PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

World J Gastroenterol 2007 February 14; 13(6): 939-944
World Journal of Gastroenterology ISSN 1007-9327

RAPID COMMUNICATION

Construction of recombinant attenuated Salmonella typhimurium DNA vaccine expressing *H pylori* ureB and IL-2

Can Xu, Zhao-Shen Li, Yi-Qi Du, Yan-Fang Gong, Hua Yang, Bo Sun, Jing Jin

Can Xu, Zhao-Shen Li, Yi-Qi Du, Yan-Fang Gong, Hua Yang, Bo Sun, Jing Jin, Department of Gastroenterology, Changhai Hospital, Second Military Medical University, Shanghai 200433, China
Supported by the National Natural Science Foundation of China, No. 30170427
Correspondence to: Dr. Can Xu, Department of Gastroenterology, Changhai Hospital, Second Military Medical University, Shanghai 200433, China. xxccan@sohu.com
Telephone: +86-21-25070539 Fax: +86-21-25074635
Received: 2006-08-08 Accepted: 2006-12-21

Abstract

AIM: To construct a recombinant live attenuated Salmonella typhimurium DNA vaccine encoding *H pylori* ureB gene and mouse IL-2 gene and to detect its immunogenicity *in vitro* and *in vivo.*

METHODS: *H pylori* ureB and mouse IL-2 gene fragments were amplified by polymerase chain reaction (PCR) and cloned into pUCmT vector. DNA sequence of the amplified ureB and IL-2 genes was assayed, then cloned into the eukaryotic expression vector pIRES through enzyme digestion and ligation reactions resulting in pIRES-ureB and pIRES-ureB-IL-2. The recombinant plasmids were used to transform competent *E. coli* DH5α, and the positive clones were screened by PCR and restriction enzyme digestion. Then, the recombinant pIRES-ureB and pIRES-ureB-IL-2 were used to transform LB5000 and the recombinant plasmids extracted from LB5000 were finally introduced into the final host SL7207. After that, recombinant strains were grown *in vitro* repeatedly. In order to detect the immunogenicity of the vaccine *in vitro*, pIRES-ureB and pIRES-ureB-IL-2 were transfected to COS-7 cells using Lipofectamine^{TM}2000, the immunogenicity of expressed UreB and IL-2 proteins was assayed with SDS-PAGE and Western blot. C57BL/6 mice were orally immunized with 1×10^8 recombinant attenuated Salmonella typhimurium DNA vaccine. Four weeks after vaccination, mice were challenged with 1×10^7 CFU of live *H pylori* SS1. Mice were sacrificed and the stomach was isolated for examination of *H pylori* 4 wk post-challenge.

RESULTS: The 1700 base pair ureB gene fragment amplified from the genomic DNA was consistent with the sequence of *H pylori* ureB by sequence analysis. The amplified 510 base pair fragment was consistent with the sequence of mouse IL-2 in gene bank. It was confirmed by PCR and restriction enzyme digestion that *H pylori* ureB and mouse IL-2 genes were inserted into the eukaryotic expression vector pIRES. The experiments *in vitro* showed that stable recombinant live attenuated Salmonella typhimurium DNA vaccine carrying ureB and IL-2 genes was successfully constructed and the specific strips of UreB and IL-2 expressed by recombinant plasmids were detected through Western blot. Study *in vivo* showed that the positive rate of rapid urease test of the immunized group including ureB and ureB-IL-2 was 37.5% and 12.5% respectively, and was significantly lower than that (100%) in the control group ($P < 0.01$).

CONCLUSION: Recombinant attenuated Salmonella typhimurium DNA vaccine expressing UreB protein and IL-2 protein with immunogenicity can be constructed. It can protect mice against *H pylori* infection, which may help the development of a human-use *H pylori* DNA vaccine.

Key words: *H pylori*; DNA vaccine; ureB gene; Salmonella typhimurium

Xu C, Li ZS, Du YQ, Gong YF, Yang H, Sun B, Jin J. Construction of recombinant attenuated Salmonella typhimurium DNA vaccine expressing *H pylori* ureB and IL-2. *World J Gastroenterol* 2007; 13(6): 939-944

http://www.wjgnet.com/1007-9327/13/939.asp

INTRODUCTION

H pylori infection can lead to chronic gastritis, peptic ulcer disease, and is also a risk factor for gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma[1-4]. More than 50% of the human population worldwide are infected with *H pylori*. About 10%-20% of all the patients have severe diseases such as gastric or duodenal ulcer and gastric cancer. The current therapy, based on the use of proton-pump inhibitor and antibiotics[5-7], is efficacious but faces potential problems like patient compliance, increasingly reported antibiotic resistance, and side effects such as abdominal pain, nausea, diarrhea[8]. Vaccination of humans against *H pylori*

infection may be an effective and economic approach to its control.

