Vaccine 28 (2010) 5220–5227

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Identification of B-cell epitopes in urease B subunit of *Helicobacter pylori* bound by neutralizing antibodies

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ARTICLE INFO

Article history:
Received 2 February 2010
Received in revised form 4 May 2010
Accepted 26 May 2010
Available online 9 June 2010

Keywords:
Helicobacter pylori
B-cell epitope
Urease B subunit
Neutralizing antibody

ABSTRACT

To identify linear B-cell epitopes of urease B (UreB), a series of 19 partially overlapping fragments of the UreB gene were expressed. Three MAbs against UreB of *Helicobacter pylori* (*H. pylori*), A1H10, A3C10, and B3D9, were tested for their reactivity to the truncated proteins by Western blot and enzyme-linked immunosorbent assay (ELISA). Three linear B-cell epitopes were identified covering a stretch of 15 amino acid (aa) residues and localized in the aa regions 158–172, 181–195, and 349–363 of UreB. ELISA also showed that the three synthetic peptides containing epitope sequences (UP32: GGGTGPADGTNATTI, UP35: WMLRAAEEYSMNLGF, and UP38: TLHDMGIFSITSSDS) were recognized by the corresponding MAbs and *H. pylori* positive sera from *H. pylori* infected patients. Mice immunized with glutathione S-transferase (GST) fusion peptides showed that epitope-specific antibodies were capable of inhibiting urease enzymatic activity. These results should be useful in clinical applications and highlight the potential importance of these epitopes as the targets for development of epitope-based vaccines against *H. pylori*.

1. Introduction

Helicobacter pylori (*H. pylori*), a spiral shaped, microaerophilic, Gram-negative bacterium, has been isolated from the gastric and duodenal mucosa of patients with gastroduodenitis and peptic ulcers by Marshall and Warren [1]. It has been classified as a group I carcinogen by the International Agency for Research on Cancer and evidence of *H. pylori* as a gastrointestinal pathogen has steadily accumulated [2–4]. Infection with *H. pylori* is highly associated with chronic active gastritis, gastric ulceration, and duodenal ulceration, lymphoma of mucosa-associated lymphoid tissue, and the development of gastric cancer [5–8]. Currently, proton pump inhibitor-based triple therapy is effective in the majority of patients with gastrointestinal symptoms. However, this eradication treatment has several disadvantages, especially, the high cost of the treatment [9–11]. Therefore, developing an effective prophylactic vaccination has been suggested as a superior strategy over the use of antibiotics for the control of *H. pylori* infection.

Urease is a major protein constituent of *H. pylori* and localized in both the cytoplasm and on the cell surface. With a molecular weight of ≈580 kDa and two distinct subunits, UreA (29.5 kDa) and UreB (66 kDa), this enzyme plays an important role in the pathogenesis of *H. pylori* infection because ammonia produced by urease can neutralize gastric acid, producing high pH conditions beneficial for promoting colonization of the stomach [12]. UreB is considered a potential antigen for the development of prophylactic and therapeutic vaccines against *H. pylori* infection, and evidence has shown that UreB is a major target recognized by the antibodies from *H. pylori* infected patients [13]. Previous studies have found that monoclonal antibody (MAb) against *H. pylori* urease has the ability to inhibit enzymatic activity [14–16], which is important in preventing *H. pylori* infection, whereas urease-specific polyclonal antibodies generated by immunization with *H. pylori* urease protein did not induce this inhibitory effect [14].

It is well known that vaccines take advantage of the surface antigen epitopes to stimulate the body's immune response, producing resistance to foreign pathogens. Thus, the acquisition of antigen epitopes is a very important step in vaccine development. Previous studies from this laboratory have developed eight MAbs against UreB of *H. pylori* which involve three different antigen determinants [17]. The aim of this study was to identify the epitopes used by MAbs A1H10, B3D9, and A3C10 and to evaluate their antigenicity to further the understanding of these proteins for application in vaccine design.

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doi:10.1016/j.vaccine.2010.05.067

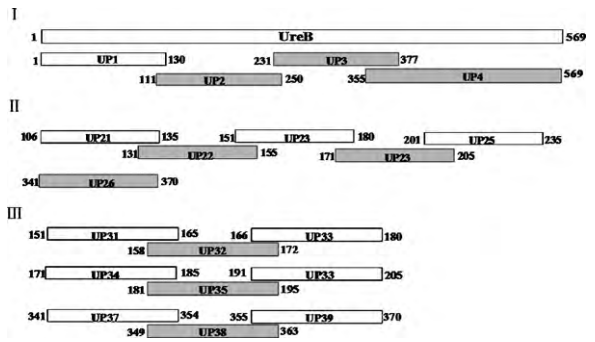

Fig. 1. Schematic diagram showing truncated fragments derived from UreB of *H. pylori* 26695 and their relative positions. Letters, aa positions of UreB; peptide names same as in Table 1; bars, peptides of truncated UreB; peptides negative in Western blot with MAbs A1H10, B3D9, or A3C10 shown in white; peptides positive under same conditions shown in gray.

2. Materials and methods

2.1. Bacterial strains and culture conditions

H. pylori strains NCTC 11637 and 26695 were cultured on *H. pylori* selective agar plates with 5% defibrinated sheep blood and antibiotics at 37 °C under microaerophilic conditions with 5% O_2, 10% CO_2, and 80% N_2. After 3–4 days of culture, colonies were scraped and washed twice with cold phosphate buffered saline (PBS) at pH 7.0 and pelleted by centrifugation at 10,000 × *g* and 4 °C for 10 min. A portion of the pellet was resuspended in PBS, disrupted by sonication, and centrifuged as above for 20 min. The resulting supernatant, containing urease, was then used for urease inhibition assay, ELISA, and Western blotting. Another portion of the pellet was used for preparing genomic DNA.

