Generation of a monoclonal antibody against AgI/II, a cellular surface protein of *Streptococcus mutans* GS5

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Abstract

Most of oral streptococci express the Antigen I/II (AgI/II) proteins, cell wall anchored adhesions. AgI/II protein binds to salivary agglutinin glycoprotein, a component of tooth pellicle and to ligands in other bacteria. These associations play important roles in bacterial colonization. Recently, it was reported that diverse host molecules also interact with AgI/II protein and that these interactions induce inflammatory responses from host cells. Among mutans streptococci containing -type hemolytic activity, *Streptococcus mutans* is a causative agent for dental caries. Compared with many other strains of *S. mutans*, GS-5 strain is unique in that this bacterium expresses truncated secretory AgI/II protein due to the nonsense mutation in the *agI/II* gene. This indicates that *S. mutans* GS-5 has a different clinical role and a recent report supported this idea based on the results from clinically isolated *S. mutans* strains. Previously, we had cloned *agI/II* gene from *S. mutans* GS-5 and generated recombinant N-terminal AgI/II protein. In this study, we further produced a hybridoma line expressing anti-AgI/II monoclonal antibodies named as 1C11A. This antibody showed high sensitivity to AgI/II protein in Western blot and ELISA. This new reagent will provide a basis for investigating the mechanisms of AgI/II-related diseases.

Key words : *Streptococcus mutans*, Antigen I/II, Monoclonal antibody

Ⅰ. INTRODUCTION

Members of normal oral flora, especially streptococci, have been suggested as causing agents for dental caries[1] and systemic inflammatory disease[2] such as bacteremia[3], infective endocarditis[4], and rheumatic diseases[5]. *Streptococcus mutans* (*S. mutans*) is known as a major causative bacterium for dental caries and responsible for over 20% viridian streptococci-induced endocarditis[6]. A surface protein of S. mutans is called as antigen I/II (AgI/II) or many other names (Pac[7], SpaP[8], B[9], SR[10], IF[11], P1[12], MSL-1[13]) and appears to be related with bacterial attachment to saliva-coated pellicle[14]. AgI/II polypeptides or AgI/II-specifc antibodies have been investigated as antibacterial activities for passive or active immunization[15-18]. As a member of modulin family, AgI/II protein is also involved in inducing immunomodulatory effects on diverse cells, leading to inflammatory diseases[19]. AgI/II-specifc antibodies can be also utilized for diagnostic tools for dental or systemic diseases using sera or saliva[20].

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Domains of AgI/II protein

More than fourteen genes encoding AgI/II proteins from six species of streptococci have been sequenced and the size of AgI/II proteins is 1310 ~ 1653 amino acid (aa) with a common structure (Fig. 1). Each AgI/II protein has a signal peptide (SP) (~38 aa), one to four repeats of alanine-rich (A-rich) domain (82 aa), one to three repeats of proline-rich domain (39 aa), and a bacterial cell wall anchoring motif (CWA). N-terminal (N), variable central (V), and C-terminal (C) domains are located between SP, A-rich, P-rich, and CWA domains.

Ligands for AgI/II protein

Saliva agglutinin glycoprotein (SAG) or gp340 is a major host receptor for AgI/II protein and belongs to scavenger receptor cystein-rich (SRCR) glycoprotein family, secreted by the parotid gland[21,22]. gp340 of saliva plays an innate immunity by clearing streptococci but also acts as receptor for streptococci after being incorporated into the tooth pellicle[23]. The A-rich domain of AgI/II protein is known to bind to gp340 but its precise mechanism is not yet understood. AgI/II protein is also related to interbacterial association and C-terminal region of *Streptococcus gordonii* AgI/II is known to associate with *Phophyromonas gingivalis*[24,25].

Collagen, fibronectin, laminin, and fibrinogen are other host receptors for AgI/II protein[26-31] and the associations of AgI/II and these receptors have been reported to induce pro-inflammatory effects from monocytes, epithelial cells, endothelial cells, synoviocytes[32-35]. Recently, it was shown that TNF was overproduced in monocytes in the presence of AgI/II N-terminal domain and the V domain of this region had a lectin-like structure and bound to fucosylated and sialylated carbohydrates on monocytic receptors[36,37].

