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Participation of glutathione in the elimination of *Porphyromonas gingivalis in vivo*

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Introduction: Glutathione is involved in immune responses such as cell proliferation and bactericidal activity. The aim of this study was to see whether glutathione influences the intraperitoneal elimination of *Porphyromonas gingivalis* in *Fusobacterium nucleatum-immunized* mice.
Methods: Mice were immunized with *P. gingivalis* or *F. nucleatum*, and then *P. gingivalis* was inoculated into the peritoneal cavity of the mice. After various lengths of time, the numbers of bacteria were determined by a colony-forming assay and by polymerase chain reaction. The effect of glutathione on the elimination of *P. gingivalis* was explored by changing the intracellular glutathione level. Furthermore, we examined the effects of glutathione on the peritoneal levels of interferon-γ, a macrophage activator, and of nitrite, a derivative of nitric oxide that acts as an antimicrobial agent when produced by macrophages and neutrophils.
Results: Inoculated *P. gingivalis* was eliminated more rapidly from *F. nucleatum*-immunized mice than from *P. gingivalis*-immunized mice. Interferon-γ levels in peritoneal lavage fluid and glutathione levels in peritoneal exudate cells were higher in *F. nucleatum*-immunized mice than in *P. gingivalis*-immunized mice. When *P. gingivalis*-immunized mice were given glutathione monoethylester (a derivative of glutathione that is converted to glutathione intracellularly through hydrolysis) into the peritoneal cavity, the elimination of *P. gingivalis* was accelerated. On the other hand, when *F. nucleatum*-immunized mice were given L-buthionine-[*S*,*R*]-sulfoximine (an inhibitor of glutathione synthesis) into the peritoneal cavity, the elimination of *P. gingivalis* was suppressed.
Conclusion: In *F. nucleatum*-immunized mice, glutathione may have a key role in the defense against *P. gingivalis* infections.

Key words: elimination; *Fusobacterium nucleatum*; glutathione; *Porphyromonas gingivalis*

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Periodontal disease is caused by anaerobic bacteria in subgingival plaque. *Porphyromonas gingivalis* is a gram-negative, anaerobic rod that is recognized as the main pathogen associated with adult periodontitis (22). *Fusobacterium nucleatum* is a gram-negative, obligatorily anaerobic, spindle-shaped rod that is frequently isolated from the subgingival plaque of patients with periodontal disease (12–15).

In previous experiments, neutrophils that were derived from non-immunized mice phagocytosed *P. gingivalis* and *F. nucleatum* under anaerobic conditions *in vitro*, but they did not kill the bacteria (17). Furthermore, serum from mice immunized with *P. gingivalis* or *F. nucleatum* had no bactericidal activity by itself and no effect on the bactericidal activity of neutrophils (17).

There are some reports about T-cell responses in mice infected with these bacteria. *P. gingivalis* infection predominantly induces T helper type 1 (Th1) responses and *F. nucleatum* infection induces Th2 responses (3, 4, 6, 7). On the other hand, our previous study showed that higher Th2 responses were demonstrated in mice immunized with *P. gingivalis* compared with mice immunized with

F. nucleatum. In *P. gingivalis*-immunized mice, the level of interleukin-4 (IL-4) in lesions was significantly higher than in *F. nucleatum*-immunized mice, but the levels of IL-12 and interferon-γ (IFN-γ) were significantly lower than in *F. nucleatum*-immunized mice (10).

Glutathione is the most abundant non-protein sulfhydryl-containing compound present in eukaryotic cells and is involved in immune responses. Glutathione plays a role as an antioxidant for reactive oxygen species and intracellular glutathione levels decrease in activated neutrophils (1). Glutathione regulates activation-dependent proliferation of human T lymphocytes (24) and large granular lymphocytes (21). Glutathione levels in antigen-presenting cells play a major role in determining whether Th1 or Th2 cytokine response patterns predominate (16, 19). Macrophages with high contents of intracellular glutathione enhance leishmanicidal activity (2). In addition, the growth of mycobacteria in macrophages is controlled by intracellular levels of glutathione and nitric oxide (26). However, the relationship between the lesional glutathione level and the survival of periodontal bacteria after infection has not been clarified.

In the present study, we examined the effect of glutathione on *P. gingivalis* elimination from the peritoneal cavity of *F. nucleatum*-immunized and *P. gingivalis*-immunized mice using drugs that modulate intracellular glutathione levels, glutathione monoethylester (GSH-OEt) and L-buthionine-[*S*,*R*]-sulfoximine (BSO). GSH-OEt is a cell-permeable derivative of glutathione and undergoes hydrolysis by intracellular esterases, thereby increasing the intracellular level of glutathione. BSO is a specific inhibitor of glutathione synthesis and causes depletion of intracellular glutathione. We also measured the peritoneal levels of IFN-γ, a cytokine promoting macrophage activation, and nitrite, a derivative of nitric oxide that acts as an antimicrobial agent when produced by macrophages. We used a low virulence strain of *P. gingivalis*, FDC381, because we were concerned about causing the early death of mice through the use of a high virulence strain that might be obstructive for the observation of defense responses in mice.

