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Original

Production of antibody against a synthetic peptide of *Porphyromonas gingivalis* 40-kDa outer membrane protein

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Abstract: ***Porphyromonas gingivalis*** **has been implicated as a major pathogen in periodontal diseases. We previously cloned a 40-kDa outer membrane protein (OMP) gene from *P. gingivalis* 381 and succeeded in producing sufficient quantities of the recombinant protein for experimental use. Since antibodies against the recombinant (r) 40-kDa OMP have potent ability to kill *P. gingivalis* cells by complement activation and opsonization, r40-kDa OMP has been the subject of considerable interest as a possible vaccine candidate. In this study, in order to develop a component vaccine, the immunodominant domain in 40-kDa OMP was identified. Peptides corresponding to portions of the N-terminal regions of 40-kDa OMP were synthesized chemically and their immunoreactivities with antibody against r40-kDa OMP were tested. The 16-mer peptide, LDDEYKERVFQTFVHY, was found to react strongly with the antibody. Furthermore, a rabbit antibody was prepared by immunization with the 16-mer peptide, cross-linked with dehydrofolate reductase, and its immunoreactivity was then examined. In a BIAcore experiment the antibody clearly reacted with r40-kDa OMP as well as *P. gingivalis* strains. These findings suggest that the 16-mer synthetic peptide may be useful for development of a component vaccine against *P. gingivalis*. (J. Oral Sci. 45, 111-116, 2003)**

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Introduction

Periodontitis is a destructive inflammatory disease of the supporting tissues of the teeth associated with subgingival infection by a large variety of gram-negative bacteria and it is a major cause of tooth loss (1). Among the oral, anaerobic, Gram-negative bacteria that have been recognized as prominent members of subgingival dental plaque, *Porphyromonas gingivalis* has been implicated as an important pathogen in adult periodontitis (2).

Since immunization against whole *P. gingivalis* cells inhibited the progression of periodontitis (3), vaccination could become an important immunotherapy to help prevent periodontal diseases. However, it is also possible to induce periodontal destruction by immunization; i.e., the immune system has the potential to induce destructive changes (4). The demand for a safer vaccine has led to the development of a component vaccine. In the development of a defined and specific component vaccine, it is essential, therefore, to identify the key virulence factors of the pathogen. The role of the outer membrane proteins in induction of a protective immune response has been studied extensively in various bacterial infections (5).

Previously, we have succeeded in gene cloning a 40-kDa outer membrane protein (OMP) from *P. gingivalis* 381 (6) and identified the 40-kDa OMP as a co-aggregation factor (7,8). We reported that rabbit polyclonal antibody against r40-kDa OMP had opsonic activity on human neutrophil function during phagocytosis of *P. gingivalis* (9).

In this study, in order to develop a peptide component vaccine, we attempted to identify an epitope in the 40-kDa

OMP from *P. gingivalis*. Furthermore, a specific antibody for the synthetic peptide was raised and the immunoreactivity of the antibody against 40-kDa OMP and *P. gingivalis* cells was examined.

Materials and Methods

Chemical peptide synthesis

The peptides were designed using the deduced amino acid sequence of r40-kDa OMP (10) and they were synthesized chemically by a solid phase method using an Fmoc strategy peptide synthesizer (PSSM-8, Shimadzu, Kyoto, Japan). To purify the synthetic peptide, a sample was applied to a reverse-phase, high-pressure liquid chromatography (HPLC) column (TSK gel ODS120T, 0.64 × 25 cm, particle size, 5 μm, Shimadzu Industry). The peptide was eluted using a linear gradient of acetonitrile (0 ~ 45%) in 0.01% (v/v) trifluoroacetatic acid at a flow rate of 1 ml/min.

Preparation of antibodies

The r40-kDa OMP was purified by the method reported previously (11). The plasmid pQE-19 that expresses dihydrofolate reductase (DHFR) with a 6 × His tag was purchased from Qiagen (CA, USA). The recombinant dihydrofolate reductase was purified from cell free extracts of *Escherichia coli* K-12 host harboring pQE-19 using Ni-NTA affinity chromatography (Qiagen). A portion of the synthesized peptides was conjugated with DHFR by the method of cofactor-dependent chemical cross-linking using sodium hydrogen carbonate and carbodiimide hydrochloride (8). After dialysis of the peptide-DHFR conjugates against PBS, the solution was sterilized using a Millipore filter (0.22 μm), and used as the antigen.

Antisera against r40-kDa OMP or the peptide-DHFR conjugates were obtained by immunizing rabbits at several sites on the back with an injection of the purified r40-kDa OMP or the peptide-DHFR conjugates in incomplete Freund's adjuvant, once a week, for four weeks. Three days after the last injection, blood was drawn from an ear vein. Immune serum was passed through a DE-52 column (Whatman, Maidston, Kent, U.K.) and used in the experiment.

