Fig.2

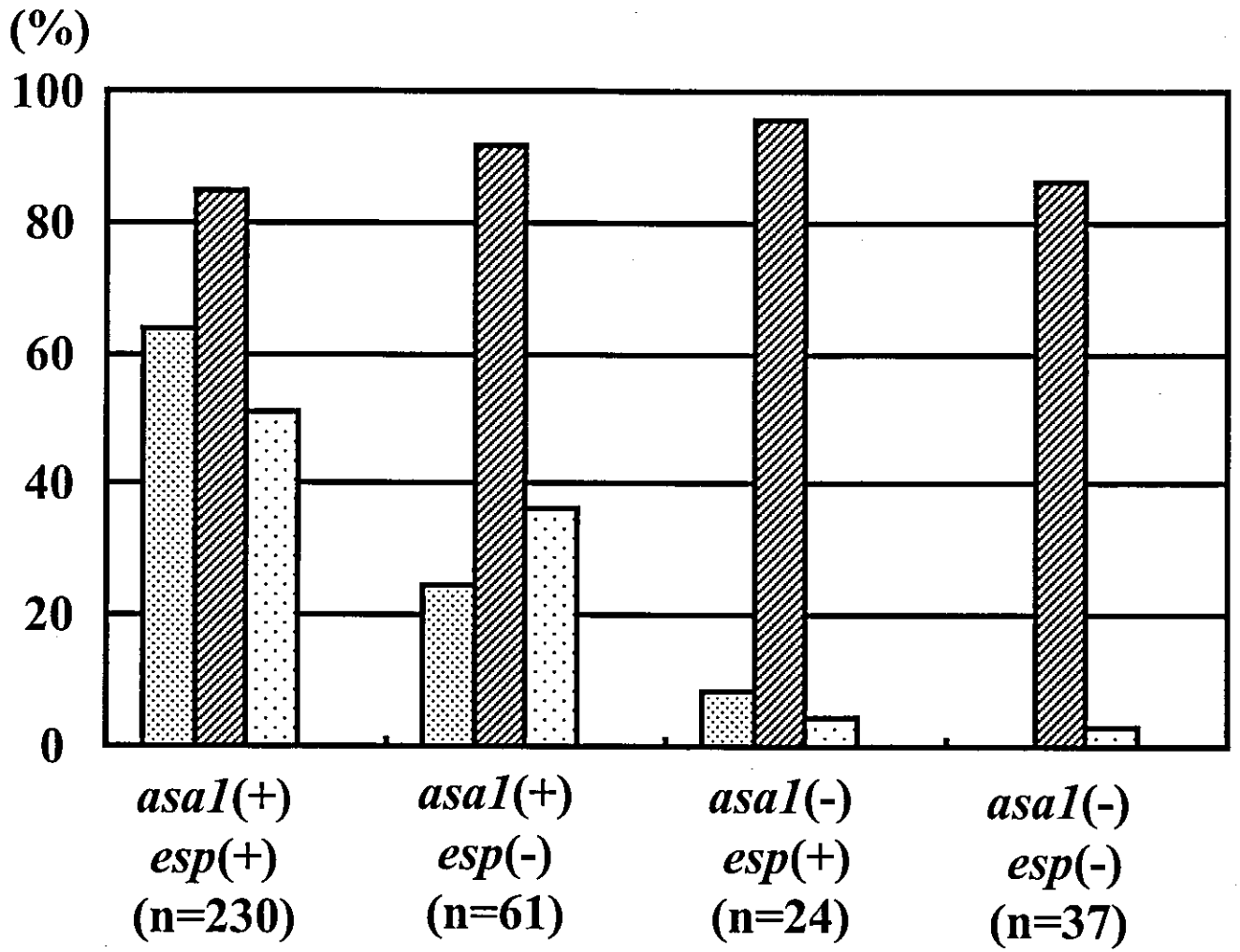
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Fig.3

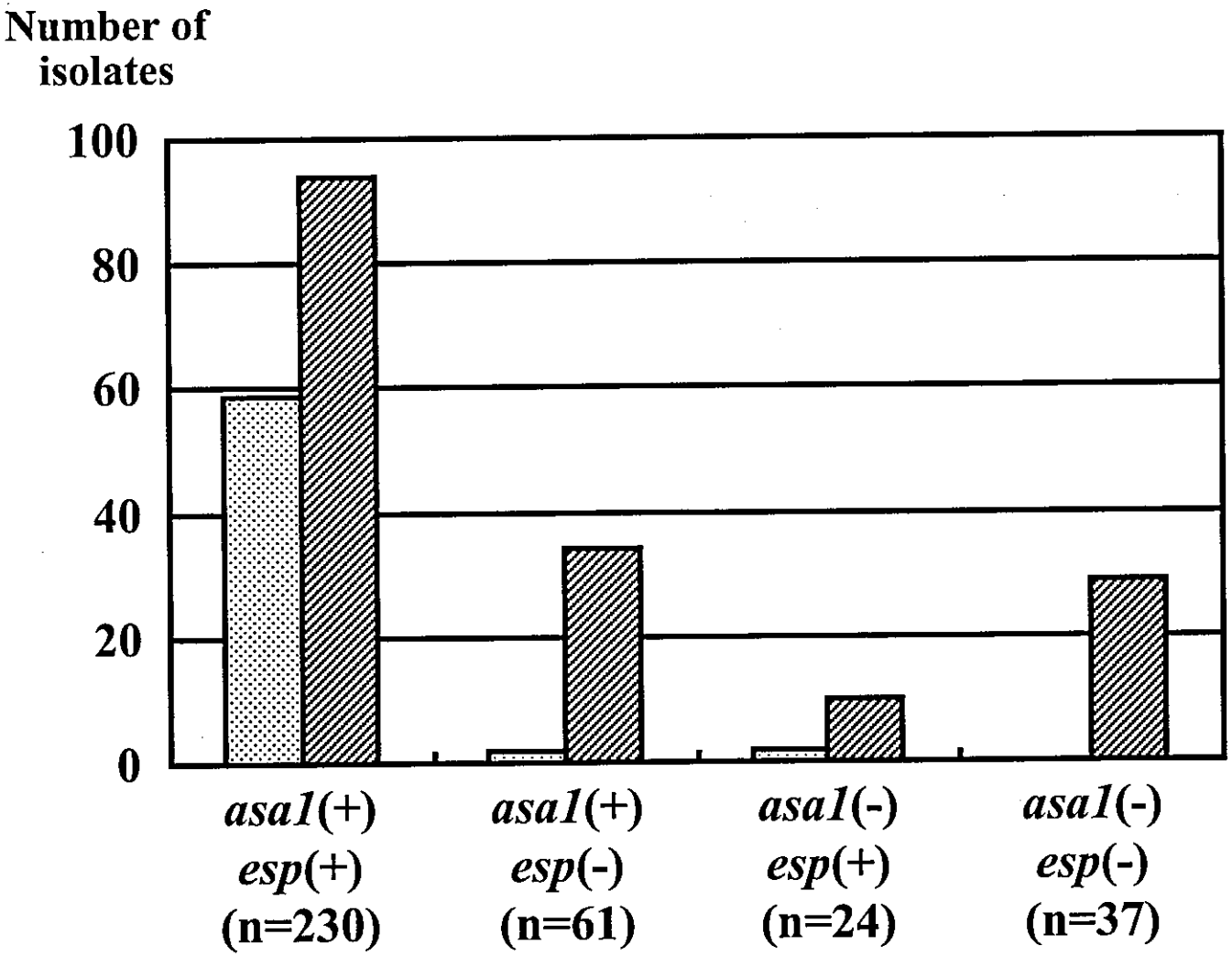
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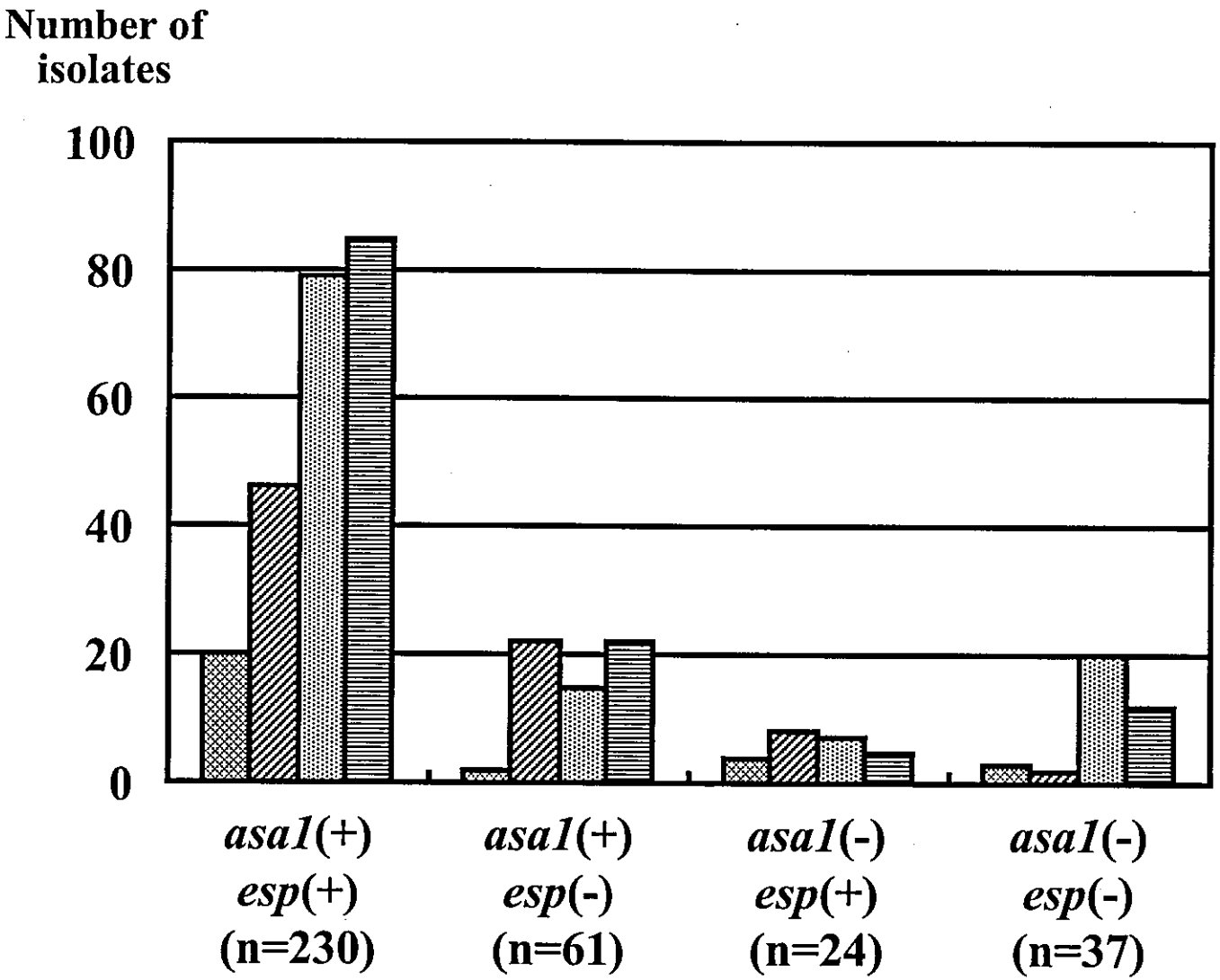
Fig.4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# Detection of periodontal pathogen *Porphyromonas gingivalis* by loop-mediated isothermal amplification method

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**Abstract**

A method for nucleic acid amplification, loop-mediated isothermal amplification (LAMP) was employed to develop a rapid and simple detection system for periodontal pathogen, *Porphyromonas gingivalis*. A set of six primers was designed by targeting the 16S ribosomal RNA gene. By the detection system, target DNA was amplified and visualized on agarose gel within 30 min under isothermal condition at 64 °C with a detection limit of 20 cells of *P. gingivalis*. Without gel electrophoresis, the LAMP amplicon was directly visualized in the reaction tube by addition of SYBR Green I for a naked-eye inspection. The LAMP reaction was also assessed by white turbidity of magnesium pyrophosphate (a by-product of LAMP) in the tube. Detection limits of these naked-eye inspections were 20 cells and 200 cells, respectively. Although false-positive DNA amplification was observed from more than $10^7$ cells of *Porphyromonas endodontalis*, no amplification was observed in other five related oral pathogens. Further, quantitative detection of *P. gingivalis* was accomplished by a real-time monitoring of the LAMP reaction using SYBR Green I with linearity over a range of $10^2$–$10^6$ cells. The real-time LAMP was then applied to clinical samples of dental plaque and demonstrated almost identical results to the conventional real-time PCR with an advantage of rapidity. These findings indicate the potential usefulness of LAMP for detecting and quantifying *P. gingivalis*, especially in its rapidity and simplicity.