AgI/II protein of *S. mutans*

Compared with AgI/II from other species of *S. mutans*, AgI/II protein of *S. mutans* GS-5 has a unique feature lacking part of the C domain and the CWA motif[38]. Therefore, AgI/II of *S. mutans* GS-5 consists of 1158 aa with the apparent molecular weight of 155 kDa due to the insertion of an adenine around the 3470 nucleotide position. For this reason, *S. mutans* GS-5 produces a secreted and truncated AgI/II protein that has been utilized as a source of vaccine candidates[39,40]. The secretory AgI/II polypeptide of *S. mutans* GS-5 may indicate another possible role in systemic inflammation. Recently, high-risk strains of S. mutans for bacteremia were clinically isolated and these isolated strains have a lower molecular weight of AgI/II protein[41]. These results imply that the smaller secreted AgI/II of *S. mutans* may cause a

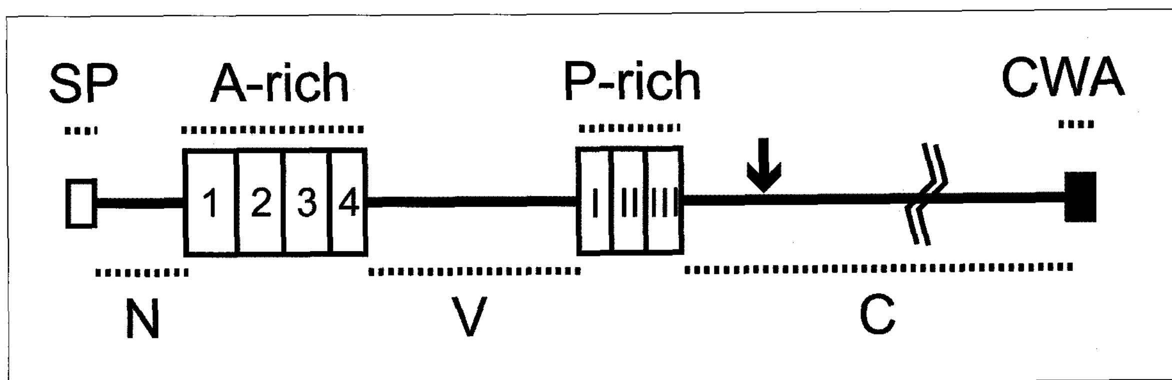

Fig. 1. Graphical representation of domains in *S. mutans* AgI/II protein. Indicated are the signal peptide (SP), alanine-rich domain (A-rich), proline-rich domain (P-rich), and cell wall-anchoring region (CWA). N-terminal (N), variable (V), and C-terminal (C) regions are also indicated. Arrow indicates the last codon of the smaller AgI/II polypeptides from *S. mutans* MT8148 or *S. mutans* Guy's, while the broken line indicates the last codon of the truncated AgI/II lacking part of the C domain and the whole CWA domain of *S. mutans* Ingbritt 162 or *S. mutans* GS-5.

specific effect in clinical situations.

In order to understand the mechanism of diseases related to AgI/II activity of *S. mutans* GS-5, we have generated monoclonal antibodies (mAbs) against AgI/II protein. Previously, we had cloned the genes of *agI/II* from *S. mutans* GS-5, subcloned the N-terminal fragment of *agI/II* gene (*agI/II-N*), generating recombinant AgI/II (rAgI/II), and producing anti-AgI/II polyclonal antibodies. In this study, we generated a hybridoma yielding AgI/II-specific mAbs using rAgI/II. This AgI/II-specific mAb was tested for Western blot or enzyme-linked immuno-sorbent assay (ELISA) for AgI/II protein.

Ⅱ. MATERIALS AND METHODS

Materials

Unless otherwise mentioned, all chemicals and plastic wares were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Falcon Labware (Frankline Lakes, NJ, USA), respectively.

