Bone is continuously remodeled by bone formation and resorption, and the cooperative bone metabolism is precisely regulated to maintain homeostasis. Osteoclasts, which are responsible for bone resorption in bone metabolism, are multinucleated cells originating from hematopoietic precursor cells of the monocyte/macrophage lineage. One of the key factors for osteoclastogenesis, which is induced by osteoblasts, is receptor activator of nuclear factor kappa B ligand (RANKL), a member of the tumor necrosis factor (TNF) family [Anderson et al., 1997; Wong et al., 1997; Lacey et al., 1998; Yasuda et al., 1998]. RANKL was found to be expressed by T-cells [Wong et al., 1997; Josien et al., 1999; Kong et al., 1999] and B-cells [Li et al., 2000] as well as osteoblastic/stromal cells and to be essential for osteoclast differentiation. Receptor activator of nuclear factor kappa B (RANK), which is one of TNF receptor family members, is expressed in osteoclasts and their precursor cells as the receptor of RANKL [Josien et al., 1999; Li et al., 2000; Choi et al., 2001]. Downstream signaling through RANK is essential for osteoclastogenesis.

Osteoclasts are differentiated through multiple steps that include cell fusion at the latest step of differentiation, yielding multi-nuclear cells. Dendritic cell-specific transmembrane protein (DC-STAMP) was recently found to play a role in the cell fusion of osteoclasts, which seems to support the activity of bone resorption by osteoclasts [Kukita et al., 2004; Yagi et al., 2005]; however, the factors involved in and the precise mechanism of the cell fusion are unknown.

Deviation from the normal conditions of bone resorption results in bone diseases such as osteopetrosis, osteoporosis, and bone resorptive infectious disease. Periodontal disease is the most frequent bone resorptive infectious disease and is thought to be caused mainly by infection with Gram-negative bacteria. Such an infectious and pathological condition induces inflammation, resulting in bone resorption. A major bacterial factor for inflammation has been believed to be lipopolysaccharide (LPS), a main component of the cell surface of Gram-negative bacteria. LPS has the ability to induce proinflammatory cytokines such as TNF-α, interluekine-1β (IL-1β), and interluekine-6 (IL-6) in various kinds of cells [Wang and Ohura, 2002]. Bacterial components such as LPS or various cytokines elicited in infectious lesions may modulate physiological osteoclastogenesis, leading to a pathological bone resorptive condition.

In the present study, we investigated what bacterial components or cytokines affect osteoclastogenesis at the stage of cell fusion, what receptor molecules are involved in the cell fusion, and what signaling pathways are necessary for the cell fusion.

## MATERIALS AND METHODS

### Reagents

Anti-nuclear factor of activated T-cells c1 (NFATc1) (7A6), anti-phospho extracellular signal-regulated kinase (ERK) (E-4), anti-TRAF6 (H-274) and anti-cellular homolog of v-src (c-Src) were purchased from Santa Cruz (Santa Cruz, CA). TNF-α, anti-TNF-α, osteoprotegrin ligand (OPG), and transforming growth factor-β (TGF-β) were from R&D Systems Inc. (Mineapolis, MN). Anti-ERK, anti-p38 MAPK (p38) (5F11), anti-phospho-p38 (28B10), anti-Akt, anti-phospho-Akt, and anti-phospho-c-Jun NH2-terminal kinase (JNK) antibodies were from Cell Signaling Technology (Beverly, MA). RANKL was from Peprotech EC Ltd (London, United Kingdom). LY294002, PD169316, SB203580, SP600125, PP2, U0126, and PD98059 were purchased from Calbiochem Corp. (La Jolla, CA). M-CSF was from Kyowa Hakko Kogyo Co., Ltd (Tokyo, Japan). RANKL was from Peprotech (Rocky Hill, NJ). cDNA of mouse kidney was from Clontech. Minimal essential medium α modification (α-MEM) and all other reagents including LPS, PGN, IL-1β, and IL-6 were obtained from Sigma (St. Louis, MO).

