and bacterial substitution were analyzed by comparing bacterial substitution between these two groups.

Statistical analysis

Differences in detection frequencies of bacteria were compared by χ^2 test using the statistical software StatFlex (Artech, Osaka, Japan). In all analyses, $P<0.05$ was taken to indicate significance.

Results

Bacterial substitution of oral mucosa after HCT

The bacteria identified on the oral mucosa before and after HCT are shown in Table 2. The detection frequencies of CoNS increased significantly, while those of bacteria that comprise the normal flora, such as *Streptococcus* species, decreased significantly with time after HCT. Significant changes were observed in detection frequencies of CoNS and α-*Streptococcus* species from before to 3 weeks after HCT (14.5% to 53.3% and 92.7% to 53.1%, respectively; both $P<0.05$, χ^2 test). The percentage of subjects carrying bacterial components of the normal flora was significantly decreased, while that of subjects carrying bacteria not normally associated with the normal flora was significantly increased (Fig. 1).

Antibiotic use and bacterial substitution

Detection frequencies of α-*Streptococcus* species and CoNS in the short-term and long-term antibiotic use groups are shown in Fig. 2. Both groups showed significant decreases in α-*Streptococcus* species and increases in CoNS, as shown in Fig. 2 (*$P<0.05$, χ^2 test). According to the course of time, these changes got clear in long-term antibiotic use group, and significant differences between the short-term and long-term antibiotic use groups are observed 7–21 days after HCT (*$P<0.05$, χ^2 test).

Type of HCT (conventional HCT or RIST) and bacterial substitution

Detection frequencies of α-*Streptococcus* species and CoNS in the conventional HCT and RIST groups are shown in Fig. 3. There were no significant differences in detection frequencies of α-*Streptococcus* species and CoNS between conventional HCT and RIST, as shown in Fig. 3, while significant bacterial substitution was observed in both HCT types, as shown in Table 2 (*$P<0.05$, χ^2 test).

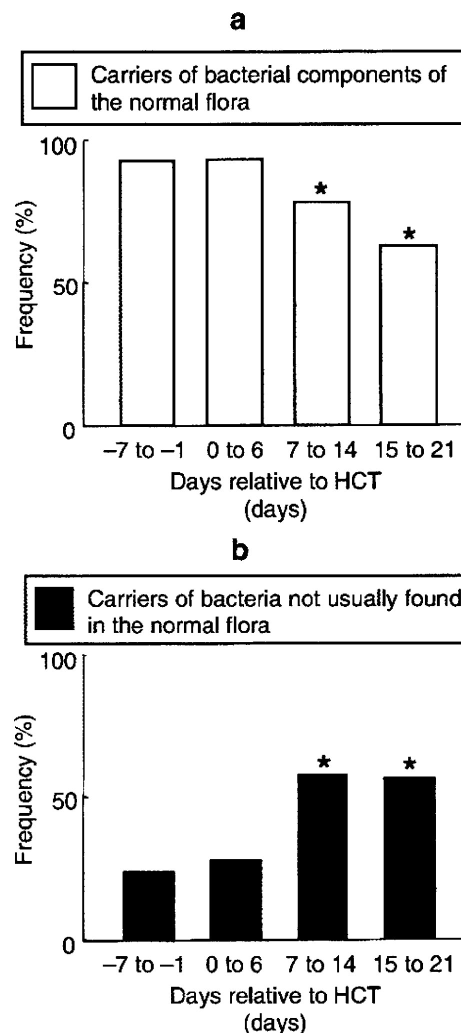

Fig. 1 Bacterial substitution on the oral mucosa after the HCT period. Carriers of bacterial components of the normal flora (**a**) and bacteria not usually found in the normal flora (**b**) according to the days relative to HCT are shown. *$P<0.05$, χ^2 test: compared with day −7 to −1. The percentage of subjects carrying bacterial components of the normal flora was significantly decreased, while that of subjects carrying bacteria not normally associated with the normal flora was significantly increased

Discussion

Bacterial substitution of mainly CoNS for streptococci occurs frequently on the oral mucosa after HCT. Approximately 75–85% of bone marrow transplantation recipients experience mucositis, and in some studies oral mucositis was the most common and most debilitating side effect reported [14, 15], which is consistent with our recent findings [12]. As described in the Introduction, the trajectory of oral mucositis of the subjects in the present study could be referred to from our previous report, as the

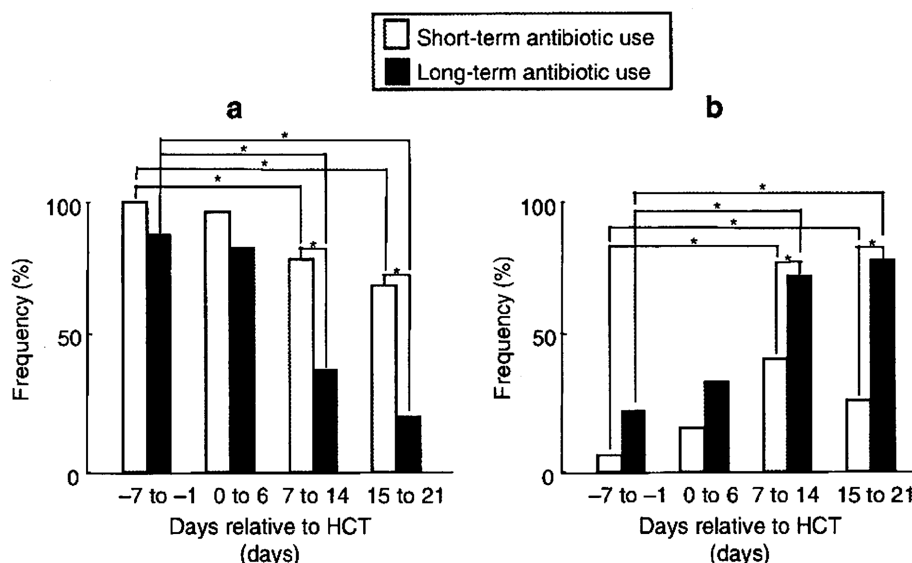

Fig. 2 Influence of antibiotic use on bacterial substitution on the oral mucosa before and after HCT. Detection frequencies of **a** α-*Streptococcus* species and **b** CoNS in the long- and short-term antibiotic use groups, according to the number of days relative to HCT are shown (*$P<0.05$, χ^2 test). Both group showed significant decreases in α-*Streptococcus* species and increases in CoNS. According to the course of time, these changes got clear in long-term antibiotic use group, and significant differences between the short-term and long-term antibiotic use groups are observed 7–21 days after HCT (*$P<0.05$, χ^2 test)

present patient population was a subset of those enrolled in our previous study [12]. The injured mucosa with mucositis may be involved in not only bacteremia caused by bacteria composing the normal flora, such as *Streptococcus* species, but also by those not associated with the normal flora, especially CoNS.

We expected bacterial substitution on the oral mucosa to occur because many different types and amounts of antibiotics are often used after HCT. The results indicated tendencies for bacterial substitution to occur in the high antibiotic use group, while no significant differences were observed in HCT types (conventional HCT and RIST). On the other hand, the differences in bacterial substitution between high and low antibiotic use groups were below our expectations. Interestingly, bacterial substitution was also observed even in two subjects with no antibiotic use. Anticancer treatment regimens, such as chemotherapy and/or irradiation, damage the salivary glands and cause hyposalivation [16, 17]. We reported that HCT also leads to the development of oral dryness [18]. Not only antibiotic use but also oral dryness may contribute to bacterial substitution. Almost all CoNS detected were estimated to be *Staphylococcus epidermidis*, although many of the reports on bacterial identification from our clinical laboratory were limited to the genus level both because of examination capacity and clinical necessity. The conditions of the oral mucosa with hyposalivation may be similar to those of the skin, which would promote such bacterial substitution of CoNS, which are the dominant bacteria composing the skin flora, as streptococci usually comprise the oral mucosal flora.

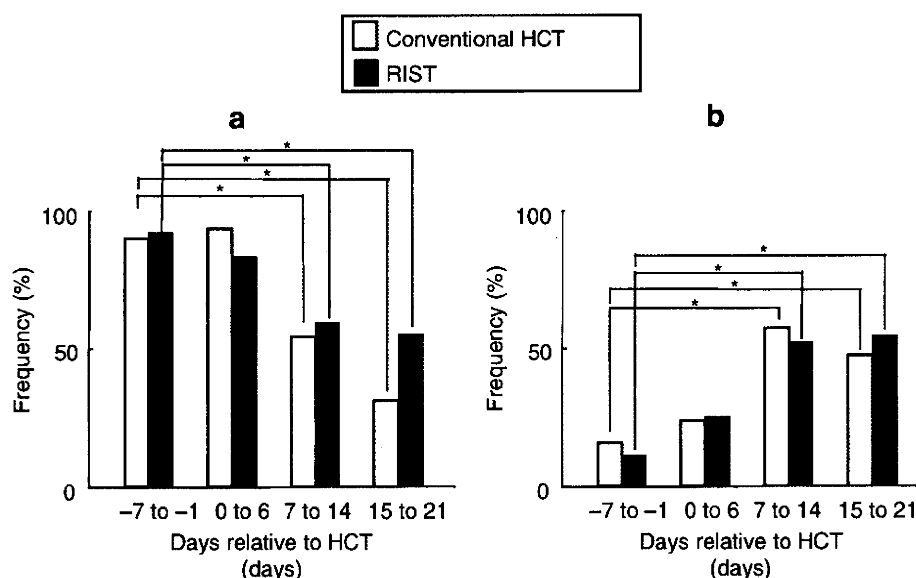

Fig. 3 Influence of HCT type (conventional HCT or RIST) on bacterial substitution on the oral mucosa before and after HCT. Detection frequencies of **a** α-*Streptococcus* species and **b** CoNS in the conventional HCT and RIST groups, according to the number of days relative to HCT are shown (*$P<0.05$, χ^2 test). There were no significant differences in detection frequencies of α-*Streptococcus* species and CoNS between conventional HCT and RIST, while significant bacterial substitution was observed in both HCT types, as shown in other table and figures

The widespread use of indwelling central venous catheters (CVC) is recognized as a significant risk factor for infections due to CoNS [19]. Management of CVC in CoNS bacteremia has been discussed previously [20]. On the other hand, DNA analysis demonstrated that the first episode of bacteremia due to *S. epidermidis* originated in the mouth, whereas a second episode involving another strain of *S. epidermidis* was derived from the CVC, recently [21]. Our findings support the suggestion that oral mucositis is a strong predictor of oral streptococci bacteremia and that CoNS bacteremia is clearly associated with mucositis [22]. We agree with the opinion that the impact of damage to the oral mucosal barrier is greatly underestimated [22, 23]. Therefore, we would like to emphasize the importance of infection control on the oral mucosa as intensive oral care in patients undergoing HCT, although the effective use of antibiotics is also important. In the present study, we also examined the relationship with detection of CoNS in blood culture. However, the detection of CoNS from blood culture was limited to five times throughout the present study, and therefore the number of cases was too small to determine the relationships between CoNS detected from the oral environment and blood. Although the reasons for the lower detection rates of CoNS from blood culture are not yet clear, these observations may have been due to our intensive oral care.