Mice

Four to five weeks old mice were purchased. BALB/c mice (Orient, Inc., Korea) were maintained by feeding sterile food and water *ad libitum*.

Bacterial culture

S. mutans GS-5 was grown in BHI medium for 16 h at 37℃ in an anaerobic chamber and 200㎕ of overnight culture was transferred into 5 ml of a fresh medium, leaving for further incubation.

Expression of AgI/II in bacterial expression system

In order to express about N-terminal half fragment of AgI/II protein (AgI/II-N) in *E. coli* system, we utilized pQE-*agI/II-N* that contained *agI/II-N* gene in pQE vector[42]. Recombinant AgI/II-N protein was expressed and purified according to the manufacturer's manual (Qiagen, Hilden, Germany). In brief, pQE-agI/II-N was transformed into *E. coli* M15. The transformant was grown until mid-log phase and induced by adding isopropylthio-β-D-galactoside (IPTG). Three hours later, pelleted cells were resuspended and sonicated. Insoluble debris was separated by centrifugation and then the cleared lysates were loaded into pre-equilibriated Ni-NTA beads. The column was washed and the bound materials were eluted. The eluted fractions were dialyzed against PBS buffer and stored at 4℃ until next use.

Generation of hybridoma expressing anti-AgI/II antibodies

Polyclonal antisera against AgI/II were generated following a method described previously[43]. Once the presence of anti-AgI/II antibodies was confirmed, the immunized mouse was sacrificed and splenocytes were prepared. Hybridomas were generated by previous protocol by fusing isolated splenocytes and myeloma Sp2/0-Age14 with polyethylene glycol 1500 (Roche, Penzberg, Germany). The fused hybridomas were selected by HAT (Sigma). Hybridomas were also carefully screened under the microscope and finally by ELISA using recombinant AgI/II-N protein. Some clones had positive results from ELISA. These positive clones were expanded and grown by limiting dilution to obtain hybridoma cell lines. The subclones were again screened by the similar way. This procedure was repeated at least twice. One hybrimoda cell line, 1C11A, was finally selected, further grown, and stored. Anti-AgI/II antibodies were also produced in a large quantity by two methods, culture supernatants from 1C11A and mice ascites obtained by injecting 1C11A. The titre of 1C11A mAb was measured by ELISA or Western blot using rAgI/II-N protein.

ELISA

About 0.1 ml of culture supernatant of hybridoma cell was added to the ELISA plate containing adsorbed antigen. After 1 h incubation, the plate was washed three times with PBST (10 mM PBS, pH 7.0, 0.05% Tween-20). Anti-mouse goat antibodies conjugated with peroxidases were added to the washed plate and then the plate was washed three times with PBST. Absorbance at 405 nm was measured after color developed by adding enzyme substrate.

Ⅲ. RESULTS

Purification of *S. mutans* GS-5 rAgI/II-N protein

In our previous study, *agI/II* gene was amplified by PCR using *S. mutans* GS-5 genomic DNA and N-terminal half fragment was subcloned into pQE expression vector to yield His-tagged recombinant N-terminal half of AgI/II protein (rAgI/II-N). As shown in Fig. 2, rAgI/II-N was purified by Ni-NTA column with more than 90% purity from the soluble fraction of bacterial lysates, while some rAgI/II-N remained in the insoluble fraction. The antigenicity of rAgI/II in mice was confirmed with the production of anti-AgI/II polyclonal antibodies and the rAgI/II protein was also utilized to produce hybridoma cell lines expressing anti-AgI/II monoclonal antibodies (mAbs).

Selection of hybridomas

After checking the presence of anti-AgI/II antibodies in immunized mice, we isolated splenocytes from the same immunized mice and fused these splenocytes with SP2/0-age14 myeloma cell lines to yield hybridomas expressing anti-AgI/II antibodies. After fusion, most of cells died away under the HAT-containing selection medium but few cells survived to form colonies (Fig. 3). After about one week of selection, only hybridoma-containing colonies reach to a colony of over one thousand cells but neighboring feeder cells or unfused cells was almost gone (Fig. 4).