### Cell Culture

The murine monocytic cell line RAW264.7 (ATCC, Rockville, MD) was cultured in a humidified incubator with an atmosphere of 5% $CO_2$ in air at 37°C and maintained on uncoated plastic dishes of 9 cm in diameter in α-MEM containing 10% (v/v) heat-inactivated fetal calf serum (FCS) with 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. For subculture, the cells were resuspended with 10 ml of fresh medium by mild pipetting and transferred to a 15 ml conical tube. After sitting for 5 min, the upper 14.5 ml fraction was removed, and aggregated cells were collected from the bottom and seeded into

a fresh dish containing 12 ml of fresh medium. For osteoclastogenesis experiments, the indicated number of cells was seeded on tissue culture plates in the presence or absence of RANKL and chemical reagents.

### Tartrate-Resistant Acid Phosphatase (TRAP) Staining

Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C for more than 60 min. The cells were then treated with 0.2% Triton X-100 in PBS at room temperature for 5 min, followed by rinsing twice with PBS. Finally, the fixed cells were stained with 0.01% naphthol AS-MX phosphate (Sigma) and 0.05% fast red violet LB salt (Sigma) in the presence of 50 mM sodium tartrate and 90 mM sodium acetate (pH 5.0) for 15–60 min and then rinsed twice with PBS.

### Measurement of TRAP Intensity

Following TRAP staining, the plates were scanned by a transparent light scanner, and the red color image was extracted from the scanned image using the Photoshop (Adobe Systems Inc., San Jose, CA) computer program. The intensity of the red color image was measured using National Institutes of Health (NIH) Image computer program and is represented as TRAP intensity in this paper.

### Cell Proliferation Assays

Cell proliferation was measured using a Cell Counting Kit (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Similar to the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl 2H-tetrazolium bromide] assay, this kit measures intracellular mitochondrial dehydrogenase activity in living but not in dead cells by forming water-soluble formazan dye with the tetrazolium compound WST-1, a sodium salt of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate.

### Immunoblotting

Cells were seeded at $5 \times 10^6$ cells/well on a 24-well plate and kept in a humidified incubator with an atmosphere of 5% $CO_2$ in air at 37°C for 4 h. The cells were stimulated with RANKL or/and other chemical reagents and incubated for the indicated time. After incubation, the cells in the 24-well plate were rinsed twice with ice-cold PBS, followed by addition of 50 μl of sodium dodecyl sulfate (SDS)-sample buffer [1% SDS, 100 mM dithiothreitol (DTT), 10% glycerol, 0.0025% bromophenol blue, 62.5 mM Tris/HCl, and pH 6.8] containing 1 mM sodium orthovanadate (protein tyrosine phosphatase inhibitor), 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1μg/ml chymostatin, and 1 μg/ml leupeptin. The whole cell lysate was then treated in ice-cold water by sonication (two 15-s pulses with a 60-s interval) (Bioruptor UDC-200T, Cosmo Bio, Tokyo, Japan). After boiling for 5 min, 4–7 μl of the lysate (20 μg of protein) was subjected to SDS- polyacrylamide gel electrophoresis (PAGE), and the proteins in the gel were transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was incubated in 5% skim milk, 25 mM Tris/HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween-20 (TBST) at room temperature for 1 h, washed twice with TBST for 5 min, and then incubated with an antibody at 2000–4000 × dilution in TBST at 4°C for 16 h. The membrane was washed three times with TBST for 10 min, incubated with a horse radish peroxidase (HRP)-conjugated second antibody at 4000–8000 × dilution in TBST at room temperature for 1 h, washed vigorously five times for 10 min, and subjected to chemiluminescence (ECL-plus, Amersham Pharmacia Biotech, Piscataway, NJ) to visualize HRP. In some experiments for reprobing, the membrane was stripped of antibody with Reblot Plus Strong (Chemicon, CA) according to the manufacturer's instructions.

