

RESEARCH ARTICLE

Serum antibody response to group II chaperonin from *Methanobrevibacter oralis* and human chaperonin CCT

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Methanobrevibacter oralis is a suspected periodontal pathogen and in this study the serum responses to human CCT and the *M.oralis* group II chaperonin were studied in patients with periodontitis and autoimmune diseases. Interestingly, both diseased groups showed elevated responses to one or more CCT subunits, suggesting the potential importance of cross-reactivity in autoimmune diseases.

Keywords

Archaea; periodontitis; chaperonin; auto-antibody; autoimmune disease.

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Abstract

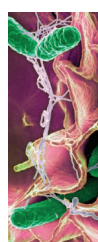
Both group I (HSP60) and group II (CCT) chaperonins are targets of autoantibodies. Autoimmune reactions to HSP60 have been well characterized, while immune reactions to group II chaperonin have not been clarified. *Methanobrevibacter oralis* is a suspected periodontal pathogen with group II chaperonin. In this study, serum responses to *M.oralis* chaperonin, human HSP60, and CCT subunits were examined using sera from patients with periodontitis and autoimmune diseases. In comparison with healthy controls, periodontitis patients showed significantly higher responses to CCT4 and CCT8 on dot blot analysis. Signals for CCT3 and CCT8 in autoimmune disease patients were significantly higher than in controls. Significant differences were also demonstrated by Western blotting in anti-CCT4 response in both patient groups. All subjects showed strong reactivity to *M.oralis* chaperonin and faint signals to human HSP60. Autoantibodies were raised against CCT rather than HSP60; and CCT3, CCT4, and CCT8 were shown to be the main targets. Host immune systems may be frequently exposed to chaperonins of *Archaea* in various habitats. Although further studies of the cross-reactivity between *M.oralis* chaperonin and human CCT are required, anti-CCT autoantibodies may be involved in the pathogenesis of periodontitis and autoimmune diseases.

Introduction

Archaea are microorganisms classified as one of the primary domains distinct from bacteria and eukaryotes (Woese *et al.*, 1990). They are ubiquitous organisms widely distributed in nature. *Methanobrevibacter* is a major archaeal species isolated from the human oral cavity (Belay *et al.*, 1988), gastrointestinal tract (Karlin *et al.*, 1982), and vagina (Belay *et al.*, 1990). A series of reports suggested that the methanogenic *Archaea*, *Methanobrevibacter oralis* and *M.oralis*-like phylotypes, are involved in the pathogenesis of periodontitis (Kulik *et al.*, 2001; Lepp *et al.*, 2004; Yamabe *et al.*, 2008; Matarazzo *et al.*, 2011; Horz *et al.*, 2012).

Periodontitis is a chronic inflammatory disease caused by polymicrobial infection by microorganisms in subgingival

plaque. Some gram-negative anaerobes and spirochetes are closely associated with the disease and are referred to as periodontal bacteria (Socransky *et al.*, 1998). In addition to these periodontal bacteria, *Archaea* dominated by *M.oralis* in the plaque shown an increase in relation to the severity of periodontitis (Lepp *et al.*, 2004) and are suspected periodontal pathogens. Methanogenic *Archaea* are suggested to be potential hydrogen competitors of treponemes and may serve as syntrophic partners with other members of the subgingival crevice flora (Lepp *et al.*, 2004). *Synergistes* spp. are possible syntrophic partners of the methanogens (Vianna *et al.*, 2009). High ratios of *Archaea* and sulfate-reducing bacteria have been reported to be associated with the severity of periodontitis (Vianna *et al.*, 2008).



Most previous reports discussed the pathogenic roles of *Archaea* in synergistic or antagonistic relations with other periodontal bacteria as described above. Distinct from these reports, we attempted to elucidate the *Archaea*-related pathogenesis of periodontitis through the host immune responses. Immunoglobulin G (IgG) antibodies against the components of *M. oralis* are produced in sera from patients with periodontitis (Yamabe *et al.*, 2008). One of the antigenic molecules recognized by patient sera was identified as group II chaperonin, which shows a relatively high level of amino acid sequence identity and cross-reactivity with human chaperonin-containing T-complex polypeptide (CCT) (Yamabe *et al.*, 2010).

