Effect of aging on BCG immunostimulation of *Porphyromonas gingivalis* infection in mice

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**ABSTRACT**

The need for population care increases with the age of the population. Pneumonia is the fourth leading cause of death in Japan, yet the risk of pneumonia could be reduced by eliminating opportunistic infection sources such as oral bacteria (*e.g.* *Porphyromonas gingivalis*). Previously, we reported removal of *P. gingivalis* by macrophages during the early stages of cellular immunity, although neither neutrophils nor antibodies participated in the antimicrobial activity. BCG is a live vaccine against tuberculosis, and is thought to maintain cellular immunity as the antigen remains *in vivo* for longer periods. In this experiment, we attempted to clarify the relationship between aging and the elimination of *P. gingivalis* by examining the protective capacity of BCG against *P. gingivalis* infection in mice of various ages. In young mice, the reduction in numbers of *P. gingivalis* was accompanied by increased IFN-γ and IL-12 levels, and nitric oxide was continuously produced. The augmentation of bactericidal activity, namely the effects of the vaccine, was clear in young mice, but weaker in older mice. Activation of cellular immunity was not observed in older mice, even when boosters were administered.

Pneumonia, which is the fourth leading cause of death in Japan (23), results in more deaths among cancer patients than terminal-stage cancer. Among the elderly, many cases of pneumonia occur due to aspiration of indigenous oral bacteria (4), but the causative organism is not considered to be aggressive. The oral bacterium *Porphyromonas gingivalis* is a gram-negative, anaerobic rod that is the main pathogen associated with adult periodontitis (33). *P. gingivalis* has recently attracted attention as the pathogenic bacterium found in systemic disorders such as bacterial endocarditis (11, 22, 25, 37) and arteriosclerosis (8, 17) as well as pneumonia (4, 27, 31). *P. gingivalis* possesses the protease gingipain, which degrades antibody-complement complexes and confers resistance to phagocytosis (5, 9) and antibiotics (3, 30), enabling it to become an opportunistic infection source.

BCG is an attenuated strain of a probiotic bovine bacterium that was first produced by Calmette and Guérin (7) for the prevention of tuberculosis, and is still in widespread use. The antimicrobial properties of macrophages activated by BCG are non-specific, and long-term effective immunization has been reported (18, 21, 22, 36). Although many studies have investigated the immunization efficacy of BCG on tubercle bacilli, few have explored the relationship between vaccination efficiency and aging.

Previously, we examined immune responses to oral bacteria and showed that stronger Th1 responses occurred in mice immunized with *Fusobacterium nucleatum* compared to *P. gingivalis* (19). In addition, we confirmed that *P. gingivalis* was removed by macrophage participation during the early stages of cellular immunity (16), although neither neutrophilic leukocytes nor antibodies took part in the antimicrobial activity (26). Accordingly, we questioned

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whether the induction of cellular immunity which decreases the number of oral bacteria causing an opportunistic infection could prevent pneumonia in the elderly.

In the present study, we investigated whether the efficacy of the BCG vaccination was affected by age, for how long the vaccination immunity effects continue in young mice, and whether these effects are enhanced by BCG boosters.

**MATERIALS AND METHODS**

**Bacteria.** *P. gingivalis* FDC381 was cultured in Gifu anaerobic medium (GAM; Nissui Pharmaceutical Co., Tokyo, Japan) under anaerobic conditions using an AnaeroPack Kenki (Mitsubishi Gas Chemical Co., Tokyo, Japan) for 48 h at 37°C. Bacteria were harvested by centrifugation, and were washed twice with phosphate-buffered saline (PBS). Bacterial cells were resuspended in PBS at an optical density of 1.5 at 540 nm, corresponding to approximately $1 \times 10^{10}$ colony-forming units (CFU) of *P. gingivalis* per mL. Suspensions were maintained anaerobically at room temperature until use. BCG was purchased from Japan BCG Supply Co. (Tokyo, Japan) and the bacterial number was adjusted for immunity after resuspension according to the manufacturer’s instructions.