Materials and methods

Bacteria

P. gingivalis FDC381 and *F. nucleatum* ATCC25586 were cultured in Gifu anaerobic medium (Nissui Pharmaceutical Co., Tokyo, Japan) under anaerobic conditions using an AnaeroPack Kenki (Mitsubishi Gas Chemical Co., Tokyo, Japan) for 48 h at 37°C. The bacteria were harvested by centrifugation and washed twice with phosphate-buffered saline (PBS). The bacterial cells were resuspended in PBS to an optical density at 540 nm of 1.5, corresponding to approximately 1×10^{10} and 1×10^{9} colony-forming units (CFU) per ml of *P. gingivalis* and *F. nucleatum*, respectively. The suspensions were stored in anaerobic conditions at room temperature until use.

Mice

Specific pathogen-free 6- to 7-week old male ICR mice were purchased from SLC Co. (Shizuoka, Japan) and housed under conventional conditions. All animal procedures were approved by the Laboratory Animal Committee of The Nippon Dental University School of Life Dentistry at Niigata.

Immunization of mice

Mice were immunized with either *P. gingivalis* or *F. nucleatum*. Briefly, mice were injected with 2×10^{9} CFU of *P. gingivalis* or 2×10^{8} CFU of *F. nucleatum* in 0.2 ml PBS, twice intraperitoneally and once subcutaneously at weekly intervals. Two weeks after the third injection, mice were used for the experiments. None of the mice died during the immunization period.

P. gingivalis elimination assay in mouse peritoneal cavity and sample preparation

Both immunized mice and control mice were injected intraperitoneally with 2×10^{9} CFU of *P. gingivalis* in 0.2 ml PBS and sacrificed after various times. Then, the mouse peritoneal cavity was lavaged with 2 ml PBS. The peritoneal lavage fluid was collected and a 20-μl aliquot was immediately used in a colony-forming assay of *P. gingivalis*. The remaining fluid was centrifuged at 400 ***g*** for 5 min. The supernatant was stored at −80°C until used for the determination of nitrite and IFN-γ. The cell pellet (peritoneal exudate cells) was resuspended in 1 ml PBS and stained with Türk solution (Wako Pure Chemical Industries Ltd., Osaka, Japan). Total and differential cell counts were performed using a Bürker–Türk hemocytometer. Leukocyte cell types (neutrophils, lymphocytes, and macrophages) were determined based on nuclear staining and size.

Colony-forming assay

A 20-μl aliquot of the peritoneal lavage fluid was mixed with 180 μl 0.4% Tween-20, and the mixture was pipetted for 1 min to lyze the peritoneal exudate cells. Serial 10-fold dilutions of the lysate containing *P. gingivalis* ingested in peritoneal exudate cells were made in sterile Gifu anaerobic medium, and 10 μl of each dilution was spread on Anaero Columbia RB plates (Nippon Becton Dickinson Ltd., Tokyo, Japan). The plates were incubated under anaerobic conditions at 37°C for 5 days and the colonies were counted. The relative number of CFU was calculated as the CFU at each time-point divided by the CFU at 0 h.

Detection of *P. gingivalis* DNA in peritoneal exudate cells by polymerase chain reaction

P. gingivalis that were ingested by peritoneal exudate cells were detected by polymerase chain reaction (PCR) using specific primers for the 16S ribosomal RNA gene. Mouse and bacterial genomic DNA was isolated from peritoneal exudate cells (2×10^{6} cells) using a Wizard genomic DNA purification kit (Promega, Madison, WI) according to the manufacturer's instructions. The DNA was dissolved in 20 μl of 10 mM Tris–HCl, 1 mM ethylenediaminetetraacetic acid (pH 7.5). The PCR mixture (25 μl) contained 1 μl DNA, 1 × PCR buffer (Finnzyme OY, Espoo, Finland), 0.5 units Tbr EXT DNA polymerase (Finnzyme), 0.2 mM each of dATP, dCTP, dGTP, and dTTP, and 0.5 μM each of *P. gingivalis* 16S ribosomal RNA gene-specific primers (forward; 5′-TGT AGA TGA CTG ATG AAA ACC-3′, reverse; 5′-ACG TCA TCC CCA CCT TCC TC-3′, amplicon size, 197 base pairs) (25). The PCR was performed in a GeneAmp PCR System 9600 (Applied Biosystems, Foster City, CA). After an initial denaturation at 94°C for 5 min, the amplification was performed for 28 cycles, with each cycle consisting of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 1 min, and a final incubation at 72°C for 4 min. PCR products were analyzed by 2% agarose gel electrophoresis. After ethidium bromide staining, the gels were photographed under ultraviolet light. The detection level for the PCR products was determined using DNA prepared from known CFU numbers of *P. gingivalis*, and the detection limit of PCR was 1×10^{3} CFU.

Quantification of glutathione content in peritoneal exudate cells

The content of glutathione in peritoneal exudate cells was measured using a Total Glutathione Quantification Kit (Dojindo Molecular Technologies Inc., Gaithersburg, MD) according to the manufacturer's instructions. Peritoneal exudate cells (1×10^6 cells) were washed twice with PBS and resuspended in 80 µl of 10 mM HCl. The suspension was frozen at −80°C and then thawed at 4°C. After repeating the freezing and thawing process, 20 µl of 5% sulfosalicylic acid was added to the suspension and the sample was centrifuged at 800 ***g*** for 10 min. The protein-free supernatants were used for measuring glutathione concentrations. The concentration was determined using a standard curve generated with serial dilutions of glutathione.