Chaperonins (Cpn) are molecules with a molecular mass of approximately 60 kDa that assist in the efficient folding of newly synthesized and stress-denatured polypeptide chains (Ellis & van der Vies, 1991; Hartl, 1996). Cpn are divided into two groups, group I and group II (Horwich & Willson, 1993; Kubota *et al.*, 1994). The amino acid sequences are conserved within but not between groups (Kubota *et al.*, 1995). Group I Cpn are also known as heat shock protein 60 (HSP60) or GroEL in bacteria. Bacteria have group I but not group II Cpn. Most archaeal species possess group II Cpn alone, while eukaryotes have both types. Cross-reactivity between bacterial GroEL and human HSP60 is known in relation to autoimmune reactions (Lamb *et al.*, 1989). Attention has been focused on group I Cpn that provides a link between infectious disease and the autoimmune response. Only one previous report has suggested the involvement of anti-CCT autoantibodies to rheumatic autoimmune diseases (Yokota *et al.*, 2000). In this report, cross-reaction between bacterial GroEL and human CCT is discussed as a possible cause of autoimmune reactions. Archaeal group II Cpn are not nominated for the cross-reactive antigens of human CCT, probably due to the absence of a concept of archaeal infection. We hypothesized that exposure to group II Cpn of *Archaea* in periodontal lesions may be a trigger or modulator of autoimmune reaction to human CCT due to cross-reaction. In the present study, as the first step to investigate this hypothesis, serum antibody responses to the archaeal Cpn and human CCT were examined in patients with periodontitis and autoimmune disease.

Materials and methods

Serum samples

Sera were obtained from 30 patients with advanced periodontitis (11 women and 19 men, age: 51.9 ± 12.6 years). Clinical diagnosis of periodontitis was based on the criteria described previously (Armitage, 1999). Sera were also obtained from 31 patients with autoimmune diseases (24 women and seven men, age: 54.6 ± 14.5 years) including 14 with rheumatoid arthritis, three with Sjögren's syndrome, two with systemic lupus erythematosus, one with Stevens–Johnson syndrome, one with Crohn's disease, one with Behçet's disease, one with adult Still's disease, one with hyperthyroidism, one with primary

macroglobulinemia, and one with IgA nephropathy. Eighteen healthy subjects were examined as controls (10 men and eight women; mean \pm SD age, 35.2 ± 7.4 years). Diagnosis of rheumatoid arthritis (Arnett *et al.*, 1988), Sjögren's syndrome (Vitali *et al.*, 1993), systemic lupus erythematosus (Tan *et al.*, 1982), Stevens–Johnson syndrome (Bastuji-Garin *et al.*, 1993), adult Still's disease (Yamaguchi *et al.*, 1992), primary macroglobulinemia (Dimopoulos *et al.*, 2005), and IgA nephropathy (Tomino, 2002) was based on published criteria. Crohn's disease, Behçet's disease, and hyperthyroidism were diagnosed based on the guideline defined by Ministry of Health, Labour and Welfare of Japan. The use of human subjects in this investigation was approved by the Ethical Committee of Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences (approval no. 624).

Cpn antigens

Recombinant human CCT1, CCT5, and CCT6 were purchased from Abnova Co. (Taipei, Taiwan) and used for dot blot analysis. Recombinant HSP60 (Stressgen, Ann Arbor, MI) was also purchased and used for dot blot and Western blotting analysis. Recombinant human CCT3, CCT4, CCT8, and *M. oralis* Cpn were constructed as described below and used for dot blot and Western blotting analysis.

