). However, the protective efficiency of a vaccine consisting of a single recombinant antigen is limited. To improve the immunoprotective effect, a multivalent antigen vaccine containing the different antigens participating in different aspects of the pathogenesis of the infection may well be superior to a single antigen vaccine.

H. pylori adheres tightly to mucus-secreting gastric epithelial cells by means of adhesion A (HpaA), which is essential for *H. pylori* colonization in mice and the pathogenesis of *H. pylori*-induced gastroduodenal disease (Carlsohn *et al*., 2006). Urease hydrolyzes urea to ammonia and carbon dioxide, thereby neutralizing gastric acid and promoting pathogen colonization (Marshall *et al*., 1990; Eaton *et al*., 1991). Urease is composed of A and B subunits. The urease B subunit, which is an excellent vaccine candidate, protects against *H. pylori* infection in mice (Smythies *et al*., 2005). HspA possesses a unique domain at its C terminus that is absent from other known heat-shock homologs of eukaryotic organisms (Ferrero *et al*., 1995). It is an essential factor for the urease maturation and is involved in the pathogenesis of *H. pylori* infection. Thus HpaA, UreB, and HspA may well serve as potential vaccine candidates of *H. pylori*. Therefore, our aim is to develop a multivalent antigen vaccine comprised of HspA, UreB, and HpaA, which are all involved in the virulence associated with *H. pylori.*

Nowadays, multivalent antigen vaccines are often developed with several antigens used in combinations (Rossi *et al*., 2004; Wu *et al*., 2008), which have some drawbacks, such as problems with the expression of each of the recombinant proteins. Therefore, we tried to construct a fusion protein containing all three antigens. However, the UreB protein is too big (569 aa, molecular weight ~66 kDa), making a fusion protein very difficult to express and purify. Therefore, we chose an antigenic fragment instead of the whole protein. The structure of the urease has been determined and the active

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site is UreB (Ha *et al*., 2001). Based on bioinformatics to analyze the structure and antigenicity of UreB, we determined that a fragment from residues 250 to 387 (encoding by 414 bp DNA segment, called UreB414) was responsible for enzyme activity and retained good antigenicity. Therefore, we expressed the recombinant UreB414 (rUreB414) and constructed a trivalent fusion vaccine based on HspA, HpaA, and UreB414. The immunoprotection afforded by this vaccine against *H. pylori* was evaluated in BALB/c mice.

Materials and Methods

Screening the antigenic fragment of UreB

The UreB amino acid sequence was acquired from NCBI protein database (accession number: AAD07143). Based on the DNAstar software and PredictProtein (http://cubic.bioc.columbia.edu/predictprotein), together with the secondary structure and X-ray crystal analysis, we analyzed the antigenicity of UreB and determined a 138 aa stretch from aa 250 to aa 387 as an antigen fragment. This fragment was called UreB414, since it is encoded by a 414 bp long DNA.

Construction of rUreB414 and recombinant HspA-HpaA-UreB414 (rHHU)

Genomic DNA extracted from *H. pylori* strain ATCC 26695 was used as a template to amplify the UreB414 gene with primers of P1 and P2 (Table 1). The gene encoding UreB414 was cloned into a pET28a (+) expression system and expressed in *Escherichia coli* BL21. The trivalent fusion protein that included HspA, HpaA, and UreB414 was designed as shown in Fig. 2A, and was generated by overlap extension PCR reactions with the protein linker, PAVPPP (Table 1). The plasmid pET-28a (+)-HspA, pMD18-HpaA (which we had previously constructed), and pET-28a (+)-UreB414 were used as templates to amplify the genes of HspA, HpaA, and UreB414 with the primers of P3 and P4, P5 and P6, and P7 and P2, respectively (Fig. 2A). The primers P4 and P5, and P6 and P7 had an overlapping complementary sequence (underlined), which resulted in the DNA sequence encoding the linker sequence (PAVPPP) between each protein (Table 1). After the first round of PCR, a second round of PCR was performed using the PCR products of HspA and HpaA as templates, and P3 and P6 as primers, to obtain the DNA encoding HspA-HpaA (HH) fusion protein. The third round of PCR was performed using PCR product of HH and UreB414 as templates, and P3 and P2 as primers, to amplify the gene encoding HHU. The amplified HHU fragment was ligated to the expression vector pET-28a (+) and transformed into *E. coli* BL21. Recombinant proteins were induced by the addition of IPTG at 1 mM, and were purified by affinity and ion-exchange chromatography. Protein purity was determined by SDS-PAGE.