Modulation of glutathione in peritoneal exudate cells

To examine whether changes in the intracellular glutathione levels of peritoneal exudate cells affect *P. gingivalis* elimination, mice were treated with GSH-OEt (Sigma Chemical Co., St Louis, MO) to increase glutathione or with BSO (Wako) to deplete glutathione. Briefly, the *P. gingivalis*-immunized mice received 1 ml of 10 or 100 mM GSH-OEt (10 or 100 µmol GSH-OEt) intraperitoneally and were then inoculated with 2×10^9 CFU of *P. gingivalis* immediately. Similarly, the *F. nucleatum*-immunized mice received 1 ml of 0.2 or 1 mM BSO (0.2 or 1 µmol BSO) intraperitoneally and were then inoculated *P. gingivalis* immediately.

Measurement of IFN-γ concentration in peritoneal lavage fluid

The IFN-γ concentration in the supernatant of the peritoneal lavage fluid was determined using an Endogen Mouse IFN-γ enzyme-linked immunosorbent assay Kit (Endogen Inc., Woburn, MA) according to the manufacturer's instructions.

Nitrite analysis

The nitric oxide production from peritoneal exudate cells was detected by measuring nitrite levels. Briefly, 100 µl of the supernatant of the peritoneal lavage fluid was mixed with 100 µl Griess reagent (9) in flat-bottom, 96-well plates. After 10 min, the absorbance at 550 nm was measured using a microplate reader (MTP-22, Corona Electric Co., Ibaraki, Japan). The nitrite concentration was determined using a standard curve generated with serial dilutions of sodium nitrite.

Statistical analysis

Data are expressed as mean ± standard error for six mice at each time-point. Statistical analysis was performed using Welch's test. Differences were considered significant at a P value of <0.05.

Results

Elimination of *P. gingivalis* from the peritoneal cavity of mice

Every immunized mouse demonstrated a significantly higher serum antibody titer to the sonicated antigens of inoculated bacteria than that at the beginning of each experiment (data not shown). To examine the elimination of *P. gingivalis* from the mouse peritoneal cavity of each group after its inoculation, we carried out colony-forming assays with the peritoneal lavage fluid. In the *F. nucleatum*-immunized mice, the elimination of *P. gingivalis* had occurred by 24 h after *P. gingivalis* inoculation, whereas in the *P. gingivalis*-immunized mice and in the control mice, the CFU of *P. gingivalis* remained unchanged from 6 h to 24 h (Fig. 1A). The elimination was ascertained by PCR using DNA prepared from peritoneal exudate cells and primers for the *P. gingivalis* 16S ribosomal RNA gene. The amounts of PCR product were almost the same in all groups until 12 h after *P. gingivalis*

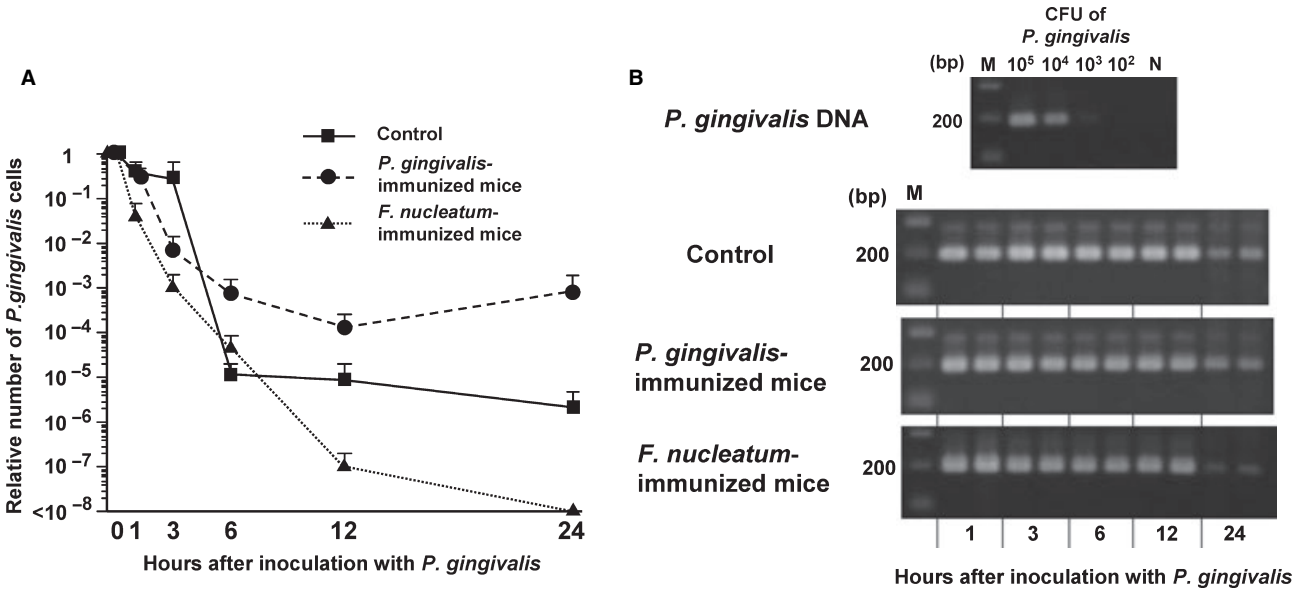

Fig. 1. (A) Reduction of *Porphyromonas gingivalis* cell number in the peritoneal cavity of mice after *P. gingivalis* inoculation. *P. gingivalis*-immunized, *Fusobacterium nucleatum*-immunized, and control mice were inoculated with *P. gingivalis* (2×10^9 CFU/mouse) intraperitoneally. The number of live *P. gingivalis* in the peritoneal cavity at various times was determined by a colony-forming assay. Data are expressed as means ± SE ($n = 6$). (B) Detection of *P. gingivalis* in peritoneal exudate cells by polymerase chain reaction (PCR) using *P. gingivalis*-specific 16S ribosomal RNA gene primers. DNA prepared from known numbers of *P. gingivalis* was used as standard. DNA of peritoneal exudate cells was obtained from two mice at 1, 3, 6, 12, and 24 h after *P. gingivalis* inoculation into each group. PCR products were analyzed by agarose gel electrophoresis. Lane M, 100-base-pair DNA ladder marker. Lane N, a negative control from PCR without DNA.