*Keywords:* Loop-mediated isothermal amplification; *Porphyromonas gingivalis*; Periodontitis

## 1. Introduction

*Porphyromonas gingivalis*, an oral black-pigmented anaerobic Gram-negative bacterium, has been frequently isolated from diseased lesions of periodontitis and strongly implicated as a key etiological agent of periodontal diseases [1–3]. For microbiological diagnosis of periodontal diseases, several techniques such as immunoassay, biochemical tests and hybridization using nucleotide probes have been developed [4]. However, since all these methods required bacterial culture, isolation and identification of anaerobic species such as *P. gingivalis*, have been laborious and time-consuming for routine clinical examinations. Recently advances of molecular biological techniques enabled culture-independent methods, such as polymerase chain reaction (PCR) [5–7]. Although PCR is very sensitive and highly specific, special regents and apparatus such as

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thermalcycler are needed. Therefore, this examination is centralized in highly sophisticated facilities and still time consuming.

Loop-mediated isothermal amplification (LAMP), a novel DNA amplification method has originally developed by Notomi et al. [8]. The method relies on autocycling strand displacement DNA synthesis by the *Bst* DNA polymerase large fragment. The amplification products are stem-loop DNAs with several inverted repeats of the target and exhibit cauliflower-like structure with multiple loops. The LAMP reaction can be conducted under isothermal condition ranging from 60 to 65 °C. The specificity is attributable to a set of two specially designed inner and outer primers that recognize six distinct sequences. Continuous amplification under isothermal condition produces extremely large amount of target DNA as well as large amount of by-product, magnesium pyrophosphate, within 30–60 min. Therefore, the method demonstrates high sensitivity and enables simple visual (naked-eye) judgment of the reaction through a color change of mixture with SYBR Green I [9] or a white turbidity of magnesium pyrophosphate [10]. Quantitative detection of LAMP is possible by a real-time monitoring of the turbidity [10–12] or fluorescence of ethidium bromide [13]. Further, it has recently been reported that LAMP can be accelerated using additional primers, termed loop primers [13]. Therefore, the LAMP may be promising in rapid detection of microorganisms [9,11,12,14]. In addition, since the method requires only one type of enzyme and special apparatus is not needed, the method would be suitable for routine examinations at dental chair-side. In this study, we evaluated the potential of LAMP for the development of simple and rapid detecting system for periodontal pathogen *P. gingivalis*.

## 2. Materials and methods

### 2.1. Bacterial strains and culture conditions

The bacterial strains used in this study were as follows: *P. gingivalis* FDC 381, *P. gingivalis* ATCC 33277, *P. gingivalis* W83, *P. gingivalis* W50, *Porphyromonas endodontalis* ATCC 35406, *Prevotella intermedia* ATCC 25611, *Fusobacterium nucleatum* ATCC 25586, *Campylobacter rectus* ATCC 33238, *Actinobacillus actinomycetemcomitans* Y4 and *Escherichia coli* K12. *P. gingivalis*, *P. endodontalis*, *F. nucleatum*, and *P. intermedia* were cultured in the modified GAM broth (Nissui Seiyaku, Inc., Tokyo, Japan) at 37 °C in an anaerobic box (Model ANX-1; Hirasawa Works, Tokyo, Japan) containing 80% $N_2$, 10% $H_2$ and 10% $CO_2$. *A. actinomycetemcomitans* and *C. rectus* were cultivated according to the methods described previously [15,16]. *E. coli* was cultured aerobically in Luria–Bertani medium. The cells were harvested by centrifugation at 10,000*g* for 20 min at 4 °C, and cell numbers were determined using Petroff–Hauser counting chamber and phase-contrast microscope.

### 2.2. DNA extraction

Total bacterial DNAs were extracted from cultivated strains and clinical dental plaque samples by using InstaGene Matrix (Bio-Rad Lab. CA, USA) according to the instructions. Plaque samples and cultivated bacteria were suspended in 1 ml of phosphate-buffered saline [PBS (–)] (Gibco BRL, MD, USA), and were pelleted and resuspended in 100 μl of InstaGene Matrix. The suspension was incubated at 56 °C for 30 min and then 100 °C for 8 min. After the incubation, the suspension was centrifuged and 2 μl of the resulting supernatant was used for the detection of *P. gingivalis* by LAMP and the conventional real-time PCR [7]. To examine a detection limit, specificity, and a quantitative range of the LAMP, DNAs were prepared from various numbers of *P. gingivalis* as well as other oral pathogens.

### 2.3. Clinical samples

Sampling of subgingival plaque from periodontitis patients was performed at Okayama University Hospital of Medicine and Dentistry after obtaining informed consent. Samples were obtained by inserting paper points (#45, Zipperer, Germany) into periodontal pockets as described previously [17], and DNAs were extracted from the plaques as described. The plaque DNA samples were used for clinical microbiological diagnosis by conventional real-time PCR as reported previously [7]. By this method, the number of *P. gingivalis* in plaque samples was quantified. The remaining plaque DNA samples were stored at −20 °C. Ten plaque DNA samples containing various numbers of *P. gingivalis* were selected randomly from the stocks for the analysis by LAMP.

### 2.4. Primers for LAMP

A set of six primers for LAMP was designed to target *P. gingivalis* 16S ribosomal RNA gene (16SrDNA). For the designing, Primer Explorer version 2 (Fujitsu, Tokyo, Japan) was used at the Net Laboratory website (http://www.venus.netlaboratory.com/partner/lamp/index.html). A forward inner primer (FIP), a backward inner primer (BIP) and two outer primers (F3 and B3c) were used for a basic LAMP method, and additional two primers (loop primers, LFc and LB) were used for an accelerated LAMP. Further, to confirm the strict requirement of each primer, the LAMP was performed in the absence of one of the inner or outer primers. The primers' sequences and the locations were indicated

in Fig. 1. The F1c, B2c, B3c and LFc in the figure denoted complementary sequences. The FIP consisted of a sequence of F1c and a sense sequence of F2 (5′-F1c-F2-3′). The BIP consisted of a sequence of B1 and a B2c (5′-B1-B2c-3′).

### 2.5. LAMP reaction

The LAMP reaction for the detection of *P. gingivalis* was carried out using a Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Tochigi, Japan) in 25 μl volume. The reaction mixture contained 40 pmol each of FIP and BIP, 5 pmol each of F3 and B3c primers, 2 μl of extracted DNA, 1 μl of *Bst* DNA polymerase and 12.5 μl of reaction mix prepared in the kit. For the acceleration of the LAMP reaction, 20 pmol of each LFc and LB was added to the reaction mixture. The reaction mixture was incubated at 60, 62, 64 or 66 °C for 30 or 60 min. After the incubation, the reaction was terminated by heating the reaction mixture at 80 °C for 2 min.

### 2.6. Detection of LAMP products

For naked-eye detection, 1.0 μl of $10^{-1}$ or $10^{-3}$-diluted SYBR Green I (Takara Bio Inc., Otsu, Japan) was added to the reaction mixture, and the color change was observed. The inspection of the tube containing the $10^{-3}$-diluted SYBR Green I was performed under UV light (302 nm wave length). The color change of $10^{-1}$-diluted SYBR Green I was judged under natural light. Prior to the addition of SYBR Green I, white turbidity of the reaction mixture by magnesium pyrophosphate (by-product of LAMP) was also inspected.

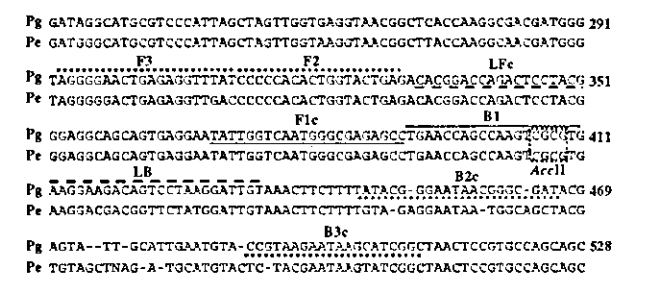


Fig. 1. Target sequence of 16SrDNA and primers for the LAMP. The nucleotide sequence of 16SrDNA of *P. gingivalis* (Accession No. L16492) was used for designing the primers and was shown with the sequence of *P. endodontalis* (L16491). A forward inner primer (FIP), a backward inner primer (BIP) and two outer primers (F3 and B3c) were used for a basic LAMP method, and additional two loop primers (LFc and LB) were used for an accelerated LAMP. The F1c, B2c, B3c and LFc denoted complementary sequences. The FIP consisted of a sequence of F1c and F2 (5′-F1c-F2-3′). The BIP consisted of a sequence of B1 and B2c (5′-B1-B2c-3′). The numbers at the right side indicate the base position of the 16SrDNA. The restriction enzyme *Acc*II was used for digestion of the LAMP product, and the recognition site was surrounded by box.

For the electrophoretic analysis, 2 μl of reaction mixture was loaded on 2% agarose gel. The gel was stained with ethidium bromide (1 mg $ml^{-1}$) and assessed photographically under UV light (302 nm). To confirm a structure of the LAMP product, the amplicon was digested with *Acc*II (Takara Bio Inc.) and was subjected to the electrophoresis.

For quantitative detection, real-time monitoring of LAMP was performed using GeneAmp® 5700 Sequence Detection System (PE Applied Biosystems, Foster, CA, USA). SYBR Green I was used as a source of fluorescence. Original solution of SYBR Green I was diluted ($3 \times 10^{-3}$), and 2 μl of the diluted SYBR Green I was added to the LAMP reaction mixture. All amplifications and detections were carried out in MicroAmp optical 96-well reaction plate with optical caps (PE Applied Biosystems). Accumulation of LAMP products was detected by monitoring the increase in fluorescence of dsDNA-binding SYBR Green at every 30 s for 40 min under isothermal condition at 64 °C. The data of real-time LAMP were analyzed using the GeneAmp® 5700 SDS software (PE Applied Biosystems).

### 2.7. Real-time PCR

Real-time PCR was performed for quantitative detection of *P. gingivalis* as described previously [7]. GeneAmp® 5700 Sequence Detection System (PE Applied Biosystems) was used for monitoring the fluorescence from dsDNA-binding SYBR Green. The PCR mixture contained 2 × SYBR Green PCR Master Mix (PE Applied Biosystems), 20 pmol of forward and reverse primer and 2 μl of extracted DNA. Both forward (5′-cttgacttcagtggcggcag-3′) and reverse primer (5′-aggaagacggttttcacca-3′) was designed from the species-specific regions of 16SrDNA. Thermocycling program was 40 cycles of 95 °C for 15 s and 60 °C for 1 min with an initial cycle of 95 °C for 10 min. The data was analyzed using the GeneAmp® 5700 SDS software (PE Applied Biosystems).