In conclusion, bacterial substitution of mainly CoNS for streptococci occurred frequently on the oral buccal mucosa after HCT.

Acknowledgments This study was partially conducted as the education curriculum, Practice in Dental Sciences, in Okayama University Dental School. We thank Ms. Hitomi Ono and Mr. Hidetaka Ideguchi, students at Okayama University Dental School, for data analysis. This study was supported by a Grant-in-Aid for Young Scientists (Start-up #20890138 and B #22791836), from the Japan Society for the Promotion of Science (JSPS) (YS) and FY 2010 Researcher Exchange Program between JSPS and The Netherlands Organization for Scientific Research.

Conflicts of interest The authors declare that there were no conflicts of interest in this study.

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Antigenic group II chaperonin in *Methanobrevibacter oralis* may cross-react with human chaperonin CCT

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Keywords: *Archaea*; chaperonin; *Methanobrevibacter oralis*; periodontitis
Accepted 26 August 2009

SUMMARY

***Methanobrevibacter oralis* is an archaeal species frequently isolated from sites of severe periodontitis. However, its pathogenic roles remain unclear. Here, we aimed to isolate group II chaperonin from *M. oralis* and examine its antigenicity. The genes encoding two chaperonin subunits (Cpn-1 and Cpn-2) were cloned from *M. oralis* using polymerase chain reaction and genome walking procedures. Recombinant proteins Cpn-1 and Cpn-2 were generated, and the reactivities of sera from patients with periodontitis were examined by Western immunoblotting. The open reading frames of Cpn-1 and Cpn-2 genes consisted of 1641 and 1614 base pairs, respectively. Putative ATP-binding domains conserved among the chaperonin family were observed in both genes. The deduced amino acid sequences of the two genes showed 28.8–40.0% identity to each of the subunits of human CCT (CCT1–8). Thirty and 29 of 36 patients' sera reacted with the recombinant Cpn-1 and recombinant Cpn-2, respectively. Western immunoblotting using antiserum against human CCT subunits indicated that anti-CCT3 and anti-CCT8 antibodies recognized recombinant Cpn-1. In addition, anti-CCT1, CCT3, CCT6, and CCT8 antibodies recognized an antigen of approximately 60 kDa in *M. oralis*. The results suggested that the chaperonin subunits of *M. oralis* were antigenic molecules that were recognized by periodontitis patients and that may cross-react with human chaperonin CCT.**

INTRODUCTION

Archaea are microorganisms classified as one of the primary domains distinct from bacteria and eukaryotes (Woese *et al.*, 1990). It is now clear that *Archaea* are ubiquitous organisms and are closely associated with plants and animals, including humans. *Methanobrevibacter* is one such major genus isolated from the human oral cavity (Belay *et al.*, 1988), gastrointestinal tract (Karlin *et al.*, 1982), and vagina (Belay *et al.*, 1990). Despite the ubiquity and close association with humans, no pathogenic *Archaea* have yet been identified, and there is controversy regarding whether any actually exist

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(Cavicchioli *et al.*, 2003; Eckburg *et al.*, 2003; Jangid *et al.*, 2004).

Periodontitis is an inflammatory disease caused by polymicrobial infection of oral microorganisms in subgingival dental plaque. Some gram-negative anaerobic rods and spirochetes in the plaque are closely associated with the disease (Socransky *et al.*, 1998) and are generally referred to as periodontal bacteria. In addition to periodontal bacteria, *Methanobrevibacter* species have been identified in cases of periodontitis (Ferrari *et al.*, 1994; Kulik *et al.*, 2001; Vianna *et al.*, 2008). Lepp *et al.* (2004) reported a relation between relative abundance of the *Methanobrevibacter* population and the severity of chronic periodontitis. We have also reported isolation of *Methanobrevibacter oralis* and *M. oralis*-like phylotypes from sites of severe periodontitis (Yamabe *et al.*, 2008). Although no studies have conclusively identified *Archaea* as causative agents of periodontitis, these previous reports suggest the potential pathogenic role of *Archaea* in this disease.

Heat shock proteins (Hsp) are highly conserved through evolution and have essential roles as molecular chaperones that assist in the efficient folding of newly synthesized and stress-denatured polypeptide chains (Ellis & van der Vies, 1991; Hartl, 1996). Among the members of the Hsp family, those with an approximate molecular mass of 60 kDa are called chaperonins (Cpn) and are divided into two groups, group I and group II (Horwich & Willson, 1993; Kubota *et al.*, 1994). They are similar in architecture to the oligomeric ring complexes and function as molecular chaperones. The amino acid sequences of Cpn are similar within the group, but not between the groups (Kubota *et al.*, 1995). Group I Cpn are also termed Cpn60, Hsp60, or GroEL, and are generally found in the bacterial cytosol as well as in mitochondria and chloroplasts. Under conditions of stress, such as heat shock, the Cpn molecules also appear at the cell surface (Alard *et al.*, 2007). Group II Cpn are found in the archaeal and eukaryotic cytosol. The archaeal group II Cpn are also known as thermosomes, and most archaeal Cpn complexes consist of two subunit proteins. Group II Cpn of eukaryotes are known as CCT (chaperonin containing T-complex polypeptide) and the human CCT complex consists of eight subunit proteins (Phipps *et al.*, 1991; Frydman *et al.*, 1992; Kubota *et al.*, 1995). Eukaryotes and some *Archaea* possess both groups of Cpn (Klunker *et al.*, 2003).

In addition to the chaperone role, bacterial Hsp60 (group I) are known to be highly antigenic molecules (Zugel & Kaufman, 1999a,b). Hsp60 are common antigens among bacterial species and serve as major immunogens in protection from the pathogenesis of infectious diseases. On the other hand, several immune disorders, such as rheumatoid arthritis, are thought to be triggered by these molecules (Feige & van Eden, 1996; van Eden *et al.*, 1998; Zugel & Kaufman, 1999a,b). As a result of the considerably high degree of primary amino acid sequence identity (molecular mimicry), immune responses against the infection-derived Hsp60 occasionally target human Hsp60 (group I), and cross-reactivity has been implicated in autoimmune or inflammatory diseases (Kiessling *et al.*, 1991). In periodontitis, Hsp60 of *Porphyromonas gingivalis* is known to be antigenic (Maeda *et al.*, 1994, 2000) and has been reported to promote the pathogenesis of atherosclerosis (Choi *et al.*, 2004; Ford *et al.*, 2005, 2007). However, there have been no reports of antigenicity of group II Cpn in any microorganisms.

We have attempted to elucidate the pathogenic role of *M. oralis* through the host immune response, and reported that immunoglobulin G (IgG) antibodies against this microorganism were detected in sera from patients with periodontitis. The sera recognized antigenic bands of approximately 60–70 kDa (Yamabe *et al.*, 2008). In the current study, for the first step of elucidating the antigenic property of *M. oralis*, group II Cpn were isolated and the antigenicity was examined. This is the first report identifying an antigenic molecule from *Archaea* recognized by the human immune system.

METHODS

Archaeal strains

M. oralis DSM 7256 was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). The purchased cells in liquid medium were pelleted and washed twice with phosphate-buffered saline (Invitrogen, Carlsbad, CA). The washed cells were subjected

directly to DNA extraction or were used as antigens for Western immunoblotting without subculture.

DNA extraction and amplification of genomic DNA

Genomic DNA was extracted from *M. oralis* by the method described by Stauffer *et al.* (Stauffer *et al.*, 1981). The extracted DNA was amplified using a REPLI-g Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The amplified genomic DNA was used for genome walking and polymerase chain reaction (PCR) as described below.

Detection and cloning of chaperonin genes

We attempted to amplify partial fragments of both group I and group II Cpn genes from genomic DNA of *M. oralis* by PCR. The PCR for amplification of the group I Cpn gene was performed as described previously (Goh *et al.*, 1996; Hill *et al.*, 2004) using a set of universal primers based on the common sequence of the genes. For amplification of the group II Cpn genes, primers were designed based on two registered sequences of group II Cpn subunits of *Methanobrevibacter smithii* (accession number: NC_009515, regions: 209884–211539 and 784085–785704). Two primer sets were designed from one of the Cpn genes (region: 209884–211539) and a set of primers was designed from another gene (region: 784085–785704). The nucleotide sequences of the primers for group II Cpn are shown in supplementary Figs S1 and S2. The contents of the PCR mixtures (50 μl) were 2.5 units of Ampli*Taq* Gold, 1 × buffer (Applied Biosystems, Foster, CA), 2.5 mm $MgCl_2$, 0.4 μm of forward and reverse primers, 0.2 mm deoxynucleotide triphosphates, and 100 ng of *M. oralis* genomic DNA. The PCR parameters included an initial incubation at 94°C for 9 min to activate the Ampli*Taq* Gold DNA polymerase, followed by 35 cycles of denaturation at 94°C for 0.5 min, annealing at 50°C for 0.5 min, and extension at 72°C for 2 min, and a final extension at 72°C for 7 min. The PCR products were separated on 1.5% agarose gels and were purified from the gels using a QIAEX®II Gel Extraction Kit (Qiagen, Hilden, Germany). The purified fragments were then cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA) for sequencing.

