PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroenterol 2004;10(16):2340-2343
World Journal of Gastroenterology
 ISSN 1007-9327

Antigen epitope of *Helicobacter pylori* vacuolating cytotoxin A

Xiu-Li Liu, Shu-Qin Li, Chun-Jie Liu, Hao-Xia Tao, Zhao-Shan Zhang

Xiu-Li Liu, Shu-Qin Li, Chun-Jie Liu, Hao-Xia Tao, Zhao-Shan Zhang, Bejing Institute of Biotechnology, Beijing 100071, China
Supported by the National High Technology Research and Development Program of China (863 Program), No. 2001AA215161
Correspondence to: Zhao-Shan Zhang, Bejing Institute of Biotechnology, 20 Dongdajie Street, Fengtai District, Beijing 100071, China. zhangzs@nic.bmi.ac.cn
Telephone: +86-10-66948834 **Fax**: +86-10-63833521
Received: 2004-02-11 **Accepted:** 2004-02-24

Abstract

AIM: To construct and select antigen epitopes of vacuolating cytotoxin A (VacA) for nontoxic VacA vaccine against *Helicobacter pylori* (*H pylori*) infection.

METHODS: Eleven VacA epitopes were predicted according to VacA antigenic bioinformatics. Three candidates of VacA epitope were constructed through different combined epitopes. The candidate was linked with *E. coli* heat-labile enterotoxin B (LTB) by a linker of 7 amino acids, and cloned into plasmid pQE-60 in which fusion LTB-VacA epitope was efficiently expressed. To test the antigencity of the candidate, 6 BALB/c mice were treated with the fusion LTB-VacA epitope through intraperitoneal injection. To explore the ability of inhibiting the toxicity of VacA,cantiserum against the candidate was used to counteract VacA that induced HeLa cells to produce cell vacuoles *in vitro*.

RESULTS: Serum IgG against the candidate was induced in the BALB/c mice. *In vitro*, the three antisera against the candidate efficiently counteracted the toxicity of VacA, and decreased the number of cell vacuoles by 14.17%, 20.20% and 30.41% respectively.

CONCLUSION: Two of the three candidates, LZ-VacA1and LZ-VacA2, can be used to further study the mechanism of vacuolating toxicity of VacA, and to construct nontoxic VacA vaccine against *H pylori* infection.

Liu XL, Li SQ, Liu CJ, Tao HX, Zhang ZS. Antigen epitope of *Helicobacter pylori* vacuolating cytotoxin A. *World J Gastroenterol* 2004; 10(16): 2340-2343
http://www.wjgnet.com/1007-9327/10/2340.asp

INTRODUCTION

Pathogenic strains of *Helicobacter pylori (H pylori)* release a M_r 95 000 protein toxin in the growth medium. Growing evidence indicates that VacA is a major virulence factor in *H pylori* long-term infection leading to gastroduodenal ulcers[1-5]. This toxin induces formation of vacuoles in the cytosol of cells, therefore it has been named vacuolating toxin[6-11]. VacA is thus considered as a therapeutic vaccine for individuals infected with *H pylori*. The *VacA* gene encodes a protoxin approximately M_r 140 000, which belongs to the family of secreted proteins. During the process of VacA secretion, a M_r 95 000 mature toxin is exported. A large oligomeric complex appear as 'flower' with M_r 900 000 which is composed of 6-7 VacA monomers[12-15]. When exposed to the acidic situation, the oligomeric complexes were assembled into monomers and the toxicity of VacA was enhanced[16-18]. After VacA exerted its effect on the cells for 90 min, vacuoles were formed[19]. The vacuoles induced by VacA were acidic. Intracellular vacuolation is believed to induce cell damage and eventually apoptosis, which might lead to release of necrotic factors *in vivo* and therefore contribute to the establishment of a chronic inflammatory response. Because vacuolating cytotoxin A is difficult to express, purify and construct the combined vaccine, we studied the antigen epitopes to reduce the toxin.

MATERIALS AND METHODS

E. coli JM109, pFS2.2 and Hela cell were preserved in our laboratory; pQE-60 was a gift of professor Hou-Chu Zhu, Bejing Institute of Biotechnology, Beijing, China; *H pylori* Sydney strain (*HP SS1*), was a gift of professor Min-Hu Chen, Sun Yat-sen University, Guangzhou, China.

*Bam*H I, *Eco*R V, *Nco* I, *Hin*d III, Pyrobest Taq DNA polymerase and T_4 DNA ligase were purchased from TaKaRa Biotechnology corporations.