Construction of recombinant human CCT subunits and *M. oralis* Cpn

Recombinant human CCT3, CCT4, CCT8, and *M. oralis* Cpn were constructed using the wheatgerm cell-free translation system (CellFree Sciences, Ehime, Japan) as described previously (Singh *et al.*, 2012). The mRNA was extracted from THP-1 cells using an mRNA isolation kit (Qiagen, Hilden, Germany), and cDNA was synthesized using Super Script III[®] (Invitrogen, Carlsbad, CA) and oligo dT primer by the standard protocol. The whole length of each gene for CCT3, CCT4, and CCT8 was amplified by PCR with the following primers containing restriction sites for the subsequent cloning step: CCT3 forward 5'-GC-GTCTCGAG(XhoI)GATGGGCCATCGTCCAGTG-3', CCT3 reverse 5'-CCTTGACTAGT(SpeI)ACTCACTCCTGGCCAGCA-3'; CCT4 forward 5'-CGGAGCTCGAG(XhoI)ATGCCGAGAATG T-3', CCT4 reverse 5'-TGGTACTAGT(SpeI)CAGTTATCCAGATTA-3'; CCT8 forward 5'-CAGGCTCGAG(XhoI)ATGGCGCTTCACGTTCCC-3', CCT8 reverse 5'-TAAGACTAGT(SpeI)TCAATCATTTTGGTCAT-3'. PCR was performed in a volume of 50 μ L using Ex-Taq polymerase (Takara, Shiga, Japan) under the conditions recommended by the manufacturer with 20 pmol of forward and reverse primers and 100 ng of the cDNA. The reaction conditions were 35 cycles of denaturation at 98 °C for 60 s, annealing at 58 °C for 30 s, and extension at 72 °C for 2.5 min, with a final extension step at 72 °C for 10 min. The amplified genes were digested with XhoI and SpeI. The digested gene fragments were cloned into the corresponding sites of the expression vector pEU-E01-GST-TEV (CellFree Sciences) in frame with the gene encoding

N-terminal glutathione S-transferase (GST). Similarly, the *M. oralis* Cpn gene was amplified by PCR from the genomic DNA of DSM 7256 strain and cloned into the expression vector. The primers were as follows: forward 5'-TGATTCTC-GAG(XhoI)GGCACAAGGTCAACCAA-3', reverse 5'-ATT-TACTAGT(SpeI)CTACATCATTGGAGGCA-3'.

Synthesis and purification of the recombinant proteins were performed by CellFree Sciences. Briefly, transcription and translation were performed using a CFS-TRI-1240G kit (CellFree Sciences) and the constructed expression vector described above. Transcription was carried out at 37 °C for 6 h in a reaction buffer containing 100 ng μL^{-1} expression vector, 1 unit per μL SP6 RNA polymerase, 1 unit per μL RNase inhibitor, 2.5 mM NTPs, and 1 \times transcription buffer supplied with the kit. Translation was carried out using 5.5 mL of SUB-AMIX (proprietary buffer containing all 20 amino acids) plus 250 μL of transcription products, 1 μL of creatine kinase (20 mg mL^{-1}), and 250 μL of WE-PRO1240G supplied with the kit for 16 h at 17 °C. Glutathione Sepharose 4B gel (GE Healthcare Bio-Sciences, Rydalmere, NSW, Australia) was used for purification of the synthesized GST-tagged proteins. Sample fractions at each purification step were analyzed by SDS-PAGE.

Dot blotting

The dot blot technique was used to analyze the serum responses to human CCT1, CCT3, CCT4, CCT5, CCT6, CCT8, HSP60, and *M. oralis* Cpn. The recombinant proteins were diluted with TBS (10 mM Tris-HCl buffer, pH 7.5, 0.9% NaCl) at a final concentration of 125 $\mu\text{g mL}^{-1}$. Aliquots of 4 μL of the diluted proteins were spotted onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). After the spots had dried, the membranes were blocked with 5% (wt/vol) skimmed milk in TBS (M-TBS). The blocked membranes were then incubated with patients' sera at a dilution of 1 : 1000 in M-TBS for 12 h at room temperature. The membranes were washed with TBS containing 0.05% Tween 20, and antibody bound to the membrane was detected with horseradish peroxidase-conjugated goat anti-human IgG antibody (Chemicon International, Temecula, CA). The intensity of the spot signals was analyzed using ImageJ (National Institutes of Health: NIH) (Kaczmarek *et al.*, 2004). The quantified spot signals were normalized relative to the phosphate-buffered saline (PBS) spot.