Genome walking

To obtain the full sequence of the Cpn genes, a PCR-based genome walking method (Siebert *et al.*, 1995) was performed. For the genome walking, a Universal GenomeWalker Kit (Clontech Laboratories, Mountain View, CA) was used according to the manufacturer's instruction manual. Briefly, amplified genomic DNA of *M. oralis* was digested with *Dra*I. Following digestion, the GenomeWalker adaptor provided with the kit was ligated to each end of the endonuclease-digested DNA for construction of the adaptor-ligated GenomeWalker library. For PCR of gene walking in each of 5′-flanking and 3′-flanking regions, two gene-specific primers were designed based on the nucleotide sequences of the cloned partial fragments of Cpn genes (shown in Figs S1 and S2). The primary PCR contained the GenomeWalker library as a template with the outer gene-specific primers and the first adaptor primer provided with the kit. The primary PCR product was then used as a template for nested PCR with the inner gene-specific primer and the second adaptor primer. Both PCR amplifications were performed using Advantage Genomic Polymerase Mix (Clontech Laboratories) under the conditions recommended by the manufacturer. The amplified DNA fragments were cloned to the pCR2.1 vector (Invitrogen, Carlsbad, CA) and were sequenced.

DNA sequencing and database search

DNA sequencing was performed using a BigDye® cycle sequencing kit (Applied Biosystems) and an automated DNA sequencer (3130xl Genetic Analyzer; Applied Biosystems). The sequence data were used to query GenBank and microbial genomes held at the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) using the blast sequence homology search program. Per cent sequence identities between *M. oralis* Cpn and other homologues of Cpn were analysed using Genetyx software (version 8; Genetyx, Tokyo, Japan).

Construction of recombinant protein

The recombinant Cpn of *M. oralis* (rCpn) was constructed using a pET Directional TOPO® Expression Kit (Invitrogen, Carlsbad, CA) according to the

manufacturer's instructions. Briefly, a partial open reading frame of the Cpn gene of *M. oralis* was amplified by PCR and inserted into the plasmid vector pET101/D-TOPO® (Invitrogen). The sequences of primers and the positions in the Cpn genes are shown in Figs S1 and S2. For directional cloning, four nucleotides (cacc) were added to the 5′ end of the forward primers, and the reverse primers were designed to allow the PCR product in frame with the histidine (His)-tag in the vector. *Escherichia coli* Rosetta™ 2 strain (DE3) (Novagen, Darmstadt, Germany) was transformed with the recombinant plasmid and was cultivated in Luria–Bertani broth supplemented with 50 μg ampicillin per ml and 1 mm isopropylthio-β-d-galactoside. The recombinant protein was purified from *E. coli* using a Qiagen Ni-NTA Fast Start Kit (Qiagen, Hilden, Germany) based on His-tag technology. Fractions eluted from the Ni-NTA columns were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and the gel region corresponding to rCpn was excised. The recombinant proteins in the excised gel fragment were then eluted and recovered from the gel using an Electro Eluter model 422 (Bio-Rad, Hercules, CA), and subjected to SDS–PAGE and Western immunoblotting.

SDS–PAGE and Western immunoblotting

SDS–PAGE and Western blotting analysis were performed as described previously (Kokeguchi *et al.*, 1989). Sera from patients with periodontitis were used at a final dilution of 1 : 200 with 5% (weight/volume) skimmed milk in Tris-buffered saline (10 mm Tris–HCl buffer, pH 7.5, 0.9% NaCl; M-TBS). Anti-human CCT1 (Abnova Co, Taipei, Taiwan), CCT3, CCT5, CCT6, and CCT8 polyclonal antibodies (Proteintech Group Inc.) were used at a dilution of 1 : 500 in M-TBS. Anti-penta·His-tag antibody (Qiagen) was used at a dilution of 1 : 1000 in TBS containing 3% bovine serum albumin. *E. coli* transformed with the Cpn genes of *M. oralis*, rCpn, recombinant human CCT1 subunit (Abnova Co, Taipei, Taiwan), human gingival fibroblasts, or *M. oralis* were used as antigens. Whole-cell lysates of *E. coli*, human gingival fibroblasts, and *M. oralis* were prepared as described previously (Maeda *et al.*, 2000; Yamabe *et al.*, 2008). Horseradish peroxidase-conjugated goat anti-human IgG antibody (Chemicon International, Inc.), goat anti-mouse IgG antibody (Millipore) or donkey anti-rabbit IgG antibody (GE Health Care, Pittsburgh, PA) were used at a dilution of 1 : 5000 with M-TBS for detection.

Serum samples

Sera from 36 patients were selected from the serum sample collection of the Okayama University Hospital of Medicine and Dentistry. Serum IgG antibody titer to the periodontal pathogens had been examined previously for clinical diagnosis (Murayama *et al.*, 1988). The sera selected in this study had elevated IgG antibody titers (greater than the mean + 2SD of the healthy controls) to the sonication extract of *P. gingivalis*, a potent pathogen in periodontitis, suggesting that patients had considerable periodontitis lesions (Murayama *et al.*, 1988). Six periodontally healthy subjects were also examined as controls. The use of human subjects in this investigation was approved by Okayama University Hospital Ethics Committee (approved no. 624).

RESULTS

Cloning and sequencing of *M. oralis* Cpn genes

PCR using universal primers for the group I Cpn gene yielded no amplification products (data not shown). However, PCR using primers designed from group II Cpn genes of *M. smithii* successfully amplified the genes, yielding reaction products of approximately 900, 1000, and 1450 base pairs (bp) (data not shown).

The three PCR products were cloned and sequenced. The nucleotide sequences of the two PCR products of 900 and 1000 bp overlapped each other by 602 bp; i.e. PCR using two primer sets designed from the same Cpn gene of *M. smithii* amplified the identical gene from *M. oralis* (Cpn-1 gene: Fig. S1). The remaining PCR product of 1450 bp had a distinct sequence (Cpn-2 gene: Fig. S2). By genome walking, DNA fragments containing 5′-flanking and 3′-flanking regions of the cloned partial Cpn-1 and Cpn-2 genes were amplified, and the complete nucleotide sequences of the Cpn-1 and Cpn-2 genes were determined (Figs S1 and S2). The open reading frames of the Cpn-1 and Cpn-2 genes consisted of 1641 bp (encoding 546 amino acids) and 1614 bp (encoding 537 amino

acids), respectively. The deduced amino acid sequences of both Cpn-1 and Cpn-2 genes contained four putative ATPase domains (Kubota *et al.*, 1995). The nucleotide sequences of the genes have been registered with the DNA Data Bank of Japan (*Cpn-1* gene: AB376229; *Cpn-2* gene: AB455150).

Homology search

A blast sequence homology search did not find any sequences identical to Cpn-1 or Cpn-2 genes in GenBank or Microbial Genomes at NCBI. Among the registered sequences in the database, Cpn-1 and Cpn-2 showed the highest levels of amino acid identity to each of the Cpn genes of *M. smithii* from which PCR primers were designed for amplification. The identity of Cpn-1 to one of the *M. smithii* Cpn was 83.9%, and Cpn-2 showed 91.1% identity to another *M. smithii* Cpn. Cpn-1 and Cpn-2 showed amino acid identities of 17.6% and 21.5% to human Hsp60 (group I), respectively. The identities to *E. coli* GroEL were 21.6% (Cpn-1) and 20.7% (Cpn-2). Amino acid identities of Cpn-1 to each of the human CCT subunits ranged between 28.8 and 37.8%, while Cpn-2 showed amino acid identities ranging from 31.4 to 40.0% to the CCT subunits (Table 1). CCT3 and CCT4 showed the greatest levels of sequence identity with Cpn-1 and Cpn-2 among the human CCT subunits, respectively. The amino acid sequences of CCT3 and CCT4 were aligned with Cpn-1 and Cpn-2, respectively, as shown in supplementary Figs S3 and S4.

Amino acid sequence identity between *M. oralis* Cpn and other Cpn homologues in *Archaea* ranged from approximately 40 to 90%. Representative results of the homology search in *Archaea* are shown in Table 2.

Construction and purification of recombinant proteins

A partial Cpn-1 gene fragment (849 bp from the start codon) and Cpn-2 gene fragment (780 bp from the start codon) were cloned into the expression vector (Figs S1 and S2). Protein profiles of *E. coli* transformed with the expression vector and rCpn during the purification steps were shown in Fig. 1. On SDS–PAGE analysis, no significant differences were found between control *E. coli* and those transformed with either the Cpn-1 or Cpn-2 gene. However, Western blotting analysis using anti-penta·His-tag antibody detected the recombinant protein in both transformants. *E. coli* transformed with the Cpn-1 gene expressed proteins of 42 kDa, 35 kDa, and 42 kDa that were reactive with anti-penta·His-tag antibody, while those transformed with the Cpn-2 gene expressed a protein of 40 kDa. The recombinant proteins of 42 kDa (rCpn-1) and 40 kDa (rCpn-2) expressed in *E. coli* were purified by Ni-NTA columns and gel elution.

Table 1 Amino acid sequence identity between *Methanobrevibacter oralis* chaperonin (Cpn) and human CCT subunits (%)

Cpn subunit	CCT1	CCT2	CCT3	CCT4	CCT5	CCT6	CCT7	CCT8
Cpn-1	36.6	33.1	37.8	37.4	36.4	28.8	34.1	35.1
Cpn-2	37.4	33.4	38.4	40.0	39.0	31.4	38.1	33.3

Accession numbers: *M. oralis* Cpn-1 (AB376229), Cpn-2 (AB455150), CCT1 (CAA37064), CCT2 (NP-006422), CCT3 (NP-005989), CCT4 (NP-006421), CCT5 (NP-036205), CCT6 (NP-001009186), CCT7 (NP-006420), CCT8 (NP-006576).