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

**Immunization.** Experiment 1: BCG-immunized mice. Mice in the Young age group (aged 6–7 weeks), and the Old age group (aged 12–14 months) were injected once subcutaneously in the flank with $1 \times 10^7$ CFU of BCG in 0.2 mL PBS. Three weeks after the injection, mice were used for experiments.

Experiment 2: BCG-early immunized mice. Mice aged 6 weeks were injected once subcutaneously in the flank with $1 \times 10^7$ CFU of BCG in 0.2 mL PBS. After 12–14 months, mice were used for experiments.

Experiment 3: BCG-boosted mice. Mice aged 6 weeks were injected once subcutaneously in the flank with $1 \times 10^7$ CFU of BCG in 0.2 mL PBS. After 12–14 months, mice were used for experiments.

None of the mice died during the immunization period in any of the experiments. At the end of the experimental periods, all mice were sacrificed, and BCG administration was confirmed by swelling of the inguinal lymph node at the site of the BCG injection.

**P. gingivalis elimination assay.** All mice were injected intraperitoneally with $2 \times 10^9$ CFU *P. gingivalis* in 0.2 mL PBS, and sacrificed at various times after injection. The peritoneal cavity was then lavaged with 2 mL PBS. Peritoneal lavage fluid was collected and a 20-μL aliquot immediately used in a colony forming assay for *P. gingivalis*. The remaining fluid was centrifuged at $400 \times g$ for 5 min. The supernatant was stored at −80°C until use for determination of nitrites and cytokines. 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 according to nuclear staining and size. Peritoneal exudate cells were re-centrifuged after cell counts for detection of *P. gingivalis* DNA by polymerase chain reaction (PCR).

**Polymerase chain reaction (PCR).** *P. gingivalis* DNA in peritoneal cell exudates was detected by PCR using specific primers for the 16S ribosomal RNA gene. Mouse and bacterial genomic DNA were isolated from cells using the Wizard genomic DNA purification kit (Promega, Madison, WI) according to the manufacturer’s instructions. DNA was dissolved in 10 mM Tris-HCl and 1 mM EDTA (pH 7.5). The PCR mixture (25 μL) contained 1 μL DNA (20–200 ng), 1 × Ex Taq buffer (Takara Bio Inc., Shiga,
was measured using a microplate reader (MTP-22; Corona Electric Co., Ibaraki, Japan). Nitrite concentration was determined using a standard curve generated using serial dilutions of sodium nitrite.

**Statistical analysis.** Data are expressed as means ± standard error for six mice at each time point. Statistical analysis was performed using the ANOVA test. Differences were considered to be significant at a *P*-value of < 0.05.

**RESULTS**

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

To examine the elimination of *P. gingivalis* from the mouse peritoneal cavity of both groups after inoculation, we carried out colony-forming assays with peritoneal lavage fluid. In BCG-immunized mice of the Young age group, elimination of *P. gingivalis* occurred within 24 h of *P. gingivalis* inoculation, whereas in non-immunized control mice of the Young age group, *P. gingivalis* CFU remained unchanged from 24 h to 48 h (Fig. 1). On the other hand, in mice of the Old age group, *P. gingivalis* was eliminated comparatively more slowly, at 48 h, after the inoculation. In the BCG-immunized, BCG-early immunized and BCG-boosted mice of the Old age group, there was no difference in survival rate of *P. gingivalis* compared with non-immunized controls.

**Concentrations of cytokines in peritoneal lavage fluid.** Concentrations of IFN-γ, IL-12, IL-4, IL-1β, IL-6 and TNF-α in the supernatant of peritoneal lavage fluids were determined using Endogen Mouse IFN-γ, IL-12, IL-4, IL-1β, IL-6 and TNF-α ELISA Kits (Endogen Inc., Woburn, MA), according to the manufacturer’s instructions.