Once many wells had well-established colonies, the supernatants from hybridomas were further screened by ELISA in order to detect the presence of anti-AgI/II antibodies. As shown in Fig. 5, three out of eighty samples had less than 0.2 value of OD405 reading and this range was similar to the level of negative control (16 buffer only reactions). Fifty six samples had values at 0.8 or 1.0 level. Nine samples had above 1.0 level and these hybridomas were selected for next round of screening. A second round of ELISA results with ninety six samples was shown in Fig. 6. There were two significantly different results from this second screening. First, sixty one samples had less than 0.2 value. Second, the remaining thirty five samples had generally higher value reaching to 3.1 of OD_{405}.

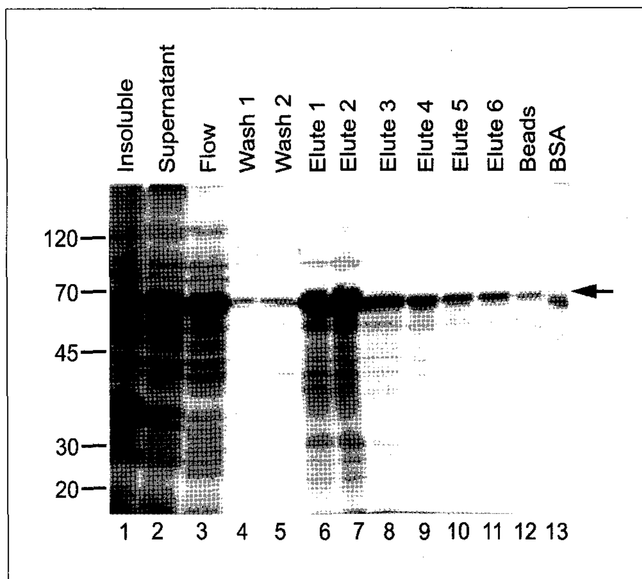

Fig. 2. Purification of rAgI/II-N protein from *S. mutans* GS-5. After sonication of rAgI/II-N-expressing bacterial lysates, insoluble pellet (lane 1) and supernatant (lane 2) are separated by centrifugation. The supernatant was then loaded to Ni-NTA column and the flow-through (lane 3) and washed twice (lanes 4 and 5). The bound proteins were eluted (lanes 6 to 11) and the remaining beads was checked (lane 12). One microgram of bovine serum albumin (BSA) was included (lane 13). The locations of size marker bands are indicated in kD, while arrow indicates the apparent location of rAgI/II-N protein in an SDS-PAGE gel.

Production of AgI/II-specific mAb from culture supernatant

After three rounds of screening, one hybridoma cell line (1C11A) was amplified, and frozen. After thawed, 1C11A cells were expanded and tested for the presence of anti-AgI/II antibodies, and then frozen again. These cycles of expansion, freezing, and thawing were repeated until the hybridoma cells were stabilized and grown well. One vial from large stock file of this hybridoma cell line was thawed and shown in Fig. 7.

Anti-AgI/II mAbs were produced by growing 1C11A until culture supernatant turned yellowish. After separating cell debris by centrifugation, the supernatant was tested to titrate AgI/II-specific activities by ELISA (Fig. 8). The culture supernatant had high activities even after 100,000-fold dilution, although buffer only control had the very low value.

Production of AgI/II-specific mAb as mice ascites

Alternatively, AgI/II-specific mAbs were obtained from the ascites by injecting 1C11A cells peritoneally into mice. Ascites was also tested to titrate anti-AgI/II activities and showed the similar level of activity compared with that of 1C11A culture supernatant (Fig. 9). Western blot with the ascites revealed that anti-AgI/II activities even after 200,000-fold dilution (Fig. 10).