Studies of *H pylori* vaccine have focused on the individual *H pylori* proteins or the whole bacterial cell sonicates as antigens which need mucosal adjuvants like cholera toxin or *E. coli* labile toxin to elicit effective protection[9-11]. However, their use in humans is hampered by extremely high toxicity of mucosal adjuvants[12,13]. Recently, DNA vaccine without such mucosal adjuvants has been demonstrated to induce both humoral and cellular immunity and is becoming a promising treatment for viral, bacterial and parasitic pathogens[14,15]. Protective immunity against HIV, influenza virus, rabies virus, malaria and tuberculosis has been shown in animal models[16-19]. DNA vaccine of *H pylori* is seldom reported.

It was reported that coexpression of cytokine genes together with antigen-encoding genes in DNA vaccination vectors can increase both humoral and cellular immune response[20,21]. No information is available about the effects of cytokine-derived adjuvant in combination with *H pylori* vaccine.

In the present study we constructed a recombinant live attenuated Salmonella typhimurium DNA vaccine carrying *H pylori* ureB gene and mouse IL-2 gene, and identified its immunogenicity in transfected COS-7 cells *in vitro* and its prophylactic immunization *in vivo*.

MATERIALS AND METHODS

Bacteria and mammalian cell line and culture conditions

Attenuated Salmonella typhimura LB5000 and SL7207 kindly provided by Professor Bruce Stocker of Stanford University, USA, were cultured in Amp (-) LB medium. *E. coli* DH5α was grown in LB medium containing 50mg ampicillin per liter. *H pylori* strain CCUG17874 (NCTC11638) kindly provided by the Italian IRIS Research Center was cultured on *H pylori* selective agar plates with 10% defibrillated sheep blood and antibiotics (Merck Company, Germany) at 37℃ under microaerophilic conditions with 5% O_2, 10% CO_2 and 85% N_2. COS-7 cell line and recombinant plasmid pCIneo-IL-2 were provided by the Department of Immunology, Secondary Military Medical University of China. The mammalian expression vector pIRES was purchased from Clontech, USA.

PCR amplication of ureB and IL-2 gene fragments

Genomic DNA of *H pylori* was extracted as previously described using CTAB[22]. According to the complete DNA sequence of *H pylori* published and multiple clone sites of pIRES, the primers to amplify ureB containing *Nhe I* site in P1 and *Xho I* site in P2 respectively were designed (P1: 5' GCTAGCCACCATGAAAAAGATTAGCAGAAAAG 3', P2: 5' CTCGAGCTAGAAAATGCTAAAGAGTTG CGCC 3'). The primers to amplify IL-2 containing *Sal I* site in P3 and *Not I* site in P4 were designed (P3: 5' GTC-GACCACCATGTACAGCATGCAGCTCG3', P4: 5' GCGGCCGCTTATTGAGGGCTTGTTGAGATG 3'). Amplication conditions for ureB were as follows: at 95℃ for 5 min, then 35 cycles at 95℃ for 30 s, at 55℃ for 50 s and at 72℃ for 90 s, followed by 10 min at 72℃. Amplication conditions for IL-2 were at 95℃ for 5 min, then 30 cycles at 95℃ for 30 s, at 55℃ for 40 s and at 72℃ for 1 min, followed by 5 min at 72℃. The amplified products were analyzed on 1.2% agarose gels stained with ethidium bromide.

Sequencing analysis of ureB and IL-2

The PCR products were separated using a QIAquick gel extraction kit (QIAGEN, CA, USA). Purified ureB and IL-2 fragments were each subcloned into TA cloning vector pUCmT (Takara, Dalian, China) resulting in pUCmT-ureB and pUCmT-IL-2, then the nucleotide sequences of ureB and IL-2 were analyzed using an automatic sequencer.

Construction of recombinant pIRES-ureB and pIRES-ureB-IL-2

Fragments of *Nhe I* and *Xho I*-digested pUCmT-ureB were inserted into the *Nhe I*/*Xho I* site of eukaryotic expression vector pIRES, through a serial of enzyme digestion and ligation reactions resulting in pIRES-ureB. Fragments of *Sal I* and *Not I*-digested pUCmT-IL-2 were inserted into the *Sal I*/*Not I* site of pIRES-ureB. The recombinant pIRES-ureB and pIRES-ureB-IL-2 were all confirmed by PCR and restriction enzyme digestion.

