RESEARCH LETTER

Distribution of *Archaea* in Japanese patients with periodontitis and humoral immune response to the components

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Abstract

There is controversy regarding the existence of archaeal pathogens. Periodontitis is one of the human diseases in which *Archaea* have been suggested to have roles as pathogens. This study was performed to investigate the distribution of *Archaea* in Japanese patients with periodontitis and to examine the serum IgG responses to archaeal components. Subgingival plaque samples were collected from 111 periodontal pockets of 49 patients (17 with aggressive periodontitis and 32 with chronic periodontitis), and 30 subgingival plaque samples were collected from 17 healthy subjects. By PCR targeting the 16S rRNA gene, *Archaea* were detected in 15 plaque samples (13.5% of total samples) from 11 patients (29.4% of patients with aggressive periodontitis and 18.8% of patients with chronic periodontitis). *Archaea* were detected mostly (14/15) in severe diseased sites (pocket depth $\geq$6 mm), while no amplicons were observed in any samples from healthy controls. Sequence analysis of the PCR products revealed that the majority of *Archaea* in periodontal pockets were a *Methanobrevibacter oralis*-like phylotype. Western immunoblotting detected IgG antibodies against *M. oralis* in eight of the 11 sera from patients. These results suggest the potential of *Archaea* (*M. oralis*) as an antigenic pathogen of periodontitis.

Introduction

Archaea are microorganisms classified as one of the primary domains distinct from bacteria and eukaryotes (Woese *et al.*, 1990). They are ecologically widespread and are known to thrive even in extreme environments, such as hot springs, salt lakes and submarine volcanic habitats (Barns *et al.*, 1994, 1996; DeLong, 1998). 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 the close association with humans, no pathogenic *Archaea* have yet been identified, and it has generally been assumed that archaeal pathogens do not exist. Some previous studies suggested the linkage between methanogens and human disease (Haines *et al.*, 1977; Weaver *et al.*, 1986; Tanaka *et al.*, 1999; Kulik *et al.*, 2001). However, as no studies have conclusively identified *Archaea* as causative agents of human disease, there has been controversy regarding the existence of pathogenic *Archaea* (Cavicchioli *et al.*, 2003; Jangid *et al.*, 2004).

Periodontitis is an inflammatory disease caused by polymicrobial infection of oral bacteria in subgingival dental plaque. Subgingival plaque consists of several hundreds of mainly anaerobic bacterial species. Some Gram-negative anaerobic rods 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 were identified in cases of periodontitis as early as 1988 (Belay *et al.*, 1988). Subsequent studies revealed that the majority of *Methanobrevibacter* species in dental plaque were *Methanobrevibacter oralis* and *Methanobrevibacter smithii* (Ferrari *et al.*, 1994; Kulik *et al.*, 2001). Recently, Lepp *et al.* (2004) performed quantitative analysis of the microbial communities of subgingival plaque

and revealed the relationship between the severity of chronic periodontitis and the relative abundance of the *Methanobrevibacter* population in patients with periodontitis. They also reported that methanogens were potential hydrogen competitors of treponemes and may serve as syntrophic partners with other members of the subgingival biofilm community.

Because methanogenic *Archaea* were frequently isolated from periodontal pockets, they seem to have the potential to be pathogens involved in periodontitis. However, some questions remain to be answered, such as the distribution of methanogenic species in different communities or regions, and our major concern is whether host immune systems recognize and respond to methanogenic *Archaea* in periodontal lesions. In the present study, we detected the archaeal 16S rRNA gene in subgingival plaque samples from Japanese patients with periodontitis and examined the humoral immune responses to the methanogenic *Archaea*. This is the first report demonstrating that the human immune system recognizes archaeal components.

Materials and methods

Archaeal strains

Methanobrevibacter oralis DSM 7256 and *M. smithii* DSM 861 were 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 [PBS (−)] (Gibco BRL). The washed cells were directly subjected to DNA extraction or used as antigens for Western immunoblotting without subculture.

