

• *Helicobacter pylori* •

***Helicobacter pylori* specific immune response induced by conservative flagellin linear B-cell epitope**

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Abstract

AIM: To testify the immunogenicity of a conservative B-cell linear epitope of *Helicobacter pylori* (*H. pylori*) flagellin A.

METHODS: Different programs were used to analyze the secondary structure, molecular hydrophathy, and surface accessibility of *H. pylori* flagellin A. Linear B-cell epitopes were estimated based on the structural and physiochemical information. Analysis of residue divergence was proposed to screen a conservative linear epitope. The 29-peptide (Pep29mer) synthesized by chemical method, including the predicted conservative B-cell epitope and a known K^{2d} compatible T-cell epitope, was used to immunize mice, and then *H. pylori*-specific antibodies were detected by ELISA.

RESULTS: Based on the analyses of divergent amino acid residues, structural and physiochemical characteristics, it was strongly suggested that the short fragment NDSGGR was the core of a conservative linear epitope in flagellin A. Animals immunized by Pep29mer acquired efficient immune response. In detail, serum *H. pylori*-specific IgA and IgG1 increased significantly in immunized group, while IgG2a only had an insignificant change. *H. pylori*-specific IgA in gastrointestinal flushing fluid also increased significantly.

CONCLUSION: The conservative short fragment NDSGGR is the core of a linear B-cell epitope of flagellin A.

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Key words: *Helicobacter pylori*; Flagellin A; B-cell epitope

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INTRODUCTION

Helicobacter pylori (*H. pylori*), a spiral-shaped and microaerophilic Gram-negative bacterium is one of the most important etiologic agents of peptic ulcer, carcinoma, mucosa-associated lymphoid tissue lymphoma and other gastric diseases. Several potential virulence factors have been suggested to play a role in *H. pylori* pathogenesis^[1-3].

Flagellar motility conferred by 3-6 flagella that extend from one pole of the bacterium is related to the colonizing ability of *H. pylori* and regarded as one of the major virulent factors. Flagellin A is the major molecule of flagellar filaments and responsible for bacterial motility in *H. pylori*^[6,7]. According to the reported data, flagellin A is one of the major antigens to induce the production of IgG and IgA in serum^[8-12]. As one of the predominant antigens, it has different serotypes of flagellin antigen in many species^[13-17], but no specific flagellar serotype has been discovered in *H. pylori* so far. Therefore, much effort should be made to determine the immunological characteristics of *H. pylori* flagellin A. This experiment was carried out to estimate and testify the linear epitopes of flagellin A in order to offer a new vaccine candidate against *H. pylori*.

MATERIALS AND METHODS

Strains and sequences

H. pylori strains were isolated from gastric biopsy specimens of Chinese outpatients with peptic ulcer or gastritis and testified by urease C¹⁴-breath test, Gram stain, and colony forming assay. All strains were preserved in -20 °C refrigerator.

Sequences Hpxa 1-6 were amplified from six Chinese clinical isolates. Other sequences were retrieved from GenBank. All sequences were compared to search the divergent amino acid residues.

Programs

Programs including Gor IV, SAPS, SOSUI and PHD were used to analyze the secondary structure (especially for β -turn), hydrophathy and surface accessibility.

Design and animal immunization of synthetic Pep29mer

Pep29mer N¹-GALNNRFQIKGVELKSKNDSGDG-RLVAAN-C' (lot 10018763, Genemed Synthesis Inc., CA,

USA) was synthesized, which contains the proposed B-cell epitope of *H. pylori* flagellin A (bold) and an accepted H^{2d} compatible T-cell epitope derived from hemagglutinin light chain HA2 of *Virus influenza* (shaded)^[18]. Animals were divided into three groups (10 mice in each group): immunized, adjuvant control and nude mice control groups. The detailed strategy of inoculation is shown in Table 1. All mice were immunized subcutaneously at two sites in the abdomen. In immunized group, 6-week old female BALB/c mice were primed with 5 mg/L Pep29mer solution (lactified with complete Freund adjuvant), and then enhanced with 5 mg/L Pep29mer solution (lactified with incomplete Freund adjuvant) 2 wk later. In adjuvant control group, 6-wk old female BALB/c mice were inoculated with complete Freund adjuvant for priming, and incomplete Freund adjuvant 2 wk later for enhancement. In nude mice control group, 6-wk old female BALB/c nude mice were treated with the same procedure as in immunized group.