## 3. Results and discussion

### 3.1. Primer design and specificity of LAMP

The primers of LAMP for the detection of *P. gingivalis* were designed to target the 16SrDNA. The 16SrDNA sequence of *P. endodontalis*, a same genus *Porphyromonas* of oral pathogen, was referred to the design. Candidate sequences for the primers were nominated by the Primer Explorer software (Fujitsu) and were compared to the corresponding sequences of *P. endodontalis*. A set of primers, which was the most specific to *P. gingivalis*, was selected in the current study (Fig. 1). In the primer set, F3, B2c, B3c and LB contained the specific

sequences of *P. gingivalis* comparing to *P. endodontalis*. By using the primer set, specificity, sensitivity, and quantitativity of the LAMP for *P. gingivalis* was investigated.

A successful LAMP reaction was seen with the complete set of primers and template DNA from four strains of *P. gingivalis* (Fig. 2(a)). The LAMP products appeared as a ladder-like pattern on the agarose gel due to its characteristic structure [8]. To confirm the specific amplification, the product was digested with *Acc*II, which recognize the sequence in B1. Consequently, many bands at different size were concentrated and fragmented to approximately 65- and 130-bp length in good agreement with the predicted size (Fig. 2(a)). The results demonstrate that the LAMP specifically amplified the target sequence in the form of stem-loop DNAs with various stem length and cauliflower-like structures with multiple loops. No difference was seen among the strains tested (data not shown).

Prior to the serial experiments, optimum reaction temperature was determined. The reactions of LAMP for *P. gingivalis* were performed under isothermal condition at 60, 62, 64 or 66 °C using extracted DNA from four strains of *P. gingivalis* ($10^2$ cells) prepared in this study. Although significant difference was not observed, the LAMP product amplified at 64 °C exhibited slightly larger amount of DNA as compared to others (data not shown). The series of experiments were performed at 64 °C hereafter. Since no difference was seen among the strains of *P. gingivalis* in any experiments, the results of FDC 381 strain were representatively shown in the figures.

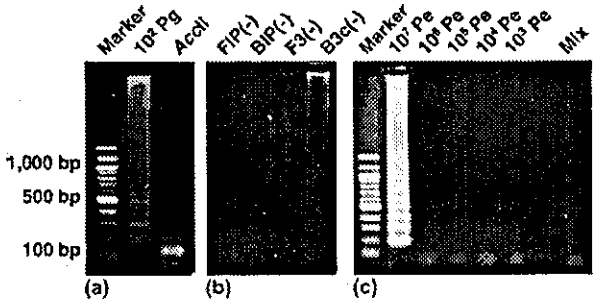


Fig. 2. (a) Electrophoretic pattern of the LAMP. The basic LAMP was carried out using extracted DNA from $10^2$ cells of *P. gingivalis* (Pg), and the LAMP product was electrophoresed on 2% agarose gel. A digested sample of the LAMP product with *Acc*II was simultaneously electrophoresed. The accelerated LAMP demonstrated identical result (data not shown). No difference was seen among the four strains tested, and the result of FDC 381 strain was representatively shown. (b) Requirement of primers for the LAMP. The target sequence was tried to amplify in the absence of one of the inner (FIP and BIP) or outer primers (F3 and B3c). Template DNA was prepared from $10^7$ cells of *P. gingivalis*. No amplification was seen in the absence of one of the inner primers. No significant amplification was seen in the absence of outer primers. (c) Specificity of the LAMP. Extracted DNAs from *P. endodontalis* ($10^3$–$10^7$) were used as templates for specificity testing of the accelerated LAMP. No amplicon was seen up to $10^6$ cells. The amplicon was detected from $10^7$ cells of *P. endodontalis*. In the last lane (Mix), reaction sample of the accelerated LAMP using the template DNA extracted from $10^7$ cells mixture of *P. intermedia*, *F. nucleatum*, *C. rectus*, *A. actinomycetemcomitans*, and *E. coli* was loaded.

The specificity of the LAMP was examined by using related oral pathogens as templates. In addition, to examine the requirement of each primer, the LAMP was performed in the absence of one of the inner or outer primers. No amplicon was seen in the absence of FIP, BIP or F3 primer, and very slight amplicon was detected in the absence of B3c primer (Fig. 2(b)). Extracted DNA mixture from $10^7$ cells of *P. intermedia*, *F. nucleatum*, *C. rectus*, *A. actinomycetemcomitans*, and *E. coli* was used as a template for the specificity testing of the LAMP, and no amplicon was observed (Fig. 2(c)). These results demonstrated that the each primer was required for the LAMP, and the reaction was specific to *P. gingivalis*. Even though a set of primers was designed in consideration of *P. endodontalis*, DNA was amplified from the extracted DNA of *P. endodontalis* in more than $10^7$ cells (Fig. 2(c)). This may influence the results of clinical plaque samples. However, since the cell numbers more than $10^7$ is extremely rare through the employed sampling method [7], the influences are considered to be small. By targeting the 16SrDNA of a multi-copy gene, high sensitivity would be expected. In addition, LAMP was a high specific method that required six distinct sequences for the amplification. Therefore, the 16SrDNA was employed for the first trial of LAMP, even though the 16SrDNA sequence was similar among genus *Porphyromonas*. Sufficient results were obtained in the current study, however, for more specific amplification, the target DNA may need to be changed. Species-specific genes responsible for the virulence or pathogenesis, such as fimbrilin (*fimA*) [18,19], would be candidates.

### 3.2. Sensitivity and quantitativity of LAMP

The LAMP amplicons were detected by agarose gel electrophoresis, naked-eye inspection, and real-time monitoring using GeneAmp® 5700 Sequence Detection System. The result of electrophoretic detection was shown in Fig. 3. The extracted DNA was prepared from the 200 cells of *P. gingivalis*, and serial 10-fold dilution of the extracted DNA was used for sensitivity testing. The accelerated LAMP using loop primers amplified the target within 30 min from the 10-fold dilution sample. The detection limit of the LAMP system was estimated to be 20 cells at the sampling stage before DNA extraction. Copy number of 16SrDNA in *P. gingivalis* is registered to be four on the website of TIGR (The Institute for Genome Research) database, and since 2 μl out of the 100 μl-DNA extracts was used for the LAMP, detection limit in one reaction tube was estimated to be near upon two copies. The basic LAMP required 60 min to detect the 200 cells of *P. gingivalis*. The

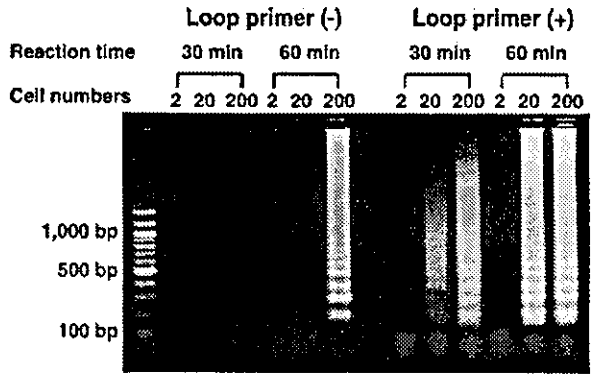


Fig. 3. Detection limit of the LAMP. Extracted DNA was prepared from various cell numbers (2, 20 and 200 cells) of *P. gingivalis* and was used as template for the basic [loop primer (−)] or the accelerated LAMP [loop primer (+)]. The reaction of LAMP was carried out at 64 °C for 30 or 60 min. The target DNA from 20 cells was amplified detectably on 2% agarose gel within 30 min by the accelerated LAMP.

loop primers prepared in this study dramatically accelerated the LAMP reaction and increased the sensitivity. The accelerated LAMP demonstrated an equivalent or higher sensitivity with the previously published PCR methods [5,6] or real-time PCR [7,20,21] in shorter time. Generally, the LAMP yields extremely large amount of DNA, and this enables naked-eye inspection [9,10]. The results of naked-eye inspections were shown in Fig. 4. The color of reaction mixture of successful LAMP exhibited green by the addition of SYBR Green I, whereas the original orange color did not change in the control tube. By using UV light, positive and negative reaction was more clearly distinguished. The naked-eye inspection with SYBR Green I demonstrated equivalent sensitivity to agarose gel electrophoresis both under UV and natural light (less than 20 cells). The sensitivity of inspection by white turbidity, a simpler eye judgment, was inferior to the electrophoresis. Two hundreds cells were required to definitely identified the reaction as positive. Although quantitative detection is difficult, the eye inspection was quite simple and rapid. Therefore it may facilitate the application of LAMP, especially in the place such as a dental chair-side.

For quantitative detection, real-time monitoring of the LAMP reaction was performed using loop primers. DNA extracts from $10^2$ to $10^6$ cells of *P. gingivalis* were subjected to the real-time LAMP. The increase in the fluorescent of SYBR Green I bound to the amplified double-strand DNA was scanned and measured by GeneAmp® 5700 Sequence Detection System at every 30 s. The scanning datum was shown in Fig. 5. The detection was linear over the rage examined, and all of the reaction reached plateau in 33 min. It has been reported that the real-time monitoring of LAMP reaction was achieved by measuring magnesium pyrophosphate [10–12] or fluorescence of ethidium bromide [13,22] with

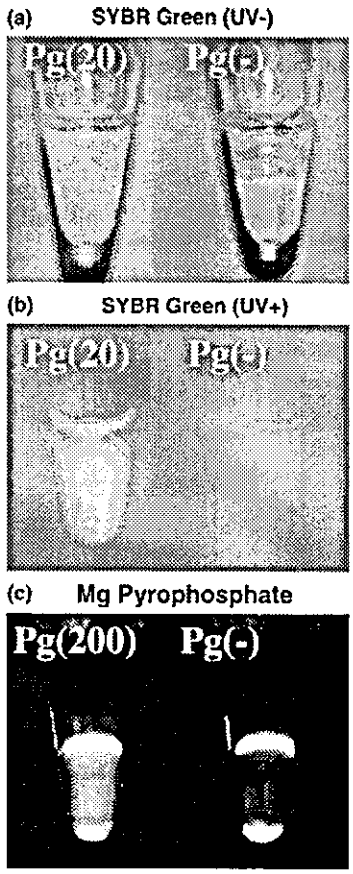


Fig. 4. Naked-eye inspection. Positive and negative reactions of the accelerated LAMP were distinguished by simple eye inspection. The original orange color of SYBR green I turned to be green in the positive reaction mixture (a), and the fluorescence of DNA-binding SYBR green I was visually detected under UV light (b). The detection limit of both naked-eye inspections with SYBR green I was less than 20 cells (Pg20). The positive reactions were shown with the negative control (Pg−). The LAMP reaction was also inspected through white turbidity of the tube caused by magnesium pyrophosphate, a by-product of LAMP (c). The detection limit of the white turbidity was less than 200 cells (Pg200).

equal level of sensitivity and quantitativity to the conventional real-time PCR [7,20,21]. By using the SYBR Green I, similar results were obtained in the current study. Although special apparatus, such as a turbidimeter or fluorescent detection system is required, the real-time LAMP will allow a quantitative detection of periodontal pathogens with an advantage of rapidity comparing to the conventional real-time PCR [7,20,21].

### *3.3. Examination of clinical dental plaque sample*

The real-time LAMP and the conventional real-time PCR [7] were applied to the clinical dental plaque samples. The original 2 μl of extracted DNA sample and 2 μl of diluted sample ($10^{-1}$) were run in duplicate. The number of *P. gingivalis* examined by the both methods was

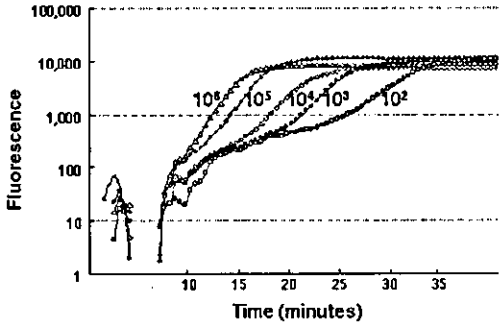


Fig. 5. Real-time monitoring of LAMP reaction. The accelerated LAMP was performed, and generated fluorescence intensity of DNA-binding SYBR Green I was monitored at every 30 s. Template DNAs were prepared from $10^2$ to $10^6$ cells of *P. gingivalis* and were used as standards for quantitative detection of *P. gingivalis* in clinical plaque samples.