inoculation. However, the level of PCR product obtained from the samples of the *F. nucleatum*-immunized mice was less than that of the *P. gingivalis*-immunized mice or that of control mice at 24 h (Fig. 1B).

Total cell and differential cell count of peritoneal exudate cells

Figure 2 shows the total cell and differential cell counts in the mouse peritoneal cavity at various times after inoculation with *P. gingivalis*. In *F. nucleatum*-immunized mice, the total cell number increased to $2.7 \times 10^7 \pm 0.9 \times 10^7$ at 6 h and $4.0 \times 10^7 \pm 1.7 \times 10^7$ at 12 h (Fig. 2A), and the neutrophil number also increased to $2.1 \times 10^7 \pm 0.8 \times 10^7$ (79.4% of total cells) at 6 h and $2.7 \times 10^7 \pm 1.3 \times 10^7$ (67.9% of total cells) at 12 h (Fig. 2B). The macrophage number increased to $6.1 \times 10^6 \pm 4.4 \times 10^6$ at 12 h (Fig. 2D). In the *P. gingivalis*-immunized mice, the total cell number reached a maximum number of $3.2 \times 10^7 \pm 1.6 \times 10^7$ at 12 h (Fig. 2A), and the neutrophil number reached a peak of $2.0 \times 10^7 \pm 1.3 \times 10^7$ at 12 h (Fig. 2B). The macrophage number increased to $8.6 \times 10^6 \pm 5.3 \times 10^6$ at 12 h and $9.0 \times 10^6 \pm 4.5 \times 10^6$ at 24 h (Fig. 2D). No significant change was observed in the lymphocyte number in mice of all groups (Fig. 2C).

Level of IFN-γ in the peritoneal cavity

IFN-γ levels in the mouse peritoneal cavity were measured after inoculation of *P. gingivalis* because IFN-γ is known to activate macrophages. At 6 h, the level of IFN-γ in the *F. nucleatum*-immunized mice was higher than that in the *P. gingivalis*-immunized mice (10.0 ± 3.4 ng and 5.0 ± 0.0 ng per mouse, respectively) (Fig. 3). IFN-γ was barely detectable in control mice.

Intracellular level of glutathione in peritoneal exudate cells

Figure 4 shows the change of the glutathione level in 1×10^6 of peritoneal exudate cells after the inoculation of *P. gingivalis*. In the *F. nucleatum*-immunized mice, the maximum level of glutathione was 28.1 ± 1.0 μM at 12 h. This level was significantly higher than those in the control and *P. gingivalis*-immunized mice (5.0 ± 0.3 and 8.2 ± 0.6 μM at 6 h, respectively).

Effect of GSH-OEt on *P. gingivalis* elimination and IFN-γ levels in the peritoneal cavity of *P. gingivalis*-immunized mice

The glutathione levels of peritoneal exudate cells of *P. gingivalis*-immunized mice treated with 100 μmol GSH-OEt were significantly higher at 6 h and 12 h (14.2 ± 1.2 and 20.3 ± 0.7 μM,

