Antigen epitope of Helicobacter pylori vacuolating cytotoxin A

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INTRODUCTION
Pathogenic strains of Helicobacter pylori (H pylori) release a M, 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, 140 000, which belongs to the family of secreted proteins. During the N process of VacA secretion, a M, 95 000 mature toxin is exported.

RESULTS
Vacuole formation was observed in HeLa cells treated with VacA. Serum IgG against the candidate was induced. In vitro, the three antisera against the candidate efficiently counteracted the vacuolation 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 VacA. A large oligomeric complex appear as ‘flower’ with M, 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 IM109, pFS2.2 and Hela cell were preserved in our laboratory; pQE-60 was a gift of professor Hou-Chu Zhu, Beijing 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.

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...
The sequence of LZ-VacA1 was:

TAC 5'---AGG GAT CCC TCT TCC gct tat aag gat Y P Q D P S S G Y K D
aaa cct aag gat aaa cct agt aac aac acg caa aat aat K P K D K P S N T T Q N N
gct aat aat aac GAT---3'
R T T R D

The sequence of LZ-VacA2 was

TAC 5'---AGG GAT CCC TCT TCC gtt tat aag gat Y P Q D P S S G Y K D
aaa cct aag gat aaa cct agt aac aac acg caa aat aat K P K D K P S N T T Q N N
gct aat aat aac GAT---3'
R T T R D

The sequence of LZ-VacA3 was

TAC 5'---AGG GAT CCC TCT TCC ccc gaa gaa gcc aat aac cca gat aaaa ccg gat aag---3', P2: 5'---

The primers were

P1 5'---cag cag cag ctc tcc gcc gaa gaa gcc aat aac cca gat aaaa ccg gat aag---3'
P2 5'---gga aac ggt gcc gat aag gat aat aat

The DNA sequences of the candidate epitope and plasmid pFS2.2 containing LTB, were digested with restriction endonuclease Hind III; Lane 1: pLZ-QV1, pLZ-QV2 and pLZ-QV3 respectively.

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 LBV-VacA was an inclusion body by SDS-PAGE test. The inclusion body was denatured with 6 mol/L guanidine hydrochloride, and natured with dialysis. LBV-VacA infected 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), LBV-VacA1, LBV-VacA2 and LBV-VacA3 groups. The mice of each group were immunized through intraperitoneal injection of 200 µL (100 µg protein) LTB, LBV-VacA1, LBV-VacA2 or LBV-VacA3 on days 0, 14, 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 CO2 at 37 °C. Twenty-four hours before experiment, the cells were released with trypsin/EDTA and seeded in 96-well plates in 103/well. After the VacA protein was incubated with antibody to LBV-VacA1, 2, 3 and LTB for 4 h at 37 °C, we added the mixture 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 Hind III, the infused gene was successfully cloned to PQE-60 (Figure 1). LBV-VacA1 had the nucleotide acid number of 465 bp (LTB387+LZ-VacA1 78). LBV-VacA2 had the nucleotide acid number of 465 bp (LTB387+LZ-VacA2 78). LBV-VacA3 had the nucleotide acid number of 489 bp (LTB387+LZ-VacA3 102). The amino acids of three LBV-VacA were deduced from the nucleotide acid sequences. LBV-VacA1 and LBV-VacA2 had 155 amino acids, and the M, was about 17 000. LBV-VacA3 had 163 amino acids, and the molecular weight was about 17 900. The sequences of the three genes were correct by sequencing analyses.
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.

Serum antibody levels in infused protein

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, 1:1000 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-VacA3 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                   | 49.32±2.77                                 | 0               |
| Anti-LTB antibody (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   |

aP<0.05, bP<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 GOLDFKEY 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 (Original 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 antigenicity 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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