**Nitrite analysis.** NO production by peritoneal exudate cells was detected by measuring nitrates. Briefly, 100 μL supernatant of peritoneal lavage fluid was mixed with 100 μL Griess reagent (15) in flat-bottom 96-well plates. After 10 min, absorbance at 550 nm

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**Fig. 1** Reduction in Porphyromonas gingivalis cell number in peritoneal cavity of mice after *P. gingivalis* inoculation. Mice in both age groups were intraperitoneally inoculated with *P. gingivalis* (2 × 10^9^ CFU/mouse). Numbers of live *P. gingivalis* in peritoneal cavity at various times were determined by colony-forming assay. Data expressed as means ± SE (n = 6). *☆* Significantly different (*P* < 0.05) from non-immunized mice.
**Detection of *P. gingivalis* in peritoneal exudate cells**

Elimination was determined by PCR using DNA prepared from peritoneal exudate cells and primers for the *P. gingivalis* 16S ribosomal RNA gene. DNA prepared from known numbers of *P. gingivalis* was used as a standard. In the Old age group, the number of *P. gingivalis* present in a peritoneal exudate cell was low after 6 h compared with the Young age groups. There were no differences in order of the number of *P. gingivalis* in a peritoneal exudate cell at any time points within the same age group, regardless of immunity in the Young age group and Old age group except for the BCG-boosted group (Table 1). In the BCG-immunized mice of the Young age group at 12 h and all mice of the Old age group at 48 h, little *P. gingivalis* could be detected even in the undiluted effusion (Fig. 1), even though intracellular *P. gingivalis* was detectable by PCR until 48 h. Thus, even if no *P. gingivalis* grew on the colony counting plate, whole or fragmented *P. gingivalis* in a peritoneal exudate cell were still detectable by PCR. In this case, *P. gingivalis* in a peritoneal exudate cell may still be intact but are unculturable on colony counting plates when removed from the peritoneal cavity at 24 h or 48 h. We performed colony-forming assay and PCR method for the above-mentioned reasons.

**Total cell and differential cell count of peritoneal exudate cells**

Fig. 2 shows the total and differential cell counts in mouse peritoneal cavities at various times after inoculation with *P. gingivalis* in the Young and Old age groups. In all mice in the Young age groups, total cell numbers peaked between 6 and 12 h. The increase in total peritoneal exudate cell number reflects an increase in neutrophil numbers. In BCG-immunized mice, macrophages increased significantly to $1.36 \pm 0.66 \times 10^7$ at 48 h, in parallel with the total cell number increase. However, in all mice in the Old age groups, no significant changes were observed in total cell, neutrophil, lymphocyte or macrophage numbers when compared with non-immunized mice.

**Levels of IFN-γ in the peritoneal cavity**

IFN-γ levels in the mouse peritoneal cavity were measured after inoculation with *P. gingivalis*, as IFN-γ is known to activate macrophages (Fig. 3). In the Young age group, IFN-γ levels in BCG-immunized mice were significantly higher at 6 h ($4.67 \pm 1.98$ ng/mouse). In both age groups, IFN-γ levels of non-immunized mice peaked at 12 h. In the Old age group, all mice showed low IFN-γ levels.

**Levels of IL-12 in the peritoneal cavity**

In the Young age group, levels of IL-12 in BCG-immunized mice were significantly higher between 12 and 24 h ($21.16 \pm 5.4$ and $26.02 \pm 9.86$ pg/mouse, respectively) compared with non-immunized control mice (Fig. 4). In the Old age group, no significant differences were observed in any mice.

**Levels of inflammatory cytokines in peritoneal cavity**

Levels of IL-1β in BCG-immunized mice were significantly higher between 12 and 24 h ($44.32 \pm 17.5$ and $44.33 \pm 15.8$ pg/mouse, respectively) compared with non-immunized control mice in the Young age group.