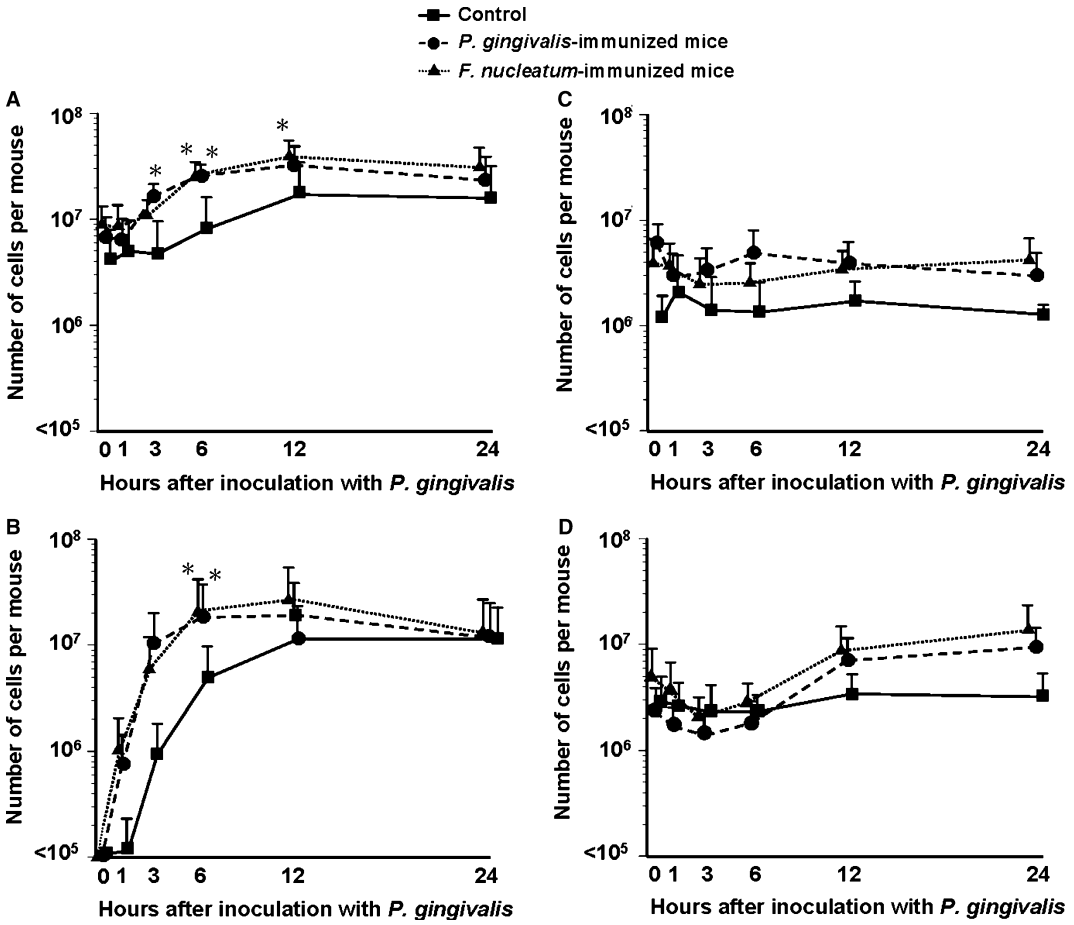

Fig. 2. Total cell count and differential cell count of peritoneal exudate cells after *Porphyromonas gingivalis* inoculation. *P. gingivalis*-immunized mice, *Fusobacterium nucleatum*-immunized mice, and control mice were inoculated with *P. gingivalis* (2×10^9 CFU/mouse) intraperitoneally. Peritoneal exudate cells were obtained at various times after the inoculation and were stained with Türk solution. Cell counting was performed with a hemocytometer. (A) Total cells, (B) neutrophils, (C) lymphocytes, and (D) macrophages. Data are expressed as means ± SE ($n = 6$). *Significantly different ($P < 0.05$) from control mice.

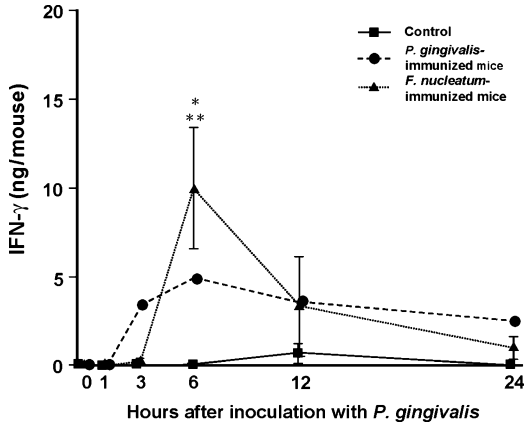

Fig. 3. Interferon-γ (IFN-γ) levels in peritoneal lavage fluid after *Porphyromonas gingivalis* inoculation into the peritoneal cavity. Data are expressed as means ± SE ($n = 6$). *Significantly different ($P < 0.05$) from control mice; **significantly different ($P < 0.05$) from *P. gingivalis*-immunized mice.

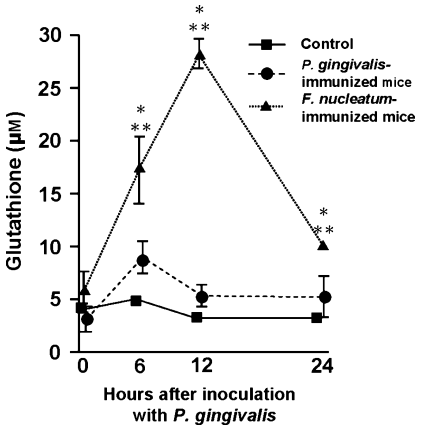

Fig. 4. Intracellular glutathione levels of peritoneal exudate cells (1×10^6 cells) harvested at various times after the *Porphyromonas gingivalis* inoculation into the peritoneal cavity. Data are expressed as means ± SE ($n = 6$). *Significantly different ($P < 0.05$) from control mice; **significantly different ($P < 0.05$) from *P. gingivalis*-immunized mice.

respectively) compared with those of untreated *P. gingivalis*-immunized mice (8.2 ± 1.3 and 4.4 ± 0.6 μM, respectively) (Fig. 5A). The bacteria were eliminated more effectively, when mice were treated with 100 μmol GSH-OEt (Fig. 5B). At 6 h, the peritoneal IFN-γ level in *P. gingivalis*-immunized mice treated with 100 μmol GSH-OEt was significantly higher compared with that of untreated *P. gingivalis*-immunized mice (15.2 ± 4.1 and 5.0 ± 0.1 ng/mouse, respectively) (Fig. 5C). There was no difference in the IFN-γ level at 12 and 24 h between these two groups. Treatment with 10 μmol GSH-OEt had no effect on intracellular glutathione levels, the elimination of *P. gingivalis*, or the IFN-γ levels.