Table 2 Amino acid sequence identity (%) of chaperonin (Cpn)[1] between *Methanobrevibacter oralis* and other *Archaea* species

Archaeal species	*M. smithii*	*M. thermautotrophicus*	*Methanosphaera stadtmanae*	*Archaeoglobus fulgidus*	*Thermococcus kodakarensis*	*Haloarcula marismortui*
M. oralis Cpn-1	84 and 58	60 and 59	57 and 45	52 and 51	53 and 53	51 and 46
M. oralis Cpn-2	92 and 58	81 and 70	79 and 45	64 and 63	65 and 63	61 and 55

[1]Two Cpn subunits were compared with each other.

Accession numbers: *M. oralis* Cpn-1 (AB376229), Cpn-2 (AB455150), *Methanobrevibacter smithii* (NC-009515), *Methanobrevibacter thermautotrophicus* (NC-000916), *M. stadtmanae* (NC-007681), *A. fulgidus* (NC-000917), *T. kodakarensis* (NC-006624), *H. marismortui* (NC-006396).

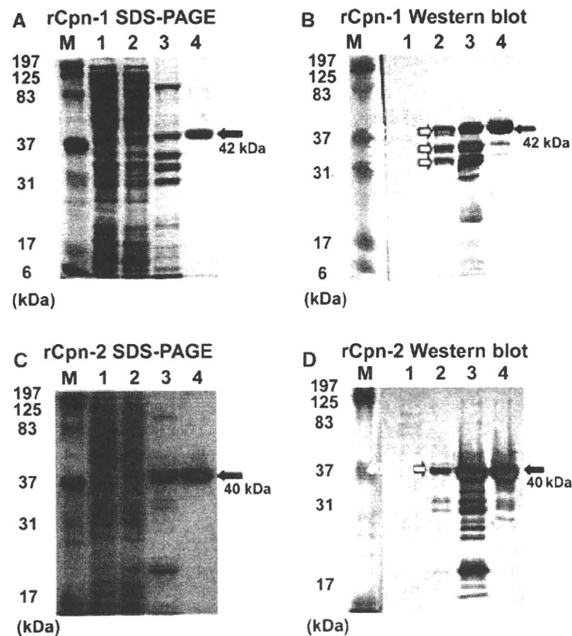

Figure 1 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting analysis of recombinant chaperonin 1 (rCpn-1) and rCpn-2. (A) and (B) showed the results of SDS–PAGE and Western blotting analysis of rCpn-1 during the purification steps, respectively. (C) and (D) show the results of SDS–PAGE and Western blotting analysis of rCpn-2, respectively. Distinct expression bands of rCpn-1 and rCpn-2 were not seen in protein profiles of the transformant *Escherichia coli* on SDS–PAGE, while anti-penta·His-tag antibody detected the recombinant proteins in each *E. coli* (white arrows). *E. coli* transformed with the Cpn-1 gene expressed proteins of approximately 32 kDa, 35 kDa, and 42 kDa that reacted with anti-penta·His-tag antibody (B). The 42-kDa recombinant protein was purified as rCpn-1. *E. coli* transformed with the Cpn-2 gene expressed a recombinant protein of approximately 40 kDa (white arrow in D) and was purified as rCpn-2. Final purification samples of rCpn-1 and rCpn-2 are indicated by black arrows. Lane M, molecular weight marker; lane 1, control *E. coli* (5 μg); lane 2, *E. coli* transformed with either the Cpn-1 or Cpn-2 gene (5 μg); lane 3, Ni-NTA column-purified fraction (1 μg); lane 4, final purification sample (500 ng).

Reactivity of sera from patients against rCpn

The reactivities of sera from patients with periodontitis against rCpn-1 and rCpn-2 were examined by Western immunoblotting (Fig. 2). Sera from 30 and 29 of the total of 36 patients reacted with rCpn-1 and rCpn-2, respectively. Two and three of six control sera showed weak reactivity to rCpn-1 and rCpn-2, respectively.

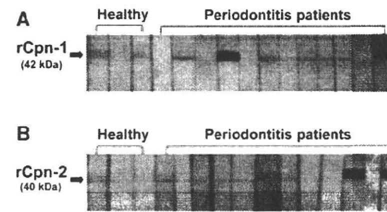

Figure 2 Serum reaction to *Methanobrevibacter oralis* chaperonin (Cpn). Reactivities of sera from patients against recombinant Cpn-1 (rCpn-1) (A) and rCpn-2 (B) were examined by Western immunoblotting. Sera from 36 patients with periodontitis and six healthy subjects were tested, and the representative results are shown. Two of six healthy controls and 30 of 36 patients showed reactivity to rCpn-1. Sera from three of the six controls and 29 of the 36 patients showed reactivity against rCpn-2. Each membrane strip contained approximately 50 ng rCpn-1 or rCpn-2.

Cross-reactivity between *M. oralis* Cpn and human CCT subunits

Cross-reactivity between *M. oralis* Cpn and human CCT was examined using anti-human CCT1, CCT3, CCT5, CCT6, and CCT8 polyclonal antibodies and rCpn of *M. oralis*. Western blotting analysis indicated that anti-human CCT3 and CCT8 antibodies reacted with rCpn-1 (Fig. 3), whereas none of the anti-human CCT antibodies reacted with rCpn-2 (data not shown).

The reactivities of anti-human CCT antibodies against the whole-cell lysate of *M. oralis* and human

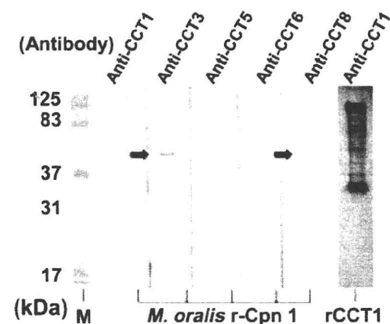

Figure 3 Reactivity of anti-human chaperonin (CCT) antibodies against recombinant chaperonin 1 (rCpn-1). Commercially available antibodies against human CCT1, CCT3, CCT5, CCT6, and CCT8 were used for Western immunoblotting to examine the reactivity against rCpn-1 and rCpn-2. Anti-CCT3 and anti-CCT8 antibodies reacted to rCpn-1, whereas none of the antibodies reacted to rCpn-2 (data not shown). Each lane contained approximately 50 ng rCpn-1. Recombinant human CCT1 (rCCT1) was loaded in the right lane, and the reactivity of anti-human CCT1 antibody was tested as a control. Lane M, molecular weight marker.

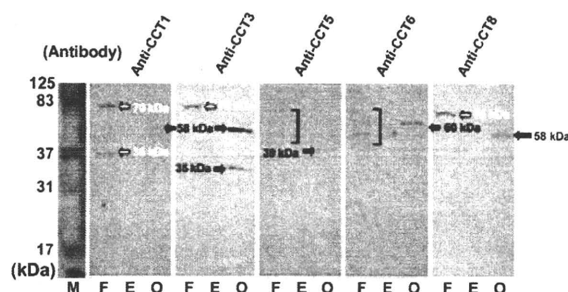

Figure 4 Reactivity of anti-human chaperonin (CCT) antibodies against *Methanobrevibacter oralis* cell lysate. The reactivities of anti-human CCT antibodies (anti-CCT1, anti-CCT3, anti-CCT5, anti-CCT6, and anti-CCT8) against whole-cell lysate of *M. oralis* (lane O) and human fibroblasts (lane F) were examined by Western immunoblotting. Anti-CCT1, anti-CCT3, anti-CCT6, and anti-CCT8 antibodies reacted to antigens of approximately 60 or 58 kDa of *M. oralis*. Anti-CCT3 antibody also recognized a 35-kDa antigen in *M. oralis*, and anti-CCT5 antibody recognized an antigen of molecular mass 39 kDa distinct from *M. oralis* Cpn. The lysate of human fibroblasts was used as a control containing CCT subunits. Anti-CCT1 and anti-CCT3 antibodies reacted to a fibroblast antigen of approximately 70 kDa. Anti-CCT1 antibody also recognized a 36-kDa antigen, and anti-CCT8 antibody reacted to an antigen of approximately 65 kDa in fibroblasts. Anti-CCT5 and anti-CCT6 antibodies recognized multiple fibroblast antigens at around 60 kDa (brackets). Each lane contained approximately 2 μg of protein. Lanes E and M, empty lane and molecular weight marker, respectively.

fibroblasts were examined by Western immunoblotting (Fig. 4). Each of the anti-human CCT antibodies reacted with the human fibroblast antigens (control) at around 60–70 kDa. In addition to the antigen of approximately 70 kDa, anti-human CCT1 antibody also recognized a 36-kDa fibroblast antigen. Anti-CCT5 and CCT6 antibodies detected multiple fibroblast antigens at around 60 kDa. In the reaction against *M. oralis*, anti-CCT1, CCT3, and CCT8 antibodies recognized an antigen of approximately 58 kDa in *M. oralis*. In addition, anti-CCT6 antibody recognized an antigen of approximately 60 kDa in *M. oralis*. In addition to the 58-kDa antigen, anti-CCT3 antibody detected another antigen of approximately 35 kDa. Anti-CCT5 antibody recognized only a 39-kDa antigen in *M. oralis*.

DISCUSSION

Methanobrevibacter species were identified in cases of periodontitis as early as 1988 (Belay *et al.*, 1988). However, their pathogenicity and influence on the pathogenesis of periodontitis have not been clarified. Recently, Lepp *et al.* (Lepp *et al.*, 2004) reported that *M. oralis* and similar species were the predominant microorganisms in microflora of sites of severe periodontitis. They also demonstrated that the relative abundance of *Treponema* species was significantly lower in sites with methanogenic *Archaea* than in those sites without methanogenic *Archaea*. Lepp *et al.* (2004) explained the pathogenic role of methanogenic *Archaea* through their influence on the microbial communities of subgingival plaque. Apart from their hypothesis, we are trying to determine the pathogenicity through antigenicity and host immune responses. We have recently reported that sera from patients with periodontitis recognized components of *M. oralis* including major 60–70 kDa antigens (Yamabe *et al.*, 2008). As Cpn with a molecular mass of 60–70 kDa are predicted to be highly expressed in *Archaea* (Karlin *et al.*, 2005), we aimed to identify the Cpn from *M. oralis* and characterize the antigenic property through the serum reaction of patients and the cross-reactivity with human CCT.