Table 1
Cell numbers of *P. gingivalis* in plaque samples

| Plaque sample | Real-time PCR | Real-time LAMP |
|---|---|---|
| 1 | Not detected[a] | Not detected[a] |
| 2 | Not detected[a] | Not detected[a] |
| 3 | 165 | 202 |
| 4 | 388 | 480 |
| 5 | 1019 | 2609 |
| 6 | 2019 | 3206 |
| 7 | 23374 | 35277 |
| 8 | 24249 | 28371 |
| 9 | 190308 | 264144 |
| 10 | 404672 | 208534 |

[a] Less than 20 cells.

shown in Table 1. The number of *P. gingivalis* determined by the real-time LAMP was well corresponded to that analyzed by the real-time PCR. Although non-specific amplification was observed in *P. endodontalis* as described above, the influence was not practically seen in the current study. The set of primer seems to have sufficient specificity for the clinical examination. In addition, the results suggested that DNA contamination of other bacterial species did not influence the reaction of LAMP. On the other hand, slightly larger difference was seen in samples of 5 and 10 comparing to other samples because of unknown reason. Since the LAMP and real-time PCR are quite sensitive methods, small technical errors in experimental manipulations such as a pipetting might influence the results seriously.

Our final goal is to establish a simple and rapid examination system for periodontal pathogens in places such as private clinics or dental chair-side. In the current study, we employed the LAMP for the purpose. It demonstrated high efficacy and specificity, which was attributable to continuous amplification under isothermal condition with four primers. Comparing to the PCR or real-time PCR, only required device was water bath or heat block, and the most practically important benefit was the rapidity. Other than the LAMP, nucleic acid sequence-based amplification (NASBA) [23] and self-sustained sequence replication (3SR) [24] have been reported as isothermal amplification methods so far. Although the methods do not require thermal cycler, they are reported to be less specific, due mainly to low temperature (40 °C) for amplification and therefore requiring elaborate method for detection. The LAMP seems to have great advantage in its detection. Naked-eye inspection, electrophoresis analysis, and real-time monitoring of the reaction will be used according to the situation of the dental office.

## Acknowledgements

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Oral Microbiology and Immunology

# Role of helper T cells in the humoral immune responses against 53-kDa outer membrane protein from *Porphyromonas gingivalis*

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Outer membrane protein with a 53-kDa molecular weight (Ag53) isolated from *Porphyromonas gingivalis* evokes strong humoral immune responses in many periodontitis patients. To examine the effects of cytokines produced by Ag53-specific Th cells on the IgG production against Ag53, we established Ag53-specific Th-cell lines from patients with early onset periodontitis and from healthy volunteers. We then developed a mixed lymphocyte culture system between Ag53-specific Th cells and auto- or allo-derived T-cell-depleted leukocytes produced from the subjects whose HLA class II haplotypes were completely matched. Interferon-γ production was observed in all Th cell lines from patients and healthy subjects. As for Th2 type cytokines, interleukin (IL)-4, IL-5, IL-6 and IL-10 production varied greatly in Th cells regardless of the periodontal condition of the donor. Only Th cell lines with a high Th2/Th1 ratio induced Ag53-specific IgG production when cocultured with T-cell-depleted leukocytes. Thus, the difference in Th2/Th1 balance may regulate the Ag53-specific IgG production.

Key words: B cell; class II HLA; cytokine profile; IgG antibody; outer membrane protein; periodontitis; *Porphyromonas gingivalis*; Th cell line

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*Porphyromonas gingivalis* is the most frequently implicated pathogen in periodontal diseases, possessing many kinds of possible antigens recognized by human T cells (4, 19). Among them, the 53-kDa outer membrane protein (Ag53), one of the highly immunogenic proteins originally isolated from the vesicle surface of *P. gingivalis* 381, reacts strongly with sera from many patients with periodontitis (8, 10). Recently, analytical studies of epitopes recognized by T and B cells from early onset periodontitis patients have revealed that there is a common region on Ag53 recognized by both T and B cells in early-onset periodontitis patients, and that the major B-cell epitope is located within the major T-cell epitope on Ag53 (13, 14). This suggests that a limited region on Ag53 is essential for developing Ag53-specific immune responses, leading to the production of specific antibodies. However, the production of antibodies against Ag53 differs greatly even among periodontitis patients, although *P. gingivalis* was detected as the predominant microorganism in their periodontal pockets (3). Therefore, it is suggested that, besides T- and B-cell epitopes, other factors are involved in the establishment of the individually distinct production of IgG antibody against Ag53.

Generally, the difference in helper T (Th) cell subsets specific for antigenic proteins is one of the possible determinants in the production of IgG antibody from B cells, since the antigen-specific antibody production requires the help of Th cells, including the expression of CD40 ligand (CD40L) and the secretion of Th2-type cytokines. Th cells are classified into Th1 and Th2 cells on the basis of their cytokine profiles. Th1 cells secrete Th1-type cytokines such as interleukin (IL)-2 and interferon (IFN)-γ and are associated with cell-mediated immune responses, whereas Th2 cells secrete Th2-type cytokines such as IL-4, IL-5, IL-6, and IL-10

and are associated with the antibody production from B cells. The profile of cytokines produced by Th cells is antigen specific, and varies among individuals. Thus, cytokine profiles of Th cells are likely to be crucial in antibody production.

In the present study, we examined the cytokine profiles of the Ag53-specific Th-cell line established from early-onset periodontitis patients and from periodontally healthy subjects, and the effects of these Th cells with different cytokine profiles on the production of Ag53-specific IgG in response to Ag53.

## Material and methods

### Antigen preparations

Purified Ag53 protein from *P. gingivalis*, maltose binding protein-Ag53 fusion protein (MBP-Ag53) and Ag53-derived peptides were used as antigens. The purified Ag53 protein was prepared from whole cells of *P. gingivalis* FDC381 according to the method described by Kokeguchi et al. (9). MBP-Ag53 was generated by using a Protein Fusion and Purification System (New England Biolabs Inc., Beverly, MA) according to the method described by Ohyama et al. (13). In short, XL-1 Blue *Escherichia coli*-expressed MBP-Ag53 was sonicated and purified by MBP-specific affinity chromatography, followed by ion-exchange chromatography and gel-filtration chromatography. Ag53-derived peptides were prepared according to the method described by Ohyama et al. (13). Briefly, 45 peptides (sequential 11 amino acids overlapping peptides composed of 21 amino acids) representing the complete Ag53 sequence were synthesized based on the amino acid sequence of Ag53 reported by Hongyo et al. (6), using a solid-phase simultaneous multiple peptide synthesizer PSSM-8 (Shimadzu, Kyoto, Japan) based on F-moc strategy. All peptides were purified using C18 reverse-phase HPLC (Waters, Milford, MA). A mixture of these peptides was used as the Ag53 antigen.

### Peripheral blood mononuclear cell donors

Ag53-specific Th-cell lines were established from peripheral blood mononuclear cells (PBMCs) of early-onset periodontitis patients (D1–D6) and healthy individuals (D7–D22). Informed consent was obtained from each donor prior to this study. The PBMC donors included six Japanese early-onset periodontitis patients (five men, mean age: 27.8 ± 7.4 years), and 16 healthy Japanese individuals (14 men, mean age: 30.1 ± 4.3 years). Early-onset periodontitis patients were diagnosed according to the description of Murayama et al. (12).

### Lymphocyte preparation

Ag53-specific Th-cell lines were generated by the methods described in our previous study (13). PBMCs ($1 \times 10^5$/well) were stimulated with 5 μg/ml MBP-Ag53 in RPMI 1640 medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 2 mM L-glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 10% pooled, heat-inactivated autologous plasma in 96-well flat-bottomed culture plates (Falcon, Becton Dickinson, Lincoln Park, NJ). PBMCs were isolated from heparinized blood by Ficoll-Paque gradient density centrifugation. After 7–9 days, irradiated (3000 cGy) autologous PBMC ($1.5 \times 10^5$/well) pulsed with mixed-overlapping Ag53-derived peptides (0.5 μM each) as antigen-presenting cells, and human rIL-2 (50 U/ml), human rIL-4 (10 U/ml), were added to the culture wells carrying T-cell blasts, and the cells were maintained for a further 7 days. After specificity assay against the mixed-overlapping Ag53-derived peptides, aliquots of growing cultures specific to Ag53 were combined as the Th-cell line. To maintain the Th-cell activity, antigen-presenting cells, rIL-2, and rIL-4 were added to the culture wells every 6 days.

T-cell-depleted leukocyte fractions were obtained from PBMCs of the donors and were used as the cell fractions enriched with antibody-producing cells. PBMCs were incubated with 150-fold higher amounts of neuraminidase-treated sheep red blood cells (Immune-Biological Laboratories, Gunma, Japan) for 1 h on ice. The cell fraction at the interface after Ficoll-Paque centrifugation was collected to isolate T cells forming rosettes with sheep red blood cells. The T-cell-depleted lymphocytes were obtained by the depletion of $CD2^+$ lymphocytes from the interface-cell fraction, using Dynabeads M450 Pan-T (Dynal, Oslo, Norway), and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated pooled human serum from each donor, penicillin (100 U/ml), and streptomycin (100 μg/ml). The T-cell-depleted leukocyte fraction exhibited < 1% $CD3^+$ cells as determined by a flow cytometer (EPICS XL, Coulter, Hialeath, FL).

### Lymphocyte culture to induce Ag53-specific antibody

T-cell-depleted leukocyte fractions were cultured with autologous or allogeneic Ag53-specific Th cells to induce Ag53-specific IgG antibodies. Freshly isolated T-cell-depleted lymphocytes ($4 \times 10^5$/well) were cultured with Ag53-specific Th cells ($2 \times 10^5$/well) in 48-well plates (Coster, Cambridge, MA) with recombinant Ag53 proteins (final concentration: 5 μg/ml) in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 units/ml of penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (Irvine Scientific, Santa Ana, CA). After 3 days, culture supernatants were collected to measure cytokine levels produced from the cells in the response to Ag53. After 5 days of culture, the medium was changed for fresh medium without antigens and the cells were further cultured. After an additional 9 days, culture supernatants were collected for quantification of the Ag53-specific IgG titer.

### Quantification of Ag53-specific IgG

Ag53-specific IgG antibodies in the donors' sera and the culture supernatants were measured by the enzyme-linked immunosorbent assay (ELISA) according to the method described in our previous study (12). ELISA plates (Dynateck Laboratory, Plochingen, Germany) were coated with 10 ng/μl of purified Ag53 protein in NaH$CO_3$/$Na_2CO_3$ buffer (pH 9.6). After an overnight incubation at 4°C, the plates were washed four times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (0.05% T-PBS: 0.02% $NaN_3$, 136.9 mM NaCl, 1.5 mM $KH_2PO_4$, 8.1 mM $Na_2HPO_4$, 2.7 mM KCl, pH 7.4). The plates were then incubated for 2 h at 37°C after 200-fold diluted sera or undiluted culture supernatants were added to the wells. For the detection of specific IgG, 1 μg/ml biotin-conjugated mouse anti-human IgG (Sigma Chemical Co, St. Louis, MO) was added (2 h, 37°C). After washing with 0.05% T-PBS four times, 1 μg/ml alkaline-phosphatase-labeled streptavidin was added and the plates incubated for 1 h at 37°C. After incubation, the plates were washed four times with 0.05% T-PBS. The plates were incubated for 30 min at 37°C after the substrate was added. The substrate consisted of 2.7 mM p-nitrophenyl phosphate (Sigma) in diethanolamine buffer (9.7% diethanolamine, 0.02% $NaN_3$, 0.5 mM $MgCl_2$, pH 9.8). Absorbance at 405 nm was measured by using Novapath Mini Reader (Bio-Rad Laboratories, Hercules, CA). Titers of Ag53-specific IgG were expressed as arbitrary units on the basis of the pooled standard serum obtained from periodontal healthy subjects.