Subgingival plaque samples

Sampling of subgingival plaque from periodontitis patients was routinely performed for clinical diagnosis of periodontal disease at the Okayama University Hospital of Medicine and Dentistry with each patient's informed consent. Plaque samples were obtained from the periodontal pockets by inserting paper points (#45; Zipperer, Germany) as described previously (Nishimura *et al.*, 1990), and the DNA was extracted. The plaque DNA samples were used for clinical microbiological examinations, and the remaining samples were stocked at $-30\,^{\circ}C$. From the stocks, 111 plaque DNA samples were randomly selected and used for the present study. The 111 plaque samples were from 49 patients with periodontitis, consisting of 17 patients with aggressive periodontitis (early-onset periodontitis) and 32 patients with chronic periodontitis (adult-type periodontitis). Thirty plaque samples from 17 healthy individuals were also selected from the stock for the present study. Sampling sites of 111 periodontal pockets included 28 relatively healthy sites (pocket depth ≤ 3 mm), 15 periodontitis sites (4 mm $\leq$ pocket depth $\leq$ 5 mm) and 68 severe periodontitis sites (pocket depth ≥ 6 mm).

DNA extraction

InstaGene Matrix (Bio-Rad) was used for DNA extraction from archaeal strains, *Escherichia coli* XL1-Blue (Stratagene) and clinical dental plaque samples according to the manufacturer's instructions. Briefly, plaque samples, archaeal strains and *E. coli* cells were suspended in 1 mL of PBS (−) (Gibco BRL), and then pelleted and resuspended in 100 μL of InstaGene Matrix. The suspensions were incubated at 56 °C for 30 min and then at 100 °C for 8 min. After incubation, the suspension was centrifuged and 2-μL aliquots of the resulting supernatants were used as templates for PCR and real-time PCR.

PCR

Detection of *Archaea* from subgingival plaque was performed by PCR. The archaeal 16S rRNA gene was amplified using forward (5′-TCCAGGCCCTACGGG-3′) and reverse primers (5′-YCCGGCGTTGAMTCCAATT-3′) specific for the archaeal 16S rRNA gene (DeLong, 1992; Lepp *et al.*, 2004). DNA-extracted samples from subgingival plaque, *E. coli* (negative control) and *M. oralis* (positive control) were used as templates. The contents of the PCR mixtures (50 μL) were 2.5 U of AmpliTaq Gold, 1 × included buffer (Perkin-Elmer), 2.5 mM $MgCl_2$, 0.4 μM of forward and reverse primers, 0.2 mM dNTPs, and 2 μL of extracted DNA template. Archaeal 16S rRNA genes were amplified under the following conditions: 35 cycles at 95 °C (30 s), 58 °C (30 s) and 72 °C (60 s), followed by a 3-min extension step at 72 °C. Aliquots of 5 μL of each PCR product were subjected to electrophoresis on 1.5% agarose gels. The gels were stained with ethidium bromide (1 mg mL^{-1}) and assessed under UV light.

Sequencing and homology search

Archaeal species in subgingival plaque samples were identified based on the 16S rRNA gene sequence of the PCR product. The PCR products were separated by agarose gel electrophoresis, and the 16S rRNA gene bands were excised from the gel and purified using a QIAEX®II Gel Extraction kit (Qiagen). The purified DNA was cloned into a PCR2.1®-TOPO® plasmid vector (Invitrogen) in accordance with the manufacturer's instructions. The insert DNA was sequenced using an ABI Prism BigDye terminator cycle sequencing ready reaction kit (Perkin-Elmer) and an automated DNA sequencer (ABI PRISM 377; Perkin-Elmer). The sequence data obtained were used for a BLAST sequence homology search against the GenBank database. A total of 25 clones were isolated and analysed from amplified 16S rRNA gene

fragments from five subgingival plaque samples (five clones from each plaque sample).