In the present study, two group II Cpn genes were identified in *M. oralis* but group I Cpn was not identified. Although some archaeal species possess both groups of Cpn, most *Archaea* possess only group II Cpn (Klunker *et al.*, 2003). Two genes for the group II Cpn subunits were found in the genome of *M. smithii* (NC_009515), and most group II Cpn of *Archaea* consist of two subunits (Kubota *et al.*, 1995). These findings, taken together, suggest that *M. oralis* has group II Cpn consisting of two subunits, but not group I Cpn.

We determined the complete nucleotide sequences of the two group II Cpn genes of *M. oralis*. The sequence identities between *M. oralis* Cpn and human CCT subunits ranged from 28.8 to 40.0% (Table 1), and conserved peptides (three to nine amino acids) were found throughout the length of the sequence (Figs S3 and S4). These findings of structural analysis suggest the possibility of immunological cross-reactivity based on molecular mimicry between human and *M. oralis* Cpn. In addition, Cpn homologues show high levels of sequence identity among archaeal species (Table 2: approximately 40–90%). *Archaea* are ubiquitous organisms prevalent in animals, including humans. Cpn may have the potential to be common antigens among archaeal strains similar to Hsp60 among bacterial strains.

To examine the antigenicity of *M. oralis* Cpn, rCpn-1 and rCpn-2 were constructed. As distinct bands of rCpn were not seen on SDS-PAGE analysis of the transformant *E. coli*, the levels of expression were estimated to be low. Both Cpn genes of *M. oralis* contained codons rarely used in *E. coli*, such as AUA and CUA. The Rosetta strain supplied with tRNA genes for these codons was used as the *E. coli* host strain (Novy *et al.*, 2001). However, efficient expression of the recombinant protein may still be difficult because of the codon usage of the Cpn genes of *M. oralis*. The Cpn-1 gene-transformed *E. coli* expressed recombinant proteins of 32, 35, and 42 kDa that reacted with anti-penta·His-tag antibody. The molecular mass of rCpn-1 was estimated to be about 42 kDa, and the His tag was fused to the C-terminus of rCpn-1. Therefore, the 32-kDa and 35-kDa recombinant proteins were probably truncated at the N-terminus. These molecules may be translated from the downstream methionine codons.

Most of the sera from patients reacted with rCpn-1 and rCpn-2, suggesting that Cpn-1 and Cpn-2 of *M. oralis* are highly antigenic molecules. Among them, five sera showed very strong reactivity to either or both of the rCpn (three of them were representatively shown in Fig. 2). These patients were possibly exposed to *M. oralis* and Cpn in periodontal lesions. Interestingly, all these high responders had severe periodontal lesions (bone loss score: 26.4–54.2%). A study for relations between serum reactivity to the Cpn and the clinical status of patients will be a good strategy for elucidation of *M. oralis* on the pathogenesis of periodontitis. Since an elevated IgG antibody titer against *P. gingivalis* was a criterion for the serum selection, the relations could not be cleared in the current study. Quantitative analysis of the serum reaction and various categories of patients will be required for the elucidation. In previous studies, membrane lipids of methanogens were shown to have strong adjuvant activity (Conlan *et al.*, 2001; Krishnan *et al.*, 2001). However, there have been no previous reports of antigenic molecules from *Archaea*. Our recent report indicated that sera from patients with periodontitis recognized the components of *M. oralis* (Yamabe *et al.*, 2008), and one of the antigenic molecules was identified in the current study. Although definite roles are still under elucidation, *Archaea* with strong adjuvant and antigenic molecules are probable modifier of inflammation in periodontal lesion. Weak reactions were seen in healthy controls. These reactions may have been to the result of exposure to other *Archaea*, which resulted in cross-reactivity among their Cpn. *M. smithii* in the gut is the most likely candidate for the source of cross-reactive Cpn.

Human CCT consists of eight subunit proteins. In the present study, cross-reactivity between *M. oralis* Cpn and human CCT subunits was examined using rCpn and five commercially available antibodies against each human CCT (subunits 1, 3, 5, 6, and 8). These antibodies were produced by immunizing animals with the recombinant CCT subunits (whole or approximately half-length). Anti-CCT 2, 4, and 7 antibodies were also obtained from commercial sources. However, they were produced by immunizing animals with short CCT peptides that did not include homologous sequences between *M. oralis* Cpn and human CCT. As the recognition epitopes were limited in the short sequences, anti-CCT 2, 4, and 7 antibodies without the possibility of cross-reactivity were excluded in the present study. Western blotting analysis suggested that CCT3 and CCT8 were cross-reactive antigens of *M. oralis* Cpn-1 with molecular mimicry. In contrast, none of the anti-CCT antibodies examined reacted with rCpn-2. Cpn-2 may not cross-react with human CCT subunits 1, 3, 5, 6, and 8. However, as neither of the rCpn examined here included the C-terminal regions, cross-reactivity in the truncated regions should be examined in future studies. In addition, polyclonal antibodies against CCT2, 4, and 7 are required to examine cross-reactivity of the uninvestigated subunits in the present study. CCT4, which showed the highest level of sequence identity to Cpn-2 (40.0%), is the most promising target for such studies.

In addition to rCpn, the reactivities of anti-human CCT antibodies against whole-cell lysate of *M. oralis* were examined. Each of the antibodies against the CCT subunits detected the cross-reactive antigens in *M. oralis*. As anti-CCT3 and anti-CCT8 antibodies reacted with rCpn-1, the 58-kDa antigen detected by these antibodies was thought to be Cpn-1 in *M. oralis*. The 58-kDa and 60-kDa antigens detected by anti-CCT1 and anti-CCT6 antibodies were not definitely identified as Cpn of *M. oralis*. However, because of the molecular weights and sequence homology between the CCT subunits and *M. oralis* Cpn, the

detected antigens were suggested to be *M. oralis* Cpn. In addition to CCT3 and CCT8, CCT1 and CCT6 may cross-react with *M. oralis* Cpn. Taken together with the reactivity to rCpn, anti-CCT1 and anti-CCT6 antibodies may recognize C-terminal regions of either Cpn-1 or Cpn-2. The 35-kDa and 39-kDa antigens detected by anti-CCT3 and anti-CCT5 antibodies with the distinct molecular mass of Cpn may have molecular mimicry with the human CCT subunit. Whole-cell lysate of human fibroblasts was used as control antigen including human CCT subunits. Anti-CCT1, CCT3, and CCT8 antibodies recognized antigens of approximately 65–70 kDa in fibroblasts, suggesting that they were very specific antibodies. In contrast, anti-CCT5 and CCT6 antibodies recognized multiple antigen bands at around 60 kDa. As the human CCT subunits show approximately 30% amino acid sequence identity with each other, anti-CCT5 and anti-CCT6 antibodies may cross-react with other CCT subunits. The 36-kDa fibroblast antigen detected by anti-CCT1 antibody may have cross-reactive epitopes with human CCT1.

The relation between autoimmune diseases and group I Cpn has been the subject of a great deal of discussion (Kiessling *et al.*, 1991; Wu & Tanguay, 2006). With regard to periodontal disease, it has been reported that Hsp60 from periodontal pathogens cross-react with human Hsp60 and may cause an autoimmune reaction to human Hsp60 (Choi *et al.*, 2004; Mandal *et al.*, 2004). The autoimmune reaction against Hsp60 was suggested to be involved in the pathogenesis of periodontitis and atherosclerosis (Ueki *et al.*, 2002; Ford *et al.*, 2005, 2007). In contrast, there have been very few studies regarding the autoimmune reaction against group II Cpn (human CCT), although human CCT was reported to be an immune target in patients with autoimmune disease or cancer (Yokota *et al.*, 2000; Schmits *et al.*, 2002). Yokota *et al.* (2000) reported that there were greater differences between sera from autoimmune disease patients and controls in CCT-reactive antibodies than in anti-group I Cpn (Hsp60) antibodies, suggesting that CCT may be a useful diagnostic antigen with which to detect the autoantibodies prevalent in patients with rheumatic disease. They hypothesized that infection with bacteria may cause an immune response to Hsp60 and result in the production of antibodies against epitopes structurally related to human CCT, and showed that the CCT-reactive autoantibodies recognized conformational epitopes conserved among CCT and Hsp60 (group I) members. Despite the low level of amino acid sequence identity (15–20%), bacterial Hsp60 was considered to be a cross-reactive antigen to human CCT because of their similar three-dimensional architecture. *Archaea* with group II Cpn have not yet been accepted as pathogens exposed to the host immune system, and the antigenicity of their Cpn has not been reported previously. However, recent reports of *Archaea* in patients with periodontitis (Lepp *et al.*, 2004; Yamabe *et al.*, 2008) and the antigenic properties of *M. oralis* Cpn revealed in the present study strongly suggest that *M. oralis* was prevalent in periodontal lesions and that the Cpn were cross-reactive antigens of human CCT recognized by the host immune system.

ACKNOWLEDGEMENTS

This study was supported by Grants-in-aid for Scientific Research (B17390502 to SK and C21592624 to HM) from the Japan Society for the Promotion of Science.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Nucleotide and deduced amino acid sequence of the Cpn-1 gene.

Figure S2. Nucleotide and deduced amino acid sequence of the Cpn-2 gene.

Figure S3. Sequence alignment of Cpn-1 with human CCT subunits.

Figure S4. Sequence alignment of Cpn-2 with human CCT4.