Isopropyl β-D-thiogalactoside (IPTG), Freund's adjuvant and sheep anti-mouse IgG-HRP were purchased from Sigma corporations, RPMI 1640 and newborn calf serum were purchased from Hyclone corporations.

BALB/c female mice: 6-8 wk old, SPF.

Bioinformatic analysis and design for candidates of VacA antigen epitope

According to the protein characteristics of hydrophilicity, hydrophobicity, secondary structure, accessibility, flexibility and antigenicity, 9 VacA antigen epitopes in the amino acid site of 34-810 were predicted by the antigen-analyzing software GOLDKEY (Table 1).

Table 1 Site and amino acid sequences of predicted VacA epitopes

Site of amino acid	Amino acid sequences of predicted epitopes
35-46	AEEANKTPDKPD
61-66	PHKEYD
146-154	KDSADRTTR
297-317	GYKDKPKDKPSNTTQNNANNN
335-338	NSAQ
446-450	TDTKN
566-568	SGE
734-737	NNNR
746-748	TDD
766-768	DNY
799-806	TPTENGGN

Three candidates of VacA antigen epitope were designed by combining part of 9 predicted epitopes. The candidate LZ-VacA1 was composed of amino acids 35-36 and 146-154, LZ-VacA2 included amino acids 297-317, and LZ-VacA3 contained

amino acids 61-66, 446-450, 734-737, 746-748, 766-768, and 799-806. The gene sequence of candidate epitope was fused with a 7-amino acid linker (YPQDPSS). The nucleotide acid and amino acid sequences of three candidate epitopes were as following.

The sequence of LZ-VacA1 was:

```
TAC  CCT  5'---CAG  GAT  CCG  TCT  TCC  gcc  gaa  gaa  gcc
 Y    P       Q     D    P    S    S    A    E    E    A
aat   aaa  acc cca   gat  aaa  ccc  gat  aag  gat  agt  gct  gat
 N    K    T   P     D    K    P    D    K    D    S    A    D
cgc   acc  acg  aga   GAT---3'
 R    T    T    R     D
```

The sequence of LZ-VacA2 was

```
TAC  CCT  5'---CAG  GAT  CCG  TCT  TCC  ggt  tat  aag  gat
 Y    P       Q     D    P    S    S    G    Y    K    D
aaa   cct  aag  gat   aaa  cct  agt  aac  acc  acg  caa  aat  aat
 K    P    K    D     K    P    S    N    T    T    Q    N    N
gct   aat  aat  aac   GAT---3'
 A    N    N    N     D
```

The sequence of LZ-VacA3 was

```
TAC  CCT  5'---CAG  GAT  CCG  TCT  TCC  cct  cac  aag  gaa
 Y    P       Q     D    P    S    S    P    H    K    E
tac   gac  acg  gat  acc  aaa  aac  aac  aat  aac  cgc  act  gat
 Y    D    T    D    T    K    N    N    N    N    R    T    D
gac   gac  aat  tac  acg  cct  act  gag  aat  ggt  ggc  aat  GAT---3'
 D    D    N    Y    T    P    T    E    N    G    G    N    D
```

Synthesis of DNA sequence of candidate epitopes and insertion of plasmid pFS2.2

Two splicing sequences were designed according to the nucleotide acid sequences of the candidate epitope and linker. These sequences were synthesized artificially and spliced by PCR reaction. PCR reaction solution containing 5 μL 10× PCR buffer, 0.25 μL pyrobest Taq polymerase (5 U/μL), 1 μL P1 and P2 primer (50 ng/μL), 4 μL dNTP, H_2O to 50 μL. Five PCR cycles were performed, each at 95 °C for 30 s, at 60 °C for 30 s, at 72 °C for 15 s.

LZ-VacA1 primer sequence was P1: 5'--cag gat ccg tct tcc gcc gaa gaa gcc aat aaa acc cca gat aaa ccc gat aag--3', P2: 5'---atc tct cgt ggt gcg atc agc act atc ctt atc ggg ttt atc tgg gg---3'.

LZ-VacA2 primer sequence was P1: 5'--cag gat ccg tct tcc ggt tat aag gat aaa cct aag gat aaa cct agt aac acc--3', P2: 5'---atc gtt att att agc att att ttg cgt ggt gtt act agg ttt atc c---3'.

LZ-VacA3 primer sequence was, P1: 5'--cag gat ccg tct tcc cct cac aag gaa tac gac acg gat acc aaa aac aac aat aac cgc act gat--3', P2: 5'---atc att gcc acc att ctc agt agg ggt gta att gtc gtc atc agt gcg gtt att gtt gt---3'.

