Rapid Detection of *S. Mutans* Surface Antigen I/II Using a Sensitive Monoclonal Anti-Ag I/II Antibody by ELISA

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The cell-surface protein antigen I/II (Ag I/II) is expressed in oral streptococci, which are known as the causative agent of a number of diseases including dental caries, endocarditis, gingivitis, and periodontal disease. Consequently, monoclonal antibodies (MAb) capable of recognizing the streptococcal Ag I/II protein could be a useful tool for the diagnosis and cure of these diseases. In this study, a previously generated monoclonal anti-Ag I/II antibody, ckAg I/II, was used to detect a small amount of *Streptococcus mutans* (*S. mutans*) surface antigen Ag I/II. The ckAg I/II was proved to be very sensitive and able to detect as little as 1 ng of recombinant Ag I/II protein within 5 min and Ag I/II in saliva within 10 min, as well as native Ag I/II in 20 μ L of culture supernatant by ELISA. These results suggest that ckAg I/II can be used as a fast and efficient diagnostic tool to detect Ag I/II.

Introduction

ORAL STREPTOCOCCI ARE RELATED TO A NUMBER OF health complications including dental caries, infective endocarditis, gingivitis, periodontal diseases, rheumatic diseases, and infection of the brain and liver.^(1–8) Most indigenous species of oral streptococci produce cell surface adhesion molecules belonging to the Ag I/II family of proteins, which interact with diverse salivary components, lysozyme, amylase, proline-rich proteins, and agglutinin.^(9–13) Consequently, these proteins mediate the adhesion of oral streptococci to tooth surfaces and other bacteria. Streptococcal Ag I/II protein also interacts with host receptors, including collagen, fibronectin, laminin, and fibrinogen, and induces and/or contributes to the pro-inflammatory effects of monocytes, epithelial cells, endothelial cells, and synoviocytes.^(14–16)

S. mutans lacking the Ag I/II surface protein has been found to be resistant to phagocytosis by polymorphonuclear leukocytes in a mouse model, thereby causing sustained inflammation. It has been reported that about 3% of clinical bacterial strains obtained from patients with infective endocarditis possess a low molecular weight Ag I/II protein.⁽¹⁷⁾ Another study showed that *S. gordonii* absorbs proline-rich salivary proteins recognized by *Candida albicans* that act as receptors for fungal and bacterial-fungal interactions and influence antibiotic resistance and the host response to infection.⁽¹⁸⁾ Collectively these findings indicate that the detection of Ag I/II molecules is important in analyzing the relationship between disease and oral streptococci.

The use of monoclonal antibodies (MAbs) is a fast and selective diagnostic tool for the detection of certain cell com-

ponents.^(5,19–21) Often, sensitive antibodies are critical elements in the rapid detection of organisms and a useful tool for curing disease.⁽²²⁾ In the present study, we investigated rapid detection of small amounts of *S. mutans* surface antigen I/II using a sensitive monoclonal anti-Ag I/II antibody, ckAg I/II, by ELISA.

Materials and Methods

Preparation and purification of recombinant Ag I/II and monoclonal anti-Ag I/II antibody

Recombinant Ag I/II protein was prepared and purified as described previously.⁽²³⁾ In brief, the N-terminal fragment (118–2273) of the *S. mutans* GS-5 Ag I/II gene was amplified by PCR and inserted into a pQE vector (pQE-Ag I/II-N). The vector was induced to produce Ag I/II protein with isopropyl- β -D-thiogalactopyranoside (IPTG), which was purified by Ni-NTA affinity chromatography. The size of the protein was determined by SDS-PAGE. This same protein was used as the immunogen to generate hybridoma-producing anti-Ag I/II MAb.⁽²³⁾ The ckAg I/II MAb was purified using a protein G column (Thermoscientific, Rockford, IL) as recommended by the manufacturer. The MAb isotype was determined as IgG1 using a commercially available isotyping kit (Sigma Chemical Co., St. Louis, MO), per the manufacturer's instructions.

