Introduction

Numerous studies have demonstrated that some viruses and bacteria including mycoplasmas could be related to the pathogenesis of arthritis\(^1\)-\(^3\). Mycoplasma species have been incriminated as causative agents of arthritis since they were found in rats and mice with natural arthritis\(^1\). Williams et al. reported the presence of *Mycoplasma fermentans* (*M. fermentans*) in the SF of patients with rheumatoid arthritis (RA), but these
results could not be reproduced in further investigations probably because of the fastidious growth requirements of the microorganism. Recently, molecular biology tools have enabled us to detect and isolate \textit{M. fermentans} in joints of patients with RA and other joint disorders, and the results suggest that \textit{M. fermentans} may play a role in the development of joint disease.

Some mycoplasmas may act as nonspecific mitogens of B and T cells, by producing superantigen. Recent studies have shown the presence of \textit{M. fermentans} DNA and specific antibodies to the bacteria in the SF of patients with RA. \textit{M. fermentans} possesses a potent immunomodulator product named macrophage activating lipopeptide 2 (MALP-2) which activates macrophage to release various proinflammatory cytokines which may in turn increase the neutrophilic infiltrate into the joints. We have found that \textit{M. fermentans} derived lipoprotein can induce the production of cytokines (TNF-\alpha and IL-1) and matrix degrading enzyme through interaction with its receptor, Toll like receptor (TLR)-2.

Mycoplasmas are common inhabitants of the human respiratory and genitourinary tracts where they produce chronic infections, and they are able to invade other tissues and in some cases induce autoimmune responses. The pathogenesis of some human mycoplasmal infections in the joints is poorly understood. Even though this microorganism has been isolated in the joints, it is not clear how the bacteria enter the body and how they reach the joints. Antonio et al. experimentally reproduced the arthritogenic ability of \textit{M. fermentans} isolated from the human respiratory tract by injecting them into the trachea. They found that live organisms settled down in the joints and induced arthritis. These observations will shed light on the role of \textit{M. fermentans} and/or its antigen in arthritis.

In this study, we evaluated whether infection with \textit{M. fermentans} is associated with internal derangement (ID) and osteoarthritis (OA) of the temporomandibular joint (TMJ). We also describe our results regarding the detection of DNA derived from \textit{M. fermentans} and the specific immunoreactivity of the patient SF and sera against mycoplasmal antigen.

### Materials and Methods

#### 1. Samples

We studied 115 TMD patients including 81 ID and 34 OA patients. All patients gave informed consent before the study commenced. Sera from some TMD patients as well as craniomaxillofacial diseases other than TMD were also subjected to the experiments. All synovial fluid samples were collected at Yamagata University, with which Dr. Shibata was formerly affiliated. Study approval was obtained from the Ethics Committee of the Medical Faculty, Yamagata University, Yamagata. The average age of the patients was 37 years old (range 14-70 years). The patients were screened for \textit{M. fermentans} infection by detection of its DNA and by determination of antibody response to \textit{M. fermentans} in SF and sera. SF was collected by puncture with a 23-gauge needle into the superior joint space from an infero-lateral approach. By directly aspirating the SF, we could obtain 0.2 - 0.5 ml of undiluted SF from the TMJ. All samples were stored at $-30^\circ$ C until processing.

#### 2. Bacterial Strain and Culture Condition

\textit{M. fermentans}, strain PG18, was obtained from American Type Culture Collection (Rockville, MD, USA). It was grown in PPLO broth (Difco Laboratories, Detroit, MI, USA) supplemented with 20\% (v/v) horse serum (GIBCO Life Technologies, Inc., Grand Island, NY, USA), 10\% (w/v) yeast extract (Difco), 1\% (w/v) D-glucose, 0.002\% (w/v) phenol red, and penicillin G (1,000U/ml). Cultures were incubated at 37\(^\circ\) C for 48 h under anaerobic condition. The cells were harvested by centrifuging the cultures at 15,000 \times g for 15 min, washed three times with sterile PBS, and suspended in sterilized water.