Table 1. Primers used for construction of recombinant proteins

Name	Primer sequence
P1	5′-*CCATGG*ACACTTTGAATGAAGCCG-3′ (*NcoI*)
P2	5′-*CTCGAG*AAATTCTTTTTTGTTTTTGTCAG-3′ (*XhoI*)
P3	5′-*CCATGG*AGTTTCAACCATTAGGAG-3′ (*NcoI*)
P4	5′-TAGGTGGTGGTACAGCAGGGTGTTTTTTGTGATC-3′ (linker)
P5	5′-CCCTGCTGTACCACCACCTAATTACCATCCA-3′ (linker)
P6	5′-CAGGTGGAGGTACTGCAGGAACCTTAATAAACCCAG-3′ (linker)
P7	5′-TCCTGCAGTACCTCCACCTGACACTTTGAATGAA-3′ (linker)

The italic letters mean restriction enzyme sites. The underlined represent the sequence encoding the linker (PAVPPP).

Production of antibody against rUreB414

Rabbits were immunized subcutaneously with rUreB414 emulsified in Freund's adjuvant 3 times at 2-week intervals. The blood samples were collected on day 5 after the last immunization. Antibody was purified on a Sepharose A column (Pharmacia, UK) according to the manufacturer's instructions.

Inhibition assay of *H. pylori* urease activity by antibodies

H. pylori was sonicated to prepare whole bacterial lysates. Lysates containing urease (80 μg/ml, 50 μl) together with 50 μl serial dilutions of anti-UreB414 antibody (100 μl/well) were added to 96-well plates. Normal rabbit IgG was used as a negative control. After incubating the plate at 4°C overnight, urease reagent (50 μl PBS containing 500 mM urea and 0.2 g/L phenolsulphonphthalein, pH 6.8) was added to each well and the plates were incubated at 37°C. The OD550 was measured after 30 min.

Western blotting

The immunoreactivity of purified rHHU was detected by western blotting. Purified rHHU was separated on 15% SDS-PAGE and electroblotted onto nitrocellulose (NC) membranes. The membranes were incubated with rabbit anti-rHspA, rHpaA or rUreB as the primary antibodies for 1 h at 37°C. The antibodies were prepared by immunizing rabbits subcutaneously with recombinant proteins emulsified in Freund's adjuvant 3 times at 2-week intervals separately. The blood samples were collected on day 5 after the last immunization. HRP-conjugated goat anti-rabbit IgG was used as the second antibody (1:10,000). The binding reaction was detected by the addition of the diaminobenzidine (DAB) color development reagent (0.05% diaminobenzidine, 0.005% hydrogen peroxide in distilled water).

Bacterial strain and culture

$VacA^+/CagA^+$ *H. pylori* strain CCS9803 (China Chongqing Strain 9803) was recovered from a patient who suffered from chronic antrum gastritis in Chongqing, China, in 1998. The isolated strain was adapted to colonize the gastric mucosa of BALB/c mice by 5 passages *in vivo* to obtain the CCS9803/B5 strain. This strain was cultured in brain-heart infusion (BHI) plates containing 10% rabbit blood under microaerobic conditions at 37°C for 2 days. The bacteria were harvested and resuspended in BHI, with adjustment to 2×10^9 colony forming units (CFU)/ml before inoculation.

Vaccination and challenge infection

SPF female BALB/c mice aged 6-8 weeks were purchased from the Experimental Animal Center of the Third Military Medical University. All animal experiments were approved by Animal Ethical and Experimental Committee of the Third Military Medical University. To evaluate the effect of different types of recombinant *H. pylori* antigens, BALB/c mice were divided randomly into the following 4 groups (n=40 per group) and vaccinated with different antigen together with $LT_{R72DITH}$ adjuvant intragastrically: I, rHHU; II, mixed proteins (rHspA+rHpaA+rUreB414); III, rUreB414; IV, PBS. All the animals

were immunized on days 0, 7, 14, and 28. Ten days after the last immunization, the mice (n=10 per group) were killed to assay the immune responses. The remaining mice were orogastrically inoculated twice at daily intervals with 5×10^8 CFU *H. pylori*. Four weeks after the challenge, the animals were killed and their stomachs were removed.