In vitro transfection and expression of UreB and IL-2

Recombinant plasmids of pIRES-ureB and pIRES-ureB-IL-2 were transfected into COS-7 cells respectively in order to detect the proteins expressed by pIRES-ureB and pIRES-ureB-IL-2. COS-7 cell line was cultured at 37℃, in Dulbecco's modified Eagle's medium supplemented with 10% FBS (Gibco-BRL, UK), 100 U/mL penicillin and 100 μg/mL streptomycin, 15 mmol/L HEPES, 2 mmol/L *L*-glutamine. The mixture of pIRES-ureB and Lipofectamine ™2000 (Invitrogen, USA) or pIRES-ureB-IL-2 and Lipofectamine™2000 were added to the COS-7 cells. Forty-eight hours after transfection, the cells were washed with PBS and protein extraction reagent was added. The lysate was collected and centrifuged at 12000 × *g* for 5 min at 4℃.

Supernatant containing proteins was electrophoretically analyzed in a 10% polyacrylamide gel, subsequently electrotransferred onto nitrocellulose membranes. Nonspecific binding sites were blocked with 2% bovine serum albumin (BSA), then rabbit anti-*H pylori* or rabbit anti-IL-2 antibodies and peroxidase-labeled anti-rabbit immunoglobulin G (IgG) were added. The antigens were visualized by chemiluminescence (Bio-Rad, Germany) according to the manufacturer's instructions.

Construction of recombinant attenuated Salmonella typhimurium carrying H pylori ureB and mouse IL-2 genes

Recombinant pIRES-ureB and pIRES-ureB-IL-2 were used to transform attenuated Salmonella typhimurium LB5000 for methylation decoration using calcium chloride, then the recombinant plasmids extracted were transformed into the final host strain SL7207 by electroporation. The attenuated Salmonella typhimurium SL7207 carrying ureB gene and SL7207 carrying both ureB and IL-2 genes were

cultured in LB medium to 80 generations. The recombinant plasmids in transformed SL7207 were isolated from every 10 generations and identified by restriction enzymes and PCR.

Immunization

Prior to immunization, mice were left overnight without solid food and 4 h without water. One hundred μL of 3% sodium bicarbonate was given orally using a stainless steel catheter tube to neutralize the stomach pH. Immediately after that, mice in the vaccination group were orally given 5×10^8 live attenuated salmonella typhimurium DNA vaccine ureB or ureB-IL-2 in a total volume of 200 μL, mice in the control group were given 200 μL PBS. Mice had free access to water and food after immunization.

Challenge of mice

Four weeks after immunization, all mice were challenged with 200 μL *H pylori* SS1 (1×10^7 CFU). Before challenge, all mice were prepared as described above.

Assessment of H pylori in gastric tissue

Four weeks after challenge, all mice were sacrificed and the stomach was carefully removed from each mouse under aseptic conditions. The stomach was washed 3 times in sterile PBS to remove food residues. A part of the antral region was used for rapid urease test to measure the urease activity.

Statistical analysis

Differences in *H pylori* strain-induced urease activity in stomach of immunized and non- immunized mice were evaluated using a two-tailed Fisher's exact test.

RESULTS

Sequence analysis of ureB and IL-2

Sequence analysis of ureB and IL-2 showed that the sequences of amplified fragments were consistent with those of *H pylori* ureB and mouse IL-2 published in the gene bank respectively.

Recombinant plasmids confirmed by PCR and restriction enzyme digestion

After pUCmT-ureB and pIRES were digested by both *Nhe* I and *Xho* I, a 1700 bp fragment of ureB was directly cloned into *Nhe* I/*Xho* I site of pIRES, resulting in a recombinant plasmid pIRES-ureB. IL-2 was inserted into the *Sal* I/*Not* I site of pIRES-ureB to get pIRES-ureB-IL-2. Both PCR and restriction enzyme digestion demonstrated that recombinant plasmids contained the ureB and IL-2 genes. PCR and restriction enzyme digestion products were analyzed on agarose gel (Figure 1).