Immunoblot analysis

Proteins were separated by SDS-PAGE then transferred to a nitrocellulose membrane in a transfer buffer (20% (v/v) methanol, 50 mM Tris, 40 mM glycine). The mini slab SDS-PAGE gel (7 × 8 cm; 1.0 mm thick) was composed of a 7% (w/v) separation gel and 4.5% (w/v) tracking gels. The chemically synthesized peptides or r40-kDa OMP were dot-blotted onto a nitrocellulose membrane. The nitrocellulose membrane was soaked in buffer (50 mM Tris-HCl; 0.15 M NaCl, pH 7.3) containing 5% (v/v) low fat milk (Tris-milk) and gently shaken for 30 min. The filter was then incubated with antisera (500-fold dilution in Tris-milk) for 1 h, followed by peroxidase-conjugated goat anti-mouse Ig G (1000-fold dilution in Tris-milk) at room temperature for 60 min. Immunoreactivities were detected by incubating the filter with 0.1 M Tris-HCl (pH 7.6) which contained 0.08% (w/v) 3,3'-diaminobenzidine tetrahydrochloride and 0.05% (v/v) H_2O_2.

Surface plasmon resonance (BIAcore) assay

Binding assays of antibodies with r40-kDa OMP were carried out using the BIAcore system (model 2000, Biacore AB, Uppsala, Sweden). The purified r40-kDa OMP was immobilized on a sensor chip, CM 5, using an amine coupling kit containing N-hydroxysuccinimide (NHS), N-ethyl-N'-[(3-dimethylamino)-propyl]-carbodimide hydrochloride (EDC), and ethanolamine-HCl. The binding assay was performed at 25°C in 10 mM Hepes buffer, pH 7.4, containing 0.15 M NaCl and 0.005% (v/v) P 20 surfactant (HBS-P buffer). The monoclonal antibody MAb-Pg-vc, which recognizes hemagglutinin of *P. gingivalis* (12), was used as a negative control.

The antibodies were purified on a protein G affinity

Table 1 Synthesized peptides desigened in 40-kDa OMP

Peptide	Position	Amino acid sequence	Residues
Peptide A	11 - 29	GSFKKNVVLEVFTAEWCGY	19
Peptide B	44 - 59	LDDEYKERVFQTFVHY	16
Peptide C	67 - 86	WPRVGQLFIALDQTLGIPGF	20
Peptide D	97 - 107	KGENLSIGAPI	11
Peptide E	131 - 144	TKGATPEDVCTATF	14
Peptide F	147 - 164	KVDADLIGKPLMLTAYVL	18

The peptides were designed from deduced amino acid sequence of 40-kDa OMP (10) and chemically synthesized by a solid phase method.

column (HiTrap Protein G HP, Amersham Pharmacia Biotech AB, Uppsala), dialyzed against HBS-P buffer, and then injected over the immobilized r40-kDa OMP sensor chip.

Results

In this study, we designed and synthesized peptides A, B, C, D, E, and F from the amino acid sequence data for r40-kDa OMP (Table 1). Each chemically synthesized peptide and the r40-kDa OMP were dot-blotted on nitrocellulose membranes and examined for immunoreactivities with antibody against r40-kDa OMP. As shown in Fig. 1, peptides B, C and r40-kDa OMP reacted strongly with the antibody.

Since peptide B reacted most strongly with the antibody against r40-kDa OMP, we selected peptide B as a candidate for the antigen to develop a component vaccine. We purified the synthetic peptide B using reverse phase HPLC. As shown in Fig. 2, synthetic peptide B was eluted as a single peak in the chromatograph. The purified peptide B was conjugated to DHRF and used to immunize rabbits. The antibody obtained by immunization with conjugated peptide B was designated as Ab-peptide B. To determine the immunoreactivity of Ab-peptide B, Western blot analysis was carried out. Fig. 3 shows that antibodies against r40-kDa OMP and Ab-peptide B clearly reacted with r40-kDa OMP.

Next, the immunoreactivty of the Ab-peptide B was confirmed further using BIAcore technology. When passed over the surface of the r40-kDa OMP sensor chip, Ab-peptide B was found to bind specifically to immobilized r40-kDa OMP, but the negative control, MAb-Pg-vc, did not (Fig. 4).