Ⅳ. DISCUSSION

The formation of a biofilm after bacterial infection is critical step for the pathogenesis of *S. mutans*, a causing agent for dental caries. Two classes of genes, *agI/II* and gtf, have been well studied about adhesion and colonization of *S. mutans* in the development of dental plaque. Once a bacterial biofilm was established, the bacterial growth in sessile mode has several advantages over the planktonic growth. In this mode of growth, bacteria will be better protected

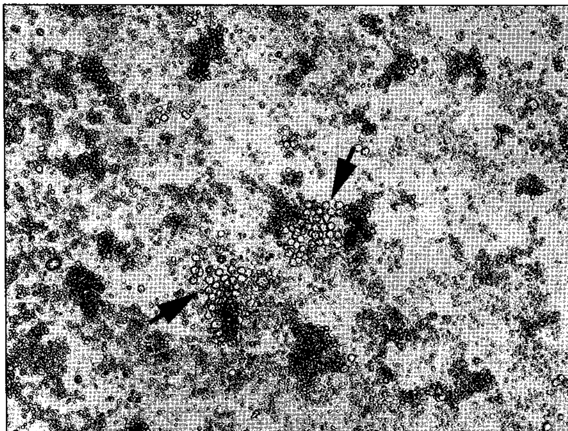

Fig. 3. Early colonization of hybridomas expressing monoclonal anti-AgI/II antibodies. Arrows indicate the colonies of hybridomas. Shown was the representative picture for early colonies after fusion between splenocytes and myelomas, or limiting dilution of hybridomas. Feeder cells or unfused cells were obviously dying out.

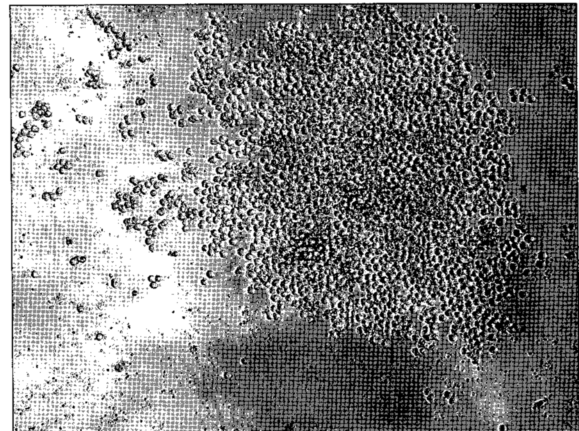

Fig. 4. Well-established colony of hybridomas expressing monoclonal anti-AgI/II antibodies. Shown was the representative picture for a colony of five hundred to one thousand hybridomas. Feeder cells or unfused cells almost disappeared.

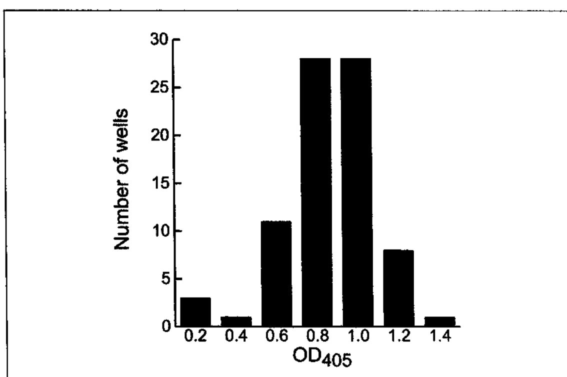

Fig. 5. Screening of hybridomas containing high-titer anti-AgI/II antibodies after initial fusion. Eighty wells of hybridomas after initial fusion were tested for anti-AgI/II antibodies by ELISA. Shown are the numbers of wells for OD_{405} intensity by groups: samples with less than 0.2 (0.2), samples with 0.2 to less than 0.4 (0.4), and so on.

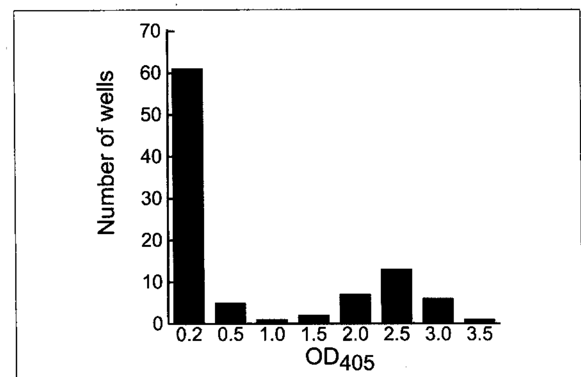

Fig. 6. Screening of hybridomas containing high-titer anti-AgI/II antibodies after limiting dilution. Ninty six wells of hybridomas after the first limiting dilution were tested for anti-AgI/II antibodies by ELISA. Shown are the numbers of wells for OD405 intensity by groups: samples with less than 0.2 (0.2), samples with 0.2 to less than 0.5 (0.5), and so on.