Microbial analysis of infection

Infection with *H. pylori* was examined using bacterial culture, a biopsy smear, a urease test and PCR. The stomach was opened along the greater curvature and cut into 4 parts for *H. pylori* culture, biopsy smear, urease test, and DNA extraction, respectively. *H. pylori* from gastric tissue was cultured on selective plates containing 10% rabbit blood agar supplemented with antibiotics. The plates were incubated at 37°C under microaerophilic conditions for 5-7 days. *H. pylori* was confirmed by its negative Gram stain, spiral morphology, catalase, oxidase, and urease activity. Bacterial smears were prepared from the gastric mucosa for Gram staining. *H. pylori* was identified by bacterial morphology and staining characteristics. The urease test was used for the rapid detection of *H. pylori* in the gastric mucosa. Urea reagent was dropped onto the gastric mucosa at room temperature, and its color change was observed. A sample was considered positive if a change of yellow-orange to pink occurred within 30 min. *H. pylori* DNA was extracted from gastric tissue by QIAGEN DNA Tissue Mini kit according to the manufacturer's instructions, and detected by PCR, as described by Thoreson *et al*. (1995). Infection was defined by microbial culture or at least 2 positive results from the other 3 tests. Data were expressed as percentage protection, which was calculated according to the following formula:

Protection rate (%) = (percentage of infection in the control group – percentage of infection in the immunized group)/percentage of infection in the control group×100%.

Determination of specific antibodies in samples by ELISA

Samples of serum, of the supernatants from extracted gastric and intestinal mucosa, and of the feces were collected on day 10 after the last immunization. Samples diluted 1:100 in PBS were added into 96-well plates (Costar) coated with *H. pylori* lysates (100 ng/well). HRP-conjugated goat anti-mouse IgG, IgG1, IgG2a, IgA or sIgA antibodies (1:10,000, Boster Company) were used as secondary antibody, respectively. Bound antibody was detected by measuring the optical density at 490 nm, developed with *o*-phenylenediamine solution (OPD) substrate.

RNA Isolation and reverse transcription (RT)-PCR

Lymphocytes from the spleens of the vaccine-immunized mice were isolated and cultured at 2×10^6 cells/well with *H. pylori* lysates (50 μg/ml) in RPMI-1640 complete medium in 24-well plates at 37°C and 5% CO_2 for 3 days. RNA was extracted with Trizol reagent (Invitrogen, UK) according to the manufacturer's instructions. Transcripts expression of interleukin (IL)-4, interferon (IFN)-γ, and β-actin was detected by RT-PCR using the primers listed in Table 2. PCR amplification comprised an initial denaturing step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 40 sec, annealing at 57°C for 40 sec, and elongation at 72°C for 40 sec. A final elongation step at 72°C for 10 min was carried out after the amplification cycles. Negative controls were included in each assay. PCR was also undertaken using non-reverse transcribed RNA as a template to confirm the absence of contaminating genomic DNA. The products of RT-PCR were electrophoresed in 2% agarose and visualized by UVP after ethidium bromide (EB) staining. Densitometric analysis of the bands was done with the gel imager program. Results are expressed as the ratio of the intensity of the band for IL-4 and IFN-γ to the intensity of the band for β-actin.

Table 2. Primers used for amplification by RT-PCR

Name	Primer sequence
β-Actin	F: 5′-ATCCGTAAAGACCTCTATGCCAACA-3′ R: 5′-GTCGCCTTCACCGTTCCAGTTT-3′
IFN-γ	F: 5′-AACTCAAGTGGCATAGATGTGG-3′ R: 5′-GACCTCAAACTTGGCAATACTC-3′
IL-4	F: 5′-TGTCATCCTGCTCTTCTTTCTC-3′ R: 5′-TGATGCTCTTTAGGCTTTCCAG-3′

Statistical analysis

The results of the antibody and cytokine levels were expressed as mean±standard deviation (SD). Student's *t*-test was used to analyze the data on antibody and cytokine expression. The *chi*-squared test was used to assess the protection rate of vaccine. A *p*-value of <0.05 was considered statistically significant.

Results

Screening the antigenic fragment of UreB

According to the secondary structure and X-ray crystallographic analysis, there are 5 α-helices from aa 250 to aa 390. Based on this and the analysis of hydrophilicity and antigenicity, we selected a fragment from aa 250 to aa 387 as an antigenic fragment of UreB, which is encoded by a 414 bp DNA segment, referred to as UreB414. UreB414 was subsequently cloned and expressed in *E. coli*.