Immunoreactivity of expressed recombinant proteins

Identification of pIRES-ureB and pIRES-ureB-IL-2 *in vitro* expression was carried out. The lysate of COS-7 cells transfected by pIRES-ureB and pIRES-ureB-IL-2 was analyzed by Western blot (Figure 2A and B). It revealed the band about 66 000 in relative molecular weight

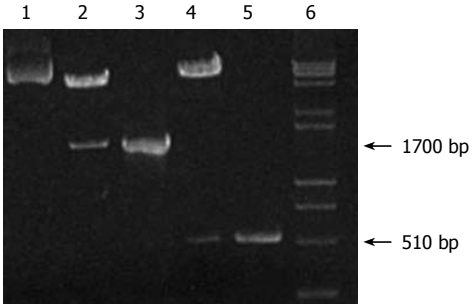

Figure 1 Agarose gel electrophoresis analysis of recombinant pIRES-ureB-IL-2. Lane 1: pIRES after digestion with Nhe I and Xho I as a negative control, lane 2: pIRES-ureB-IL-2 after digestion with Nhe I and Xho I, lane 3: PCR product of ureB, lane 4: pIRES-ureB-IL-2 after digestion with SalI and Not I, lane 5: PCR product of IL-2, lane 6: DNA Marker (DL2000 + 15000).

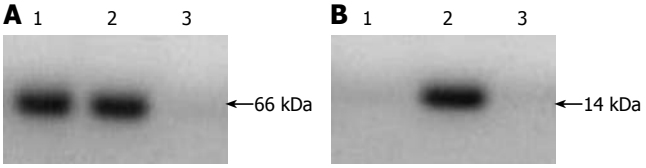

Figure 2 Western blot analysis of expressed UreB protein products (**A**) and IL-2 protein products (**B**). Lane 1: COS-7 cells transfected by pIRES-ureB, lane 2: COS-7 cells transfected by pIRES-ureB-IL-2, lane 3: COS-7 cells transfected by pIRES as a control.

corresponded to UreB protein and 14 000 to IL-2 protein, but the control transfected with pIRES had no specific band.

Recombinant attenuated Salmonella typhimurium DNA vaccine and its stability in vitro

After transformed by pIRES-ureB-IIL-2, recombinant plasmid extracted from LB5000 was used to transform SL7207. Plasmid stability is essential to assure the stable expression of antigens encoded by genes which were cloned into the plasmids. Therefore, SL7207 carrying pIRES-ureB-IIL-2 was grown *in vitro* up to 80 generations to examine the plasmid stability. The objective fragments (1.7 kb and 510 bp) could be seen on the map of agarose gel of PCR products and those of restriction enzyme digested recombinant plasmid isolated from transformed SL7207 (Figure 3A and B).

Rapid urease test results of gastric mucosa

All mice in the control PBS group were infected with *H pylori*. In contrast, 62.5% and 87.5% of mice immunized with ureB and ureB-IL-2 were resistant to *H pylori* ($P < 0.01$). The negative rate of rapid urease test in ureB-IL-2 group was significantly higher than that in ureB group ($P < 0.05$, Table 1).

DISCUSSION

DNA vaccine or genetic vaccine can induce immune response to DNA-encoded proteins after naked DNA is

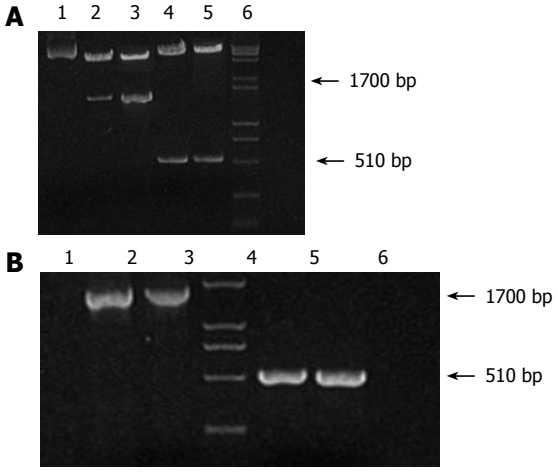

Figure 3 Agarose gel electrophoresis analysis of recombinant attenuated Salmonella typhimurium DNA vaccine strain with restriction enzyme digestion (**A**) (lane1: pIRES after digestion with Nhe I and Xho I, lanes 2-3: recombinant plasmid pIRES-ureB-IL-2 from strains of different generations after digestion with Nhe I and Xho I, lanes 4-5: pIRES-ureB-IL-2 from strains of different generations after digestion with Sal I and Not I, lane 6: DNA marker: (DL2000 + 15000) and identification of recombinant attenuated Salmonella typhimurium DNA vaccine strain carrying ureB by PCR (**B**) (lane1: product amplified from pIRES as a negative control, Lane4: Marker (DL2000); Lanes 2-3: ureB amplified from strains of different generations, lane 4: marker-DL2000, lanes 5-6: IL-2 amplified from strains of different generations.