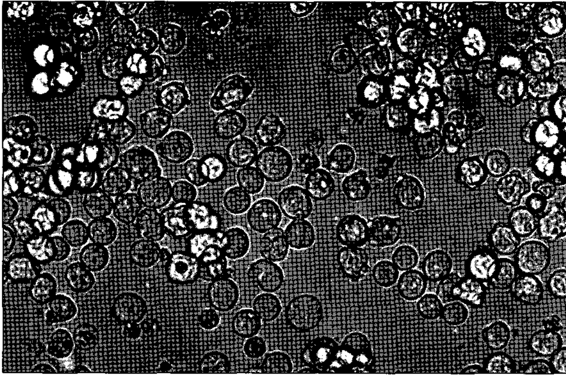

Fig. 7. Hybridoma cell line expressing monoclonal anti-AgI/II antibodies. Shown was the representative picture for a hybridoma cell line screened by several rounds of limiting dilution. The above cells survived for long-term storage of freezing-thawing cycles. The early stage of a hybridoma cell line after thawing was shown and many healthy cells were recovered from freezing conditions.

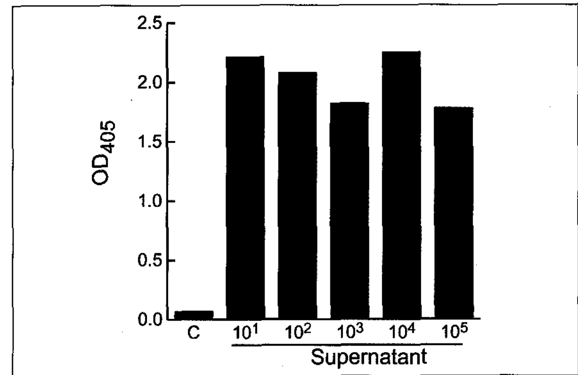

Fig. 8. Titration of anti-AgI/II antibodies from culture supernatant after the final selection of a hybridoma cell line, 1C11A. Culture supernatant from 1C11A was serially diluted by ten-fold and tested for anti-AgI/II activities by ELISA with the control of PBS solution (C).

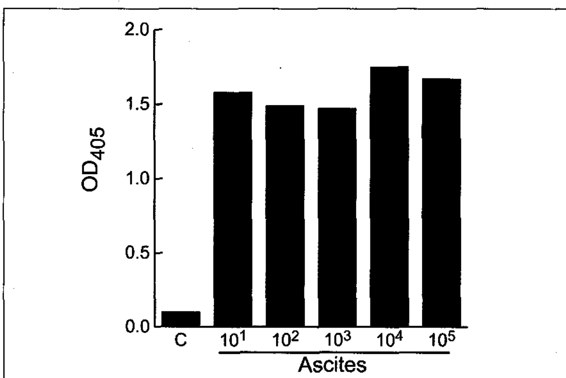

Fig. 9. Titration of anti-AgI/II antibodies from mice ascites obtained with injection of 1C11A cells. The ascites was serially diluted by ten-fold and tested for anti-AgI/II activities by ELISA with the control of PBS solution (C).