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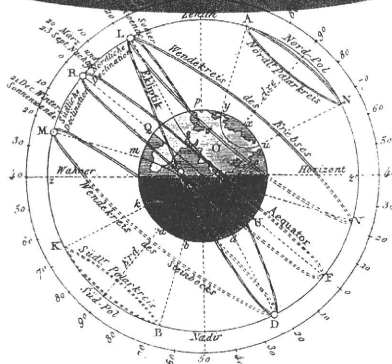
【解説】



メタン生成古細菌は歯周病の病原因子？

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口腔内に生息するメタン生成古細菌（***Methanobrevibacter***）が歯周病（歯槽膿漏）の病態に関与していることが示唆されるようになった．古細菌には，膜脂質や抗原分子において，真正細菌にはない特徴がある．これまで，歯周病の病態はグラム陰性桿菌を主体とした，いわゆる歯周病原細菌の感染とそれらに対する免疫応答から説明されていた．メタン生成古細菌の参戦によって，歯周病の病態がこれまでにはない側面から解明される可能性がでてきた．

古細菌（*archaea*）は，細菌とも真核生物とも異なる第三のドメインとして分類される微生物であり，過酷な環境でも生存可能な生物として自然界に広く分布している[1]．動植物の生体内にも生息しているが，病原性は不明で，癌などの疾患をひき起こす原因となるか否か，議論の分かれるところとなっていた[2]．ヒトの口腔内に定着することも知られていたが，最近になって，口腔内の代表的な疾患である歯周病との関連が注目されている．歯の喪失に関わる口腔二大疾患は，う蝕と歯周病であるが，とりわけ歯周病は口腔疾患としてだけではなく，糖尿病，心臓・血管疾患，妊娠障害や誤嚥性肺炎をはじめとして，様々な全身疾患と健康に影響することが知られている．本稿では，口腔内に生息する古細菌（メタン生成菌：*Methanobrevibacter*）と歯周病との関連性について，最近の知見をふまえて概説する．また，筆者らの研究グループの実験結果をもとに，古細菌研究の現状と今後の展望について紹介する．

Methanogenic Archaea Has a Potential to be a Pathogen of Periodontal Disease?
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歯周病とは

歯の周囲には**図1**に示すように，通常 1〜3 mm の歯肉溝（歯周ポケット）と呼ばれる『溝』構造が存在する．この歯肉溝に口腔内微生物が定着し，歯周組織に感染することで，歯周病が発症する．以下に，細菌因子と生体因子（免疫応答）に分けて，歯周病の病態を簡単に説明する．

1. 歯周病と細菌因子

歯の表面に付着している黄白色の塊はプラーク（歯垢）と呼ばれ，単なる食べカスではなく生きた細菌の塊である．口腔内には700種以上に及ぶ細菌種が存在するといわれ，プラーク 1 mg あたりには10億個もの細菌が存在する．健康な歯に付着しているプラークは，主に好気性菌（連鎖球菌グラム陽性桿菌）で構成されている．

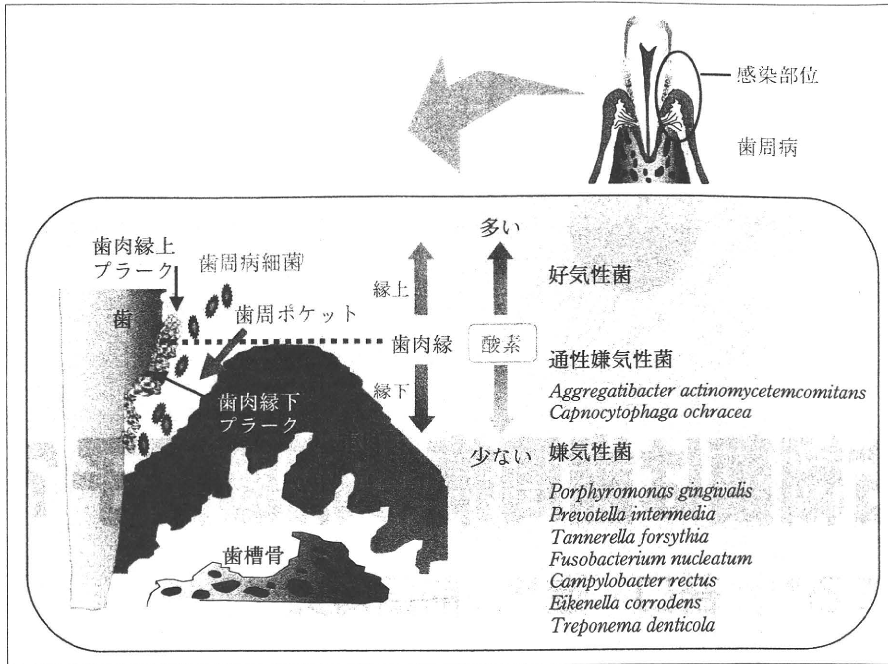

図1■歯周組織の構造と歯周病原細菌

歯と歯肉が接している部位には歯肉溝（歯周ポケット）と呼ばれる『溝』構造がある．歯肉縁下となる歯周ポケット内は酸素分圧が低い．このため，歯周ポケット内の歯垢（歯肉縁下プラーク）は嫌気性の口腔内細菌が細菌叢の主体となる．この中で，歯周病の発症と進行に深く関与している菌種が，歯周病原細菌である．図中に示している細菌種は代表的な歯周病原細菌であるが，近年の分子生物学的手法を用いた細菌叢の解析によって，古細菌をはじめとした歯周病原細菌以外の微生物が，歯周病の病態に関与している可能性がでてきた．

ところが，歯周病が進行すると歯肉溝は深くなり，歯周ポケットと呼ばれるようになる．深い歯周ポケット内では，好気性菌に代わり，酸素を嫌う嫌気性菌（主にグラム陰性桿菌）が主体となってプラーク細菌叢が構成されている．すなわち，プラークは歯肉縁上プラークと歯肉縁下プラークに分けられ，歯周病は歯肉縁下プラークを構成する嫌気性菌を原因として発症する（図1）．

プラークは多様な細菌種の共同体であり，粘着性のある多糖体に包まれ，歯面に付着するバイオフィルムと考えられている．バイオフィルムが形成されると薬剤抵抗性が高くなり，単独（浮遊）状態で存在する細菌の場合よりも約500倍強い病原性を発揮するといわれている．歯周病の治療が難しい原因のひとつは，プラークがバイオフィルムの性質をもつためである．

また歯周病は，複合感染症という側面をもっている．すなわち，歯周病は単一細菌の感染によって発症するのではなく，プラーク中に存在する多くの細菌種が感染することで発症する．その中でも数種類のグラム陰性嫌気性桿菌とらせん菌（スピロヘータ属）は，特に歯周病の病態に深く関わっており，歯周病原細菌として知られている[3]．*Porphyromonas gingivalis*, *Tannerella forsythia*, *Prevptella intermedia*, *Aggregatibacter actinomycetemcomitans*, *Treponema denticola* などが代表的な歯周病原細菌である．近年，分子生物学的手法を駆使して口腔細菌叢の解析研究が進められており[4]，歯肉縁下プラークには多くの未知の難培養細菌や *Methanobrevibacter oralis* に代表される古細菌（*archaea*）の存在することが明らかとなった[5]．それらの研究結果から，プラーク細菌叢はこれまでに考えられていた以上に複雑であること，そして歯周病原細菌と呼ばれている細菌種以外にも歯周病の病態に関与している微生物の存在していることが明らかになりつつある．

2. 歯周病と生体因子（免疫応答）

歯周病の病態には外来微生物の因子に加えて，生体因子，特に生体防御細胞の応答が深く関与している．**図2**に示すように，歯周病細菌は歯周ポケット内に定着し，持続的に歯周組織に感染する．細菌の毒素や酵素といった病原因子も歯周組織を破壊する要因になるが，組織破壊の主要因は生体の免疫応答によって起こると考えられている．すなわち，微生物によって単球・マクロファージやリンパ球などの生体防御細胞が活性化されると，免疫応答を調節する因子としてサイトカインなどの炎症性メディエーターが産生される．感染微生物を上手く排除するためには，様々な種類の生体防御細胞が連携して機能する必要がある．サイトカインは防御細胞がネットワークを形成するために必要な物質である．しかし，炎症性サイトカインの中には破骨細胞（骨を吸収する役割をもつ細胞）を活性化するものも存在する．感染量が多くなり，細菌を排除するために過剰な免疫応答が必要に

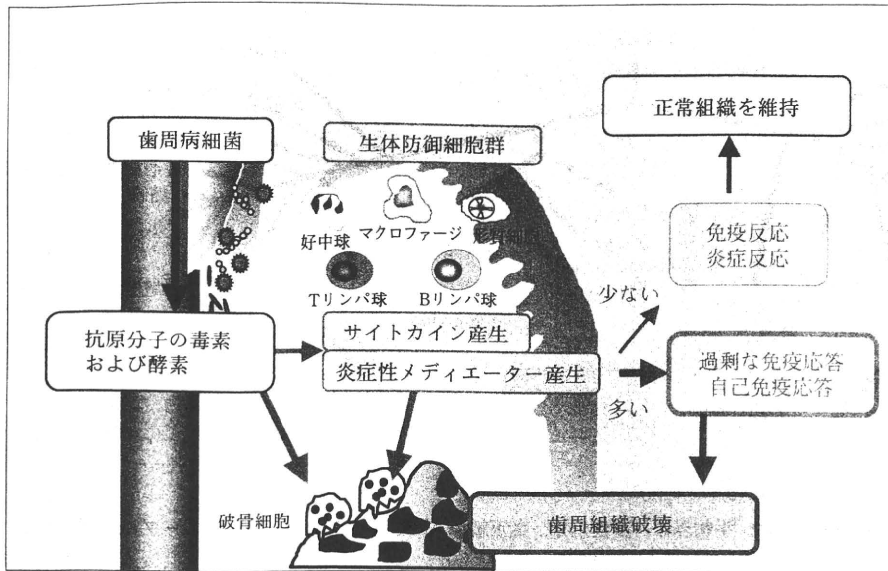

図2■歯周組織の破壊と免疫応答
歯周ポケット内に定着した微生物は，歯周組織に侵入して，生体防御細胞を活性化する．その結果，歯周組織には炎症反応（強い免疫応答）が起こり，産生された炎症性サイトカインは，破骨細胞を活性化して歯槽骨（歯の周りの骨）の吸収が起こる．ブラッシングによって細菌量をコントロールしていれば，過剰な免疫応答が起こらず，歯周組織の安定が保てる．