2.2. Expression and purification of truncated UreB

Nineteen truncated fragments of UreB were constructed (Fig. 1). The primers for amplification of the truncated fragments were designed using the *H. pylori* strain 26695 gene sequence and two restriction enzyme sites, SacI or EcoRI or BstBI and HindIII or XhoI, were introduced into the sense and antisense primers, respectively; all the sequences and locations of the primers are listed in Table 1. The amplified PCR products were cloned into the prokaryotic expression vector pET-28a (+) or pGEX-4T-2. After verification by sequencing, each of the recombinant expression constructs was transformed into *Escherichia coli* BL21(DE3) (Novagen, USA) to produce the recombinant proteins in vitro. Recombinant GST or GST fusion proteins were purified by glutathione-Sepharose 4B beads. At the same time, the UreBM (106–377, used as positive control) also was expressed in *E. coli* BL21 and purified by HisTrap FF column. All the procedure of purification was according to the manufacturer's protocol (GE healthcare, USA). The proteins were then used for subsequent Western blot analysis, immunization, and ELISAs.

2.3. Epitope mapping

2.3.1. SDS-PAGE and Western blot analysis

To identify each MAb-defined epitope, truncated recombinant proteins were separated by 15% SDS-PAGE under denaturing conditions, transferred onto nitrocellulose (NC) membranes, and then blocked with blocking buffer (5% skimmed milk dissolved in PBS, pH 7.4, with 0.05% Tween 20) at 37 °C for 2 h to prevent non-specific protein binding. The membranes were then incubated at 37 °C for 2 h with a 1:1000 dilution of each MAb, washed 3 times with PBS solution containing 0.05% Tween 20 (PBST) at room temperature, and incubated with HRP-conjugated goat anti-mouse IgG (1:50,000 dilution, Sigma–Aldrich) at 37 °C for 2 h. The binding reaction was detected using detected using HRP Western blotting analysis kit (TianGen, China).

2.3.2. ELISA analysis

The reactivity of each MAb with purified, truncated recombinant proteins GST–UP32, GST–UP35, and GST–UP38 was determined by ELISA. Purified GST protein used as negative control. Briefly, 96-well micro-ELISA plates were coated with 5 μg/mL recombinant proteins diluted in 0.1 mM carbonate buffer (pH 9.6), incubated overnight at 4 °C, washed 3 times with PBST, and 200 μl blocking buffer (PBST containing 1% BSA) added to the wells for blocking at 37 °C for 2 h. Next, 100 μl of MAbs (1:400–1:1000) or *H. pylori* positive sera (1:100) from *H. pylori* infected patients (in The General Hospital of Chinese PLA), appropriately diluted in PBST containing 0.1% BSA, was added and incubated at 37 °C for 2 h. Purified glutathione S-transferase (GST) protein or sera from healthy persons was used as a negative controls for the MAbs or patient sera, respectively. After washing the wells 3 times with PBST, HRP-conjugated goat anti-mouse IgG (1:50,000 dilution, Sigma–Aldrich) was added to the wells (100 μl/well) and after a further 1 h incubation at 37 °C the plates were again washed. An enzyme substrate solution (0.1 M citrate monohydrate, 0.2 M disodium hydrogen phosphate, 1 mg/ml OPD and 0.3% hydrogen peroxide 100 μl/well), was added and the reaction allowed to progress at room temperature for 15 min. The A_{492} was measured using an ELISA plate reader after quenching the reaction with 50 μl of 2 M H_2SO_4. All samples were analyzed in duplicate and the ELISA and means of the duplicates reported in the results.

2.4. Peptide synthesis

The synthetic peptides used are listed in Table 2. Three peptides were synthesized commercially (Scilight Biotechnology, LLC) and comprised 15 aa which included aa 158–172 (GGGTGPADGTNATTI), 181–195 (WMLRAAEEYSMNLGF), and 349–363 (TLHDMGIFSITSSDS) of UreB. The peptides, supplied as a white water soluble powder, were assessed (by HPLC) to be in excess of 90% pure and stored at a concentration of 1 mg/ml.

2.5. ELISAs for peptides

Binding of MAbs to peptides was measured by ELISA. Each peptide was coated onto micro-ELISA plates (10 μg/ml, 100 μl/well) and the subsequent steps of the assay performed as described above for the indirect ELISA. A non-specific peptide was used as a negative control to determine background responses. For example, if MAb A1H10 was used as primary antibody, peptide UP32 was used as specific sample, while peptide UP35 or UP38 was used as a control, and vice versa. *H. pylori* positive sera (1:100) from *H. pylori* infected patients was also used as the primary antibody to detect the reactivity of the peptides, with sera from healthy individuals used as a negative control. All assays were performed in triplicate.

2.6. Immunization of mice and sample collection

The antigenicity of the purified fusion proteins (GST–UP32, GST–UP35, and GST–UP38) was evaluated by immunizing 8-week-old female BALB/c mice (Experimental Animal Center of The Academy of Military Medicine Sciences, China, 5 per group) subcutaneously with 100 μg of purified proteins in an emulsion with complete Freund's adjuvant (CFA, Sigma–Aldrich) on day 0. The mice were then boosted at 2-week intervals with 100 μg of the same protein in an emulsion with incomplete Freund's adjuvant (IFA, Sigma–Aldrich) for three cycles. Control mice were immunized using the same immunization schedule and purified GST