Table 1 Strategy of animal inoculation

Grouping	Animal	Treatment
Immunized	BALB/c mice	Pep29mer 50 µg plus equal volume of adjuvant
Adjuvant control	BALB/c mice	Adjuvant only
Nude mice control	BALB/c nude mice	Pep29mer 50 µg plus equal volume of adjuvant

Preparation of *H. pylori* antigen

H. pylori strains were inoculated onto tryptic soy agar containing 70 g/L sheep blood and incubated microaerophilically at 37 °C for 2 wk. The colonies were washed and harvested with 0.1 mol/L phosphate buffered saline (PBS) and sonicated. The supernatant clear of cellular debris was collected by centrifugation. Concentration of *H. pylori* lysates was determined by spectrophotometric measurement of A₂₈₀ (UV2201, Shimadzu Corporation, Japan).

Preparation of mouse serum and gastrointestinal washing fluid

Exsanguination from caudal vein was performed on d 7, 21, 35 and 49, respectively, after priming inoculation. Serum was preserved at -70 °C with 500 mL/L glycerol till assay. Gastrointestinal washing fluid was collected as described by Elson *et al.*^[19].

ELISA measurement of antibodies in serum and gastrointestinal fluid

H. pylori-specific antibodies of serum and gastrointestinal fluid were detected by ELISA. Each well of microtiter plates (Maxisorp, Nunc, Denmark) was coated with 100 µL *H. pylori* lysate solution at the concentration of 12.5 g/L in 10 mmol/L PBS (pH 7.2) at 37 °C for 3 h and blocked by 50 g/L lipid-free milk PBS (pH 7.2) overnight at 4 °C. One hundred microliters of mouse serum was added to each well and incubated at 37 °C for 1 h at the dilution of 1:100. Horse radish peroxidase-labeled goats-anti mouse IgG₁, IgG_{2a} and IgA (cat.No. 1070-05, 1080-05 and 1040-05,

Southern Biotechnology Associates, Inc., USA) were added at the dilution of 1:6 000 and incubated at 37 °C for 1 h. Gastrointestinal washing fluid was diluted serially and 100 µL of it was added to each well to detect the titer of IgA. TMB kit (011228, Jingmei Biotech) was employed to show the results. Briefly, 50 µL of A and B solutions were added to each well for 5-30 min. The reaction was stopped by 50 µL C solution (1.2 mol/L sulfuric acid). Full cleansing using 1 g/L Tween-20 10 mmol/L PBS (PBST) was required before each step. Absorbance (A₄₅₀) was measured by a plate reader (MRP-2100, Syntex, USA). Calibrator and controls were used in each test. Negative, positive control and mice sera were assayed in duplicate. The absorbance value was presented as mean ± SD. The cut-off value was determined.

RESULTS

Structural and physiochemical characteristics

Figure 1 shows the structural and physiochemical characteristics of *H. pylori* flagellin A. α -helices were located in both termini which formed the central tube of filaments, while β -sheets and irregular coils were mainly located in the central region of flagellin A which stretched out of the filament. As a hydrophobic molecule in general, three hydrophilic peaks were found in the central region of flagellin A (residues 211-230, 261-280, and 281-300, respectively). β -turns (residues NKNRTG and NDSDGR) were found in the first and second hydrophilic peaks. Residues KNSNRTG in the first peak and DIKKNDSD in the second peak were exposed outside with high probability by analysis of surface accessibility. The segments of high surface accessibility overlapped the hydrophilic residues in the first and second peaks.



Figure 1 Structural and physiochemical characteristics of flagellin A. **A:** Sequence of antigen determinant region in the central part of flagellin A. Underlined: hydrophilic peaks; shaded: β -turn short fragment; framed: residues accessible to molecular surface with high probability; **B:** secondary structure of flagellin A. Long bar: α -helices; short bar: β -sheets; other residues: irregular coils.