Table 1 Rapid urease test results of gastric mucosa in different groups

Group	Positive (*n*)	Negative (*n*)	Total (*n*)	Negative rate (%)
PBS	10	0	10	0
ureB	9	15	24	62.5[b]
ureB-IL-2	3	21	24	87.5[a,b]

[a]$P < 0.05$ *vs* ureB group; [b]$P < 0.01$ *vs* PBS group.

injected into a host. DNA vaccines have been widely used in laboratory animals to elicit comprehensive humoral and cellular immune responses. Clinical trials have shown that DNA vaccines are safe and well tolerated[15,23]. Moreover, some reports have demonstrated that DNA vaccine could produce long-lasting immunity[24-25]. The vaccine itself is a recombinant plasmid with heat stability and does not need to be purified. It can be used to protect against and treat tumors[26-29].

However, this approach has not been fully explored in *H pylori* vaccine. The available *H pylori* vaccine is a protein vaccine, including *H pylori* whole cells or one of the recombinant proteins of *H pylori* as an antigen of the vaccine in combination with mucosal adjuvants such as cholera toxin or heat-labile toxin of enterotoxigenic *E. coli* [11,30,31]. The manufacture of such vaccines is complicated, and some mucosal adjuvants have gastrointestinal toxicity. It was reported that mucosal immunization with *Helicobacter heilmannii* urease B or *H pylori* urease, given nasally with cholera toxin, could protect BALB/c mice against *H heilmannii* infection and significantly reduce the preexisting infection[32]. However, immunization aggravates gastric corpus atrophy.

Urease is an enzyme consisting of six UreA subunits and six UreB subunits. Urease negative *H pylori* strains are unable to colonize the stomach of gnotobiotic piglets, demonstrating its role in the colonization. Urease may intervene directly or indirectly in induction of tissue damage in the stomach. Moreover, it may play an additional role in adhesion and stimulation of inflammatory cells. Urease is a highly conserved protein among gastric *H pylori* species and a potential antigen for prophylactic and therapeutic vaccines against *H pylori* infection among different animals[33-35], suggesting that it is an ideal antigen candidate for *H pylori* vaccine.

It has been shown that live attenuated bacteria carrier including attenuated strains of Salmonella *in vitro* could deliver DNA vaccines to human cells, thus allowing vaccination via mucosal surfaces and specific targeting of professional antigen presenting cells in mucosa-associated lymphoid tissue tumors[36,37].

Several studies have shown that co-administration of cytokine proteins or of cytokine-expressing plasmids can modulate the immune response to DNA vaccination[40,41]. Enhancement of antigen-specific antibody response and T cell response has been demonstrated by vaccine co-expressing DNA and immune adjuvants like IL-2 or granulocyte macrophage colony stimulating factor (GM-CSF) gene[40,41].

In this study, we constructed a live recombinant attenuated Salmonella typhimurium DNA vaccine strain expressing UreB protein and mouse IL-2 protein. The complete ureB gene fragment and mouse IL-2 gene were amplified and then sequence analysis was performed after they were cloned respectively into the TA cloning vector pUCmT. Purified ureB and IL-2 were cloned into eukaryotic expression vector pIRES. Both the enzyme digestion and PCR confirmed the successful construction of recombinant plasmid pIRES-ureB and pIRES-ureB-IL-2. Recombinant attenuated Salmonella typhimurium carrying *H pylori* ureB gene and mouse IL-2 was successfully constructed after recombinant plasmid was used to transform LB5000 and SL7207. Since the stability of the protective antigen is very important for a vaccine, we assayed the stability of the recombinant plasmid *in vitro*. PCR and restriction enzyme digestion confirmed the presence of pIRES-ureB-IL-2 in all transformed strains of SL7207 up to the 80th generation, demonstrating the stability of the recombinant plasmid in SL7207.

It was also shown *in vitro* in our present study that COS-7 cellls transfected by pIRES-ureB-IL-2 could express 66000 UreB and 14000 IL-2, but COS-7 cells transfected by pIRES could not express them. The pIRES-ureB-IL-2 DNA vaccine we constructed could express UreB protein which can react with anti-*H pylori* protein and IL-2 which can react with anti-mouse IL-2 protein.