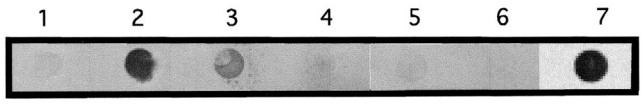

Fig. 1 Immunoblot assay of immuno-reactivity of peptides to antibody against r40-kDa OMP. synthetic peptides A-F (1-6) and r40-kDa OMP (7) were dot-blotted on nitrocellulose membrane and probed with antibody against r40-kDa OMP.

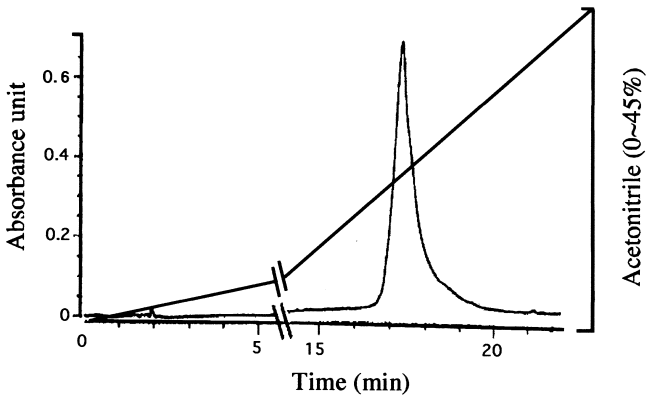

Fig. 2 The elution profile of synthetic peptide B in reverse-phase HPLC. Synthetic peptide B was applied to a reverse-phase HPLC column and eluted at 1 ml/min flow rate with 0.01% (v/v) trifluoroacetic acid containing a linear gradient of acetonitrile (0 ~ 45%).

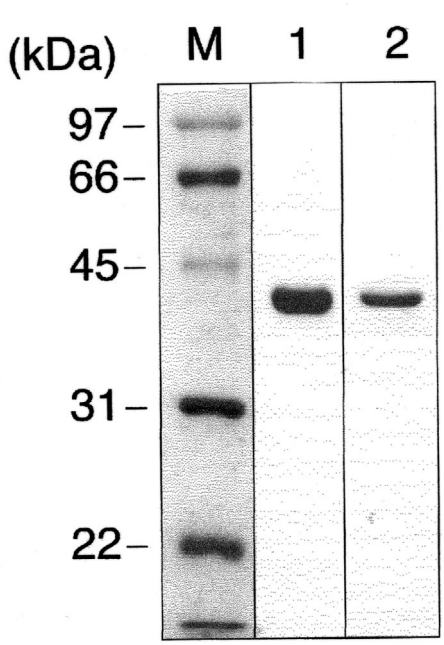

Fig. 3 Western blot analysis of Ab-peptide B. r40-kDa OMP was run on SDS-PAGE and transfered to a nitrocellulose membrane, then probed with antibody against r40-kDa OMP (Lane 1) and Ab-peptide B (Lane 2).

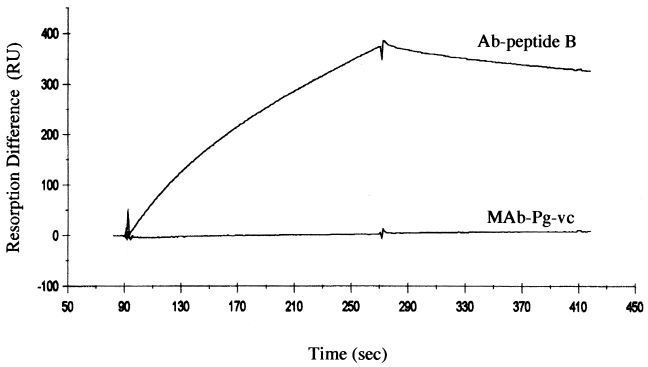

Fig. 4 BIAcore assay of immuno-reactivity of Ab-peptide B. Ab-peptide B or MAb-Pg-vc was injected over a r40-kDa OMP immobilized sensor chip and analyzed using the BIAcore system. RU, resonance unit.

When developing a component vaccine, examination of specificity for raised antibody against the target pathogen is important. We analyzed the specificity of the Ab-peptide B against pathogens associated with periodontal disease. Cell extracts of several pathogens were dotted on a nitrocellulose membrane, and the immunoreactivities of antibodies against r40-kDa OMP and Ab-peptide B were examined. As shown in Fig. 5, both antibodies reacted with r40-kDa OMP and all the *P. gingivalis* strains used, but did not react with other bacterial pathogens associated with periodontal disease.