SDS-PAGE and Western blotting

SDS-PAGE and Western blotting analysis were performed as described previously (Kokeguchi *et al.*, 1989). Sera from patients with periodontitis and autoimmune disease were used at a final dilution of 1 : 1000 with M-TBS. Antihuman CCT3, CCT4, and CCT8 polyclonal antibodies (Proteintech Group Inc., Chicago, IL) were used at a dilution of 1 : 500 in M-TBS. Anti-HSP60 monoclonal antibody (Abcam, Cambridge, UK) was used at a dilution of 1 : 1000 in M-TBS. Recombinant human HSP60, CCT3, CCT4, CCT8, and *M. oralis* Cpn were used as antigens. Horseradish peroxidase-conjugated goat antihuman IgG antibody (Chemicon

International, Inc.), goat antimouse IgG antibody (Chemicon International, Inc.), or donkey antirabbit IgG antibody (GE Healthcare BioSciences) was used at a dilution of 1 : 5000 with M-TBS for detection. The intensity of the band signals was analyzed using ImageJ (NIH) (Kaczmarek *et al.*, 2004).

Statistical analysis

Group means of the signal intensities in dot and Western blotting analysis were compared by unpaired Student's *t*-test. In all analyses, $P < 0.05$ was taken to indicate statistical significance. Correlation of signal intensities of the antibody against the *M. oralis* Cpn and CCT subunits was analyzed by Pearson's correlation test.

Results and discussion

Construction of recombinant Cpn antigens

Recombinant *M. oralis* Cpn, human CCT3, CCT4, and CCT8 were successfully expressed by the wheatgerm cell-free translation system (CellFree Sciences) and purified by affinity chromatography. Protein profiles during the purification steps are shown in Supporting Information Fig. S1. The purified proteins with a GST tag showed the expected molecular mass of approximately 80 kDa. The wheatgerm cell-free translation system is free from bacterial antigens. In this regard, the system may have an advantage for application to immunological research as compared to the recombinant protein prepared from *Escherichia coli*. On the other hand, the method is relatively expensive, and the amount of protein product is small.

Dot blot analysis

Dot blot analysis was performed in 15 patients with autoimmune disease, 10 patients with periodontitis, and 10 healthy controls. Representative results are shown in Fig. 1. Eight recombinant antigens and PBS(-) (Invitrogen) were spotted on a membrane, which was then subjected to serum reaction. Distinct signals were frequently seen on the spots of *M. oralis* Cpn, CCT3, CCT4, and CCT8. In comparison to the signals for these recombinant antigens, the signals for CCT1, CCT5, and human HSP60 were faint in most reactions for both patients' sera and those from healthy controls.

Reactivity to *M. oralis* Cpn

Spot intensity of the *M. oralis* Cpn was relatively strong compared to the other antigens tested (Fig. 2). However, contrary to our hypothesis, there were no significant differences in reaction to *M. oralis* Cpn among the subject groups, and even the healthy subjects showed strong signals to the antigen. *M. oralis* is specifically distributed in severe periodontal lesions and not in healthy sites (Lepp *et al.*, 2004; Yamabe *et al.*, 2008). The levels of amino acid sequence identity between *M. oralis* Cpn and other Cpn homologs in *Archaea* range from approximately 40% to