なった場合には，それらの炎症性サイトカインの産生量も増加し，歯槽骨が吸収されてしまう．また，微生物を貪食し殺菌する役割をもつ細胞には細菌を消化するための酵素が含まれているが，微生物を排除するために集積した防御細胞からそれらの酵素が放出されると，周囲の組織が傷害を受けることになる．さらに，場合によっては，外来微生物を排除するための免疫応答が自分自身の細胞へ向けられ（自己免疫応答），歯周組織の破壊が起こることも報告されている[6]．

歯磨きを上手に行ない，細菌量をコントロールしていると，過剰な免疫応答は起こらず，歯周組織を健康に保つことができる．歯周病細菌と呼ばれる菌は，歯周ポケットに定着しやすく，歯槽骨吸収などの組織破壊に結びつく免疫応答を誘導する性質の強い細菌種であると考えられている．先に述べたように，これまではグラム陰性嫌気性桿菌あるいは口腔スピロヘータが歯周病の原因微生物と考えられていた．ところが最近になって，メタン生成古細菌についても歯周病の病態に深く関与していることを示す研究が報告されるようになってきた．

古細菌とは

古細菌とは，原核生物でありながら，生物学的・生化学的性質が真正細菌とは大きく異なっている単細胞生物である．細胞の直径は0.5～1.5 μm，長さが1～数μmであり，細胞膜の他には膜系をもたず，細胞中にも際立った構造は認められない[1]．

古細菌という概念はWoeseとFox[7]により最初に提唱された．それまで，微生物の分類は菌の細胞の性質，形態，生育条件などの特徴で行なわれていた．しかし，こうした分類法では，分類基準の中のどれを上位の基準とするかの絶対的なルールを作成することが困難であった．このため，原核生物の進化系統を議論する理論的な基礎は存在しなかった[1]．このような背景のなかで，Woeseら[8]はrRNA遺伝子の塩基配列をもとに微生物を分類し，生物群は3つのグループ，真核生物，真正細菌，そして古細菌に大別されることを報告した（図3）．

古細菌の特徴

古細菌の特徴は大きく2つある．一つは，膜脂質構造である．他の生物がすべて，エステル脂質を膜脂質として保有しているのに対し，古細菌はグリセロールにアルコールがエーテル結合したエーテル型脂質をもっている．興味深いことに，古細菌の膜脂質はアーキアソーム(archaeosome)と呼ばれ，強いアジュバント活性（免疫賦活作用）をもつことが報告されている[9]．

もう一つの特徴として，古細菌にはそれまで真核生物に特有の性質と思われていたさまざまな生化学的・分子生物学的性質が備わっている[1]．たとえば，DNA複製酵素は，真核生物のDNAポリメラーゼαに類似したものであり，翻訳開始の反応にはフォルミル化していないメチオニルtRNAを使用すること，あるいは染色体にヒストン様タンパク質があり，クロマチン様構造を形成していることなどがあげられる．5S rRNAの塩基配列をもとにした系統解析では，古細菌は細菌よりも真核生物に類似することが明らかになっている[10]．

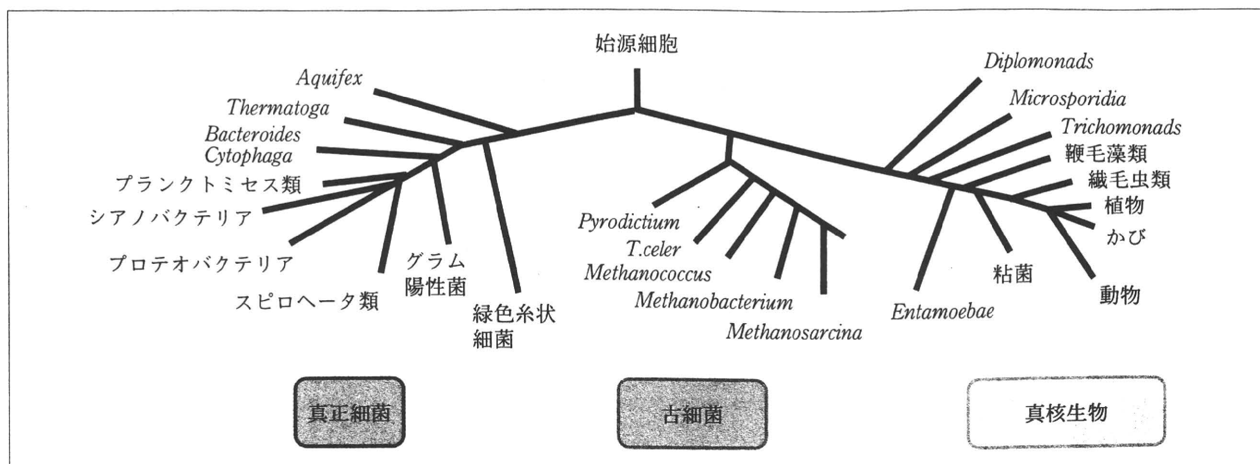

図3■生物界の系統樹（文献8から改変）
リボゾーマルRNA (rRNA) 遺伝子の塩基配列をもとに，生物界は，真核生物，真正細菌，そして古細菌の3つのグループに大別される．

メタン生成古細菌

ヒトの口腔内[11]や胃[12]からはメタン生成古細菌（メタン菌）が高頻度に検出される．メタン菌は，メタンを生成することでエネルギーを得る偏性嫌気性菌であり，湖沼，水田，海洋，動物の腸などの嫌気的環境中に生息している．メタン菌の生育には，H_2–CO_2，ギ酸，酢酸，メタノール，メチルアミン類などが必要だが，硫化ジメチルを利用するもの，あるいは，2-プロパノール，シクロペンタノール，2-ブタノールなど第二アルコールを水素供与体とし，CO_2を水素受容体として利用するメタン菌もいる[1]．

メタン菌の多くは他種生物と共存し，それらの生物からエネルギー源を供給されて，共生関係を結んでいる．しかし，同じ基質を利用する微生物が存在する場合には，メタン菌は他の生物とその基質をめぐって競合することもある．

古細菌と歯周病の関係

歯周炎局所に古細菌が存在することは，比較的古くから示唆されていた．1988年にBelayら[11]は，メタン菌である*Methanobrevibacter*属が歯周炎局所に存在することを報告している．日本人を対象とした研究においても[13]，歯周病の重度な病変部位から*M. oralis*とその類縁菌種が高頻度に検出されることが明らかになっている．古細菌の検出頻度は軽度な歯周炎部位に比べ，重度な歯周炎病巣において高く，病態との関連性が示唆されている．

近年，Leppら[5]によって歯周病変部のプラーク微生物叢が定量解析され，重度な歯周病変部においては*M. oralis*あるいはその類縁菌が微生物叢の主体となっていることが報告された．さらに彼らは，*M. oralis*量が多いプラークにおいては歯周病原細菌のひとつである*Treponema denticola*の感染量が少なくなること，また逆に*M. oralis*の量が少ない微生物叢では*T. denticola*の割合が高くなることを報告している．この現象は両菌が生育に必要な水素化合物を競合するために起こると考えられるが，*M. oralis*がプラーク微生物叢の構成に影響を与えることによって歯周病の病態に関与していることを示唆するものである．Leppらがプラーク微生物叢との関連性から古細菌の病原性を示唆したのに対して，筆者らの研究グループは古細菌 (*M. oralis*) の抗原性，あるいは古細菌に対する宿主の免疫応答から，古細菌と歯周病の関連性を明らかにしたいと考えてきた（**図4**）．

古細菌に対する免疫応答

ヒトの生体内に定着しているにもかかわらず，古細菌に対するヒトの免疫応答に関する報告はこれまでになかった．そこで筆者らは，歯周病患者から血液を採取して血清中に含まれる抗体の反応性を調べてみた．その結果，歯周病患者の血清中には古細菌*M. oralis*と特異的に反応する抗体の存在することが明らかになった[13]．抗体は外来微生物を排除する目的に，免疫応答の結果として産生される物質である．すなわち，*M. oralis*に対する抗体を歯周病患者が保有していたことは，宿主（体）に古細菌に対する免疫応答が誘導されていることを意味する．