We also demonstrated that a single dose of ureB or both ureB and adjuvant IL-2 delivered by the live attenuated Salmonella typhimurium SL7207 strain could protect C57BL/6 mice against *H pylori* colonization in the stomach. No protection was observed in the control group. Compared with ureB group, ureB-IL-2 group

appeared to induce a better protection against *H pylori* infection with a significantly higher negative rate of rapid urease test, suggesting that adjuvant IL-2 may improve the protective immunization level.

In summary, recombinant attenuated Salmonella typhimurium DNA vaccine encoding *H pylori* ureB gene and mouse IL-2 gene can express UreB and IL-2 with immunogenicity. DNA vaccine can protect against *H pylori* challenge in animal models. The therapeutic effect of DNA vaccine on *H pylori* infection and its mechanism should be further studied.

COMMENTS

Background

Studies of *H pylori* vaccine have focused on the individual *H pylori* proteins or the whole bacterial cell sonicates as antigens which need mucosal adjuvants like cholera toxin or *Escherichia coli* labile toxin to elicit effective protection. However, clinical trials have shown the intestinal toxicity of mucosal adjuvants. Recently, DNA vaccine without such mucosal adjuvants has been demonstrated to induce humoral and cellular immunity and is becoming a promising treatment for viral, bacterial and parasitic pathogens. Protective immunity against HIV, influenza virus, rabies virus, malaria and tuberculosis has been shown in animal models. DNA vaccine against *H pylori* is seldom reported.

Research frontiers

It was reported that co-expression of cytokine genes and antigen-encoding genes in DNA vaccination vectors can increase humoral and cellular immune response. No information is available about the effects of cytokine-derived adjuvant in combination with H pylori vaccine.

Innovations and breakthroughs

Recombinant attenuated Salmonella typhimurium DNA vaccine encoding *H pylori* ureB gene and mouse IL-2 gene can express UreB and IL-2 with immunogenicity. DNA vaccine against *H pylori* and adjuvant IL-2 may improve the protective immunization level.

Applications

H pylori vaccine can prevent *H pylori* infection in human beings.

Peer review

The manuscript by Dr. Xu *et al* described an interesting study on the construction of a recombinant attenuated S. typhimurium DNA vaccine for *Helicobacter pylori* infection. The authors have coexpressed *H pylori* ureB and cytokine IL-2 which can improve the potential immunogenicity and protective efficacies of the vaccine.