The DNA sequences of the candidate epitope and plasmid pFS2.2 containing LTB, were digested with restriction endonucleases *Bam*HI and *Eco*RV, and ligated with T_4 ligase. The recombinant plasmid, which included LTB-VacA gene was transformed to JM109. The positive clones were screened and named them as pLZ-SV1, pLZ-SV2 and pLZ-SV3, respectively.

Construction of expression vector and purification of LTB-VacA protein

To improve the efficiency of expression, two primers with the cloning sites *Nco*I and *Hind*III were designed to construct plasmid pQE-60 with LTB-VacA gene. The recombinant plasmid was transformed to JM109. The positive clones were screened and named them as pLZ-QV1, pLZ-QV2 and pLZ-QV3 respectively.

The primers was

P1 5'---cagccatggGTGAATAAAGTAAAATG---3'
Nco I

P2 5'---ggcaagcttGCTCGGTACTAATTAATTAG---3'
Hind III

The strain with recombinant plasmid was cultured in the Luria-Bertani broth for 3 h, induced by IPTG for 4 h, and then harvested. The targeted protein of LTB-VacA was an inclusion body by SDS-PAGE test. The inclusion body was denatured with 6 mol/L guanidine hydrochloride, and natured with dialysis. LTB-VacA infused protein was purified through the anti-LTB antibody affinity chromatography.

Immunization

Twenty-four female BALB/c mice were randomly and averagely divided into control (LTB), LTB-VacA1, LTB-VacA2 and LTB-VacA3 groups. The mice of each group were immunized through intraperitoneal injection of 200 μL (100 μg protein) LTB, LTB-VacA1, LTB-VacA2 or LTB-VacA3 on days 0, 14 and 28. On days 7, 21 and 35, blood of each mouse was collected and antibody titer was determined.

Cell Vacuolization test

VacA was purified from *H pylori* strain SS1 culture supernatant with ammonia sulfate precipitation. The preliminary experiment was performed to show the amount of *H pylori* strain SS1 culture supernatant was added when cell vacuoles were formed. HeLa cells were cultured as monolayers in flasks in RPMI 1640 containing NCS under 50 mL/L CO_2 at 37 °C. Twenty-four hours before experiment, the cells were released with trypsin/EDTA and seeded in 96-well plates in 10^3/well. After the VacA protein was incubated with antibody to LTB-VacA1, 2, 3 and LTB for 4 h at 37 °C, we added the fixture and VacA protein onto the cell surface for 6 h. Then we calculated the total cell number and cell number of vacuolization.

Statistical analysis

Data are presented as mean±SD. Analysis of variance with a two-tailed students *t*-test was used to identify significant differences. $P<0.05$ was considered statistically significant.

RESULTS

Construction of recombinant plasmid

Recombinant plasmids pLZ-VacA1, 2, 3 encoded the infused gene of LTB and VacA1, 2, 3. It was shown from the digestion map of restriction endonucleases *Nco*I and *Hin*d III, the infused gene was successfully cloned to PQE-60 (Figure 1). LTB-VacA1 had the nucleotide acid number of 465 bp (LTB387+LZ-VacA1 78). LTB-VacA2 had the nucleotide acid number of 465 bp (LTB387+LZ-VacA2 78). LTB-VacA3 had the nucleotide acid number of 489 bp (LTB387+LZ-VacA3 102). The amino acids of three LTB-VacA were deduced from the nucleotide acid sequences. LTB-VacA1 and LTB-VacA2 had 155 amino acids, and the M_r was about 17 000. LTB-VacA3 had 163 amino acids, and the molecular weight was about 17 900. The sequences of the three genes were correct by sequencing analyses.

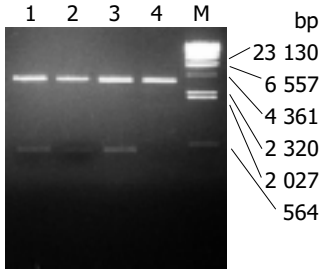

Figure 1 Digestion of recombinant plasmid. M: λDNA / *Hin*d III; Lane 1: pLZ-QV3 / *Nco* I+*Hin*d III; Lane 2: pLZ-QV2 / *Nco* I+*Hin*d III; Lane 3: pLZ-QV1 / *Nco* I+*Hin*d III; Lane 4: pQE-60 / *Nco* I+*Hin*d III.