Real-time PCR (detection of *Porphyromonas gingivalis*)

In addition to detection of *Archaea*, *P. gingivalis* in the plaque samples was detected by real-time PCR as described previously (Maeda *et al.*, 2003). Briefly, the PCR mixture contained 2 × SYBR Green PCR Master Mix (PE Applied Biosystems), 20 pmol of forward and reverse primers and 2 μL of extracted DNA sample. Both forward and reverse primers were designed according to the species-specific regions of the 16S rRNA gene (Maeda *et al.*, 2003). The thermocycling programme was 40 cycles at 95 °C for 15 s and at 60 °C for 1 min with an initial denaturation step at 95 °C for 10 min. A GeneAmp® 5700 Sequence Detection System (PE Applied Biosystems) was used to monitor the fluorescence from dsDNA-bound SYBR Green I. Data were analysed using the GeneAmp 5700 SDS software (PE Applied Biosystems).

Western immunoblotting

Humoral immune response to the archaeal components was examined by Western immunoblotting performed as described previously (Kokeguchi *et al.*, 1989). *Methanobrevibacter oralis* and *M. smithii* were disrupted by vortexing in 1.5-mL tubes with zirconia beads (FastGene™ ZircoPrep Mini; Nippon Genetics Co. Ltd) and used as antigens. Approximately 1 μg of the proteins were loaded in each lane. Sera from patients with periodontitis and healthy subjects were used at a final dilution of 1 : 500 with 5% (w/v) skimmed milk in Tris-buffered saline (10 mM Tris-HCl buffer, pH 7.5, 0.9% NaCl; M-TBS). Horseradish peroxidase-conjugated goat anti-human IgG antibody (1 : 5000 dilution with M-TBS; INC Biochemicals) was used as the secondary antibody.

Serum samples

Sera from 11 patients were selected from the serum sample collection of the Okayama University Hospital of Medicine and Dentistry. Serum antibody titres to the periodontal pathogens had been examined previously for clinical diagnosis (Murayama *et al.*, 1988). The sera from the 11 patients selected for this study had elevated IgG antibody titres (> mean + 2 SD of the healthy controls) to the sonication extract of *P. gingivalis*, a potent pathogen of periodontitis (supporting Table S1), suggesting that patients had considerable periodontitis lesions (Murayama *et al.*, 1988). Four periodontally healthy subjects were also examined as controls.

Table 1. Distribution of *Archaea* in Japanese patients

Disease type of subjects	Mean PD* (mm)	*Archaea* positive/ number of subjects
Healthy controls	–	0/17 (0%)
Aggressive periodontitis	6.98 ± 2.86	5/17 (29.4%)**
Chronic periodontitis	4.83 ± 2.04	6/32 (18.8%)
Total patients	5.72 ± 2.60	11/49 (22.4%)

*Mean PD = mean pocket depth ± SD of the sampling sites.
**$P = 0.044$ for difference from control group (Fisher's exact test).

Statistical analysis

Significant differences in frequency of *Archaea*-positive subjects between disease types were assessed by Fisher's exact test. Fisher's exact test was also used to assess differences between the groups of shallow (≤ 3 mm), moderate (4–5 mm) and deep pocket depths (≥ 6 mm) for outcomes of archaeal detection in their plaque samples.

Results and discussion

Detection of archaeal 16S rRNA gene from subgingival plaque

Archaeal 16S rRNA gene was amplified from subgingival plaque samples. According to the previous reports (DeLong, 1992; Lepp *et al.*, 2004), a PCR primer set specific for the archaeal 16S rRNA gene was used for detection. Before application to clinical samples, PCR was performed using DNA extracts of *E. coli* and *M. oralis* as templates to confirm the specificity. As expected, the 16S rRNA gene was successfully amplified from *M. oralis*, but not from *E. coli* (data not shown).

The distribution of *Archaea* in Japanese patients with periodontitis is shown in Table 1. A total of 49 patients participated in the present study, and archaeal 16S rRNA gene was detected in 11 patients (22.4%). The frequency of detection of *Archaea* in Japanese patients was lower than that reported previously. Lepp *et al.* (2004) detected the archaeal 16S rRNA gene in 36% of periodontitis patients in the United States. In the United States and the United Kingdom, about 30–60% of the adult human population are methane producers (Bond *et al.*, 1971; Haines *et al.*, 1977; Pitt *et al.*, 1980), while this figure in Japan is about 10–15% of the population (Hoshi *et al.*, 1985; Morii *et al.*, 2003). The number of methanogenic *Archaea* in the gastrointestinal tract of Japanese subjects was reported to be lower than those in other countries and was suggested to be affected by genetic factors or environmental factors (McKay *et al.*, 1981; Morii *et al.*, 2003). The low frequency of archaeal detection in subgingival plaque of Japanese patients may be due to a similar reason.