Effect of BSO on *P. gingivalis* elimination and IFN-γ levels in the peritoneal cavity of *F. nucleatum*-immunized mice

F. nucleatum-immunized mice were treated with BSO to deplete glutathione in the peritoneal exudate cells. The glutathione level of *F. nucleatum*-immunized mice treated with 0.2 μmol BSO was significantly lower at 6 and 12 h (5.6 ± 0.8 and 11.2 ± 0.7 μM, respectively) compared with those of untreated *F. nucleatum*-immunized mice (17.5 ± 1.0 and 28.3 ± 1.4 μM, respectively) (Fig. 6A). Figure 6(B) shows the elimination of *P. gingivalis* in the peritoneal cavity. In untreated *F. nucleatum*-immunized mice, *P. gingivalis* was eliminated by 24 h after its inoculation. When mice were treated with 0.2 μmol BSO, *P. gingivalis* was still detected at 24 h after inoculation. Figure 6(C) shows the IFN-γ levels of the peritoneal cavity. The IFN-γ level at 6 h in *F. nucleatum*-immunized mice treated with 0.2 μmol BSO was significantly lower than that of untreated *F. nucleatum*-immunized mice (1.9 ± 1.3 and 8.1 ± 4.8 ng/mouse, respectively). Treatment with 1 μmol BSO had the same effects as treatment with 0.2 μmol BSO on the glutathione level of peritoneal exudate cells but no effect on the elimination of *P. gingivalis* (Fig. 6A and B). Unexpectedly, treatment with 1 μmol BSO did not decrease the IFN-γ level (Fig. 6C).

Nitrite level of peritoneal lavage fluid after the modulation of glutathione level

Figure 7(a and b) shows the nitrite levels in the peritoneal lavage fluid after the inoculation of *P. gingivalis* into *P. gingivalis*-immunized mice treated with GSH-OEt and into *F. nucleatum*-immunized mice treated with BSO, respectively. There were no significant changes in the nitrite levels among any of the groups or at any time-points.

Discussion

We reported previously on bacterial elimination from the peritoneal cavity of mice immunized with the same bacteria (10). The elimination of *F. nucleatum* from *F. nucleatum*-immunized mice was faster than that of *P. gingivalis* from *P. gingivalis*-immunized mice. In the present study, we demonstrated that the elimination of *P. gingivalis* from the peritoneal cavity of *F. nucleatum*-immunized mice was also faster than from *P. gingivalis*-immunized mice (Fig. 1). This result suggests that non-specific bactericidal activity might be enhanced by immunization with *F. nucleatum*.

Neutrophils are known to be the first line of response against bacterial attacks. The significant increase in the total peritoneal exudate cell number in each immunized group at 12 h was reflected in an increase in neutrophil numbers (Fig. 2). Although there was no difference in the number of neutrophils in the peritoneal cavity of both immunized mice, over 1000-fold more *P. gingivalis* cells survived in *P. gingivalis*-immunized mice compared with in *F. nucleatum*-immunized mice. These results suggested that neutrophils did not participate in the elimination of *P. gingivalis in vivo*. We reported that mouse neutrophils did not have a bactericidal effect on *P. gingivalis in vitro* (17). Similar results have been obtained from a study using human neutrophils (unpublished data). Our findings lead us to propose that neutrophils are not especially effective at eliminating *P. gingivalis.*

In the present study, the serum antibody titer to *P. gingivalis* was increased by immunization with *P. gingivalis* but not by immunization with *F. nucleatum* (data not shown). This suggests that the antibody to *P. gingivalis* may not be involved in the elimination of *P. gingivalis*. Okuda et al. described the difficulty in eliminating *P. gingivalis* with antibodies (18). We also reported that the antibody against *P. gingivalis* has little participation in the elimination of *P. gingivalis* both in human and murine experimental systems (17).

T helper lymphocytes are classified into two major subsets, Th1 cells and Th2 cells, on the basis of their cytokine production