REFERENCES

1 **Isakov V**, Malfertheiner P. *Helicobacter pylori* and nonmalignant diseases. *Helicobacter* 2003; **8** Suppl 1: 36-43
2 **Miwa H**, Sakaki N, Sugano K, Sekine H, Higuchi K, Uemura N, Kato M, Murakami K, Kato C, Shiotani A, Ohkusa T, Takagi A, Aoyama N, Haruma K, Okazaki K, Kusugami K, Suzuki M, Joh T, Azuma T, Yanaka A, Suzuki H, Hashimoto H, Kawai T, Sugiyama T. Recurrent peptic ulcers in patients following successful Helicobacter pylori eradication: a multicenter study of 4940 patients. *Helicobacter* 2004; **9**: 9-16
3 **Takahashi S**. Long-term Helicobacter pylori infection and the development of atrophic gastritis and gastric cancer in Japan. *J Gastroenterol* 2002; **37** Suppl 13: 24-27
4 **Al-Akwaa AM**, Siddiqui N, Al-Mofleh IA. Primary gastric lymphoma. *World J Gastroenterol* 2004; **10**: 5-11
5 **Suzuki H**, Masaoka T, Nomura S, Hoshino Y, Kurabayashi K, Minegishi Y, Suzuki M, Ishii H. Current consensus on the diagnosis and treatment of H. pylori-associated gastroduodenal disease. *Keio J Med* 2003; **52**: 163-173
6 **Perri F**, Qasim A, Marras L, O'Morain C. Treatment of Helicobacter pylori infection. *Helicobacter* 2003; **8** Suppl 1: 53-60
7 **Anagnostopoulos GK**, Kostopoulos P, Margantinis G, Tsiakos S, Arvanitidis D. Omeprazole plus azithromycin and either amoxicillin or tinidazole for eradication of Helicobacter pylori infection. *J Clin Gastroenterol* 2003; **36**: 325-328
8 **Cameron EA**, Powell KU, Baldwin L, Jones P, Bell GD, Williams SG. Helicobacter pylori: antibiotic resistance and eradication rates in Suffolk, UK, 1991-2001. *J Med Microbiol* 2004; **53**: 535-538
9 **Kotloff KL**, Sztein MB, Wasserman SS, Losonsky GA, DiLorenzo SC, Walker RI. Safety and immunogenicity of oral inactivated whole-cell Helicobacter pylori vaccine with adjuvant among volunteers with or without subclinical infection. *Infect Immun* 2001; **69**: 3581-3590
10 **Jeremy AH**, Du Y, Dixon MF, Robinson PA, Crabtree JE. Protection against Helicobacter pylori infection in the Mongolian gerbil after prophylactic vaccination. *Microbes Infect* 2006; **8**: 340-346
11 **Durrani Z**, Rijpkema S. Orogastric vaccination of guinea pigs with Helicobacter pylori sonicate and a high dose of cholera toxin lowers the burden of infection. *FEMS Immunol Med Microbiol* 2003; **36**: 169-173
12 **Holmgren J**, Adamsson J, Anjuère F, Clemens J, Czerkinsky C, Eriksson K, Flach CF, George-Chandy A, Harandi AM, Lebens M, Lehner T, Lindblad M, Nygren E, Raghavan S, Sanchez J, Stanford M, Sun JB, Svennerholm AM, Tengvall S. Mucosal adjuvants and anti-infection and anti-immunopathology vaccines based on cholera toxin, cholera toxin B subunit and CpG DNA. *Immunol Lett* 2005; **97**: 181-188
13 **Pizza M**, Giuliani MM, Fontana MR, Monaci E, Douce G, Dougan G, Mills KH, Rappuoli R, Del Giudice G. Mucosal vaccines: non toxic derivatives of LT and CT as mucosal adjuvants. *Vaccine* 2001; **19**: 2534-2541
14 **Martin JE**, Sullivan NJ, Enama ME, Gordon IJ, Roederer M, Koup RA, Bailer RT, Chakrabarti BK, Bailey MA, Gomez PL, Andrews CA, Moodie Z, Gu L, Stein JA, Nabel GJ, Graham BS. A DNA vaccine for Ebola virus is safe and immunogenic in a phase I clinical trial. *Clin Vaccine Immunol* 2006; **13**: 1267-1277
15 **Schalk JA**, Mooi FR, Berbers GA, van Aerts LA, Ovelgönne H, Kimman TG. Preclinical and clinical safety studies on DNA vaccines. *Hum Vaccin* 2006; **2**: 45-53
16 **Muthumani K**, Zhang D, Dayes NS, Hwang DS, Calarota SA, Choo AY, Boyer JD, Weiner DB. Novel engineered HIV-1 East African Clade-A gp160 plasmid construct induces strong humoral and cell-mediated immune responses in vivo. *Virology* 2003; **314**: 134-146
17 **Soboll G**, Horohov DW, Aldridge BM, Olsen CW, McGregor MW, Drape RJ, Macklin MD, Swain WF, Lunn DP. Regional antibody and cellular immune responses to equine influenza virus infection, and particle mediated DNA vaccination. *Vet Immunol Immunopathol* 2003; **94**: 47-62
18 **Carvalho LJ**, Daniel-Ribeiro CT, Goto H. Malaria vaccine: candidate antigens, mechanisms, constraints and prospects. *Scand J Immunol* 2002; **56**: 327-343
19 **Ha SJ**, Jeon BY, Kim SC, Kim DJ, Song MK, Sung YC, Cho SN. Therapeutic effect of DNA vaccines combined with chemotherapy in a latent infection model after aerosol infection of mice with Mycobacterium tuberculosis. *Gene Ther* 2003; **10**: 1592-1599
20 **Chang SY,** Lee KC, Ko SY, Ko HJ, Kang CY. Enhanced efficacy of DNA vaccination against Her-2/neu tumor antigen by genetic adjuvants. *Int J Cancer* 2004; **111**: 86-95
21 **Buonocore F**, Mazzini M, Forlenza M, Randelli E, Secombes CJ, Zou J, Scapigliati G. Expression in Escherchia coli and purification of sea bass (Dicentrarchus labrax) interleukin 1beta, a possible immunoadjuvant in aquaculture. *Mar Biotechnol* (NY) 2004; **6**: 53-59
22 **Xu C**, Li ZS, Tu ZX, Xu GM, Gong YF, Man XH. Distribution of cagG gene in Helicobacter pylori isolates from Chinese patients with different gastroduodenal diseases and its clinical

and pathological significance. *World J Gastroenterol* 2003; **9**: 2258-2260

23 **Liu MA**, Wahren B, Karlsson Hedestam GB. DNA vaccines: recent developments and future possibilities. *Hum Gene Ther* 2006; **17**: 1051-1061