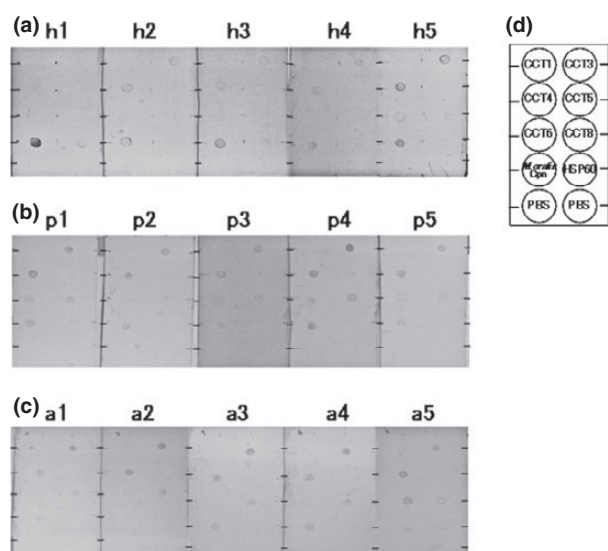


Fig. 1 Dot blot analysis. Serum IgG responses to recombinant *Methanobrevibacter oralis* Cpn, human CCT1, CCT3, CCT4, CCT5, CCT6, CCT8, and human HSP60 were examined by dot blot analysis. Representative results of sera from healthy controls (a: h1–h5), patients with periodontitis (b: p1–p5), and patients with autoimmune disease (c: a1–a5) are shown. Approximately, 500 ng of each protein was spotted on the membrane. (d) Shows a schematic map of the protein spots.

90%, and the identity was approximately 20% with *E. coli* and other bacterial GroEL (Yamabe *et al.*, 2010). Therefore, the reactions in the healthy subjects were probably cross-reaction with Cpn from other archaeal species rather than bacterial group I Cpn, GroEL. In addition to the oral cavity, the gut (Karlin *et al.*, 1982) and vagina (Belay *et al.*, 1990) are known to be methanogen habitats. *Methanobrevibacter smithii*, a dominant methanogenic *Archaea* in the gut,

possesses a Cpn with 91% sequence identity to *M. oralis* Cpn that is the best candidate for a cross-reactive antigen. In addition to periodontitis, the immunogenic properties of *Archaea* in bioaerosols were recently reported, and an immunomodulatory role in the pulmonary tract was suggested (Blais Lecours *et al.*, 2011). Methanogens are widely distributed in nature and are present even in foodstuffs (Brusa *et al.*, 1998). The human immune system may be constantly exposed to *Archaea* and their Cpn, and the group II Cpn may be common antigens among archaeal species.

Reactivity to CCT subunits and HSP60

The spot intensities to CCT3 and CCT8 were significantly stronger in the reactions of patients with autoimmune disease than in those of the healthy controls. Serum reactions of the patients with periodontitis showed significantly higher responses to the protein spots of CCT4 and CCT8 than the healthy controls. Although significant difference was not seen, recombinant CCT6 showed distinct reactions to the sera from four patients with autoimmune disease (Fig. 2). There have been very few previous reports regarding anti-CCT autoantibodies. Yokota *et al.* (2000) reported elevated levels of autoantibodies against human CCT complex in patients with autoimmune diseases, and in cancer patients, CCT was reported to be a target of serum antibodies (Schmits *et al.*, 2002). In addition to these previous reports, the present study demonstrated elevated levels of autoantibodies against CCT in patients with periodontitis and in patients with autoimmune diseases. Among the CCT subunits, CCT3, CCT4, CCT6, and CCT8 may be the main targets of these autoantibodies.

There has been a great deal of research and discussion of regarding autoantibodies to human HSP60 in relation to

