

Production of Monoclonal Antibodies Specific to FimA of *Porphyromonas gingivalis* and Their Inhibitory Activity on Bacterial Binding

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Background: The FimA of *Porphyromonas gingivalis* is a crucial pathogenic component of the bacteria and has been implicated as a target for vaccine development against the periodontal diseases. **Methods:** In this study, the purified fimbriae (FimA subunit polymers) protein was used for immunization in their native form and B hybridoma clones producing antibodies specific to FimA were established. **Results:** The monoclonal antibodies prepared from selected two clones, designated #123 (IgG2b/ kappa) and #265 (IgG1/kappa), displayed different patterns of binding activity against the cognate antigen. Both antibodies reacted with conformational epitopes expressed by partially dissociated oligomers, but not with monomer as elucidated by Western blot analysis. Ascites fluid containing the monoclonal antibodies showed the inhibitory activity against *P. gingivalis* to saliva-coated hydroxyapatite beads, an *in vitro* model for the pellicle-coated tooth surface. **Conclusion:** These results suggest that the monoclonal antibodies could be used as vaccine material against the periodontal diseases through passive immunization.

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INTRODUCTION

Periodontitis is a common oral inflammatory disease characterized by redness and bleeding of gingival tissue, resorption of alveolar bone and tooth loss (1,2). *Porphyromonas gingivalis*, a black pigmented gram-negative anaerobic bacterium, is one of the major pathogens in periodontitis (3). The viru-

lence of *P. gingivalis* has been known to be caused by various factors including hemagglutinins, cysteine proteinases, aggregation factors, lipopolysaccharides, and fimbriae and those factors are characterized to be responsible for bacterial colonization (4,5).

Several reports have indicated that fimbriae, one of the virulent factors, are important for mediating adherence to host tissues and other oral bacteria, and, consequently, are considered as a promising candidate antigen for vaccine development (6-9). This consideration was supported by the report that immunization of rat animal model with purified *P. gingivalis* fimbriae or synthetic peptide induced the protective immunity against periodontal destruction (10,11). In addition, FimA, a major subunit of *P. gingivalis* fimbriae with a molecular mass of 43 kDa, is considered as a major target for vaccine development based on the observation that monoclonal antibody against FimA blocked adhesion of the bacteria to human buccal epithelial cells (12,13).

Passive immunization using antibodies against the specific pathogens has advantages of immediate-immune response, low toxicity, and high specific activity against the infection (14). In human, passive immunization using antibody against *Streptococcus mutans* prevented bacterial recolonization for up to 2 years (15). In this study, as an attempt to develop monoclonal antibody specific to FimA for passive immunization against *P. gingivalis* infection, we established the hybridoma clones expressing anti-FimA monoclonal antibody. In addition, their inhibitory activity against the bacterial binding

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onto oral surface was assessed using hydroxyapatite bead assay.

MATERIALS AND METHODS

Purification of fimbriae protein from *P. gingivalis*

Fimbriae of *P. gingivalis* 2561 was purified according to the method described by Lee et al (16). Briefly, after harvesting cells of *P. gingivalis* strain 2561, the bacterial cells were subjected to mild ultrasonication (Vibra Cell, Model VC-600, Sonic and Materials Inc., Danbury, CT). After ultrasonication, crude fimbriae of the sonic extract were obtained by centrifugation. The fimbriae-containing supernatant was brought to 40% saturation by stepwise addition of solid ammonium sulfate and stirred at 4°C overnight. The precipitated crude fimbrial extract was dialyzed and clarified by centrifugation. The clarified crude fimbrial extract was mixed with guanidine HCl (UltraPURE, enzyme grade; BRL, Gaithersburg, MD) and subjected to gel filtration on a Sepharose CL-6B (Pharmacia Biotech, Sweden) column equilibrated with 6 M guanidine HCl. The fimbriae were purified by repeated Sepharose column chromatography. Homogeneity of the purified fimbrial protein was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Preparation of B hybridoma clones