Bacterial strain and culture conditions

To investigate Ag I/II production time, *S. mutans* GS-5 was selected and grown in brain-heart infusion (BHI) broth at 37°C

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FIG. 1. (A) Growth curve of *S. mutans* GS-5. Optical density (540 nm) of the bacterial culture was measured at various time points after a 200 mL aliquot of overnight culture was incubated in 5 mL of fresh BHI. (B) SDS-PAGE analysis of *S. mutans* GS-5 culture supernatants. *S. mutans* GS-5 culture supernatants (20 mL) were collected at various time points during bacterial growth and separated on an 8% SDS-PAGE gel followed by Coomassie brilliant blue staining. (C) Western blot analysis of *S. mutans* GS-5 culture supernatants. *S. mutans* GS-5 culture supernatants (20 mL) were collected at various time points of bacterial growth and analyzed by Western blot using ckAg I/II.

under 5% CO₂. A colony of *S. mutans* GS-5 was inoculated in 2 mL BHI broth and incubated for 16 h. Subculture was performed by inoculating 5 mL BHI with 200 µL of the 16 h culture.

Saliva sampling

Whole saliva samples were collected from 41 donors (between 26 and 39 years of age). They chewed paraffin to stimulate saliva secretion and the samples were collected into 50 mL tubes on ice and kept at 4°C until used. Consent was received from the donors and their parents.

SDS-PAGE and Western blot analysis

Twenty µL of the concentrated bacterial culture supernatant using 50% ammonium sulfate was separated on an 8% SDS-PAGE gel and then stained with Coomassie brilliant blue for molecular weight analysis. The same volume of culture supernatant obtained during each growth phase was also separated on an 8% SDS-PAGE gel and the proteins were transferred to a nitrocellulose membrane for Western blot analysis. The membrane was blocked with 5% skim milk for 1 h, incubated with ckAg I/II for 1 h, and washed. After incubation with anti-mouse IgG horseradish peroxidase-

FIG. 2. Reaction of ckAg I/II. The MAb was examined for reactivity with other components of *S. mutans*, Ag I/II (■), GTFB (●), GTFC (▲), and GTFD (▼). The plate was read at 405 nm. Data are shown as the mean ± S.E.M. of triplicate assays.

FIG. 3. Evaluation of sensitivity of ckAg I/II. (A) ckAg I/II at a 1:1000 dilution was used to detect the recombinant Ag I/II protein. (B) ckAg I/II at a 1:100 dilution was used to detect the recombinant Ag I/II protein. The plate was read at 405 nm. The results are presented as the mean values $\pm$ S.E.M. of triplicate assays from two separate experiments. ** $p < 0.01$ and *** $p < 0.001$ indicate significant differences between experimental groups and control group. (C) Detection of recombinant Ag I/II protein with purified ckAg I/II at 5 min. A 96-well plate was coated with 1 ng (■), 10 ng (●), 50 ng (▲), and 100 ng (▼).

conjugated antibody for 1 h, the membrane was developed using enhanced chemiluminescence (ECL) for 5 min.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was used to investigate the sensitivity of ckAg I/II and rapid detection of Ag I/II. A 96-well flat-bottom polystyrene microtiter plate (Nunc, Roskilde, Denmark) was coated with recombinant Ag I/II protein, 20 μ L culture supernatant, or 100 μ L of saliva at 4°C overnight and then blocked with 3% skim milk for 30 min at room temperature (RT). After washing three times with PBS, ckAg I/II that had been previously prepared⁽²³⁾ was added and incubated for 1 h at 37°C, followed by three washes with PBS. Then, a secondary alkaline phosphatase-labeled goat anti-mouse immunoglobulin G antibody (Sigma Chemical) was added and the wells were washed four times with PBS. Color was developed with an alkaline phosphatase substrate and the wells were read at 405 nm using an ELISA reader (Packard Instrument, Downers Grove, IL).

Statistical analysis

The antibody titer was expressed as the reciprocal of the geometric log₂ of the least concentrated sample that gave a basal level of absorbance of 0.07, which was the value of the PBS control. Maximum mean log₂ titers and their standard errors were calculated. Results are presented as the mean $\pm$ standard error (S.E.M.). Statistical analyses were performed using Origin 7 (Origin Lab, Northampton, MA) and Student's *t*-tests were used to compare groups. *P* values * < 0.05 , ** < 0.01 , and *** < 0.001 were considered significant.