Expression and purification of infused protein

The LTB-VacA1, 2, 3 proteins were expressed in the JM109 strain. In the SDS-PAGE, the three proteins were 14.13%, 15.51% and 14.79% of the total protein respectively. After purification with anti-LTB antibody affinity chromatography, the percentage of three proteins in the total proteins was improved to 69.26%, 70.18% and 75.35% respectively (Figure 2).

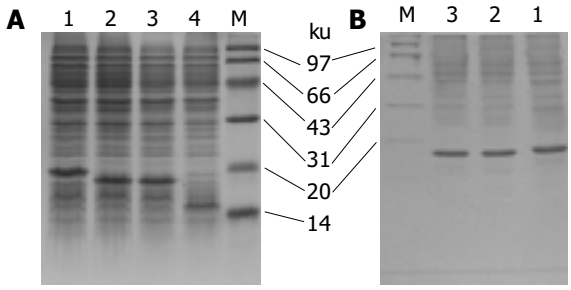

Figure 2 SDS-PAGE of LTB-VacA protein. M: Marker; Lane 1: LTB-VacA3; Lane 2: LTB-VacA2; Lane 3: LTB-VacA1; Lane 4: LTB. A: LTB-VacA proteins were expressed in the JM109; B: Purification with anti-LTB antibody affinity chromatography.

Serum antibody levels in infused protein

Intraperitoneal immunization with the infused protein and Freund's adjuvant resulted in a marked elevation of serum IgG antibody in all the 6 mice after three immunizations (Figure 3).

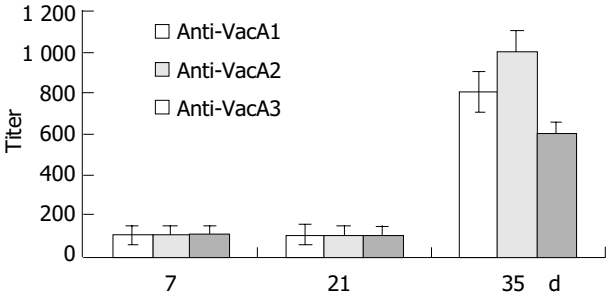

Figure 3 IgG against VacA induced by different LTB-VacA.

Seven days after the first immunization, the Level of antibody was lower. The titer of the antibody was about 1:100. Seven days after the second immunization, the titer of the antibody had no remarkable changes. But 14 d after the third immunization, the titer of the anti-LTB-VacA1 antibody, the titer of the anti-LTB-VacA2 antibody and the titer of the anti-LTB-VacA1 antibody was increased to 1:800, 1:1 000 and 1:600 respectively. The biggest value of positive and negative in the three antibodies was 3.8, 4.2 and 3.2.

HeLa cell vacuolization

The preliminary experimental results showed when we added 10, 30, 40, 80 and 100 μL culture supernatant, the cell vacuolization was produced. Because the space of wells in flasks well was limited, 100 μL VacA protein was added into the culture medium. The ratio of cell vacuolization after we added 100 μL protein was 49.52% (Figure 4).

The control serum, anti-VacA1 serum, anti-VacA2 serum and anti-VacA1 serum were incubated with 100 μL VacA antigen for 4 h. Then we added the fixture and VacA protein in the flasks wells for 6 h. The result showed the control serum and 100 μL VacA antigen could not reduce the ratio of cell vacuolization, but anti-VacA1 serum, anti-VacA2 serum, anti-VacA3 serum could decrease the ratio of cell vacuolization. The change rate of cell vacuolization is 30.41%, 20.20% and 14.17% respectively (Table 2).

Table 2 Inhibition of anti-LTB-VacA antibody on VacA toxin

Group	HeLa cells with vacuoles in total cells (%)	Change rate (%)
VacA toxin	49.52±2.77	0
Anti-LTBantibody (Control)	47.51±1.31	-4.05±1.61
Anti-LTB-VacA1 antibody	34.47±1.97	-30.41±2.41[b]
Anti-LTB-VacA2 antibody	39.52±1.69	-20.20±2.07[b]
Anti-LTB-VacA3 antibody	42.51±1.84	-14.17±2.27[a]

[a]$P<0.05$, [b]$P<0.01$ *vs* control.

DISCUSSION

Many methods could predict the epitopes of protein known as the primary structure, for example hydrophilicity scheme[20], accessibility scheme[21], antigenicity scheme[22], flexibility scheme[23,24] and secondary structure scheme[17]. The antigenic epitopes are correlated with the characteristics, number, sequence of amino acid and protein conformation. Because the different prediction methods emphasize different biological information of antigens, several methods are considered in practice. In this study we chose the GOLDKEY software developed by the Experimental Group led by professor Jia-Jin Wu to analyse the characteristics of VacA protein including hydrophilicity, hydrophobicity, secondary structure, accessibility, flexibility and antigenicity. This software could predict the liner B epitope. At last we got 11 VacA candidate epitopes.