Syngeneic BALB/c mice were purchased from Orient Bio (Sungnam, Korea) and immunized with 20 g/mouse FimA protein emulsified with complete Freund's adjuvant. The same mice were boosted with the same antigen emulsified with incomplete Freund's adjuvant. The spleen cells of immunized mice were fused with SP2/0 myeloma cells (ATCC, Manassas, VA) at a ratio 1 : 1 using PEG 1500 (Roche Diagnostics GmbH, Mannheim, Germany). The fused cells were spread onto 96-well culture plates in Dulbecco's modified Eagle's medium (Hyclone Laboratories, Logan, UT) supplemented with HAT and 20% FBS (PAA, Etobicoke, Ontario, Canada). Generated hybridoma clones were subcloned three times by limiting dilution procedure and the binding specificity of the antibodies produced by the hybridoma clones were determined through ELISA and Western blot analyses.

Determination of antibody specificity and isotypes

Specificity of antibodies produced from established hybridoma clones was determined by ELISA and Western blot analyses. For ELISA, briefly, 96-well plates were coated with

native FimA protein (65 ng/well) and blocked with 5% non-fat dried milk. After the blocking, culture supernatants of hybridoma clones were added. Following incubation, plates were washed and AP-conjugated anti-mouse antibodies were added to wells. Finally, plates were incubated with the substrate of p-nitrophenyl phosphate and absorbance at 405 nm was measured using ELISA reader (Packard Instrument, Downers Grove, IL). The isotype of antibodies produced from stabilized hybridoma clones was determined using isotyping kit according to the protocol suggested by the supplier.

Western blot analysis was conducted using two different conditions of antigen preparation were used to determine the specificity of the antibodies. In order to prepare monomeric FimA, FimA protein was incubation at 100°C for 10 min in a sample buffer containing 60 mM Tris-HCl, 2% SDS, 0.1% bromophenol blue, glycerol and 2-mercaptoethanol. In order to prepare partially depolymerized FimA, FimA protein was incubated at 80°C for 5 min in a sample buffer without 2-mercaptoethanol. Two micrograms per well of prepared FimA proteins were loaded and separated in 10% SDS PAGE. The resolved proteins were transferred to nitrocellulose membranes (Whatman, Dassel, Germany) and blocked with 5% skim milk. The blotted membranes were incubated with hybridoma culture supernatants or diluted-polysera, followed by incubation with AP-conjugated anti-mouse secondary antibodies.

Ascites fluid preparation

Three 12-week old male BALB/c mice were inoculated with 0.5 ml of pristine through an intra-peritoneal injection. After 7 days, 1×10^6 hybridoma cell producing monoclonal antibodies, which had been harvested and washed in PBS for twice, were injected peritoneally to induce ascites production.

Inhibition assay for bacterial attachment

Antibody-mediated inhibition of bacterial binding assay was performed with saliva-coated hydroxyapatite (SHA) beads as described in a previous study (17). Briefly, *P. gingivalis* 2561 grown overnight was washed in buffered KCl twice and adjusted to $OD_{600}=0.1$, which is equivalent to 1×10^8 CFU/ml. The bacterial suspension (100 μ l) was added to a series of tubes containing 2 mg SHA beads and brought to a final volume of 400 μ l with buffered KCl. Simultaneously, antibody was added to the tubes in a final dilution of 1 : 100, 1 : 200 and 1 : 400. This mixture was incubated at room temperature. After an incubation time, the reaction mixture was

layered on 1 ml of 100% Percoll in a new siliconized borosilicate tube to separate cells which were free from those bound to the SHA beads. Unbound, free *P. gingivalis* cells floating on the Percoll layer were removed and the beads with bound cells and the wall of the tube were then washed.