Discussion

Several studies have reported that immunization with whole *P. gingivalis* cells, cell extracts, or outer membrane preparations can reduce tissue destruction caused by a *P. gingivalis* challenge in animal models (13-16). Furthermore, immunization with whole *P. gingivalis* cells or sonicated cell surface extracts reduces bone loss in the rat (17) and nonhuman primate (18) models of periodontitis. Vaccination could become an important adjunct therapy, not only to help prevent site recolonization by *P. gingivalis* and/or restrict the further progression of disease but also prevent systemic diseases. Besides blocking virulent factors of periodontal pathogens, the antibodies directed to key functional epitopes might facilitate the removal of pathogens through opsonization and phagocytosis.

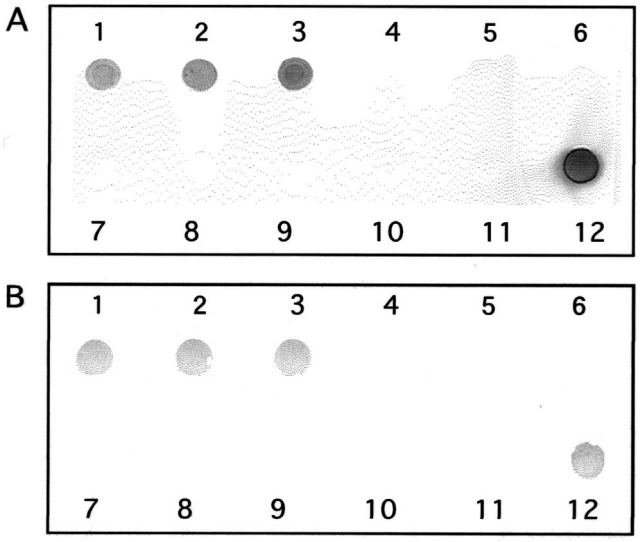

Fig. 5 Immunospecificty of Ab-peptide B. Cell extracts from 1, *Porphyromonas gingivalis* 381; 2, *Porphyromonas gingivalis* ATCC 33277; 3, *Porphyromonas gingivalis* ATCC 49417; 4, *Prevotella intermedia* ATCC 25611; 5, *Prevotella denticola* ATCC 38115; 6, *Actinobacillus actinomycetemcomitans* ATCC 29522; 7, *Treponema denticola* ATCC 35405; 8, *Fusobacterium nucleatum* ATCC 23726; 9, *Campylobacter rectus* ATCC 19246; 10, *Actinomyces viscosus* ATCC 19246; 11, *Streptococcus mutans* GS5; and 12, r40-kDa-OMP were dotted on nitrocellulose membranes, and probed with antibody against r40-kDa OMP (A) or Ab-peptide B (B).

Recently, it has been recognized that oral infections, especially periodontitis, may affect the course and pathogenesis of several systemic diseases, such as cardiovascular disease, bacterial pneumonia, diabetes mellitus, and low birth weight (19). Although the importance of systemic diseases caused by periodontitis has been recognized by the dental profession, the general public still believes that periodontal diseases do not possess the potential to affect lethal diseases. A practical vaccine for humans must be absolutely safe. It should not elicit unwanted immune responses or any other danger to human health. The current approach has been aimed at developing synthetic peptide vaccines against viral infections (20,21). Since the antibody against 40-kDa OMP has been shown to have potent ability to kill *P. gingivalis* cells (9), it is of interest to determine the epitope region in 40-kDa OMP that can be used to develop a peptide component vaccine.

In this study, in order to develop the peptide vaccine, we designed and chemically synthesized several peptides from the structure of 40-kDa OMP and examined the immunoreactivities of the synthetic peptide antibodies against r40-kDa OMP. Immunoblot analysis showed strongly reactivity with the antibody of the peptide B (16-mer, LDDEYKERVFQTFVHY). One problem with utilizing a peptide-based antigen is that, because of their small size, peptides are not likely to elicit a robust stimulation of the immune system. The inclusion of the appropriate carrier and/or adjuvant in vaccine formulations can overcome this problem. DHFR has been demonstrated to be a versatile "affinity handle" as a carrier protein for immunization with short peptides (22). The synthetic peptide B was purified by reverse-phase-HPLC and conjugated with DHFR and used as an immunogen. A peptide vaccine would only be useful if the peptide antigen gained access to the systemic immune system. Next, rabbits were immunized with synthetic peptide B-DHFR conjugates to raise antibodies. Western blot analysis and BIAcore assay clearly demonstrated that the antibody (Ab-peptide B) reacted with r40-kDa OMP. Furthermore, the antibody recognized *P. gingivalis* strains but not other oral bacteria.

These findings suggest that peptide B is satisfactory when used as an immunogen and that it could be important for the development of a component vaccine against *P. gingivalis*. Further studies that focus on the potential role

of the raised antibody in preventing infections of *P. gingivalis* are necessary.

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