#### 3. Preparation of samples for PCR

SF samples (200 \mu l) were mixed with 800 \mu l of HMW buffer and incubated at 50\(^\circ\) C for 2 h. The DNA was extracted twice with an equal volume of phenol saturated with TE buffer (10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA), and was also extracted once with phenol-chloroform-isooamyl alcohol (24:1, v/v). Sodium acetate (0.3 M) was added to the aqueous phase, and the DNA was precipitated with two volumes of
absolute ethanol. The precipitate was suspended in TE buffer.

4. PCR assay

PCR amplification was performed essentially as described previously in a total volume of 20 μl PCR solution buffer. Primers for 5'-TGA CGG GGT CAC CCA CAC TTG GCC-3' and 5'-TAG AAG CAT TTG CAC ACG ATG-3' were used for the amplification. The PCR amplification reaction utilized 1 μg of DNA derived from SF extract, 10 × PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 25 mM MgCl₂), 2.5 mM dNTPs, 3' (antisense) primer, 5' (sense) primer, and 2.5 units of Taq DNA polymerase (Takara Bio, Inc., Shiga, Japan). As a positive control, DNA from M. fermentans strain PG18 was used for amplification in each experiment. PCR was carried out in a PCR thermal cycler (Takara) with the following temperature profile: denaturation at 94°C for 2 min, primer annealing at 60°C for 30 sec in 5'-actin and at 56°C for 30 sec in M. fermentans, and polymerization at 72°C for 1 min in each cycle. Amplification was carried out at 30 cycles in 5'-actin and 45 cycles in M. fermentans. Amplification products were visualized under ultraviolet light after electrophoresis by 1.5% agarose gel (Wako Pure Chemical Industries, Osaka, Japan) followed by staining with ethidium bromide.

5. Preparation of M. fermentans lipoproteins by Triton X-114 phase separation

Mycoplasma membrane lipoproteins were prepared by the phase-partitioning method using a detergent, Triton X-114, extraction as described previously. Briefly, after light sonication, M. fermentans cells were suspended in sterilized water. The cell suspension was mixed with 20% Triton X-114 working solution and TS buffer (10 mM Tris-HCl (pH 7.4), 154 mM NaCl). The tube containing the mixture was placed on a rotator at 4°C for 60 min, and then it was centrifuged at 10,000 × g for 4 min at 4°C. After removal of the aqueous phase, the Triton phase was resuspended in TS buffer, and incubated for 5 min on ice followed by 37°C for 5 min, and then centrifuged at 10,000 × g for 5 min. Lipoprotein (TXLP) from the Triton X-114 phase was precipitated by adding methanol and used for immunoblotting after being suspended in sterile water.

6. Detection of antibody against M. fermentans by Western-blot analysis

TXLP (0.3 μg) was fractionated by SDS-PAGE on 12.5% acrylamide gel and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, England). After being blocked with Tween-PBS containing 2.5% skim milk, the membrane was probed with either SF or sera (1.5 μg protein) by means of overnight incubation at 4°C. After being probed, the membrane was washed three times with a washing buffer (20 mM Tris-HCl and 500 mM NaCl). Next, it was incubated for 1 h at room temperature with peroxidase-conjugated anti-human IgG F(ab')₂ antibody diluted to 1:1000 (Cortex Biochem, CA, USA). The membrane was then washed again in the same manner, followed by developing the blots with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). Mouse hyperimmune antiserum to TXLP and rat anti-mouse IgG₁ conjugated with peroxidase (Nippon Becton Dickinson Company Ltd., Tokyo, Japan) was used as a positive control.

7. Statistical analysis

The statistical significance of differences between experimental groups was analyzed by the t² test. A p value of less than 0.05 was considered significant.