Analysis of amino acid divergence

Three characteristic divergent residues, 206 V→I, 227 R→Q and 294 K→N, were found by comparing 16 sequences of 120 amino acid residues (182-301) in central region of flagellin A. Among them, two divergent residues, 227 R→Q and 294 K→N, were in the first and third peaks, respectively. No divergent residues were found in the second hydrophilic peak (Table 2).

Table 2 Characteristic divergences of amino acid residues in the central region of flagellin A

Sequence	Divergent residues		
	206 th	227 th	294 th
U63249 ¹	I	R	N
U63238 ¹	I	Q	N
Hpxa3	I	Q	N
U63253 ¹	I	Q	N
Hpxa1	I	Q	N
Hpxa2	I	Q	N
Hpxa5	I	Q	N
U63250 ¹	V	Q	N
U63236 ¹	V	R	K
AE0001478 ¹	V	R	K
U63224 ¹	V	R	K
AE000574 ¹	V	R	K
X60746 ¹	V	R	K
AJ009373 ¹	V	R	K
Hpxa4	V	R	K
Hpxa6	V	R	K

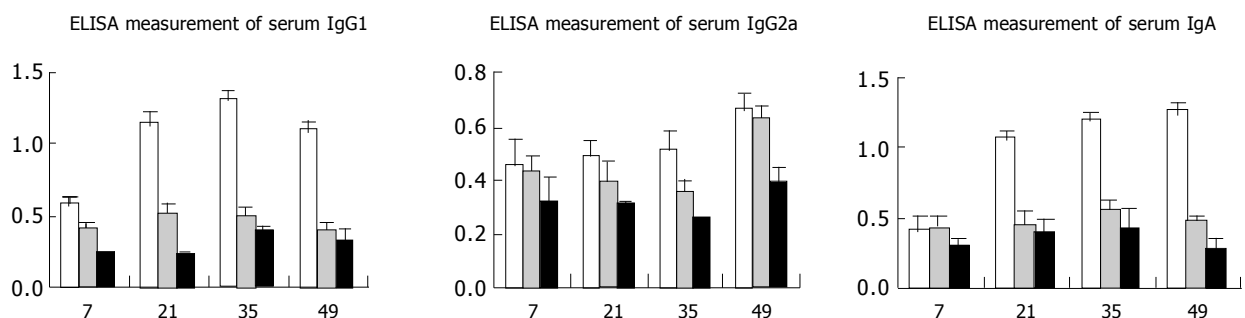
¹Sequence retrieved from GenBank.**Specific antibodies in serum and gastrointestinal fluid**

In ELISA, *H. pylori* specific IgG₁ and IgA in mouse serum rose significantly three weeks after priming inoculation, whereas only slight change of *H. pylori* specific IgG_{2a} was found in BALB/c mice (Figure 2). Measurement of antibody titer demonstrated that *H. pylori* specific IgG₁ and IgA rose significantly within the dilution of 1:64-1:1 024 (Figures 3A

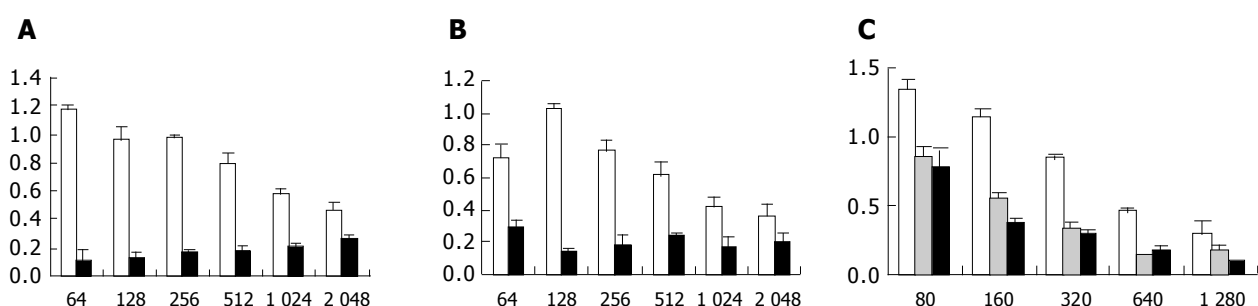
and B). In gastrointestinal washing fluid, *H. pylori* specific IgA was also detected which was significantly high in immunized group with effective titer between the dilution of 1:160-1:640 (Figure 3C).