24 **Medjitna TD**, Stadler C, Bruckner L, Griot C, Ottiger HP. DNA vaccines: safety aspect assessment and regulation. *Dev Biol* (Basel) 2006; **126**: 261-270; discussion 327

25 **Fuller DH**, Loudon P, Schmaljohn C. Preclinical and clinical progress of particle-mediated DNA vaccines for infectious diseases. *Methods* 2006; **40**: 86-97

26 **Henke A**. DNA immunization--a new chance in vaccine research? *Med Microbiol Immunol* 2002; **191**: 187-190

27 **Abdelnoor AM**. Plasmid DNA vaccines. *Curr Drug Targets Immune Endocr Metabol Disord* 2001; **1**: 79-92

28 **Donnelly J**, Berry K, Ulmer JB. Technical and regulatory hurdles for DNA vaccines. *Int J Parasitol* 2003; **33**: 457-467

29 **Reisfeld RA**, Niethammer AG, Luo Y, Xiang R. DNA vaccines designed to inhibit tumor growth by suppression of angiogenesis. *Int Arch Allergy Immunol* 2004; **133**: 295-304

30 **Guy B**, Hessler C, Fourage S, Haensler J, Vialon-Lafay E, Rokbi B, Millet MJ. Systemic immunization with urease protects mice against *Helicobacter pylori* infection. *Vaccine* 1998; **16**: 850-856

31 **Keenan JI**, Rijpkema SG, Durrani Z, Roake JA. Differences in immunogenicity and protection in mice and guinea pigs following intranasal immunization with *Helicobacter pylori* outer membrane antigens. *FEMS Immunol Med Microbiol* 2003; **36**: 199-205

32 **Dieterich C**, Bouzourène H, Blum AL, Corthésy-Theulaz IE. Urease-based mucosal immunization against Helicobacter heilmannii infection induces corpus atrophy in mice. *Infect Immun* 1999; **67**: 6206-6209

33 **Dunn BE,** Phadnis SH. Structure, function and localization of Helicobacter pylori urease. *Yale J Biol Med* 1998; **71**: 63-73

34 **Sougioultzis S**, Lee CK, Alsahli M, Banerjee S, Cadoz M, Schrader R, Guy B, Bedford P, Monath TP, Kelly CP, Michetti P. Safety and efficacy of E coli enterotoxin adjuvant for urease-based rectal immunization against *Helicobacter pylori*. *Vaccine* 2002; **21**: 194-201

35 **Hocking D**, Webb E, Radcliff F, Rothel L, Taylor S, Pinczower G, Kapouleas C, Braley H, Lee A, Doidge C. Isolation of recombinant protective Helicobacter pylori antigens. *Infect Immun* 1999; **67**: 4713-4719

36 **Thole JE**, van Dalen PJ, Havenith CE, Pouwels PH, Seegers JF, Tielen FD, van der Zee MD, Zegers ND, Shaw M**.** Live bacterial delivery systems for development of mucosal vaccines. *Curr Opin Mol Ther* 2000; **2**: 94-99

37 **Dietrich G**, Spreng S, Favre D, Viret JF, Guzman CA. Live attenuated bacteria as vectors to deliver plasmid DNA vaccines. *Curr Opin Mol Ther* 2003; **5**: 10-19

38 **Sirard JC**, Niedergang F, Kraehenbuhl JP. Live attenuated Salmonella: a paradigm of mucosal vaccines. *Immunol Rev* 1999; **171**: 5-26

39 **Nobiron I**, Thompson I, Brownlie J, Collins ME. Cytokine adjuvancy of BVDV DNA vaccine enhances both humoral and cellular immune responses in mice. *Vaccine* 2001; **19**: 4226-4235

40 **McGettigan JP**, Koser ML, McKenna PM, Smith ME, Marvin JM, Eisenlohr LC, Dietzschold B, Schnell MJ. Enhanced humoral HIV-1-specific immune responses generated from recombinant rhabdoviral-based vaccine vectors co-expressing HIV-1 proteins and IL-2. *Virology* 2006; 344: 363-377

41 **Niethammer AG**, Xiang R, Ruehlmann JM, Lode HN, Dolman CS, Gillies SD, Reisfeld RA. Targeted interleukin 2 therapy enhances protective immunity induced by an autologous oral DNA vaccine against murine melanoma. *Cancer Res* 2001; **61**: 6178-6184

S- Editor Liu Y **L- Editor** Wang XL **E- Editor** Ma WH