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<table>
<thead>
<tr>
<th>Table 1</th>
<th>Porphyromonas gingivalis cell number per single peritoneal exudate cell after <em>P. gingivalis</em> inoculation</th>
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<tr>
<td>Age group</td>
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Data expressed as means ± standard deviation (n = 6)
* Significantly different ($P < 0.05$) from Young group at 6 h.
# Significantly different ($P < 0.05$) from non-immunized mice.
§ Significantly different ($P < 0.05$) from BCG-immunized mice.
§§ Significantly different ($P < 0.05$) from BCG-early immunized mice.
Attenuation of BCG effect with aging

Fig. 2 Total and differential counts of peritoneal exudate cells after *P. gingivalis* inoculation. Non- and BCG-immunized mice in the Young age group were intraperitoneally inoculated with *P. gingivalis* ($2 \times 10^9$ CFU/mouse). Non-, BCG-, BCG-early immunized and BCG-boosted mice in the Old age group were intraperitoneally inoculated with *P. gingivalis* ($2 \times 10^9$ CFU/mouse). Peritoneal exudate cells were obtained at various times after inoculation, and stained with Türk solution. Cell counting was performed with a hemocytometer. (A) Total cells, (B) neutrophils, (C) lymphocytes and (D) macrophages. Data expressed as means ± SE ($n = 6$). ★ Significantly different ($P < 0.05$) from non-immunized mice.
Immunity improved the level of TNF-α in the mice of the Young age group, although not significantly so compared with the level in non-immunized controls (Fig. 5). Aging had no effect on the production of inflammatory cytokines.

Nitrite levels in peritoneal lavage fluid

Fig. 6 shows nitrite levels in peritoneal lavage fluid after inoculation with *P. gingivalis*. All mice constantly generated nitrites (0.5–2.5 nmol/mouse) in peritoneal lavage fluid after inoculation with *P. gingivalis*. In the Young age group, BCG-immunized
mice increased nitrite levels in the peritoneal lavage fluid, with peak levels seen between 24 and 48 h, and there was a significant difference at 24 h. In the Old age groups, there were no significant changes in nitrite levels at any time points.

DISCUSSION
In the present study, BCG inoculation was an effective immunostimulant in the Young age groups of mice (Fig. 1), reducing levels of *P. gingivalis* which can cause endogenous infection within the peritoneal cavity. However, the inoculation was not effective in the Old age group. The BCG-induced immunostimulation effect disappeared with increasing mouse age, even when a booster inoculation was administered. From these results, it appears that innate immunity cannot be reinforced by BCG vaccination further into old age. In recent years, the efficacy of BCG inoculation against tuberculosis has been debated; our experimental results show that BCG inoculation cannot be expected to reduce bacterial numbers in opportunistic infections, as well as those of the tuberculosis bacterium in the elderly.

In this study, we considered mice aged 12–14 months as the Old age group because thymus weight increased nitrite levels in the peritoneal lavage fluid, with peak levels seen between 24 and 48 h, and there was a significant difference at 24 h. In the Old age groups, there were no significant changes in nitrite levels at any time points.

DISCUSSION
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In this study, we considered mice aged 12–14 months as the Old age group because thymus weight
and cell numbers are decreased by about half, and cell subsets of thymocytes are also known to differ at 12–14 months (2). Inoculation of the Young age group with BCG led to a marked increase in total cell count in the peritoneal cavity, in line with an increase in macrophages 48 h after administration of _P. gingivalis_ (Fig. 2). In the Old age group, there were almost no changes in any cell count, notwithstanding the presence or absence of immunity (Fig. 2), which indicated that the decrease in _P. gingivalis_ in the peritoneal cavity was probably not due to increased neutrophil or macrophage counts contributing to elimination.

In the Old age group of mice in the present study, a low cell number of _P. gingivalis_ was incorporated into one peritoneal exudate cell at 6 h (Table 1), which supports the previous finding of a reduced phagocytosing ability in aged mice (39). It was estimated that, because of this, no more than $1 \times 10^8$ _P. gingivalis_ remained at 48 h in the Old age group, and that this is likely to be due to innate immunity. Esposit et al. (10) reported that alveolar macrophages of the lungs, even in aged mice, showed some ability to kill bacteria, and there was no significance compared with young mice.