The distribution of *Archaea* in periodontal pockets is shown in Table 2. Lepp *et al.* (2004) detected archaeal 16S rRNA gene in 76.6% of periodontitis sites (pocket depth ≥ 4 mm). Further, they reported a relationship between the

Table 2. Distribution of *Archaea* in periodontal pockets

Pocket depth (mm)	Disease type	*Archaea* positive	Pg positive
–	Healthy ($n = 30$)	0 (0%)	14 (46.7%)
≤ 3	Aggressive ($n = 6$)	0 (0%)	4 (66.7%)
	Chronic ($n = 22$)	0 (0%)	16 (72.7%)
	Total	0 (0%)	20 (71.4%)
4–5	Aggressive ($n = 5$)	0 (0%)	4 (80.0%)
	Chronic ($n = 10$)	1 (10%)	7 (70.0%)
	Total	1 (6.7%)	11 (73.3%)
≥ 6	Aggressive ($n = 35$)	8 (22.9%)	30 (85.7%)
	Chronic ($n = 33$)	6 (18.2%)	29 (87.9%)
	Total	14 (20.6%)†	59 (86.8%)††
Total patient sample ($n = 111$)		15 (13.5%)†††	90 (81.1%)††

Pg, *Porphyromonas gingivalis*.
†$P < 0.01$, significantly different from healthy group and ≤ 3 mm group (Fisher's exact test).
††$P < 0.001$, significantly different from healthy group (Fisher's exact test).
†††$P < 0.05$, significantly different from healthy group (Fisher's exact test).

severity of periodontitis and the relative abundance of the archaeal population in the microbial community in periodontal pockets. In the present study, the detection frequency of *Archaea* was lower than that reported previously (20.6% even in plaque samples from pocket depth ≥ 6 mm). However, as *Archaea* were mostly detected in samples from sites of severe disease (Table 2), the results of the present study suggested that *Archaea* are involved in the pathogenesis of periodontitis in accordance with the previous report. The low detection frequency may have been due to genetic or environmental differences, as described above.

The detection frequency of *Archaea* in patients with aggressive periodontitis (severe disease type of periodontitis) was significantly higher than that in healthy controls ($P = 0.044$, Table 1). In contrast, there were no significant differences in the prevalence of *Archaea* between healthy control and chronic periodontitis groups or between aggressive and chronic periodontitis groups. Although the prevalence of *Archaea* was limited to periodontitis patients, there was no significant difference between the healthy controls and the total patient group ($P = 0.053$). This may have been due to the small number of subjects included in the study. A larger-scale study is required to elucidate this point further. There was a clear relationship between the prevalence of *Archaea* and pocket depth. There was a significant difference between deep (≥ 6 mm) and shallow periodontal pockets (≤ 3 mm) ($P < 0.01$). *Archaea* were not detected in shallow pockets, even in periodontal patients. This finding suggested that pocket depth (strict anaerobic environment) of individual teeth is a more important factor for colonization than the disease status or the patient category. It is still unclear whether *Archaea* are the causative agents of periodontitis. However, detection of *Archaea* may be useful at least as a marker for sites of severe disease in cases of periodontitis.

In addition to *Archaea*, the prevalence of *P. gingivalis*, one of the most commonly implicated pathogens in periodontitis, was examined simultaneously. In accordance with the results reported previously (Tran & Rudney, 1999), *P. gingivalis* was widely distributed in periodontal pockets, even in shallow pockets or gingival sulci of healthy controls, while *Archaea* were limited to deep periodontal pockets (Table 2). There was a significant difference in the prevalence of *P. gingivalis* between healthy and deep pocket groups (≥ 6 mm) and between healthy controls and the total patient group. On the other hand, there was no significant difference between shallow and deep pocket groups.