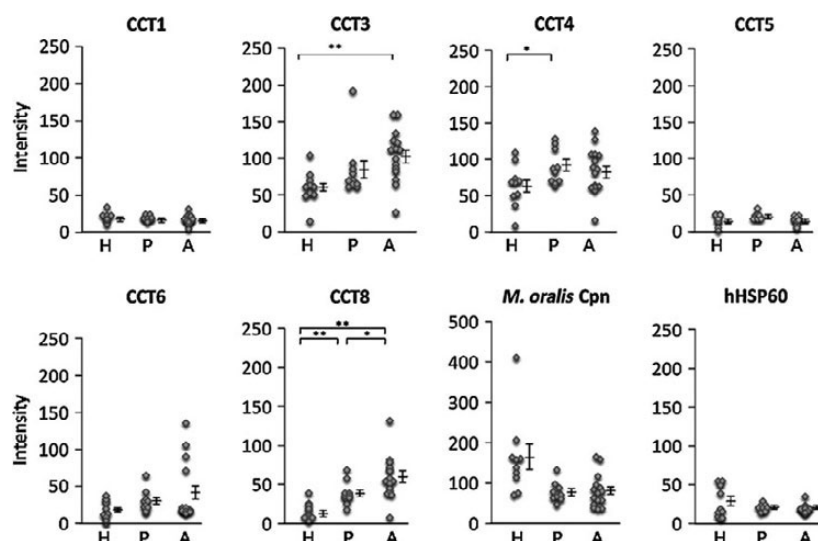


Fig. 2 Spot intensity on dot blot analysis. Signal intensity of spots that reacted to serum was quantified using ImageJ (NIH) and normalized relative to the signals of PBS spots, and the relative intensities are shown. Mean values and standard errors are shown on the right. ** $P < 0.01$, * $P < 0.05$. H, Healthy control; P, Patients with periodontitis; A, Patients with autoimmune diseases.

periodontitis, autoimmune disease, and atherosclerotic vascular diseases (Winfield & Jarjour, 1991; Yamazaki *et al.*, 2004; Alard *et al.*, 2007; Ayada *et al.*, 2009). While associations were suggested between autoantibodies and the pathogenesis of these diseases, the opposite results have also been reported (Shovman *et al.*, 2005; van Halm *et al.*, 2006). Yokota *et al.* (2000) reported that IgG autoantibody titers against CCT and HSP60 were both significantly higher in the sera of patients with rheumatic autoimmune disease than in healthy control sera. They also demonstrated greater differences between patient and control sera in CCT-reactive antibodies than in anti-HSP60 antibodies. Consistent with this report, significant differences were seen in the reactions against CCT subunits, but no significant difference was seen between patients and controls in the reaction against HSP60. Autoantibodies against HSP60 may be considered as natural autoantibodies (Pozsonyi *et al.*, 2009), and their roles in disease are controversial (Wu & Tanguay, 2006). Rather than HSP60, CCT may be a useful diagnostic antigen with which to detect autoantibodies prevalent in patients with autoimmune diseases as suggested by Yokota *et al.* (2000), and CCT3, CCT4, and CCT8 may be more specific diagnostic tools. Further, the results of the present study suggest the involvement of anti-CCT autoantibodies in the pathogenesis of periodontitis.

Correlations with signal intensity against *M. oralis* Cpn and CCT subunits

Methanobrevibacter oralis Cpn has been reported to be a probable cross-reactive antigen of human CCT subunits. The

sequence identities between them range from 28.8% to 40.0% (Yamabe *et al.*, 2010). Scatter diagrams to examine the possible correlations of antibody levels between *M. oralis* Cpn and CCT subunits are shown in Fig. 3. In the subject group with periodontitis, antibody levels against *M. oralis* Cpn were correlated with those against CCT3, CCT5, CCT6, and CCT8 subunits. Although serum reactions were weak, the intensities of signals to CCT5 and CCT6 were strongly correlated with the reaction to *M. oralis* Cpn. In both healthy controls and autoimmune disease patients, levels of antibodies to *M. oralis* Cpn were not positively correlated with the levels against CCT subunits (Figs S2 and S3). As the sample number was small, definitive conclusions cannot be drawn. However, these results suggest that the generation of anti-CCT antibody may be related to that of anti-*M. oralis* Cpn in patients with periodontitis, suggesting a cross-reaction between them. In contrast, generation of anti-CCT autoantibodies may not be related to anti-*M. oralis* Cpn antibody in patients with autoimmune disease. In patients with autoimmune disease, cross-reaction may not be responsible for autoantibody generation, and another mechanism of host immune disorder must be assumed. Because various types of autoimmune disease were included in the current study, further investigations of the cross-reaction are required for each types of the disease.