Results

1. Detection of M. fermentans in SF

Characterization of the patients and the percentage of SF samples positive for M. fermentans DNA are shown in Table 1. M. fermentans DNA was detected in 43 (37.4%) out of 115 samples from patients with TMD. SF samples from 32 (39.5%) of 81 patients with ID were positive for the DNA. Also, samples from 11 (32.4%) of 34 patients with OA were positive for M. fermentans DNA. There was no difference in positive rates of M. fermentans DNA between ID and OA. No difference was observed also in the percentage positive for M. fermentans DNA between males and females. Representative results of the PCR analyses are shown in Fig. 1. In ID patients, however, the positive rate of M. fermentans DNA was different between patients aged
over 40 years and those of under 40 years. Thus, the detection rate of *M. fermentans* DNA was found to be significantly higher in elderly ID patients than in younger ones (Table 2). In OA, no differences in age were demonstrated between patients positive or negative for *M. fermentans* DNA (Table 2).

Table 1  Characterization of patients and positive rate of *M. fermentans* DNA in SF of TMD patients

<table>
<thead>
<tr>
<th>Type of TMD</th>
<th>Total sample tested</th>
<th>M. fermentans DNA positive samples¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of samples</td>
<td>Mean are (yr)</td>
</tr>
<tr>
<td>ID²</td>
<td>81</td>
<td>28 (14-63)</td>
</tr>
<tr>
<td>OA³</td>
<td>34</td>
<td>46 (16-70)</td>
</tr>
<tr>
<td>ID + OA</td>
<td>115</td>
<td>37 (14-70)</td>
</tr>
</tbody>
</table>

¹ *M. fermentans* DNA was detected by PCR analysis using specific primers reported by Watanabe et al. (Ref. 15). ² ID: Internal derangement, ³ OA: Osteoarthritis

Table 2  Prevalence of *M. fermentans* DNA in various TMJ arthropathies

<table>
<thead>
<tr>
<th>Age²</th>
<th>No. of patients</th>
<th>No. of SF samples¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID³</td>
<td>40 ≥ 66</td>
<td>21 (31.8%)</td>
</tr>
</tbody>
</table>

¹ *M. fermentans* DNA was detected by PCR analysis using specific primers reported by Watanabe et al. ² SF samples were divided into two categories according to the patient’s age, under 40 and over 40 years of age. Statistical analyses were performed by the χ² test. Values significantly different (P < 0.05) are shown by an asterisk. ³ ID: Internal derangement, ⁴ OA: Osteoarthritis.

2. Detection of Anti-*M. fermentans* antibody in SF and sera

Verification of the specific IgG antibodies to *M. fermentans*-derived lipoproteins was obtained by Western-blot analyses. The use of peroxidase-conjugated anti-human IgG F(ab')2 antibody instead of using anti-human whole Ig as secondary antibody enabled us to detect more easily *M. fermentans* specific antibodies in SF and sera (data not shown). Positive reactivities to *M. fermentans* lipoproteins with different molecular sizes (56 kDa, 48 kDa, 38 kDa and 29 kDa) were observed in SF samples from TMD patients (Fig. 2 A, B), and designated as p56, p48, p38, and p29, respectively. No difference in reactivities by age (over 40 or under 40), sex, and disease categories (ID or OA) was demonstrated between patients positive and negative for *M. fermentans* DNA (Fig. 2 A and B). The sera taken from patients with diseases other than TMD positively reacted with *M. fermentans* lipoproteins as depicted in Fig. 3. The sera from TMD patients also exhibited similar reactivity to *M. fermentans* lipoproteins irrespective of the disease categories, but prominent reactivity to p56, p48, and p29 was uniformly observed (Fig. 3). The reactivity pattern of
the SF to various *M. fermentans* lipoproteins was absolutely different from that observed in the sera.