### Measurement of cytokine production

Antigen-induced cytokine production from the Ag53-specific Th-cell line was assayed by culturing the T cells ($5 \times 10^4$/well) in 96-well flat-bottomed culture plates (Falcon) in the presence of the mixed-overlapping Ag53-derived peptides (0.1 μM each), and 3000 cGy-irradiated autologous PBMCs ($1.5 \times 10^5$/well). After 3 days, the culture supernatants were collected for quantification of cytokines by the IFN-γ ELISA kit (Otsuka, Tokyo, Japan), Quantikine human IL-4 and IL-6 Immunoassay Kits (R & D Systems, Minneapolis, MN), and human IL-5 and IL-10 ELISA kits (Endogen, Woburn, MA) according to the manufacturers' instructions. The sensitivity of each assay was as follows: 10 Pg/ml for IFN-γ, 10 Pg/ml for IL-4, 10 Pg/ml for IL-5, 1 Pg/ml for IL-6, 15 Pg/ml for IL-10.

### Statistical analysis

The correlation between the amounts of Ag53-induced cytokine and serum Ag53-specific IgG titers of each donor was determined by analyzing correlation factors using Student's *t*-test.

The ratio of each Th2 cytokine to IFN-γ (Th2/Th1) in the culture supernatants of Ag53-specific Th-cell lines was calculated, and the differences in the total amounts of the cytokines and in the ratio of Th2/Th1 between the subjects were determined by the Mann–Whitney *U*-test.

## Results

### Serum anti-Ag53 IgG titers of the subjects

First, the serum anti-Ag53 IgG titers of all donors were measured. Each titer was indicated as arbitrary ELISA units assuming that the serum IgG titer of donor #7

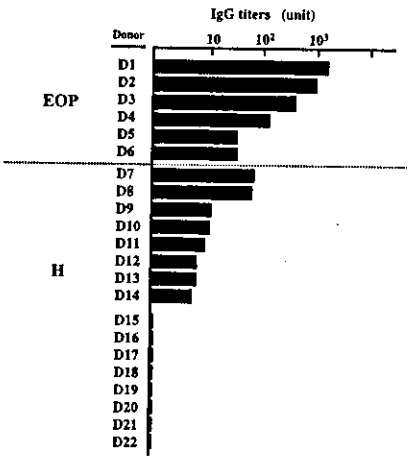


*Fig. 1.* Serum anti-Ag53 IgG titers of all subjects as measured by ELISA and described in Material and methods. Dotted line separates the donors into two groups, a patient group and a healthy control group (D1–D6, early-onset periodontitis patients; D7–D22, periodontal healthy individuals). Each titer was indicated as arbitrary ELISA units assuming that the serum anti-Ag53 IgG titer of donor #7 (D7) was '100' as a standard. The serum anti-Ag53 IgG titers of early-onset periodontitis patients were significantly higher than those of healthy subjects ($P < 0.05$, Mann–Whitney *U*-test). EOP, early-onset periodontitis patients. H, healthy patients.

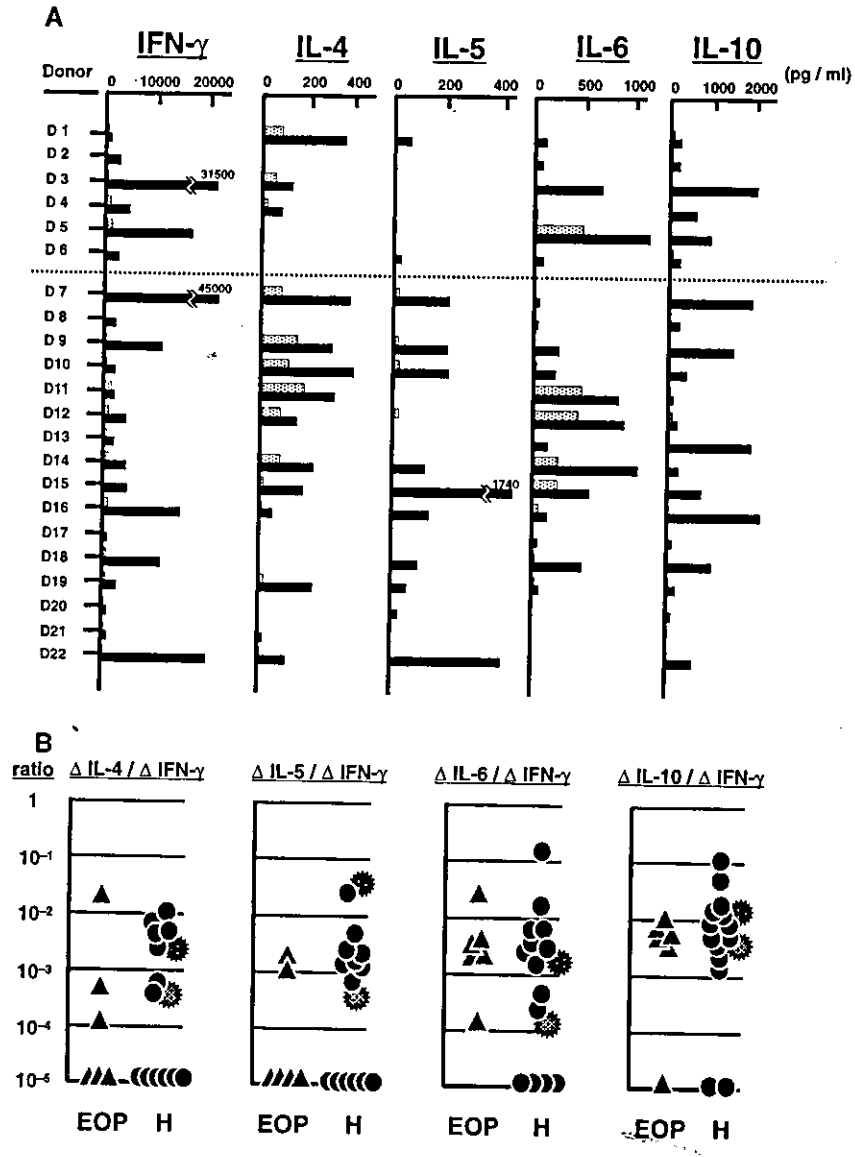


*Fig. 2.* Cytokine profile of Th-cell line specific to Ag53. A) Amounts of cytokine produced from Ag53-specific Th-cell line. The profile of cytokines produced from 22 Ag53-specific Th-cell lines was determined by measuring each cytokine level including IL-4, -5, -6, and -10 and IFN-γ in the supernatants after the culture of the lines with irradiated autologous PBMC pulsed with (■ black bar) or without (▨ speckled bar) Ag53-derived peptides. The amounts of cytokine were indicated as the mean value of duplicate cultures. Patient and healthy control groups are separated by the dotted line (D1–D6, early-onset periodontitis patients; D7–D22, periodontally healthy individuals). B) Th2/Th1 cytokine ratios of Ag53-specific Th-cell line. The ratio of each Th2-type cytokine including IL-4, -5, -6, and -10 to the Th1-type cytokine, IFN-γ, all of which were produced from the Th-cell lines, was calculated and demonstrated. The ratios of all Th2 cytokines to IFN-γ (Th2/Th1) in Th-cell lines established from D15 (●) were significantly higher than those in Th-cell lines from D7 (●) ($P < 0.05$, Mann-Whitney *U*-test). All experiments were done in duplicate, and the mean calculated. Each ratio was calculated following subtraction of the mean value of the produced cytokines in the absence of the antigen from the mean value of the cytokine in the presence of antigen. The experiment was repeated four times and similar results were obtained. The representative data of four separate experiments are shown. EOP, early-onset periodontitis patients. H, healthy patients.

(D7) was '100' as a standard (Fig. 1). Serum anti-Ag53 IgG titers of early-onset periodontitis patients were significantly higher than those of healthy subjects ($P < 0.05$, Mann–Whitney *U*-test).

#### Profiles of cytokines produced from Ag53-specific Th-cell line

The profile of cytokines produced from 22 Ag53-specific Th-cell lines was determined by measuring each cytokine level. These include IL-4, -5, -6, and -10 and IFN-γ in the culture supernatant after the coculture of the cell lines with irradiated autologous PBMC pulsed with Ag53-derived peptides (Fig. 2A). The Th1-type cytokine IFN-γ was produced from all Th-cell lines, whereas the production of Th2-type cytokines greatly varied among the Th-cell lines. The ratio of Th2-type cytokines (IL-4, -5, -6, and -10) to the Th1-type cytokine IFN-γ in each T-cell line was calculated, and is shown in Fig. 2B. The total amounts of each cytokine and the Th2/Th1 ratios did not differ significantly in early-onset periodontitis patients and periodontally healthy subjects. Correlation analysis also indicated that there was no positive or negative relationship between any of the Ag53-induced cytokine levels from T-cell lines and the serum Ag53-specific IgG titers.

#### Ag53-specific Th-cell line with high Th2/Th1 ratio induces production of Ag53-specific IgG

Fortunately, it was found that periodontally healthy donors #7 (D7) and #15 (D15) shared completely matched HLA class II haplotypes (DRB1*0101–DQA1*0101–DQB1*0501/DRB1*0406–DQA1*0301–DQB1*0302), and that the Th-cell line established from these two subjects had quite different cytokine productivity patterns. The ratio of all Th2/Th1 (IL-4/IFN-γ, IL-5/IFN-γ, IL-6/IFN-γ and IL-10/IFN-γ) from D15 was significantly higher than that from D7 ($P < 0.05$, Mann–Whitney *U*-test) (Fig. 2B). Thus, it was suspected that D15 produced higher amounts of Ag53-specific IgG. However, as shown in Fig. 1, the serum IgG titer specific to Ag53 was much higher in D7 than in D15.

To see whether this difference in cytokine profile is actually reflected in Ag53-specific antibody production, we employed the mixed lymphocyte culture system between Ag53-specific Th cells and auto- or allo-derived T-cell-depleted leukocytes from D7 and D15(Fig. 3). When Th cells from D7 showing a lower Th2/Th1 ratio were cultured with T-cell-depleted lymphocytes from either D7 or D15, no Ag53-specific IgG was detected in the culture supernatants. However, Ag53-specific IgG was produced from the T-cell-depleted leukocyte fraction in both D7 and D15 when Th cells from D15 with higher Th2/Th1 ratio were employed, suggesting that the induction of Ag53-specific IgG depends on the Th2/Th1 ratio.