### Real-Time PCR

Total RNA was prepared using an RNeasy Mini Spin Column (Quiagen) according to the manufacturer's instructions, and the cDNA was reverse-transcribed by SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). Using an M × 3005PTM Real-time PCR System and Brilliant SYBR Green QPCR Mastermix (Stratagene), the reverse-transcribed cDNA was amplified and quantifed with specific primers according to the manufacturer's instructions. The primer sequences used for amplification were as follows: mouse GAPDH, 5′-ACCCAGAAGACTGTGGATGG-3′ and 5′-CACATTGGGGGTAGGAACAC-3′; NF-ATc1, 5′-TCATCCTGTCCAACACCAAA-3′ and 5′-TCACCCTGGTGTTCTTCCTC-3′; DC-STA-MP, 5′-GGGCACCAGTATTTTCCTGA-3′ and 5′-TGGCAGGATCCAGTAAAA-

GG-3′; and TRAP, 5′-CAGCAGCCAAGGAG-GACTAC-3′ and 5′-ACATAGCCCACACCGT-TCTC-3′. The relative amounts of each mRNA were normalized by the GAPDH expression.

## RESULTS

### LPS-Mediated Inhibition in the Initial Step of Osteoclast Differentiation

LPS, a major component of the cell wall of Gram-negative bacteria, has been reported to strongly induce inflammation, which is thought to cause bone destruction. LPS induces bone resorption when administered in vivo; however, it suppresses osteoclast formation from bone marrow macrophages in vitro cell cultures. In order to determine what step of osteoclastogenesis is influenced by LPS, we examined whether LPS affected RANKL-induced osteoclastogenesis when it was present in RAW264.7 cell culture at different time periods after treatment with RANKL. In a previous study, we found that 1–3 μM U0126, a MAPK-ERK kinase (MEK)/ERK inhibitor, accelerated osteoclastogenesis of RAW264.7 cells in the presence of RANKL but that 10 μM of U0126 suppressed osteoclastogenesis [Hotokezaka et al., 2002]. In culture conditions with or without 2 μM U0126, cells were treated with LPS for different periods of 12 h during the total 48-h culture. In both culture conditions, LPS suppressed osteoclastogenesis when added for the first 12 h, and the