3. Differences in the specificity to certain lipoproteins detected in SF and sera of the identical patient

The reactivity pattern of the SF to various *M. fermentans* lipoproteins was absolutely different from that observed in the sera. Among lipoproteins from *M. fermentans*, immunoreactivity to p48 and p29 was conspicuously observed both in SF and sera taken from the identical patient. Interestingly, the immunoreactivity to p56 was much lower in the SF than that in the sera, and conversely the relative reactivity to p48 was much stronger in the SF than that in the sera (Table 3 and Fig. 4). Similar reactivity patterns of the SF and the sera in Western blotting were unequivocally observed in other patients. The relatively high reactivity of the SF against p48 and p29 lipoproteins suggests local production of IgG antibodies specific for p48 and p29 in the TMJ.

**Discussion**

We detected *M. fermentans* DNA in SF of 37.4% of patients with TMD. Our results are in accordance with those of Henry et al., who found *M. fermentans* DNA in a similar percentage in the TMJ posterior bilaminar tissue from patients with TMD. They also reported the isolation of DNAs of several bacterial species from TMJ tissue. Although the primer sequence, RW004 and RW005, used to detect *M. fermentans* which was reported by Henry et al. and ourselves involved the insertion-like sequence that had been shown to also detect *M. orale*, there was no big difference in the positive rate of the DNA between the SF and the tissue. In contrast, Watanabe et al. detected a much higher prevalence of *M. fermentans* DNA (61%) in SF of patients with closed lock symptoms of the TMJ.
compared to that of ours. There was no difference between PCR-positive and -negative patients regarding sex and disease categories of the TMJ (e.g., internal derangement or osteoarthritis). The presence of *M. fermentans* DNA in SF, however, does not seem to be contamination, since we did not detect this DNA randomly distributed among patients. The higher incidence of *M. fermentans* DNA in elderly patients who suffered from ID suggests a causal relationship of this organism to ID.

Mycoplasmas, including *M. fermentans*, are excellent candidates for induction of arthritis in humans; *M. fermentans* can induce experimental arthritis in animals, and its cell membrane extracts (lipoproteins) are shown to induce the production of various kinds of proinflammatory cytokines by human macrophages, including TNF-α, IL-1β and IL-6, which are key cytokines in the development of RA as well as other arthropathies including TMD. Especially, these cytokines are associated with synovitis in ID and osteoarthritic changes of the TMJ. Our previous study revealed that *M. fermentans*-derived membrane fraction (TXLP) upregulates TNF-α and IL-1β production from monocytes, and it also stimulates gene transcription of matrix metalloproteinase (MMP)-3 in rabbit synoviocyte cell line. Thus the high prevalence of *M. fermentans* DNA in elderly ID patients may explain the possibility that they can induce or perpetuate inflammatory reaction in the TMJ.

Kaufmann et al. recently identified a well-defined lipopeptide, named 2-kDa macrophage-activating lipopeptide (MALP-2), from *M. fermentans*. This lipopeptide with N-terminal cysteine residue bound to a diacylated glyceride residue through a thioester linkage was reported as a potent activator of mammalian cells. Various mycoplasmal lipoproteins have their active site...
on N-terminal moiety, the structure of which is similar to MALP-2\textsuperscript{23}. As for the ligands for recognizing the lipoproteins and MALP-2, TLRs have been recently discovered\textsuperscript{24}. Among 10 identified TLRs, TLR2 has been shown to be essential for the recognition of distinct bacterial components, such as peptidoglycan (PGN), lipoarabinomannan (LAM), and lipoprotein (LP)\textsuperscript{25}. Subsequent to ligand binding, TLR-2 initiates signaling that leads to the activation of transcription factor NF-\textsuperscript{κ}B, which also initiates the production of reactive oxygen species, initiation of the proinflammatory cytokines, and upregulation of costimulatory molecules\textsuperscript{25}. Thus, it is probable that once an inflammatory process of the TMJ synovium is somehow induced by immune reaction to a certain bacterial antigen or by mechanical overloading, inflammatory cytokines will be produced with a pattern similar to RA.