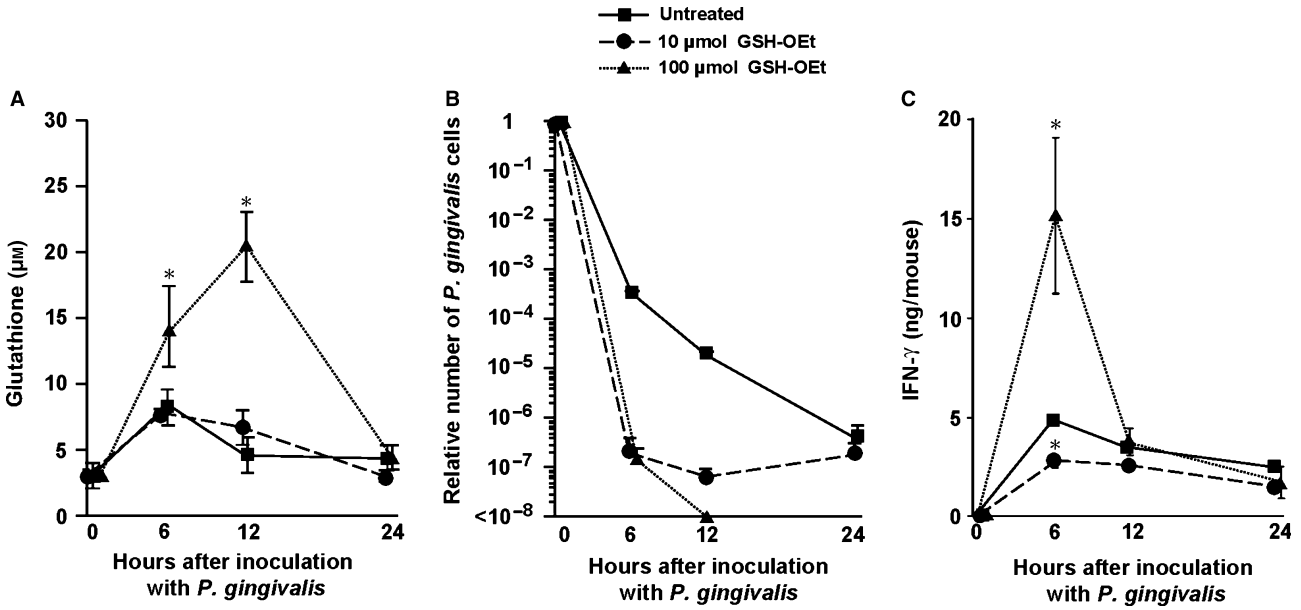

Fig. 5. Effect of GSH-OEt on the glutathione levels of peritoneal exudate cells (A), on the elimination of *P. gingivalis* from the peritoneal cavity (B), and on the IFN-γ levels in peritoneal lavage fluid (C). *P. gingivalis*-immunized mice were administrated intraperitoneally with GSH-OEt and inoculated immediately with 2×10^9 CFU of *P. gingivalis*. ■: Untreated mice, ●: Mice treated with 10 μmol GSH-OEt, and ▲: Mice treated with 100 μmol GSH-OEt. Data are expressed as means ± SE ($n = 6$). *Significantly different ($P < 0.05$) from untreated mice.

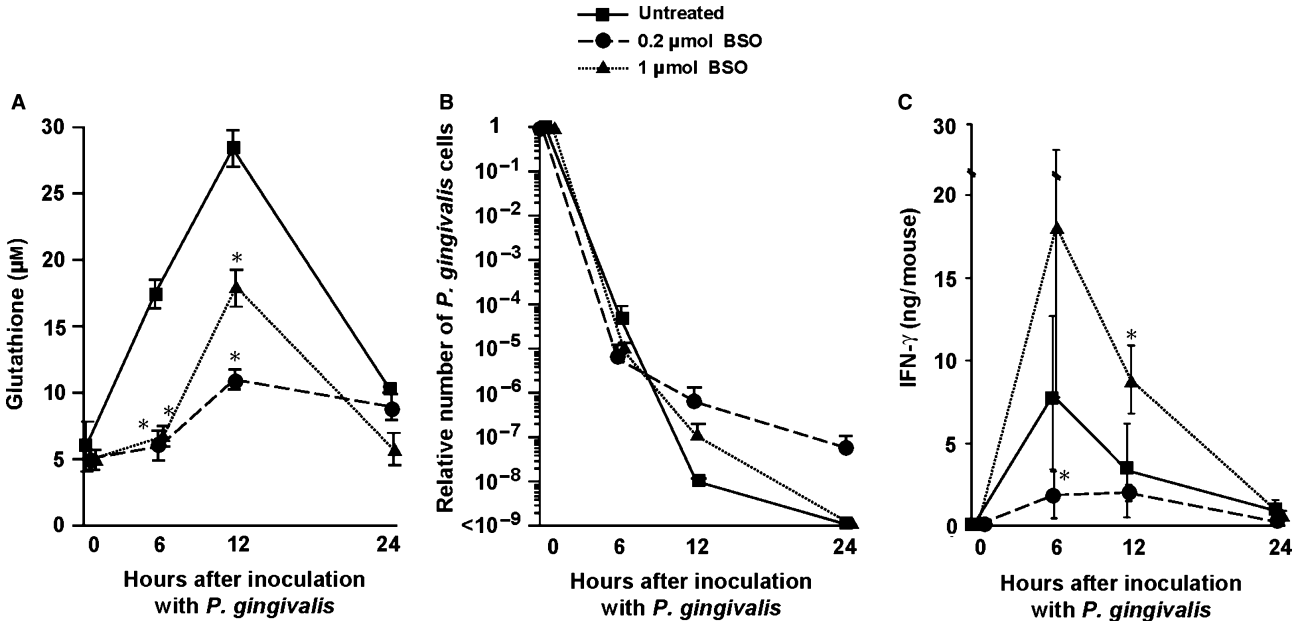

Fig. 6. Effect of BSO on the glutathione levels of peritoneal exudate cells (A), on the elimination of *P. gingivalis* from the peritoneal cavity (B), and on the IFN-γ levels in peritoneal lavage fluid (C). *F. nucleatum*-immunized mice were administrated intraperitoneally with BSO and inoculated immediately with 2×10^9 CFU of *P. gingivalis*. ■: Untreated mice, ●: Mice treated with 0.2 μmol BSO, and ▲: Mice treated with 1 μmol BSO. Data are expressed as means ± SE ($n = 6$). *Significantly different ($P < 0.05$) from untreated mice.