Sequencing and homology search of amplified 16S rRNA gene

Amplified archaeal 16S rRNA gene (573 bp) was cloned, sequenced and used for homology search to identify the organisms in the periodontal pockets of Japanese patients. A total of 25 clones were isolated from five plaque samples from four patients. The results of the homology search are shown in Table 3. Four different 16S rRNA gene fragments were included among the 25 clones. Most of the clones showed sequences identical to the registered sequence of *Methanobrevibacter* phylotype SBGA-1 (13/25) or *M. oralis* (10/25). In addition, two minor populations were also identified. One minor clone showed 99.8% sequence

Table 3. Identification of archaeal 16S rRNA gene from subgingival plaque

Patient	Disease type	Pocket depth (mm)	Clone number	Identified species/ sequence identity
A	Chronic	6	4	Phylotype SBGA-1*/ 100%
			1	*M. oralis***/100%
		6	3	Phylotype SBGA-1*/ 100%
			2	*M. oralis***/100%
B	Aggressive	6	1	Phylotype SBGA-1*/ 100%
			3	*M. oralis***/100%
			1	*M. oralis***/99.6%
C	Chronic	7	2	Phylotype SBGA-1*/ 100%
			2	*M. oralis***/100%
			1	Phylotype SBGA-1*/ 99.8%
D	Chronic	4	3	Phylotype SBGA-1*/ 100%
			2	*M. oralis***/100%

**Methanobrevibacter* phylotype SBGA-1, accession no. AY374553.
**Accession no. AJ001709.

identity to SBGA-1, and another showed 99.6% identity to *M. oralis* (supporting Fig. S1). *Methanobrevibacter* phylotype SBGA-1 was reported by Lepp *et al*. (2004) as the dominant *Archaea* in subgingival plaque closely associated with *M. oralis* (99.8% sequence identity). In their study, a clone library of archaeal 16S rRNA gene was constructed, and 81% of the clones were shown to be of the phylotype SBGA-1. Kulik *et al*. (2001) also reported that *M. oralis* was the predominant *Archaea* in subgingival plaque. Similar to other countries, *M. oralis*-like phylotypes were suggested to be the predominant *Archaea* in the periodontal pockets of Japanese periodontitis patients. *Methanobrevibacter* sequence associated with the ciliate *Eudiplodinium maggii* (19% of the total clones in Lepp's report) or *M. smithii* (26% of the total clones in Kulik's report) identified as minor populations in subgingival plaque in the previous studies were not found in Japanese patients. Environmental factors may be responsible for these minor differences.

Humoral immune response to *Archaea*

Serum IgG against *M. oralis* and *M. smithii* were detected by Western immunoblotting (Fig. 1). Patients' sera with elevated IgG antibody titres against *P. gingivalis* were selected and used for Western immunoblotting (supporting Table S1), implicating that the patients had considerable periodontitis (Murayama *et al*., 1988). The reactivity of sera against *M. oralis* seemed to be different between periodontitis patients and healthy controls. Sera from eight of the 11 patients showed clear reactivity to the components of *M. oralis*, while no distinct bands were seen in those from healthy controls. This result demonstrated that patients with considerable periodontitis had IgG antibodies against *M. oralis*. Because *Archaea* were detected mostly in severely diseased sites, patients with severe disease with reactive serum might be exposed to *M. oralis* or *M. oralis*-like phylotypes in periodontal lesion and produced IgG antibodies against these organisms. However, because serum and plaque samples were obtained separately in the current study, plaque samples corresponding to the serum samples were missing. Therefore, the distribution of *Archaea* in patients with the *M. oralis*-reactive serum remained unknown. Further study is needed to elucidate the correlation between the archaeal distribution and the immune response. Faint bands in the reactions of healthy subjects were suggested to be background signals, as they were observed throughout the membrane.