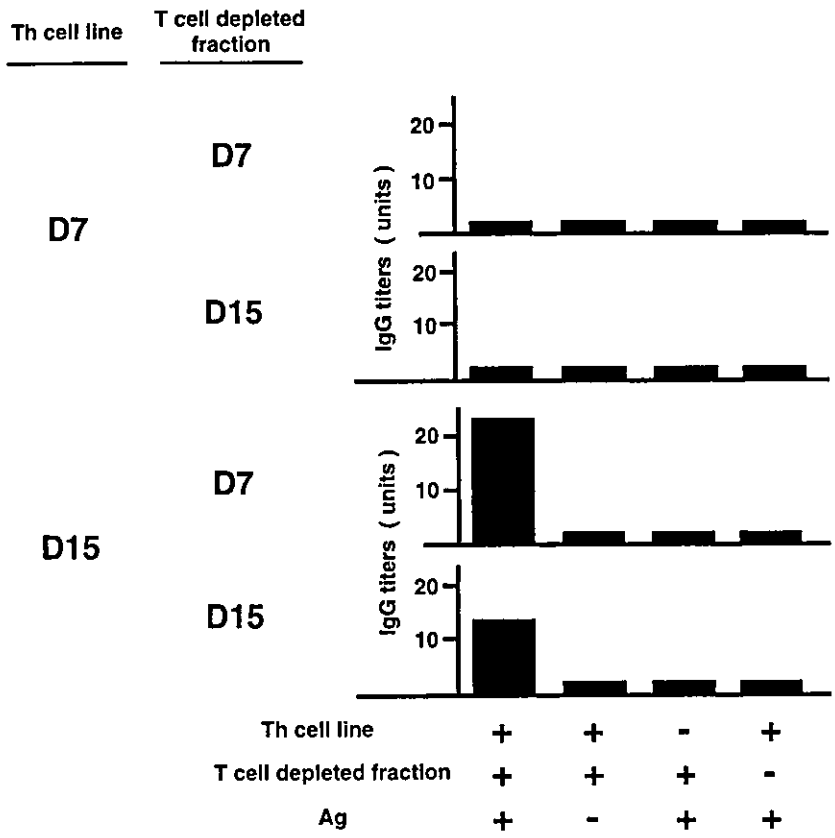


*Fig. 3.* Ag53-specific IgG production induced by the culture of Ag53-specific Th cell line with HLA-matched T-cell-depleted lymphocytes. Ag53-specific Th cells ($2 \times 10^5$/well) from periodontally healthy donors (D7 and D15) were cultured with autologous or allogeneic freshly isolated T-cell-depleted lymphocytes ($4 \times 10^5$/well) in 48-well plates with recombinant Ag53 proteins (final concentration: 5 μg/ml). Culture supernatants were collected at day 14 for quantification of Ag53-specific IgG titer. Each titer was indicated as arbitrary ELISA units assuming that the serum anti-Ag53 IgG titer of D7 was '100' as a standard. Ag, antigen.

We also examined whether PBMCs alone could induce specific antibody against Ag53 when cultured with Ag53. Following *in vitro* culture of PBMCs with the antigen protein, Ag53-specific antibody was not detected in the culture supernatant, even in the culture of PBMCs from the donors who showed a high serum antibody titer against Ag53 (data not shown).

#### Cytokine production during Ag53-specific IgG induction

Besides a high Th2/Th1 ratio, to determine the essential cytokines influencing the Ag53-specific IgG production, we measured cytokine levels in the supernatants after 3-day culture of Th cells with T-cell-depleted lymphocytes, and calculated the ratio of Th2/Th1 cytokines. Of note, no IL-4 was detected in any culture supernatants examined. Higher Th2/Th1 ratios (IL-5/IFN-γ, IL-6/IFN-γ and IL-10/IFN-γ) were observed in cultures where Ag53-specific IgG was produced than in the cultures where it was not (Fig. 4), indicating that IL-5, IL-6 and IL-10 are important cytokines inducing antibody production.

## Discussion

In this study, we established Th-cell lines specific to Ag53 and examined their cytokine profiles in response to Ag53. The comprehensive approach that we have conducted so far on Ag53 (6, 8–10, 13, 14), including the current study, is valuable for obtaining a better understanding of the immune responses specific to the pathogenic antigens. Our present study

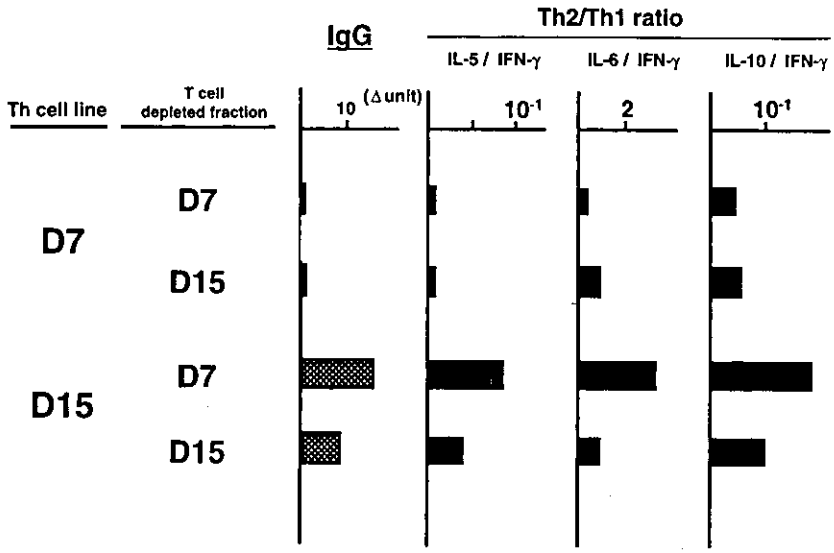


*Fig. 4.* Th2/Th1 ratio of cytokines in the mixed lymphocyte culture between Th cells and auto- or allo-derived T-cell-depleted leukocytes. Ag53-specific Th cells ($2 \times 10^5$/well) were cultured with autologous or allogeneic freshly isolated T-cell-depleted lymphocytes ($4 \times 10^5$/well) in 48-well plates with recombinant Ag53 proteins (final concentration: 5 μg/ml). Culture supernatants were collected on day 3 for quantification of cytokines and the ratio of Th2/Th1 in the culture supernatants of Ag53-specific Th-cell lines were calculated (■ black bar). The amount of Ag53-specific IgG titer was (□ speckled bar) indicated as the mean value of duplicate cultures, after subtraction of the mean value obtained from cultures without antigens. Ag, antigen.

demonstrated that IFN-γ of Th1-type cytokine was produced from all Th-cell lines obtained from periodontitis patients and healthy subjects. The Th1-type immune response plays an important role in the protection against several intracellular bacteria (5, 16). In fact, in recent studies, it was reported that *P. gingivalis* can actually invade oral epithelial cells *in vitro* or in the organ culture system (17, 18). Thus, Ag53-specific T-cell subsets exhibiting IFN-γ production in response to Ag53 may contribute to the development of specific cellular immunity against *P. gingivalis* infection. This speculation appears reasonable, as Ag53 has been observed on the vesicle surface of *P. gingivalis* (10).

We also demonstrated that Th2-type cytokine production varied in the response to Ag53. Although IL-4 and IL-5 were detected in the culture supernatants of only a limited number of subjects, IL-6 and IL-10 were frequently detected in the supernatants of most subjects. Since IL-6 and IL-10 are cytokines produced from both T cells and monocytes, the observed results in this study may comprise the total amount of the cytokines produced by both types of the cells. In the experimental systems employed in this study, it seemed difficult to distinguish exactly how much cytokine is produced by specific cell types. Further studies will be needed, such as treating the PBMCs with the protein synthesis inhibitor, e.g. emetine, when using these cells as antigen-presenting cells.

A major determinant of the development of either the Th1- or Th2-type cytokine profile is the combination of cytokines produced locally, as these cytokines not only promote each type of immunity but also suppress each other's activity. A predominance of IL-10 favors the development of Th2 cells and suppresses the expansion of Th1 cells producing IFN-γ. In our present study, a high amount of IFN-γ was often detected in the culture supernatants that also contained high amounts of IL-10. Therefore, the results are contradictory. However, we measured the amount of cytokine in the supernatants collected after short-term culture of the T cells, and therefore did not observe the polarized profile. With long-term culture, it is possible to observe the polarized cytokine profile in the culture supernatants, as either cytokine may dominate and promote either type of immunity.

It has been reported that in subjects exposed to antigens, including ovalbumin, tetanus toxoid and filarial parasite (7, 22, 24), and subjects vaccinated against influenza virus and poliovirus (20, 23, 25) their PBMCs are easily able to produce the antigen-specific antibodies following culture with each antigen. In terms of Ag53, following *in vitro* culture of PBMCs with the antigen protein, antigen-specific antibody was not detected in the culture supernatant, even in the culture of PBMCs from the donors who exhibited a high serum antibody titer against Ag53 (data not shown). However, it became possible to detect Ag53-specific IgG by employing the culture system of T-cell-depleted lymphocytes with Ag53-specific Th-cell line with a high Th2/Th1 ratio. This finding suggests that T-cell activation is required for the induction of antigen-specific antibody production. The memory T cells specific to Ag53 did not appear to be activated in the culture of PBMCs with antigen protein; the number of clones of T cells specific to Ag53 might be either too small (if present at all) to activate themselves in such an *in vitro* situation. To overcome this problem, we established an Ag53-specific Th-cell line and used it to study *in vitro* Ag53-specific IgG induction. This also has the advantage that it minimizes individual differences in the number of T-cell clones specific to Ag53 from their previous immune exposure to Ag53.

It was likely that whether Ag53 specific IgG was produced depends upon T-cell phenotypes. When the T-cell line showing a high Th2/Th1 ratio was cultured with the T-cell-depleted leukocyte fraction, IgG antibody production was detected in the culture supernatant. However, the T-cell line with a low Th2/Th1 ratio did not have the ability to induce IgG production. Thus, the Th2/Th1 ratio of Th cells may be a factor regulating the Ag53-specific IgG production in response to Ag53.

With respect to Ag53-specific IgG production, we obtained contrary results between *in vitro* assays and serum data. In *in vitro* assays, Ag53-specific IgG production was only induced when Th cells showing a higher ratio of Th2/Th1 were cultured with the T-cell-depleted leukocyte fraction. Because the assays were performed in a closed space, cytokines produced from Th cells could easily have influenced the T-cell-depleted leukocyte fraction, causing it to induce specific IgG. In addition, high amounts of Ag53 were added to the cultures. Thus, one can consider these *in vitro* assays to be a model of immunological events occurring in local periodontal lesions. However, in the case of serum IgG, the elevation of serum antibody level may require a certain amount of infection with particular antigens. We could not detect serum IgG specific to Ag53 in D15. However, if D15 is infected with Ag53, we believe that the level of serum IgG specific to Ag53 would easily elevate.

Recently, it has been reported that B-cell proliferative responses are observed in the presence of *P. gingivalis* and IL-10 (21), a phenomenon known as polyclonal B-cell activation, leading to elevated numbers of the B cells in periodontal lesions. Polyclonal B-cell activation induced by periodontopathic bacteria has been suggested to contribute to an increase in IgG antibodies in periodontal lesions (2). In the present study, however, Ag53-specific IgG production was not observed in the culture without the help of Th cells. Thus the antibody production against Ag53 occurred in an immunologically antigen-specific manner, and was not simply a result of polyclonal B-cell activation.

To distinguish the possible cytokines affecting the Ag53-specific IgG production, we evaluated the amounts of several cytokines in the culture supernatants. Surprisingly, IL-4 was not detected under our experimental conditions and may therefore not be essential for the induction of Ag53-specific antibody production. Production in the absence of IL-4 could be due to several factors. For example, endogenously produced IL-6 and IL-10 are involved in the differentiation of CD40-activated B cells and production of immunoglobulins (1), and IL-13 can also compensate the function of IL-4 (11, 15).

In conclusion, we examined cytokine profiles of Ag53-specific Th-cell lines and their effects on Ag53-specific IgG production in response to Ag53. The data suggest that differences in the profile of Th2-type cytokine may explain individual differences in antibody titer against Ag53, and that differences in the ratio of Th2/Th1 cytokines produced from Th cells regulate the production of Ag53-specific IgG.