Western blotting analysis

Attention was focused on *M. oralis* Cpn, CCT3, CCT4, and CCT8, and Western blotting analysis was performed by increasing the number of serum samples used. HSP60 was also used as a control antigen. The analysis was performed

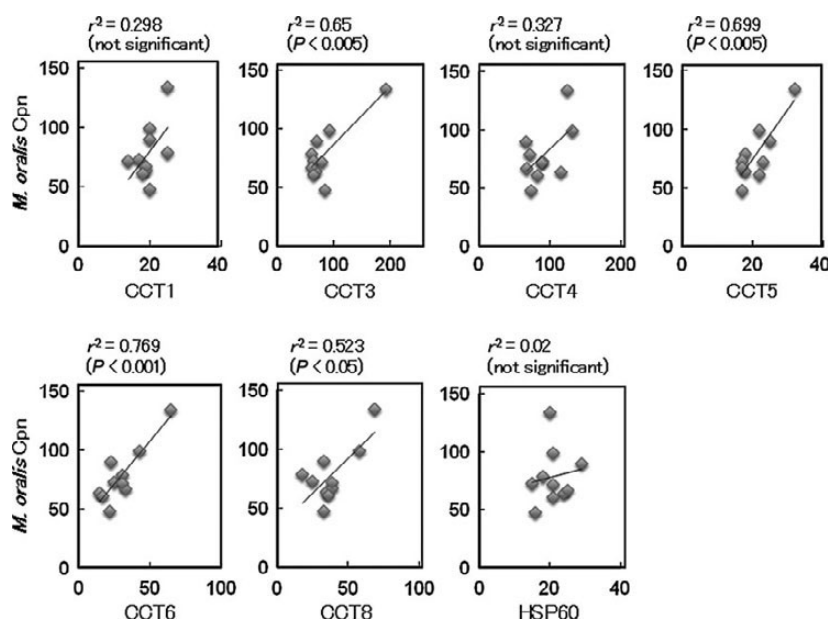


Fig. 3 Correlation between antibody levels against *Methanobrevibacter oralis* Cpn and human chaperonins (CCT subunits and HSP60) in patients with periodontitis. Antibody levels against *M. oralis* Cpn correlated with those against CCT3, CCT5, CCT6, and CCT8 subunits. In the healthy control and autoimmune disease patient groups, no positive correlations were observed between antibody levels against *M. oralis* Cpn and human chaperonins (Figs S2 and S3).

in 30 periodontitis patients, 31 autoimmune disease patients, and 18 healthy controls. Representative results are shown in Fig. 4. Strong reactivity for *M. oralis* Cpn was seen in all groups, whereas serum-reacting bands were hardly seen in the reaction of HSP60 corresponding to the results of dot blotting. CCT3, CCT4, and CCT8 showed weak reactivity with the test sera.

The intensities of the bands on Western blotting analysis were quantified, and the results are shown in Fig. 5. The intensities of the serum-reacted CCT4 bands were significantly higher in both periodontitis and autoimmune disease patients compared to healthy controls. Consistent with the results of dot blotting, signals to the *M. oralis* Cpn were stronger than those observed against the CCT antigens. However, there were no significant differences among the groups. Inconsistent with the results of dot blotting, significant differences were not seen in the reactions against CCT3 and CCT8 among the groups. In addition, no

correlations were seen between the levels of anti-*M. oralis* Cpn antibodies and any CCT subunits in any of the groups (data not shown). Previously, it was suggested that CCT-reactive autoantibodies recognized conformational epitopes (Yokota *et al.*, 2000). The results of the present study also suggest the significance of the conformational epitopes in autoimmune reaction. No distinct HSP60 band was seen in any reaction with the serum samples, and therefore, quantification of the band signal was impossible.

The results of the present study indicated that both patients and healthy subjects raised IgG antibodies against *M. oralis* Cpn. Rather than HSP60, the titers of autoantibodies against CCT were high in both periodontitis and autoimmune disease patients. Among the subunits, CCT3, CCT4, and CCT8 were the main targets of the autoantibodies. Antibody levels against *M. oralis* Cpn and CCT were correlated in patients with periodontitis, whereas no such relation was seen in autoimmune disease patients.