DISCUSSION

A single small segment (sequence recognition) or a domain (conformation recognition) could act as an antigen (antigenic epitope). The epitope of the former is a consecutive sequence (linear) and generally bends with a typical non-ordered structure (turns and/or loops) and might serve as a strategy of synthetic vaccine. The influence of the physiochemical characteristics of amino acid residues on molecular secondary structure has been studied based on the statistical propensity^[20,21]. The antigen determinant region can be determined by the analysis of molecular hydrophathy with computer program^[22]. In some reports, many short fragments predicted with hydrophathy as epitopes are proved to be by immunological methods^[23-27]. Surface accessibility is also related to antigenicity, but fewer experiments have predicted epitopes with this method. Forster *et al*^[28], have predicted three epitopes of *H. pylori* urease by surface accessibility analysis of computer-aided molecular modeling, but only one of them is proved to produce anti-holoenzyme specific antiserum. It seems that computer-aided method is limited in predicting antigenicity of flagellin A based on protein physiochemical characteristics. Van Regenmortel *et al*^[29], found that none of the scales, including hydrophathy, surface

**Figure 2** ELISA measurements of antibody subtypes in mouse serum Blue: immunized group; brown: adjuvant control group; light yellow: nude mice

control group. Abscissa: time for exsanguination after prime inoculation; ordinate: A value (mean±SD).

**Figure 3** Titer measurements of IgG₁, IgA in mice serum (A and B) and IgA in gastrointestinal fluid (C) Blue: immunized group; brown: adjuvant control group;

light yellow: nude mice control group. Abscissa: dilution of mouse gastrointestinal washing fluid; ordinate, A value (mean±SD).

accessibility or segmental motility, gives a level of correct prediction higher than 50-60%. Therefore, integration of physiochemical and structural information is required to improve the reliability and accuracy of antigenic prediction. Alix *et al.*^[30], have summarized the known algorithms, and presented predictive estimation of protein linear epitopes (PEOPLE) algorithm to predict the linear consecutive antigenic epitopes, in which enough attention is paid to the secondary structure (mainly β -turns), hydrophilicity, surface accessibility and flexibility/motility.

Flagellins are genetically diverse and intraspecific free recombination of frequent incidence. In this experiment, to disclose the antigenicity of flagellin A, β -turns, molecular hydrophathy and surface accessibility were analyzed because these characteristics greatly influence the prediction of linear epitopes. Briefly, three hydrophilic peaks were found in the central region of flagellin A. In the first and second peaks, two short segments, NKNSNRTG and NDSGDR, with typical β -turns were found. These two short segments were overlapped with residues that exposed outside the filaments. According to the composite index, the first and second peaks are more likely the proposed B-cell epitopes. Residues in the second hydrophilic peak were conservative in all the 16 *H. pylori* strains. As a conservative short segment with a consecutive epitope, short segment KNDSGDRLVAAIN might be a hopeful vaccine candidate.

In animal model, efficient *H. pylori* specific immune response was acquired in immunized group. In detail, *H. pylori*-specific IgA and IgG1 rose significantly, while IgG2a only had a slight change. *H. pylori*-specific IgA in gastrointestinal flushing fluid also had a significant change. Though more work is needed to determine the residues of this epitope, the initial results strongly suggest that there is a B-cell epitope in the short segment KNDSGDRLVAAIN.

In summary, based on the analyses of genetic divergence, molecular structure, and physiochemical characteristics, a conservative hydrophilic segment in flagellin A central region is found, which is in accordance with the quality of linear B-cell epitope. With the adjuvant of CFA/IFA, Pep29mer containing the predicted B-cell epitope can induce *H. pylori*-specific immune response in animal model. The short segment KNDSGDRLVAAIN is proved to be the core of a linear B-cell epitope in *H. pylori* flagellin A.

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