IFN-γ is known to activate macrophages and to increase the production of NO. High levels of IFN-γ were certainly present in the Young age groups of BCG-immunized mice (Fig. 3), probably activating macrophages and increasing NO production (Fig. 6), but did not markedly increase in the Old age group, even when a booster inoculation was administered. This suggests that a certain threshold value for IFN-γ production is required to produce sufficient quantities of NO to reduce _P. gingivalis_. Our experiments indicate that the increase in activated macrophages and the production of NO both contributed to the _P. gingivalis_ reduction in the Young age group. NO produced in the Young age group 12–24 h after administration of _P. gingivalis_ is thought to contribute to the reduction in the _P. gingivalis_ count, but NO produced immediately after administration of _P. gingivalis_ and the subsequent gradual production of NO are not regarded as contributing to this reduction. There was no significant difference in the number of neutrophils in the Young and Old age groups (Fig 2). Because both neutrophils and macrophages are necessary for killing bacteria _in vivo_ (13, 34), it is possible that activated neutrophils in the BCG-immunized mice in the Young age group may have contributed to _P. gingivalis_ elimination from the peritoneal cavity.

In our experiments, we inoculated mice subcutaneously with $10^7$ BCG cells. Inoculations with greater cell counts resulted in marked increases in IFN-γ and NO production but, at the same time, marked spleen enlargement was noted (data not shown). A BCG cell count of $10^7$ provided an inoculation that did not enlarge the spleen, maintaining the proportion of spleen weight (0.157–0.277 g) to body weight (47–62.5 g) at a constant ratio (0.0034–0.0057%). In our experiments, we obtained the same results as those of Murciano et al. (24), namely that IFN-γ production decreases in aged mice. The Th1-type CD4⁺-T cells comprise the main form of IFN-γ-producing T cells, but recent attention has focused on IFN-γ-producing CD8⁺-T cells (32), which are now regarded as the most reliable parameter for protective immunity due to the nature of IFN-γ as the strongest macrophage-activating cytokine (29). For this reason, IFN-γ may also be regarded as a parameter of protective immunity to _P. gingivalis_, which may act as an opportunistic source of endogenous infection.

Production of cytokine IL-12, which induces helper type 1 T cells and is believed to be involved in cellular immunity, was significantly higher in mice in the Young age group with BCG immunity, but no significant differences were observed in the Old age group (Fig. 5). Flesch et al. (12) reported that IL-12 is produced in the early stages of infection by activation of macrophages with IFN-γ and TNF-α. In this study, high levels of TNF-α were observed just after administration of _P. gingivalis_ at 0 h in all mice. It is possible that the high levels of TNF-α and IFN-γ observed in the Young age group mice significantly elevated the production of IL-12.

BCG has been successively subcultured in recent years, and investigations of the vaccine used in different countries based on the _IS6110_ and _mpt64_ markers have revealed extensive differences (6). Its pathogenicity in mice also varies and, according to Lagranderie et al., the immune activation resulting from the Japanese strain is weak (20). However, contradictory reports suggest that the protective effect of the Japanese strain against tuberculosis respiratory tract infection in guinea pigs does not differ from that of other strains (38). Differences in mouse strain (28), immunization method (1, 28) and the preparation of freeze-dried BCG vaccine (14, 28) all influence the survival of BCG numbers, and it is possible that such factors lead to differences in the evaluation of BCG cellular immunity.

This study is one of only a few to have practically demonstrated that BCG loses its effectiveness as an activator of cellular immunity with age and that
it is only effective during youth. These valuable findings substantiate the results of epidemiological research. In an increasingly elderly society, there is a need to discover new agents to activate cellular immunity that can be used in place of BCG in order to protect against opportunistic infection.

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REFERENCES