Distinct antigenic bands of *M. oralis* recognized by patients' sera were detected at various molecular weights. The molecular weights of the antigenic bands of *M. oralis* were different from those of *M. smithii,* and *M. oralis*-specific recognitions were observed in some serum reactions (lanes 10, 11 and 13 in Fig. 1). Each serum of lanes 10, 11 and 13 showed a distinct reaction to *M. oralis* but not to *M. smithii*, although both were closely resembling species. These results suggest that the patients' sera recognized *M. oralis*-specific antigens, and, therefore, the patients were supposed to be exposed to *M. oralis*. Among the antigenic bands of *M. oralis*, three antigenic bands at around 70, 17 and 15 kDa were recognized by multiple sera. Each of the reactive sera (8/11) recognized at least one of the three antigens. These bands may indicate highly antigenic molecules in *M. oralis*, and thus analysis of the antigenic bands will facilitate analysis of the role of *M. oralis* in the pathogenesis of periodontitis. No remarkable differences were seen in the reactivity of sera against *M. smithii* between patients and controls. Three antigenic bands were commonly detected among patients and controls. These results suggest that the dominant archaeal pathogen of periodontitis is *M. oralis* rather than *M. smithii* in

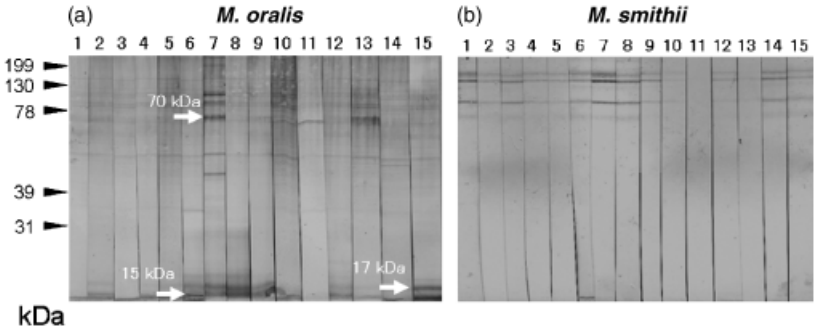

Fig. 1. Serum IgG response to *Archaea*. (a) Serum IgG responses to *Methanobrevibacter oralis* and (b) *Methanobrevibacter smithii* were examined by Western immunoblotting. Lanes 1–4: responses of sera from healthy controls, Lanes 5–15: responses of sera from periodontitis patients. Distinct antigenic bands were observed in the reaction of 8/11 patient's sera (except lanes 5, 12 and 14) against *M. oralis*, while distinct bands were not observed in the reaction of healthy controls. Arrows indicate the major antigenic bands at around 70, 17 and 15 kDa. Each of the reactive sera (8/11) recognized at least one of these antigens. There were no marked differences in the reactivity of *M. smithii* between patients and healthy controls. Each lane contained *c*. 1 μg of protein. The numbers on the left indicate molecular masses (in thousands).

the Japanese population. The reactivity of sera against *M. smithii* may be a consequence of sensitization in the gastrointestinal tract or crossreactivity with other antigens.

The pathogenic role of *Archaea* in periodontitis has been demonstrated and discussed in previous reports. Lepp *et al.* (2004) demonstrated that the relative abundance of *Archaea* increased in relationship with the severity of periodontal disease. They also demonstrated that the relative abundance of *Treponema* spp. was significantly lower in sites with than in those without *Archaea*, suggesting that syntrophic interactions between *Archaea* and other members of the microbial community may be important features of periodontitis. Further, archaeosomes, unique liposomes prepared from membrane lipids of *Archaea*, have been reported to be potent immune adjuvants (Conlan *et al.*, 2001; Krishnan *et al.*, 2001). The polar lipid from *M. smithii* has been shown to facilitate crosspresentation of $CD8^+$ T-cell response (Krishnan *et al.*, 2000) and to activate dendritic cell costimulation and cytokine production (Krishnan *et al.*, 2001; Gurnani *et al.*, 2004). In addition to these previous reports, the present study suggested an antigenic role of *Archaea* in the pathogenesis of periodontitis. It is difficult to conclude that *M. oralis* is one of the causative agents of periodontitis. However, the *Archaea* colonizing the periodontal pockets possess antigenic molecules and are therefore likely modifiers of inflammation in periodontal lesions.

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Supporting Information

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

Fig. S1. Standardized ELISA value of serum samples used for Western immunoblotting.

Table S1. Alignment of the PCR-amplified 16S rRNA gene sequence.

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