Due to the small molecular weight and the weakness of antigenicity of the antigen epitopes, the carrier or adjuvant must be linked to the epitopes[25,26]. Several epitopes were joined in series and at last got 3 VacA candidate epitopes. LTB is an excellent protein adjuvant to facilitate the organism to produce the antibody epitopes, so we chose LTB to link the 3 epitopes.

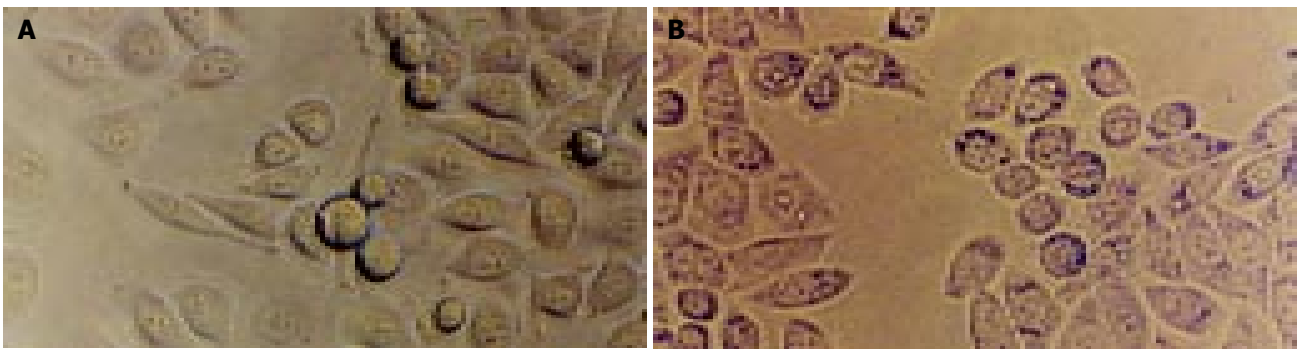

Figure 4 Microscopy of large vacuoles induced by VacA in HeLa cells. A: Normal HeLa cells; B: Vacuolated HeLa cells (Origingal magnification: ×400).

The experiment of Schodel enucleate that plasmid pFS2.2 was a carrier in which *LTB* gene could express soluble LTB and carry outer polypeptides. The experiment of Zhang showed that 21-bp nucleotide acids between LTB and the epitopes could enhance the antigencity of the epitopes. In our study, 7 peptides were used as a linker to join LTB with the epitopes.

At the beginning, LTB-VacA was cloned into plasmid pFS2.2, but the gene could not express these proteins, and then the genes were cloned into plasmid pET22b (+) again. There was a signal peptide in plasmid pET22b (+) in which the gene could express the soluble protein and secrete the protein into periplasm. The soluble proteins would be purified through the anti-LTB antibody affinity chromatography. Contrary to our wishes, the proteins in pFS2.2 were not expressed as expected. Finally, infused proteins were cloned into plasmid pQE-60 and expressed in JM109, but the expressed proteins were inclusion bodies. The inclusion bodies were denatured with guanidine hydrochloride and natured with dialysis, and LTB-VacA was purified through the anti-LTB antibody affinity chromatography.

Protein vacuolating toxin A is the only known virulence factor of *H pylori*. Ninety minutes after VacA activation, the acidic vacuoles were induced in cells. Scientific researches showed that VacA was integrated with receptors in membranes to form an anionic channel. This channel could change the permeation characteristics of the membranes, so that the cells were damaged would undergo apoptosis. In this study, all the 3 antigen epitopes of VacA could induce antibody in mice. Although the antibody could inhibit the vacuolation of Hela cells at a certain extent, they did not inhibit the vacuoles entirely. There are two reasons for this result. First, these epitopes were a part of the neutralized epitopes of VacA, antibody to these epitopes combined with VacA did not destroy the toxicity of VacA, only suppressed the toxin partly. Second, the titer of the antibody was not enough to neutralize the toxin of VacA. Next we are going to settle the problem. First, these epitopes will be joined in series for several copies so that the titer of the antibody will be improved to inhibit the toxin of VacA. Second, we will construct deficient mutations to farther verify the neutralized epitopes of VacA.

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Edited by Wang XL and Chen WW **Proofread by** Xu FM