Inhibitory effect of anti-rUreB414 antibody

To detect the antigenicity of rUreB414, we prepared the antibody against rUreB414. Incubation of *H. pylori* lysates with a serial dilution of anti-rUreB414 antibody showed that urease activity could be inhibited when the antibody was diluted below 1:400 (Fig. 1). This result confirms that rUreB414 is an antigenic fragment of UreB and has good antigenicity, which may induce neutralizing antibody against urease activity.

Construction, expression, purification, and characterization of rHHU

Since anti-rUreB414 antibody inhibits urease activity, UreB414 can be used instead of UreB to construct a recombinant trivalent fusion vaccine. Based on UreB414, we constructed a fusion protein of HHU. The gene for HHU was generated by overlap-extension PCR, cloned into pET28a (+) plasmid and expressed in *E. coli* BL21. Recombinant HHU was purified by ion-exchange and affinity chromatography, yielding a purity of >90%, as determined by SDS-PAGE. Western blotting was used to detect the immune reactivity of rHHU and the results showed that it strongly reacted with anti-rHspA, rHpaA and rUreB antibodies (Fig. 2B), whereas there was no reaction with normal rabbit serum (data not shown). The result indicated that rHHU retains good immunoreactivity of the antigenic domains of HspA, HpaA, and UreB.

Protective effect of rHHU on *H. pylori* infection

To follow the effects of vaccination of rHHU against *H. pylori*

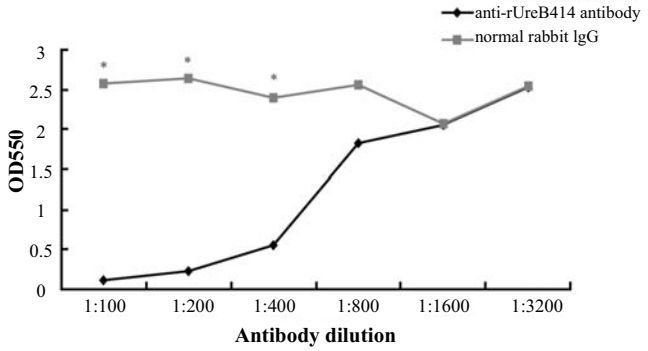

Fig. 1. Inhibition of *H. pylori* urease activity by anti-UreB414 antibodies. *H. pylori* lysates were incubated with a serial dilution of anti-rUreB414 antibody or normal rabbit IgG. Urease reagent was added to detect the activity of urease. The optical density was determined at 550 nm. The results are representative of three independent experiments.

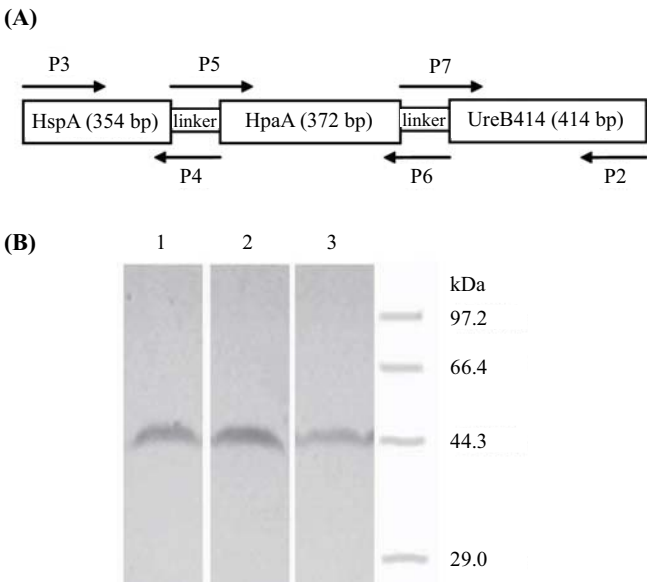

Fig. 2. Expression of rHHU. (A) The design of rHHU. (B) Immunoreactivity of rHHU was detected by Western Blotting with rabbit anti-rHspA (lane 1), rabbit anti-rHpaA (lane 2), and rabbit anti-rUreB (lane 3).

infection, BALB/c mice were immunized with rHHU, mixed proteins, rUreB414, or PBS and challenged with *H. pylori* on day 10 after the last immunization. The groups vaccinated with rHHU, mixed proteins, and rUreB414 were significantly protected compared with the PBS control group ($p<0.01$, Table 3). In addition, the rHHU-vaccinated group was significantly better protected than the rUreB414-vaccinated group ($p<0.05$). These results indicate that the trivalent fusion vaccine confers greater protection against *H. pylori* infection than a single antigen vaccine.