It has been suggested that \textit{M. fermentans} is found in SF due to passive carriage of this organism from other organs, which was normally found in the pharynx and saliva of 20-40\% as well as in 10\% of peripheral mononuclear cells of humans\textsuperscript{26}. Thus, the presence of \textit{M. fermentans} in SF could be regarded as that of an innocent bystander without pathogenic significance. However, if this were the case, we would expect to detect \textit{M. fermentans} DNA in SF of all patients irrespective of their ages and disease categories (ID or OA). Furthermore, the high prevalence of \textit{M. fermentans} in aged patients does not support this hypothesis. We believe that \textit{Mycoplasma} may not be permanently present in SF or TMJ tissue and therefore might not always be detectable. In experimental arthritis in rabbits by \textit{M. arthritidis} and in swine by \textit{M. hyorhinis}, \textit{Mycoplasma} could only be isolated from the joints for a few weeks post-infection; it then disappeared, while the chronic arthritis process continued\textsuperscript{27}. Therefore, we consider the \textit{Mycoplasma} infection in the TMJ is transient but the accumulation of its antigen in the TMJ tissue may induce a specific humoral immune response against the inspection, and the phenomenon may persist for a long time in the body.

The anti-\textit{M. fermentans} immunoreactivity patterns to the various lipoproteins in the SF were different from those of the sera (Fig. 4). This phenomenon suggests local production of antibodies specific for a certain \textit{M. fermentans} lipoprotein in the joint \textit{per se}. This also indicates that \textit{M. fermentans} or its membrane product can elicit an immune response inside the joint. Similar findings were reported regarding \textit{M. fermentans} in RA, where the bacterial antigens were detected in the joints, and levels of antibodies to the bacteria in the SF were higher than those observed in the sera\textsuperscript{4}. We did not measure the level of antibody in the SF by ELISA, but the different epitope specificities of anti-\textit{M. fermentans} antibody detected in the SF and the sera strongly suggest the joint specific humoral immune response.

It is possible that \textit{Mycoplasma} persists at another location in the body (e.g., urogenital or respiratory tract) and occasionally reaches the joint, causing exacerbation of arthritis. This is partially substantiated by the findings that \textit{M. fermentans} was detected within peripheral polymorphonuclear cells in 50\% of RA patients\textsuperscript{28}. This kind of phenomenon was described in reactive arthritis (ReA) associated with mostly enteric bacteria, such as \textit{Salmonella} and \textit{Yersinia}\textsuperscript{29,30}. However, Henry et al. recently reported that \textit{Chlamydia} as well as \textit{Mycoplasma} could cause ReA in human TMJ\textsuperscript{16}. In either case the infection might result in the release of arthritogenic antigens, in transformation of immune reactivity by releasing proinflammatory cytokines. Thus, it is possible that \textit{M. fermentans} is carried inside the body by peripheral blood cells into the joints, as shown in the phenomenon described in ReA.

As another interesting finding, we have shown that IgG antibodies present in SF of our patients have a high affinity to certain \textit{M. fermentans} membrane lipoproteins, including p48 and p29. p48 was recently shown to be an arthritogenic antigen to induce TNF-\textit{α} production from human monocytes\textsuperscript{31}. More interestingly, various \textit{Mycoplasma} species were shown to have affinity to cartilage, possibly promoting the homing of the bacteria to the joint tissue\textsuperscript{32}. In this regards, the fact that one of the \textit{M. fermentans} derived lipoproteins, p29, was shown to mediate initial binding to some type of articular cells deserves special attention\textsuperscript{33}. It is also noteworthy that \textit{M. arthritidis} was shown to share a common antigenic determinant with chondrocytes surface antigens or their products, glycosaminoglycans, suggesting molecular mimicry of mycoplasmal antigens in the pathogenesis of arthritis\textsuperscript{4}.

In conclusion, our findings suggest that \textit{M. fermentans} infection was present in some TMD patients. We also first found that anti-\textit{M. fermentans} Ab is produced locally in the TMJ, possibly reflecting
immunoreaction to this organism. It is, however, still unknown how the bacteria can influence TMD, and further direct evidence is needed to determine the validity of this conjecture.

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References