古細菌は歯周ポケットに定着し，歯周組織においては

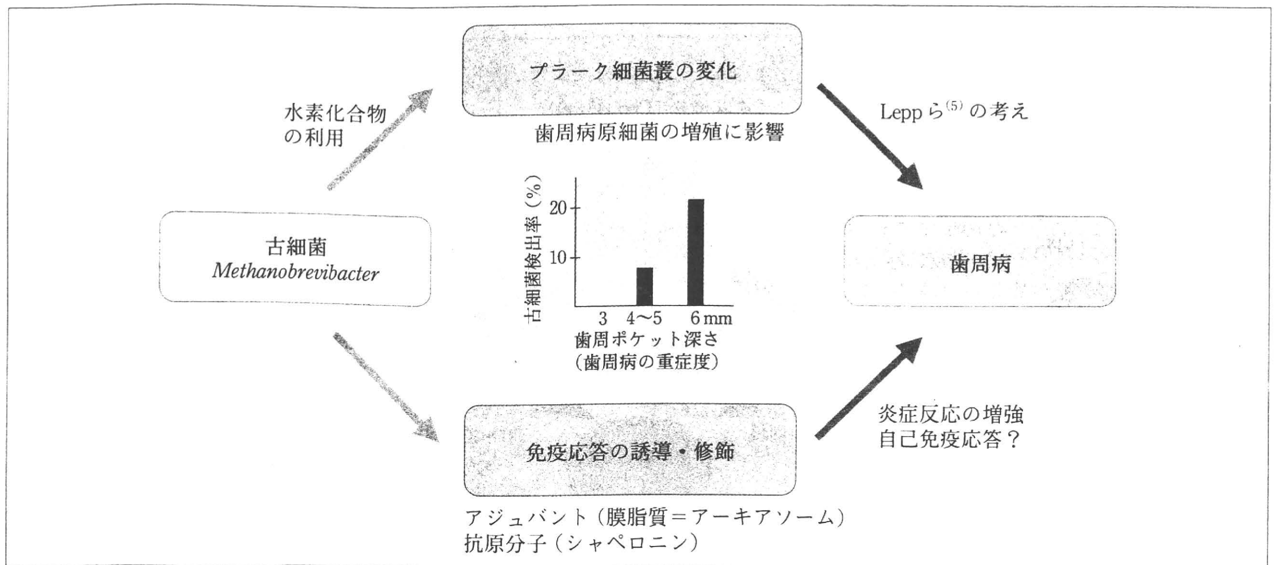

図4■古細菌と歯周病の関係
歯周病変部からはメタン生成菌である *Methanobrevibacter* 属が検出される．この古細菌は歯周病が重度であるほど高頻度に検出される．水素化合物を利用する *Methanobrevibacter* は口腔細菌種と共生，あるいは競合的な関係をもち，歯周病をひき起こすプラーク細菌叢に影響を与える．また，*Methanobrevibacter* の膜脂質には強いアジュバント活性があり，菌体成分には強い抗原性をもった分子が存在する．古細菌はプラーク細菌叢に影響を与えること，あるいは歯周炎局所の免疫応答を誘導・修飾することによって歯周病の病態に関与していると考えられる．

古細菌に対する免疫応答が誘導されていると考えられる．それでは，古細菌に対する免疫応答はどのような形で歯周病の病態に影響を与えているのだろうか？　筆者らの研究グループでは，自己免疫応答の観点から，古細菌に対する免疫応答の解析を試みている．

自己免疫応答

自己免疫応答とは，本来，外来微生物を排除するために働くはずの免疫応答が，自分自身の細胞や組織を攻撃してしまう反応のことを指す．自己免疫応答の発症機序は完全には明らかにされていないが，外来微生物との交差反応が原因のひとつと考えられている．

ヒトの免疫担当細胞は，ヒトの組織にはない，微生物だけがもつ構造（アミノ酸配列）を認識することで，それを侵入者とみなし，攻撃しようとする．通常は4〜6残基のアミノ酸配列が認識され，免疫応答が起こる．この免疫担当細胞が認識するアミノ酸配列を抗原決定基（エピトープ）と呼ぶ．微生物の多様性に対応できるように，ヒトの体の中には星の数ほどのエピトープに対応できる仕組みが備わっている．

ところが，微生物のもつ抗原性の高いタンパク質（強い免疫応答を誘導するタンパク質）に免疫応答が起こった場合，ヒトとの類似構造部分 (molecular mimicry) がエピトープとして認識される場合がある．その結果，微生物に対して惹起された免疫応答が，類似した構造をもつヒトのタンパク質に向けられ（交差反応），自己の細胞の傷害につながることになる．

歯周病と自己免疫応答

歯周病の病態には自己免疫応答が関与している場合があるが，歯周病に限らず，自己免疫応答が関与している疾患においては，熱ショックタンパク質（ストレスタンパク質）が病態の鍵のひとつと考えられている[14〜16]．ストレスタンパク質は分子量の異なる幾つかのファミリーに分類されるが，分子量が約60,000のストレスタンパク質はシャペロニン (Cpn) と呼ばれ，他のタンパク質の高次構造の形成を補助する役割をもっている．このCpn分子は進化の過程で保存されており，アミノ酸配列は種をこえて高い相同性を示す．このような分子には通常，強い免疫応答は起こらないが，微生物の保有するCpnは強い抗原性をもつ．このため，微生物のCpnとヒトのCpnに交差反応が起こり，感染症に伴って自己免疫応答が誘導されると考えられている（**図5**）．

Cpn分子はグループIとグループIIに大別され，細菌種はグループIを，古細菌種は主にグループIIを，そしてヒトは両方のCpn分子を保有している．しかし，これ

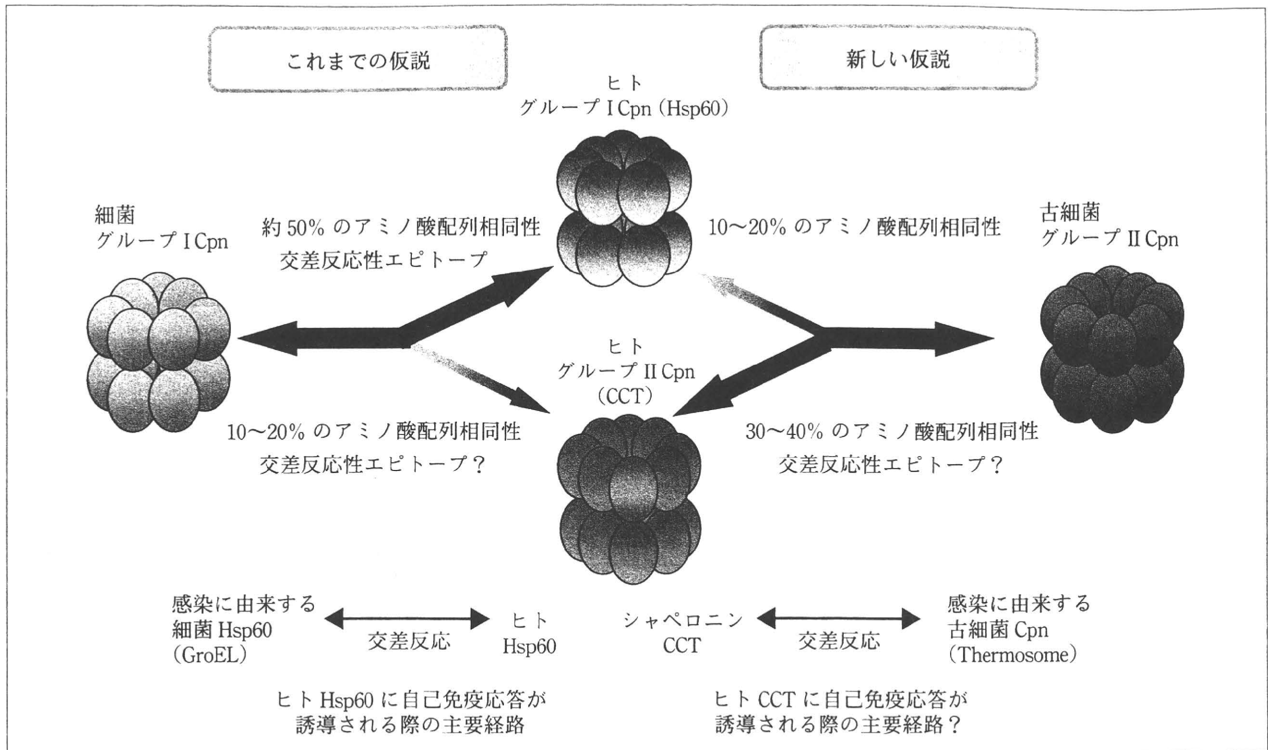

図5■シャペロニン(Cpn)の交差反応と自己免疫応答

CpnはグループⅠとグループⅡに大別される．真正細菌はグループⅠを，古細菌は主にグループⅡを，そしてヒトは両方のCpnを保有している．グループⅠCpnは熱ショックタンパク質(Heat shock protein) 60 (Hsp 60)，細菌種ではGroELの別名でも知られている．細菌とヒトのグループⅠCpnのアミノ酸配列には約50%の相同性がある．細菌のCpnには強い抗原性があり，細菌のCpnに対して誘導された免疫応答がヒトのCpnに交差反応し，自己免疫応答が誘導される場合があると考えられている（これまでの仮説）．一方，グループⅡCpnに対する自己免疫応答の報告はあるものの，その発症機序に関する研究はほとんどない．ところが，最近の知見から，古細菌のCpn（別名Thermosome）がヒトのグループⅡ Cpn(CCT)の交差反応抗原である可能性がでてきた（新しい仮説）．

までのCpnの交差反応，自己免疫応答，あるいは自己免疫疾患の発症機序に関する研究は，そのほとんどがグループⅠCpnに関するものである．これまでの研究がグループⅠを中心に展開してきた理由は，感染症をひき起こす病原微生物，すなわち真正細菌がグループⅠのCpnを保有しているためであり，感染症に起因した交差反応から自己免疫応答の発症機序が説明されやすいためだったと推察できる．

一方，ヒトのグループⅡCpnに関しては，自己免疫応答が起こっているという報告はあるものの[17]，その発症機序や疾患との関連性についてはほとんど報告がない．グループⅠとグループⅡCpnはリング状の類似した高次構造と機能をもっており，同一グループ内では，生物種が異なってもアミノ酸配列が保存されている．しかし，グループⅠとグループⅡの間ではアミノ酸配列の相同性が高くない．このため，感染症とグループⅡCpn，そして自己免疫応答とが結びつくことはなかった．

古細菌*M. oralis*の保有するCpn分子の構造

これまで，グループⅡCpnを保有している古細菌の病原性についてはまったく明らかにされていなかった．しかし，最近の歯周病と古細菌に関する一連の研究から，古細菌は歯周病変部に定着し，宿主であるヒトは古細菌に感作され，免疫応答が誘導されている可能性の高いことがわかってきた．そこで筆者らは，「古細菌のCpnはヒトのグループⅡCpnの交差反応抗原である」という仮説をたて，高頻度に歯周病変部から検出される*M.oralis*のCpn分子について，その構造と抗原性について調べてみた．

*M. oralis*についてはゲノムデータベースが確立されていないため，筆者らはまず，*M. oralis*からCpn遺伝子をクローニングして塩基配列の解析を行なった．古細菌のCpnは2つのサブユニットタンパク質で構成されており，ヒトのCpnは8つのサブユニットタンパク質で構成される16量体である．ヒトのグループⅡCpnは