Antibodies responses in immunized mice

To see the effect of immunization, specific antibodies induced by the different vaccines after last immunization were detected by ELISA. IgG, IgG1, IgG2a, and IgA antibodies in serum of the mice immunized with rHHU, mixed proteins and rUreB414 were significantly increased compared to the PBS control ($p<0.05$, Fig. 3A). sIgA levels in the groups immunized with rHHU, mixed proteins and rUreB414 were significantly higher than those in the PBS group ($p<0.05$, Fig. 3B).

Expression of IL-4 and IFN-γ mRNA in antigen-stimulated lymphocytes

To test the cellular immune responses induced by immunezation, splenic lymphocytes were separated 10 days after the last immunization and stimulated with *H. pylori* lysates for 3 days. RNA was extracted from the lymphocytes and the expression of IL-4 and IFN-γ mRNA was detected by RT-PCR. IL-4 and IFN-γ transcript levels in the stimulated lymphocyte were significantly increased in rHHU, mixed proteins and rUreB414 immunized mice compared to PBS controls ($p<0.05$, Fig. 4), indicating Th1 and Th2 cell responses were induced by rHHU immunization.

Discussion

UreB is the subunit responsible for enzyme activity of urease that is critical for virulence of *H. pylori.* The antibody against UreB capable of destroying urease activity can protect the stomach from colonization by *H. pylori*. Therefore, an antigenic fragment containing the active center of urease might serve as a good vaccine candidate. Using bioinformatics and protein prediction tools, we were able to find an antigenic fragment, UreB414, which contains the active center of urease. The rUreB414-specific antibody efficiently neutralized the activity of urease, so UreB414 was potentially useful in constructing a multivalent vaccine, since immunization with UreB414 partially protected mice from *H. pylori* infection (Table 3).

Previous studies showed that HspA and HpaA might serve as potential candidate antigens for use in vaccine against *H.*

Table 3. Effect of vaccine on *H. pylori* infection

Group	Vaccine	Adjuvant	No. of *H. pylori* positive samples	Protection rate (%)
I	rHHU	$LT_{R72DITH}$	3 of 30	89.6[ab]
II	Mixed proteins (rHspA+rHpaA+rUreB414)	$LT_{R72DITH}$	8 of 30	72.3[a]
III	rUreB414	$LT_{R72DITH}$	12 of 30	58.5[a]
IV	PBS		27 of 28	0

Percentage protection was calculated according to the formula in 'Materials and Methods'
[a] $p<0.01$ compared with group IV
[b] $p<0.05$ compared with group III

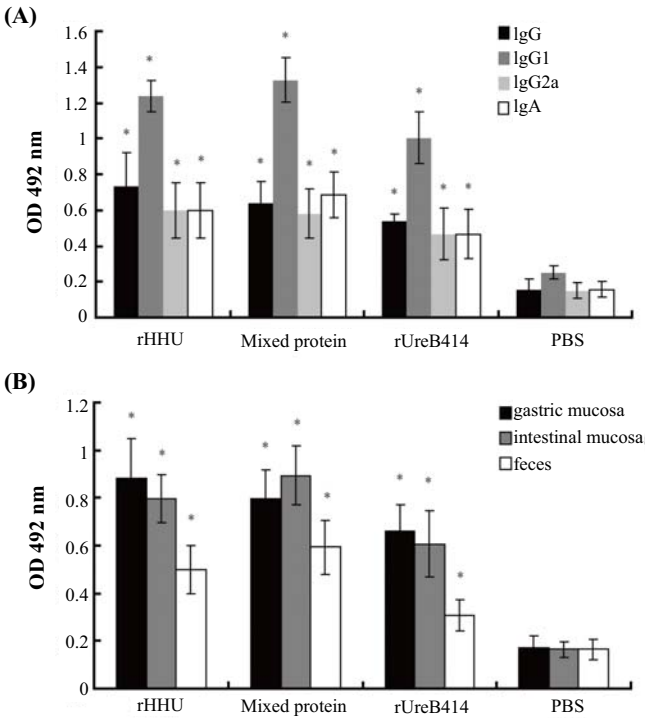

Fig. 3. Specific antibody response in vaccinated BALB/c mice. BALB/c mice were orally immunized with different vaccines 4 times and were killed on day 10 after the last immunization. Specific antibody levels were measured by ELISA. (A) Levels of IgG, IgG1, IgG2a, and IgA in serum. (B) Level of sIgA in gastric, intestinal mucosa, and feces of mice. Data were showed as Mean±SD. * $p<0.05$ vs PBS control.