pattern. Th1 cells produce IL-2 and IFN-γ and stimulate cell-mediated immunity. Th2 cells produce IL-4, IL-5, and IL-10 and stimulate humoral immunity. Therefore, the Th1/Th2 balance is important for the progression of diseases. As shown in Fig. 3, the IFN-γ level in *F. nucleatum*-immunized mice was higher than that in the other two groups of mice. The higher IFN-γ level is considered to induce a Th1-dominant state leading to macrophage activation. We speculate that macrophages contribute to the elimination of *P. gingivalis* from *F. nucleatum*-immunized mice. There was no significant difference in the number of macrophages in peritoneal exudate cells among the three groups so a change in the nature of macrophages was suggested to act in the elimination of *P. gingivalis*. Cohn reported that activated (inflammatory) macrophages in the mouse peritoneal cavity were different from resident macrophages in terms of protein content, enzyme activities, and production

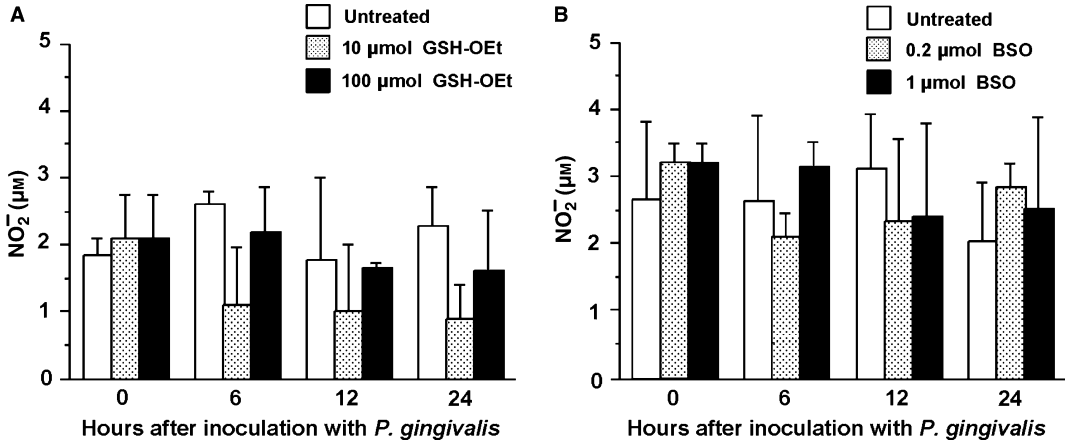

Fig. 7. Nitrite levels in peritoneal lavage fluid of *Porphyromonas gingivalis*-immunized mice injected with GSH-OEt (A) and of *Fusobacterium nucleatum*-immunized mice injected with BSO (B). Data are expressed as means ± SE (n = 6).

of reactive oxygen intermediates (5). Gordon et al. reported that the exudative macrophages were different from the resident macrophages in the expression and function of plasma membrane receptors (8). Cell-mediated immunity is known to enhance the non-specific bactericidal activity of macrophages against intracellular pathogens such as *Mycobacterium tuberculosis* or *Salmonella typhi* (11).

A dichotomy in the intracellular redox status of macrophages, reductive macrophages, and oxidative macrophages was proposed previously (16). Reductive macrophages are activated by Th1 responses and have high intracellular glutathione contents. Oxidative macrophages are induced by Th2 responses and have low intracellular glutathione contents. It was reported that macrophages in which intracellular glutathione was depleted had reduced leishmanicidal activity (2). This finding led us to verify the relationship between *P. gingivalis* elimination and the intracellular glutathione level. From our results, we speculate that repeated infection with *F. nucleatum* raises the glutathione level of the host and leads to a Th1-dominant state, thereby not only *F. nucleatum* but also *P. gingivalis* are eliminated. On the other hand, repeated infections with *P. gingivalis* do not raise the glutathione level and maintain a Th2-dominant state; thereby the elimination of bacteria is delayed.

GSH-OEt treatment of *P. gingivalis*-immunized mice and BSO treatment of *F. nucleatum*-immunized mice resulted in changes not only of the glutathione level but also of the IFN-γ level (Figs 5A and C and 6A and C). Moreover, mice with high glutathione levels showed enhanced bacterial elimination (Figs 1, 5B, and 6B). It may be speculated that higher IFN-γ levels activate macrophages and hence higher intracellular glutathione levels are induced. Nitric oxide is a potent bactericidal agent produced by activated macrophages. Stuehr et al. identified glutathione as a cofactor needed for the activation of nitric oxide synthase in macrophages (23). In the present study, no significant change in nitric oxide level was detected after the injection of a glutathione level elevator or inhibitor in any of the three groups of mice or at any of the time-points examined (Fig. 7A and B). Nitric oxide is not considered to be involved in the elimination of *P. gingivalis*.

Though it is still not clear what cells are involved in modulating the glutathione level, neutrophils might be excluded because they are not effector cells of *P. gingivalis* elimination, as mentioned above. Wedner et al. showed that the intracellular glutathione level of neutrophils after the ingestion of bacteria decreased and this state was maintained for a long time (27). Rouzer et al. reported that macrophages obtained from mice challenged with *Corynebacterium parvam* maintained a higher intracellular glutathione level than did resident macrophages (20). As shown in this study, intracellular glutathione levels of peritoneal exudate cells after *P. gingivalis* inoculation in *P. gingivalis*-immunized mice were lower than that in *F. nucleatum*-immunized mice although there were no differences in total cell or differential cell counts of peritoneal exudate cells in any of the groups of mice. Immunization with *P. gingivalis* may suppress Th1 responses and/or inhibit the synthesis of glutathione in macrophages. In future studies, we will attempt to clarify the relationship between glutathione metabolism and the activation of macrophages after immunization with these periodontopathic bacteria.

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