Quantitative real-time PCR

Quantitative real-time PCR (QRT-PCR) of bacterial cells bound to the SHA beads was performed as described previously (18). Genomic DNA was extracted from the bound bacteria in each test tube using DOKDO-prep™ Bacterial Genomic DNA Purification Kit (ELPIS-Biotech Inc., Korea) and used as template. To obtain standard curves for QRT-PCR that relates bacterial concentration to Ct, the turbidity of bacterial suspension of *P. gingivalis* 2561 grown overnight was adjusted to OD₆₀₀=0.1 (1×10⁸ CFU/ml). For amplification of *P. gingivalis* 16S rRNA gene, 3 μl of each template was added to the reaction mixture containing 10 μl of iQ™ SYBR® Green SuperMix (Bio-Rad, Hercules, CA) and 250 nM of each primer, Pg16S-F (TGT TAC AAT GGG AGG GAC AAA GGG) and Pg16S-R (TTA CTA GCG AAT CCA GCT TCA CGG), which were prepared based on the primer sequence published previously (19), in a final volume of 20 μl. QRT-PCR was carried out using MiniOpticon™ Real-Time PCR Detection System (Bio-Rad). The reaction was cycled with preliminary denaturation for 5 min at 95°C, followed by 40 cycles of denaturation at 95°C for 10 sec, annealing at 61°C for 20 sec and primer extension at 72°C for 30 sec. To confirm that a single PCR product was amplified, melting curve analysis was performed with the following condition: 65°C to 95°C with a heating rate of 0.2°C per 1 sec. The amount of bacterial DNA in each sample was quantified by interpolation of the corresponding Ct values in the standard curves. Inhibitory efficiency of monoclonal antibodies was measured by determining relative percentage of DNA from the cells bound to SHA beads in the presence of antibody to DNA from the bound cells in the absence of antibody.

Statistics

The significance of differences between means of groups was determined using the Student's t-test using Sigmaplot™ (SPSS, San Rafael, CA) and a p-value <0.05 was considered significant.

RESULTS AND DISCUSSION

P. gingivalis is one of the major pathogenic microorganisms associated with periodontitis and fimbriae of *P. gingivalis* are responsible for adherence of the bacteria onto dental surfaces. In order to develop the preventive vaccine against the *P. gingivalis* through passive immunization, monoclonal antibodies capable of inhibiting the binding of the bacteria onto dental surfaces should be developed. Consequently, we immunized the mice with FimA of *P. gingivalis* and generated hybridoma clones producing anti-FimA monoclonal antibody through fusion of spleen cells prepared from the immunized mice with Sp2/0 myeloma cells.

We initially selected 17 clones through ELISA. Since the fimbriae are FimA subunit polymers composed of a repeated-monomer form (20), different structure forms appeared by denaturing conditions. Among those, we tried to select the hybridoma clones secreting monoclonal antibody with binding activity specific to native FimA (polymer form) not to monomer form by Western blot analysis. Interestingly, all selected clones were reactive to oligomer form but not to monomer form. We assume that the injection of native form of FimA efficiently stimulated the oligomer form of FimA-specific monoclonal antibody producing hybridoma cells. In addition, prescreening through ELISA in which native form of FimA was used as coating antigen may enrich the selection of hybridoma clones producing native form of FimA-specific monoclonal antibody.

This pattern of recognition is comparable to the previous reports of specificity of antibody against fimbriae to oligomer form not to monomer form (21,22). Among those clones, two clones, namely #123 and #256, showed high affinity to FimA and subjected to further characterization (Fig. 1). Monoclonal antibody from hybridoma clone #123 was reactive more to FimA oligomers with high molecular weight than FimA oligomers with low molecular weight. When we determined the isotypes of the monoclonal antibody, #123 clone was determined to produce monoclonal antibody with IgG2b/kappa isotype and #256 clone determined to produce monoclonal antibody with IgG1/kappa. In addition, binding activity of #265 clone was turned out to be greater than that of #123 clone. These results suggested that hybridoma clones secreting monoclonal antibody specific to oligomer form of FimA are generated.

In order that the monoclonal antibody could be used as vaccine through passive immunization, the monoclonal anti-