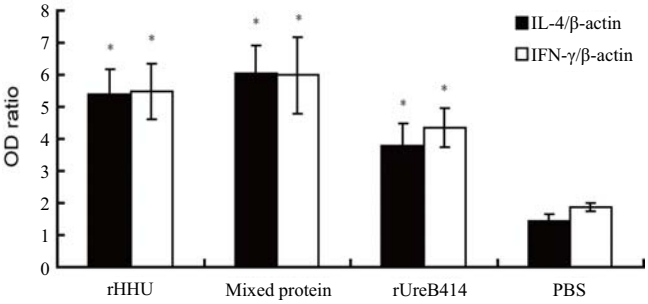

Fig. 4. IL-4 and IFN-γ mRNA expression in antigen-stimulated lymphocytes. Ten days after the last immunization, splenic lymphocytes were separated and stimulated with *H. pylori* lysates for 3 days. The expression of IL-4 and IFN-γ mRNA was detected by RT-PCR and the results expressed as the ratio of the intensity of the bands for IL-4 and IFN-γ relative to the band for β-actin. Results are showed as the Mean±SD. * $p<0.05$ vs PBS control.

pylori infection (Ferrero *et al*., 1995; Li *et al*., 2001; Sutton *et al*., 2007). Further, the trivalent vaccine (rHHU), based on HspA, HpaA, and UreB414, was constructed by fusing the 3 genes genetically. A vaccine developed in this way effectively protected mice from *H. pylori* infection. Th1 and Th2 type cell responses were also elicited by the rHHU, which may be responsible for the protection of this vaccine, since the protection of mice against *H. pylori* infection is dependent on $CD4^+$ cell responses (Ermak *et al*., 1998). We also detected *H. pylori*-specific antibody production after vaccination. Whether antibody production is responsible for the protective immunity is still controversial. It has been reported that the antibody response are not required for protection (Ermak *et al*., 1998). But some studies support that humoral immune response is important to clear *H. pylori* (Lee *et al*., 1995; Nystrom and Svennerholm, 2007). Our results showed that anti-UreB414 antibodies can inhibit urease activity, which might breakdown the microenvironment colonized by *H. pylori* and contribute to clearance of *H. pylori.*

Our previous work showed that the multicomponent vaccine (a mixture of the recombinant UreB, HspA, and HpaA) provided effective protection against *H. pylori* infection compared with a single antigen vaccine (Wu *et al.*, 2008). But the mixed subunit vaccine also had some disadvantages. It needs a lot of work to prepare each recombinant antigen. For considering the preparation of vaccine, trivalent fusion vaccine seems more economy and facility than mixed protein vaccine, suggesting that a multivalent fusion vaccine is a good way of constructing an effective vaccine against *H. pylori* infection. In addition, UreB414 was chosen to replace UreB to construct the fusion vaccine and shows good effect, indicating that an antigenic fragment can be used instead of the whole protein to construct a multivalent fusion vaccine. In further studies, other antigens can be added to the present vaccine to improve its efficiency further. To avoid the difficulties of expressing a large protein, antigenic fragments have considerable potential as substitutes for the whole protein in constructing multivalent fusion vaccines in the future.

$LT_{R72DITH}$, a mutated form of LT_{R72}, was mixed with the antigen as a mucosal adjuvant. Our previous study demonstrated that $LT_{R72DITH}$ was less toxic and had better adjuvanticity than LT_{R72} (Wu *et al*., 2008). Considering the convenience of preparation, we will construct an intramolecular adjuvant by linking the adjuvant with the antigen genetically, which will save both time and costs in preparing the extra adjuvant.

In conclusion, UreB414 was identified as an antigenic fragment of UreB. Oral administration of rHHU gave greater protection against *H. pylori* infection than the single recombinant antigen, rUreB414. The results indicate that multivalent fusion vaccines can be an effective way of combating *H. pylori* infection. The data show the value of using an antigenic fragment instead of the whole protein to construct a multivalent fusion vaccine.

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