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RESEARCH REPORTS
Biological

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# Thiazolidinedione (Pioglitazone) Blocks *P. gingivalis*- and *F. nucleatum*, but not *E. coli*, Lipopolysaccharide (LPS)-induced Interleukin-6 (IL-6) Production in Adipocytes

## ABSTRACT

An elevated level of C-reactive protein (CRP) predicts the future development of coronary heart disease. Periodontitis appears to up-regulate CRP. CRP is produced by hepatocytes in response to interleukin-6 (IL-6). A major source of IL-6 in obese subjects is adipocytes. We hypothesized that lipopolysaccharide (LPS) from periodontal pathogens stimulated adipocytes to produce IL-6, and that the production was suppressed by the drugs targeted against insulin resistance, thiazolidinedione (pioglitazone), since this agent potentially showed an anti-inflammatory effect. Mouse 3T3-L1 adipocytes were stimulated with *E. coli*, *P. gingivalis*, and *F. nucleatum* LPS. The IL-6 concentration in culture supernatants was measured. All LPS stimulated adipocytes to produce IL-6. Although pioglitazone changed adipocyte appearance from large to small, and completely suppressed *P. gingivalis* and *F. nucleatum* LPS-induced IL-6 production, *E. coli* LPS-induced IL-6 production was not efficiently blocked. Thus, pioglitazone completely blocked periodontal-bacteria-derived LPS-induced IL-6 production in adipocytes, a major inducer of CRP.

**KEY WORDS:** diabetes, adipocyte, insulin resistance, thiazolidinedione, interleukin-6.

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## INTRODUCTION

Diabetic subjects develop coronary heart disease more frequently as compared with non-diabetic subjects (Bierman, 1992). An elevated level of C-reactive protein (CRP), although often, for the most part, in a healthy reference range, has been reported to predict the future development of coronary heart disease (Ridker *et al.*, 1997). Although it is still inconclusive as to whether elevation of CRP is simply a sensitive marker of inflammation, or whether increased circulating CRP directly exhibits pathological mechanisms promoting vascular disorders, there is growing evidence that CRP directly participates in the pathogenesis of vascular disorders *via* several distinct mechanisms (Rattazzi and Kushner, 2003). CRP is produced by hepatocytes in response to interleukin-6 (IL-6) (Gabay and Kushner, 1999). One of the major sources of IL-6 is believed to be adipose tissue in obese and/or obese-diabetic subjects (Coppack, 2001). Obesity is strongly associated with increased insulin resistance. In this connection, peroxisome proliferator-activated receptor (PPAR) activator, such as thiazolidinedione, has been demonstrated to improve insulin sensitivity and is currently being widely used for therapeutic purposes in obese-diabetic subjects (Willson *et al.*, 2001). The hallmark of the pharmacological action of thiazolidinedione appears to be to increase the number of small, premature adipocytes without changing the adipose tissue mass (Okuno *et al.*, 1998). In fact, thiazolidinedione has been reported to lower the expression of tumor necrosis factor-α (TNF-α)—an important adipocytokine responsible for insulin resistance—in adipocytes (Okuno *et al.*, 1998). However, the effect of thiazolidinedione on IL-6 production in adipocytes has not yet been well-addressed.

Recent epidemiological studies have suggested that periodontal disease is associated with enhanced atherogenesis (Beck *et al.*, 1998). CRP values are elevated in severe periodontitis patients (Slade *et al.*, 2000; Noack *et al.*, 2001; Nishimura *et al.*, 2002), as measured by highly sensitive assays, and decrease with therapy (Iwamoto *et al.*, 2003). In this study, therefore, we hypothesized that (1) bacterial lipopolysaccharide (LPS) derived from periodontal pathogens stimulated adipocytes to produce IL-6, and (2) thiazolidinedione would suppress LPS-induced IL-6 production in adipocytes.

## MATERIALS & METHODS

### Preparation of Bacterial LPS

Bacterial LPS from 3 distinct micro-organisms were used. *Porphyromonas gingivalis* FDC381 LPS and *Fusobacterium nucleatum* ATCC 25586 LPS were prepared by hot-phenol water extraction as described previously (Onoue *et al.*,

2003), while *E. coli* LPS was purchased from Sigma (St. Louis, MO, USA).

### Cells and Culture Conditions

Cultured mouse 3T3-L1 cells were differentiated to mature adipocytes as described previously (Bernlohr *et al.*, 1984). Briefly, ATCC 3T3-L1 cells were cultured in growth medium containing Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc., Gaithersburg, MD, USA) and 10% bovine calf serum (SIGMA), with a change of medium every 2 days. Two-day-post-confluent cells were switched to differentiation medium (DMEM, 10% fetal calf serum, 1 μM dexamethasone, 10 μg/mL insulin, and 0.5 mM 3-methyl-1-isobutylxanthin [Sigma]) for 2 days. Thereafter, the cells were cultured in post-differentiation medium (DMEM, 10% fetal calf serum, and 10 μg/mL insulin), and the medium was changed every 2 days. We confirmed differentiation and maturation of adipocytes by staining the cells with oil red O (Sigma). Following confirmation of full maturation, these cells were cultured with indicated concentration of thiazolidinedione. Pioglitazone (Actos), which was a generous gift from Takeda Pharmaceuticals (Osaka, Japan), was used as the thiazolidinedione. The changes in the appearance of adipocytes by thiazolidinedione were observed by microscopy. To quantify the changes in adipocyte appearance, we manually traced adipocyte size (diameter) on digital photographs at high power (400x). In a second experiment, the cells were challenged with each bacterial LPS at indicated concentrations. To see the effect of thiazolidinedione on LPS-induced IL-6 production, we co-incubated LPS-challenged adipocytes with pioglitazone. Twenty-four and 48 hrs after LPS stimulation, culture supernatants were collected. IL-6 concentrations in culture supernatants were measured with the use of a commercial sandwich ELISA kit (R&D Systems, Minneapolis, MN, USA). All experiments were performed in triplicate, and the results from these triplicate experiments were subjected to statistical analysis.

### Statistical Analysis

Significance of the difference in IL-6 production in each culture condition was determined by Student's *t* test.

## RESULTS

### Thiazolidinedione Changes Adipocyte Appearance

The effect of thiazolidinedione on changes in adipocyte appearance was examined first. After we confirmed the maturation of adipocytes by staining the cells with oil red O (Fig. 1A), we co-cultured the cells with the indicated concentrations of pioglitazone. Pioglitazone changed adipocyte size from large to small in a dose-dependent manner (Fig. 1B). These changes were observed at 1 μM of pioglitazone. To quantify these changes, we compared the diameters of oil-red-stained cells (Fig. 1C). Both 1 and 10 μM of pioglitazone reduced adipocyte diameter by 40 to 50% ($p < 0.01$; Student's *t* test).

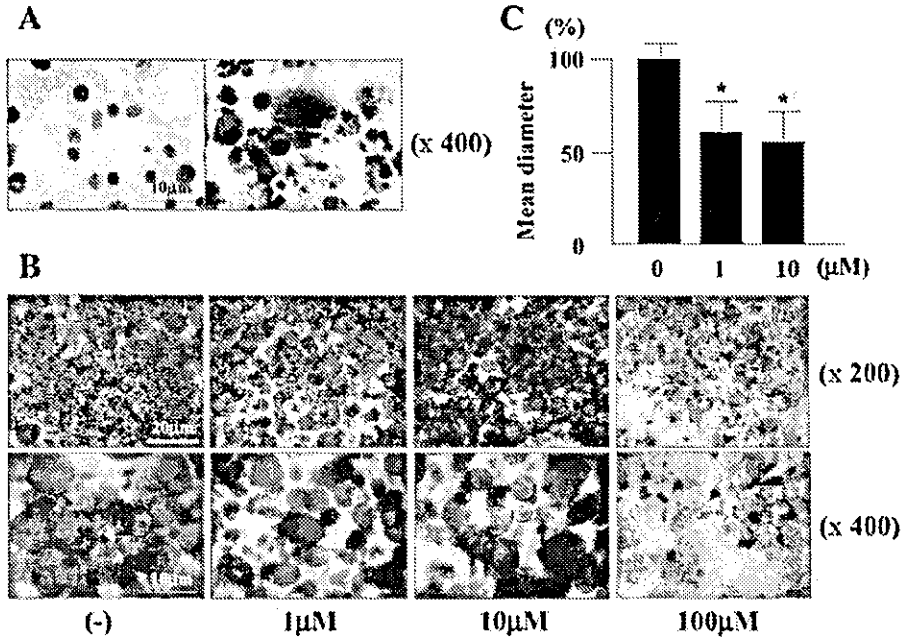


Figure 1. The effect of pioglitazone on the morphology of mouse 3T3-L1 adipocytes. Mouse 3T3-L1 adipocytes were differentiated into mature adipocytes as in MATERIALS & METHODS. We confirmed maturation by staining the cells with oil red O (A: right panel). When more than 95% of the cells were stained with oil red O, similarly prepared cells were co-incubated with indicated concentrations of pioglitazone. Twenty-four hrs later, the cells were stained with oil red O, and the morphology of the cells was observed by microscopy. Pioglitazone changed adipocyte appearance from large to small size (B). For comparison, the diameters of 10 randomly selected oil-red-positive cells *per* high-power field (400x) were measured and compared (C). The data were expressed as % diameter of the cells against the mean diameter (N = 10) of control (untreated) cells. The mean diameter of adipocytes significantly decreased by 1 μM and 10 μM of pioglitazone (100.0 ± 11.3% for pioglitazone-untreated cells, 61.7 ± 17.7% for 1 μM pioglitazone-treated cells, 54.3 ± 18.8% for 10 μM pioglitazone-treated cells, N = 10 for all groups). *$p < 0.01$, compared with the cells without pioglitazone.

### LPS Increased Il-6 Production in Adipocytes

Next, we examined the effects of bacterial LPS on IL-6 production in adipocytes (Figs. 2, 3, 4). All LPS at a concentration of 10 ng/mL or more significantly stimulated adipocytes to produce IL-6, as compared with control (medium alone) (Figs. 2, 3, 4; $p < 0.01$). *E. coli* LPS at a concentration of 1 ng/mL also significantly stimulated adipocytes to produce IL-6 (Fig. 2; $p < 0.0001$), while *P. gingivalis* LPS with 1 ng/mL did not show any effect on IL-6 production (Fig. 3). Thus, *E. coli* LPS showed the highest IL-6 productivity.

### Thiazolidinedione Completely Blocks *P. gingivalis* and *F. nucleatum* LPS-induced IL-6 Production in Adipocytes

We then examined the effect of thiazolidinedione on LPS-stimulated IL-6 production in adipocytes (Figs. 2, 3, 4). Although pioglitazone significantly suppressed *E. coli* LPS-induced IL-6 production in adipocytes, the suppression was not complete. About 30% reduction was observed ($p < 0.005$), as compared with the condition with LPS stimulation alone (Fig.

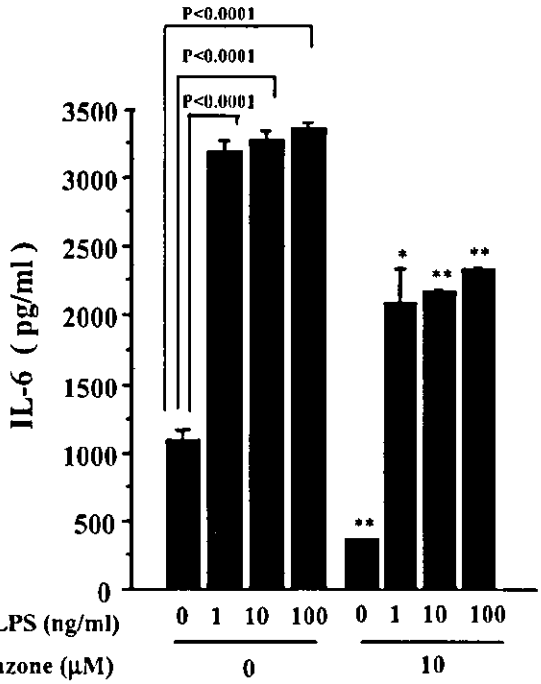


**Figure 2.** The effects of *E. coli* LPS on IL-6 production, and of pioglitazone on *E. coli* LPS-induced IL-6 production in adipocytes. Mouse 3T3-L1 adipocytes were differentiated in 24-well tissue culture plates as described in MATERIALS & METHODS. After confirming the maturation of adipocity by staining the cells with oil red O, we stimulated the cells with indicated concentrations of *E. coli* LPS. In some cultures, the cells were co-incubated with 10 μM of pioglitazone. Twenty-four hrs later, the cell-culture supernatants were harvested, and the IL-6 concentration was measured by ELISA. All experiments were done in triplicate, and statistical differences were calculated by Student's *t* test. In pioglitazone-treated cells, IL-6 production was compared with that of untreated cells when stimulated with the identical concentration of *E. coli* LPS. Mean IL-6 concentration ± standard deviation in each culture condition was calculated (1094.2 ± 68.6 pg/mL for LPS-unstimulated cells, 3189.7 ± 77.7 for 1 ng/mL of LPS-stimulated cells, 3271.5 ± 65.0 pg/mL for 10 ng/mL of LPS-stimulated cells, 3353.3 ± 53.2 pg/mL for 100 ng/mL of LPS-stimulated cells, 365.1 ± 5.0 pg/mL for LPS-unstimulated cells co-incubated with pioglitazone, 2087.4 ± 250.0 pg/mL for 1 ng/mL of LPS-stimulated cells co-incubated with pioglitazone, 2181.5 ± 16.0 pg/mL for 10 ng/mL of LPS-stimulated cells co-incubated with pioglitazone, and 2331.9 ± 11.8 pg/mL for 100 ng/mL of LPS-stimulated cells co-incubated with pioglitazone, N = 3 for all groups). *$p < 0.005$ and **$p < 0.0001$, compared with the cells stimulated with the same concentration of LPS in the absence of pioglitazone.