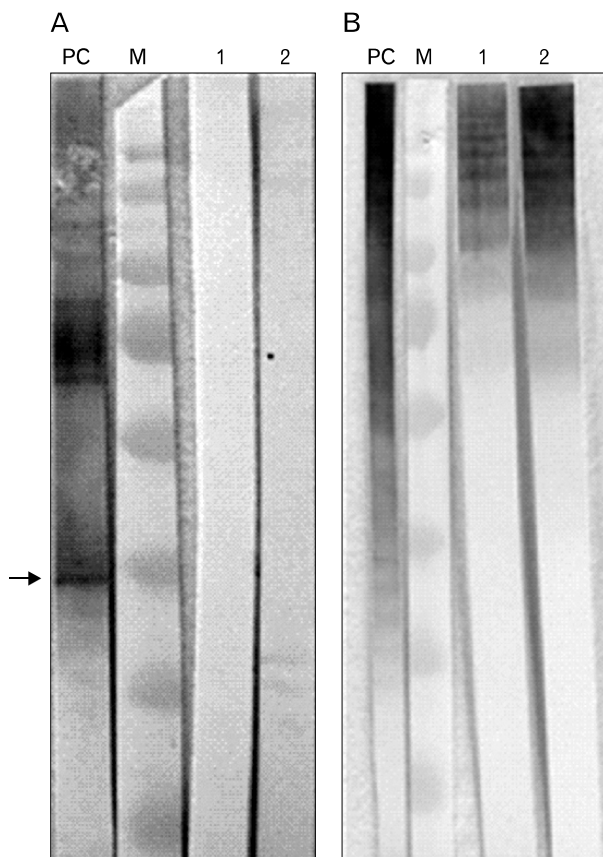


Figure 1. Binding activity of anti-FimA monoclonal antibodies. The two conditions for sample preparation were applied to display FimA antigen in Western blot analysis by incubating the strips with polyclonal antibody (1 : 5,000) prepared from the mice injected with FimA. (A) Monomerized FimA protein was prepared by treating protein with 100°C for 10 min in the absence of 2-ME and display through gel electrophoresis. (B) Partially depolymerized FimA protein was prepared by treating protein with 80°C for 5 min in the presence of 2-ME. Lane PC is purified bacterial FimA. Lane M is Prestained Protein Ladder (Fermentas, Glen Burnie, MD). Lane 1 is culture supernatant from #123 hybridoma clone. Lane 2 is culture supernatant from #256 hybridoma clone. The arrow indicated the size of FimA monomer of about 43 kDa.

body has the activity to inhibit the binding of *P. gingivalis* onto dental surfaces. For determination of the inhibitory efficiency of monoclonal antibodies against *P. gingivalis* binding, we applied the *in vitro* model system using saliva-coated hydroxyapatite (SHA) beads (17). When we prepared the ascites fluid and add the diluted ascites fluid into the reaction solution, efficiently inhibited the adherence of the bacteria onto bead surfaces (Table I). For example, 400-fold diluted monoclonal antibodies from #123 and #256 hybridoma clones significantly inhibited the binding of the bacteria onto bead surfaces of 29.5% and 41.5%, respectively. However, control as-

Table I. Inhibitory effect of ascites fluid on the binding of *P. gingivalis* to saliva-coated hydroxyapatite beads

Dilution factor	Inhibition of bacterial binding (%)	
	# 123	# 265
1 : 100	71.2 ± 5.15*	73.4 ± 2.15*
1 : 200	44.1 ± 11.65*	49.0 ± 1.29*
1 : 400	29.5 ± 9.38*	41.5 ± 5.42*

*p < 0.05.

cites fluid did not inhibited the binding of the bacteria onto SHA beads and recorded 0% inhibition rate (data not shown). Usually, there are a lot of unknown factors in ascites fluid. Therefore, further experiments should be performed to clearly characterize the nature of monoclonal antibody-mediated inhibition of bacterial binding. Nevertheless, it could be assumed that the monoclonal antibodies produced from the established hybridoma are able to efficiently inhibit the bacterial attachment since the control ascites fluids which were produced by using hybridoma clones with unrelated specific.

Collectively, these results suggested that we successfully established the hybridoma clones producing monoclonal antibody specific to FimA of *P. gingivalis* and capable of interfering with interaction between SHA beads and the bacteria. Monoclonal antibodies could be produced through several different production processes. We are currently trying to produce monoclonal antibody with safe and economical way including antibody-gene transformed plant cell suspension culture. Therefore, we cloned the antibody genes and confirmed authenticity of the cloned antibody genes through binding assay with antibodies produced from the antibody gene-transfected CHO cells (manuscript in preparation). We are currently introducing the cloned antibody genes into plant expression vector to produce the antibody through plant cell suspension expression system.

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CONFLICTS OF INTEREST

The authors have no financial conflict of interest.

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