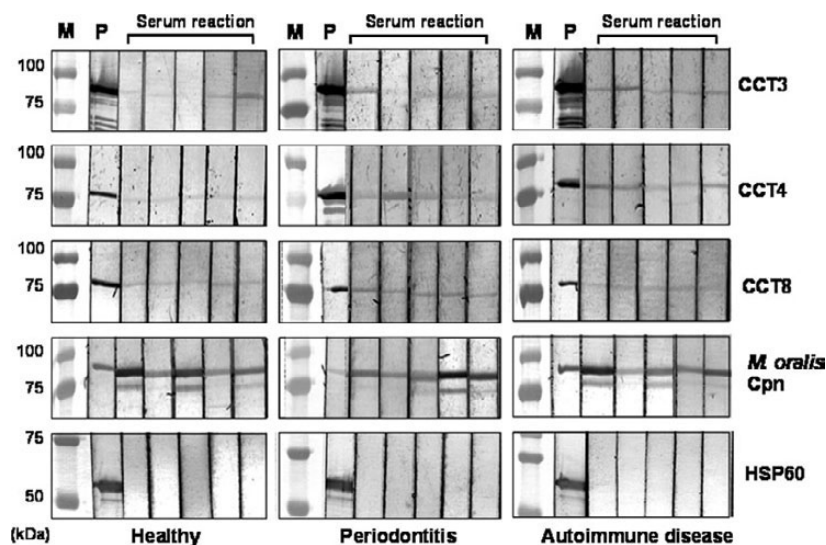


Fig. 4 Western blotting analysis. Serum IgG responses to recombinant *Methanobrevibacter oralis* Cpn, human CCT3, CCT4, CCT8, and human HSP60 were examined. Representative results of sera from healthy controls, from patients with periodontitis, and from patients with autoimmune disease are shown. Strong reactivity against *M. oralis* Cpn was seen in all groups. Distinct bands were also seen in the reactions against CCT subunits, whereas no serum-reacted band of human HSP was identified. Approximately, 200 ng of protein was blotted on a membrane strip, and sera were used at a dilution of 1 : 1000. M, Molecular weight marker; P, Positive control.

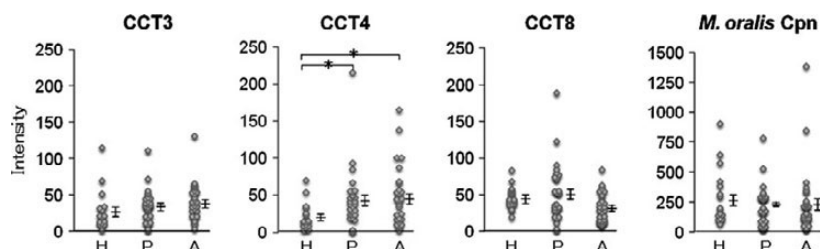


Fig. 5 Intensities of the serum-reacted bands on Western blotting analysis. Band signals on Western blotting analysis were quantified by ImageJ (NIH) and normalized relative to the background signal. Mean values and standard errors are shown on the right. * $P < 0.01$. H, Healthy control; P, Patients with periodontitis; A, Patients with autoimmune diseases.

Cross-reactivity between *M. oralis* Cpn and human CCT is still under investigation, and the roles of anti-CCT antibodies in disease are unclear. However, elevated levels of autoantibodies, which could generate immune complexes, are at least potential modulators of inflammation in injured tissues in periodontitis and autoimmune diseases.

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Supporting Information

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

Fig. S1. Protein profiles of the recombinant chaperonins.

Fig. S2. Correlation between antibody levels against *M. oralis* Cpn and human chaperonins (CCT subunits and HSP60) in Healthy group.

Fig. S3. Correlation between antibody levels against *M. oralis* Cpn and human chaperonins (CCT subunits and HSP60) in patients with autoimmune disease.