Results

To analyze and demonstrate the sensitivity of ckAg I/II, the production time of *S. mutans* GS-5 Ag I/II in its growth phase was investigated by monitoring a growth curve (Fig. 1A) generated by analyzing secreted Ag I/II in culture supernatants using SDS-PAGE and Western blot analysis (Fig. 1B, C, respectively). Ag I/II was first detected at 4 h and the amount detected increased in a time-dependent manner. However, Western blot analysis of *S. mutans* culture supernatants showed multiple bands (Fig. 1C).

The ckAg I/II used in this study was derived from the *S. mutans* GS-5 Ag I/II, and its reaction with other components of the cell that play important roles in dental caries, including GTFB, GTFC, and GTFD, was tested (Fig. 2). The ckAg I/II reacted with the Ag I/II protein, but not with the other proteins. In addition, the reaction of the ckAg I/II with three glucosyltransferases did not change regardless of the amount of MAb used.

The sensitivity of ckAg I/II was investigated using 1 ng, 10 ng, 50 ng, and 100 ng recombinant Ag I/II protein in ELISA assays at 1:1000 or 1:100 dilutions. As shown in Figure 3A, the MAb detected 1 ng at 10 min and 10 ng at 5 min when the dilution was 1:1000. When the ratio was 1:100, the MAb showed significantly enhanced sensitivity, detecting 1 ng protein at 5 min (Fig. 3B, $p < 0.001$). The sensitivity test was performed with purified MAb, which exhibited a much higher detection sensitivity than MAb-containing serum (Fig. 3C). The sensitive MAb, ckAg I/II, was used to detect Ag I/II in saliva samples collected from 41 donors, and the result

FIG. 4. Detection of Ag I/II in saliva samples. A 96-well flat-bottom polystyrene microtiter plate was coated with 200 μ L of whole saliva samples, which were collected from 41 donors. Ag I/II in the saliva was detected with 100 ng of the purified ckAg I/II and read at 405 nm. The control was PBS. Data are shown as the mean $\pm$ S.E.M. of triplicate assays.

showed that ckAg I/II could detect Ag I/II in saliva within 10 min (Fig. 4).

Discussion

Several studies have reported that oral streptococci are associated with dental caries and other diseases, including endocarditis induced by infection with some species of oral streptococci. Oral bacteria produce a family of cell surface proteins, including Ag I/II, some of which act as adhesins to mediate colonization and binding of oral streptococci to salivary pellicles.⁽⁹⁾ In this study, the monoclonal anti-Ag I/II antibody, ckAg I/II, was found to be highly sensitive. Given the involvement of Ag I/II in the initial adhesion of streptococci to oral surfaces, the formation of biofilms, and the development of oral and systemic diseases, our findings suggest that using this MAb to detect Ag I/II might facilitate the diagnosis and prevention of oral disease.

S. mutans GS-5, which is well known as a causal agent of dental caries^(3,6) and bacterial infective endocarditis, was used to initially characterize the sensitivity of ckAg I/II. Tests assessing the reaction of the recombinant Ag I/II protein and native Ag I/II in the culture supernatant of *S. mutans* GS-5 with the MAb were conducted. The purified recombinant Ag I/II appeared as a single band in SDS-PAGE analysis,⁽¹³⁾ and ckAg I/II reacted with the Ag I/II protein but not with other *S. mutans* cellular components. The Western blot analysis of *S. mutans* culture supernatants showed multiple bands (Fig. 1C), and studies have shown that the band of about 150 kDa is Ag I as it reacted with antiserum to Ag I/II, but not with antiserum to Ag II.^(12,24)

ELISA results with 1 ng, 10 ng, 50 ng, and 100 ng recombinant Ag I/II protein for 5 min and 10 min showed that the MAb was able to detect 1 ng of recombinant Ag I/II at 5 min.

In conclusion, the results of this study showed that ckAg I/II is highly sensitive. Thus, it could be a good diagnostic tool

to identify Ag I/II-related diseases since rapid diagnosis is often critical for treatment of the disease and a sensitive antibody is an important element. To our knowledge, this is the first report on the rapid detection of *S. mutans* surface antigen I/II using a sensitive monoclonal anti-Ag I/II antibody.

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Author Disclosure Statement

The authors have no financial interests to disclose.

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