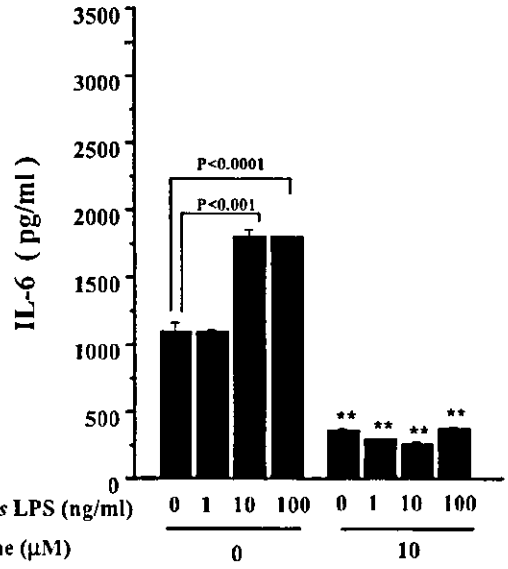


**Figure 3.** The effects of *P. gingivalis* LPS on IL-6 production, and of pioglitazone on *P. gingivalis* LPS-induced IL-6 production in adipocytes. Mouse 3T3-L1 adipocytes were differentiated in 24-well tissue culture plates as described in MATERIALS & METHODS. After confirming maturation of adipocity by staining the cells with oil red O, we stimulated the cells with indicated concentrations of *P. gingivalis* LPS. In some cultures, the cells were co-incubated with 10 μM of pioglitazone. Twenty-four hrs later, the cell-culture supernatants were harvested, and the IL-6 concentration was measured by ELISA. All experiments were done in triplicate, and statistical differences were calculated by Student's *t* test. In pioglitazone-treated cells, the IL-6 production was compared with that of untreated cells when stimulated with the identical concentration of *P. gingivalis* LPS. Mean IL-6 concentration ± standard deviation in each culture condition was calculated (1094.2 ± 68.6 pg/mL for LPS-unstimulated cells, 1097.0 ± 13.2 pg/mL for 1 ng/mL of LPS-stimulated cells, 1796.9 ± 57.7 pg/mL for 10 ng/mL of LPS-stimulated cells, 1794.7 ± 8.2 pg/mL for 100 ng/mL of LPS-stimulated cells, 365.1 ± 5.0 pg/mL for LPS-unstimulated cells co-incubated with pioglitazone, 299.2 ± 1.8 pg/mL for 1 ng/mL of LPS-stimulated cells co-incubated with pioglitazone, 267.9 ± 4.1 pg/mL for 10 ng/mL of LPS-stimulated cells co-incubated with pioglitazone, and 372.4 ± 9.5 pg/mL for 100 ng/mL of LPS-stimulated cells co-incubated with pioglitazone, N = 3 for all groups). **$p < 0.0001$, compared with the cells stimulated with the same concentration of LPS in the absence of pioglitazone.

2). In contrast, pioglitazone completely blocked *P. gingivalis* and *F. nucleatum* LPS-induced IL-6 production (Figs. 3, 4; $p < 0.0001$ at all LPS concentrations). Pioglitazone also suppressed basal IL-6 production (without LPS stimulation) at the $p < 0.0001$ level of significance.

In a separate experiment, we measured IL-6 production from the cells incubated with LPS for 48 hrs. The results were very similar to those obtained from 24-hour incubation, suggesting that IL-6 production occurred during the first 24 hrs (data not shown).

## DISCUSSION

Mild elevation of CRP has been suggested to be a sensitive marker for predicting the future development of coronary heart disease (Ridker *et al.*, 1997). This may detect early but already ongoing vascular inflammation. However, recent studies have demonstrated that CRP is not merely a sensitive marker but also actively participates in the progression of atherosclerosis by binding to LDL-cholesterol, which makes the LDL-cholesterol-CRP complex more easily taken up by macrophages, leading to the formation of foam cells. CRP also stimulates endothelial cells to express adhesion molecules as intercellular adhesion molecule-1, which makes vascular endothelium easily bind to inflammatory cells. CRP promotes smooth-muscle-cell proliferation and chemotaxis, both of which promote atherosclerotic changes (for review, see Rattazzi *et al.*, 2003).

CRP is produced by hepatocytes in response to IL-6. The major source of IL-6 in obese diabetic subjects is adipocytes, since obese subjects are characterized by increased circulating IL-6, and successful weight loss results in

decreased serum IL-6 concentration (Ziccardi *et al.*, 2002). Several reports have demonstrated that periodontal infection also up-regulates CRP (Slade *et al.*, 2000; Noack *et al.*, 2001; Nishimura *et al.*, 2002), and periodontal treatment decreases CRP (Iwamoto *et al.*, 2003). These observations suggest that severe periodontal infection results in increased IL-6 synthesis in the liver, to produce CRP. Although a previous study had reported that periodontal disease was associated with elevated circulating IL-6 concentration, the elevation was very weak and might be insufficient to stimulate hepatocytes to produce CRP (Loos *et al.*, 2000). Therefore, we hypothesized that the source of IL-6 in severe periodontitis patients should be either Kupfer cells in the liver and/or adipocytes, in the case of fatty liver. In fact, most circulating antigens are cleared in the liver by means of so-called 'hepatic clearance'. Thus, we hypothesized that infected antigens are concentrated in the liver, and that the concentration was higher than in other organs. It has been suggested that the circulating LPS concentration in some periodontitis subjects is more than 1 ng/mL (Vilkuna-Rautiainen *et al.*, 2003, unpublished observations). Therefore, at least in the liver, the LPS concentration should be higher than this concentration.

Although a previous study has suggested that adipocytes express both toll-like receptors (TLR)-2 and -4 (Lin *et al.*, 2000), it is still unclear whether the adipocytes produce IL-6 in response to bacterial LPS *via* such receptors. Therefore, in this study, we chose *E. coli* and *F. nucleatum* LPS, both of which bind TLR-4, and *P. gingivalis* LPS, whose action appears to be mediated through TLR-2 (Bainbridge and Darveau, 2001). The results indicated that activation of both TLR-2 and -4 in adipocytes induced IL-6 production, with higher productivity *via* the TLR-4-mediated pathway.

Although precise pharmacological actions of PPAR activators have not yet been fully elucidated, some previous studies have suggested that they suppress NF-κB activity by competing with the binding proteins essential for nuclear translocation (Ruan *et al.*, 2003) or by inhibiting the transcriptional regulatory functions of NF-κB (Chinetti *et al.*, 1998). Therefore, PPAR activator effectively suppresses tumor necrosis factor-α (TNF-α) production in adipocytes, an important adipocytokine responsible for insulin resistance (Uysal *et al.*, 1997). Thus, the improvement of insulin sensitivity by thiazolidinedione is at least partially explained by decreased TNF-α synthesis in adipose tissues. Moreover, thiazolidinedione has been suggested to exhibit anti-inflammatory properties (Ricote *et al.*, 1998), and to be effective not only in patients with type 2 diabetes but also in immune-related diseases such as rheumatoid arthritis (Oates *et al.*, 2002). Since monocytes from diabetic subjects were reported to over-produce TNF-α, and since this over-production has been suggested to be one of the mechanisms responsible for enhanced periodontal tissue breakdown in such subjects (Salvi *et al.*, 1997), thiazolidinedione would have an additive effect in preventing the progression of periodontal disease itself in patients with diabetes. Such a clinical study would be interesting, since this reagent has already been marketed clinically.

In conclusion, pioglitazone completely blocked periodontal-bacteria-derived LPS-induced IL-6 production in adipocytes, a major inducer of CRP.

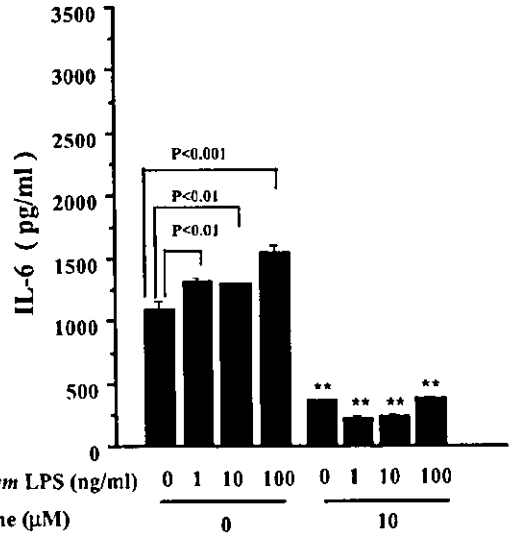


**Figure 4.** The effects of *F. nucleatum* LPS on IL-6 production, and of pioglitazone on *F. nucleatum* LPS-induced IL-6 production in adipocytes. Mouse 3T3-L1 adipocytes were differentiated in 24-well tissue culture plates as described in MATERIALS & METHODS. After confirming maturation of adipocity by staining the cells with oil red O, we stimulated the cells with indicated concentrations of *F. nucleatum* LPS. In some cultures, the cells were co-incubated with 10 μM of pioglitazone. Twenty-four hrs later, the cell-culture supernatants were harvested, and the IL-6 concentration was measured by ELISA. All experiments were done in triplicate, and the statistical differences were calculated by Student's *t* test. In pioglitazone-treated cells, the IL-6 production was compared with that of untreated cells when stimulated with the identical concentration of *F. nucleatum* LPS. Mean IL-6 concentration ± standard deviation in each culture condition was calculated (1094.2 ± 68.6 pg/mL for LPS-unstimulated cells, 1308.3 ± 33.6 pg/mL for 1 ng/mL of LPS-stimulated cells, 1283.8 ± 6.4 pg/mL for 10 ng/mL of LPS-stimulated cells, 1552.9 ± 42.7 pg/mL for 100 ng/mL of LPS-stimulated cells, 365.1 ± 5.0 pg/mL for LPS-unstimulated cells co-incubated with pioglitazone, 223.7 ± 11.8 pg/mL for 1 ng/mL of LPS-stimulated cells co-incubated with pioglitazone, 236.0 ± 5.9 pg/mL for 10 ng/mL of LPS-stimulated cells co-incubated with pioglitazone, and 383.8 ± 5.5 pg/mL for 100 ng/mL of LPS-stimulated cells co-incubated with pioglitazone, N = 3 for all groups). **$p < 0.0001$, compared with the cells stimulated with the same concentration of LPS in the absence of pioglitazone.

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