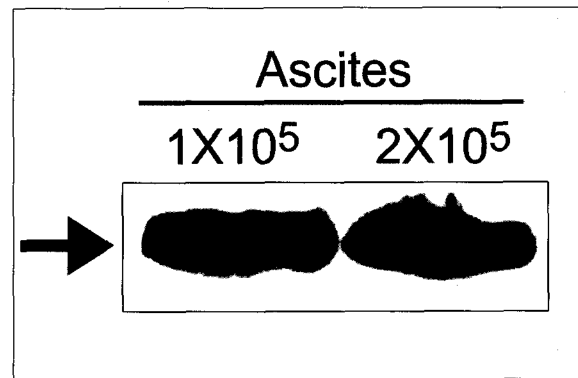

Fig. 10. Western blot with 1C11A ascites against rAgI/II-N proteins. The ascites was diluted by 100,000-fold or 200,000-fold and tested for anti-AgI/II activities by Western blot. Arrow indicates rAgI/II-N proteins.

from the host defense system such as shear force generated by fluid flow, antibiotics, antibodies, phagocytosis, and changes in pH. Another benefit for the growth in a biofilm is that it offers a favorable environment for bacterial growths in the sense of nutrient-rich, relatively stable conditions. Once the favorable niche was achieved, bacteria grow faster and generate lots of acids leading to demineralization of tooth enamel called as dental caries.

Once oral bacteria colonize in tooth pellicle, some bacteria could be released to infect other sites outside the oral cavity, leading to systemic diseases. Several inflammatory diseases may be related to *S. mutans* infection and AgI/II protein is supposed to play a role in the development of *S. mutans*-driven inflammations. In order to study the precise mechanism of AgI/II protein in the systemic diseases, we generated recombinant N-terminal fragment of AgI/II (rAgI/II-N) and anti-AgI/II mAbs. Our rAgI/II-N of *S. mutans* GS-5 covers about half of AgI/II protein and contains A-rich, V, and P-rich domains that have a certain role in inducing inflammation from

monocyte[36]. A-rich and P-rich domains are believed to interact each other to stabilize the conformation of AgI/II protein, while V domain binds to fucosylated and sialylated carbohydrates of monocyte receptor[37].

Recently, it was reported that the Ag-I/II-specific mAb named as 6-11A is capable of modulating host responses after *S. mutans* infection, when AgI/II protein coated with 6-11A mAb was injected in mice[44-47]. This kind of therapy using antigen-antibody mixture has been tested for a long time in diphtheria but recent accomplishment in *S. mutans*-related diseases will open another horizon. Together with rAgI/II-N, anti-AgI/II mAb will provide new tools in *S. mutans* GS-5 research.

In conclusion, a hybridoma line expressing 1C11A mAb was established and ELISA and Western blot assay using this new mAb clearly proved that 1C11A had a high sensitivity to AgI/II antigen. 1C11A will work as a new tool in the research fields of dental caries and other systemic diseases. We are currently investigating the roles of 1C11A in *S. mutans* colonization and systemic inflammatory effects related to AgI/II antigen or *S. mutans*.

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국문초록

Streptococcus mutans GS5의 세포막 단백질 Ag Ⅰ/Ⅱ에 대한 단항체의 생산

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치아우식증의 원인균으로 알려져 있는 *Streptococcus mutans*의 여러 균주 중 GS-5 균주는 AgI/II 유전자 내에 생기는 nonsense mutation에 의하여 절편의 분비형 AgI/II 단백질을 발현한다. 이러한 사실은 *S. mutans* GS-5가 독특한 임상 기능을 가질 수 있음을 암시하며, 최근의 보고는 임상적으로 분리된 *S. mutans* 균주를 이용한 실험 결과에 근거하여 이러한 가능성을 지지한다.

본 연구는 이전의 실험을 통하여 *S. mutans*의 agI/II 유전자를 확보한 후 재조합 단백질인 N-terminal AgI/II 단백질을 생산하였다. 이 후 하이브리도마를 통하여 1C11A라 명명되는 세포막 단백질 AgI/II에 대한 단항체를 생산하였으며, Western blot과 ELISA를 통하여 이 항체가 AgI/II 단백질에 매우 높은 특이성을 나타냄을 알 수 있었다. 새로이 얻어진 단항체는 AgI/II 와 관련된 질환의 기전을 규명하는데 기초 재료가 될 것이다.

주요어 : 연쇄상 구균, Antigen I/II, 단항체