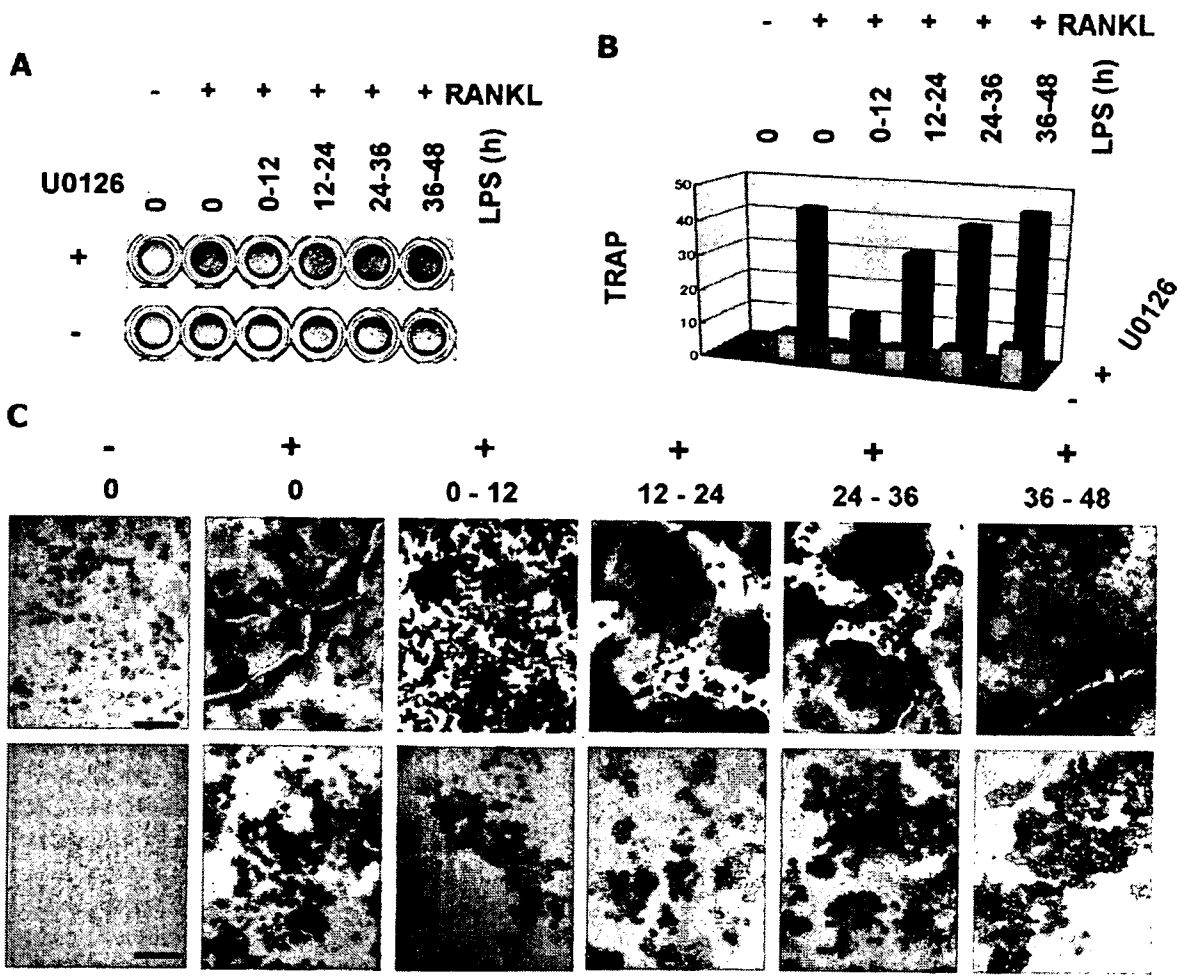



**Fig. 1.** Effect of LPS on differentiation of RAW264.7 cells into osteoclast-like cells. RAW264.7 cells in 250 μl of α-MEM were cultured in a 96-well tissue culture plate in the presence of 25 ng/ml RANKL with (upper; 16,000 cells) or without (lower; 8,000 cells) 2 μM U0126. The cells were stimulated with 25 ng/ml LPS during the indicated period in the absence of RANKL. TRAP activity was visualized by TRAP staining. **A:** culture plate scanned. **B:** measured TRAP intensity, the mean of three different determinations was plotted. **C:** cell shapes under an optical microscope. Bars indicate 50 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

suppression was markedly weaker when LPS was added at a later 12-h period (Fig. 1). Similar to the previous reports [Takami et al., 2002; Zou and Bar-Shavit, 2002; Zou et al., 2002; Hayashi et al., 2003], LPS inhibits osteoclastogenesis only in the early step of differentiation suggests that effects of cytokines or Toll-like receptor (TLR) ligand molecules such as LPS on osteoclastogenesis may vary among steps of differentiation.

### Effects of Cytokines or TLR Ligands on the Latest Step of Osteoclastogenesis

In this study, we used an enhanced culture condition in which the cells were cultured in the presence of both RANKL and U0126 since TRAP-positive multinuclear cells were generated in the culture condition within 48 h (Fig. 1). Effects of various cytokines or TLR ligands on the latest step of osteoclastogenesis and fusion of TRAP-positive mononuclear cells were investigated. First, TRAP-positive mononuclear cells were generated from RAW264.7 cells by incubation with RANKL and U0126 for 36 h. The cells were then incubated in culture medium with various cytokines or TLR ligand molecules for 12 h in the absence of RANKL. Similar to the previous reports [Lam et al., 2000; Zou and Bar-Shavit, 2002; Zou et al., 2002], LPS, PGN, and TNF-α induced cell fusion as well as RANKL (Fig. 2). Although M-CSF failed to induce cell fusion, it increased the number of TRAP-positive mononuclear cells. IL-1β had no ability to induce cell fusion in this condition. In addition, we examined IL-6 (0.1–50 ng/ml), TGF-β (0.1–50 ng/ml), phorbol myristic acetate (PMA; 0.1–10 μM), concanavalin A (0.1–10 μg/ml), amphotericin B (AmB; 0.1–10 μg/ml), and oxydized low-density lipoprotein (LDL; 0.1–10 μg/ml). None of them induced cell fusion (data not shown) although PMA, AmB, and oxydized LDL are known as inducers of proinflammatory cytokines in macrophages [Stuart and Hamilton, 1980; Cleary et al., 1992; Pollaud-Cherion et al., 1998]. These results suggested that cell fusion in the latest step of osteoclastogenesis might be induced by treatment with RANKL, TNF-α, LPS, and PGN but not by treatment with others.

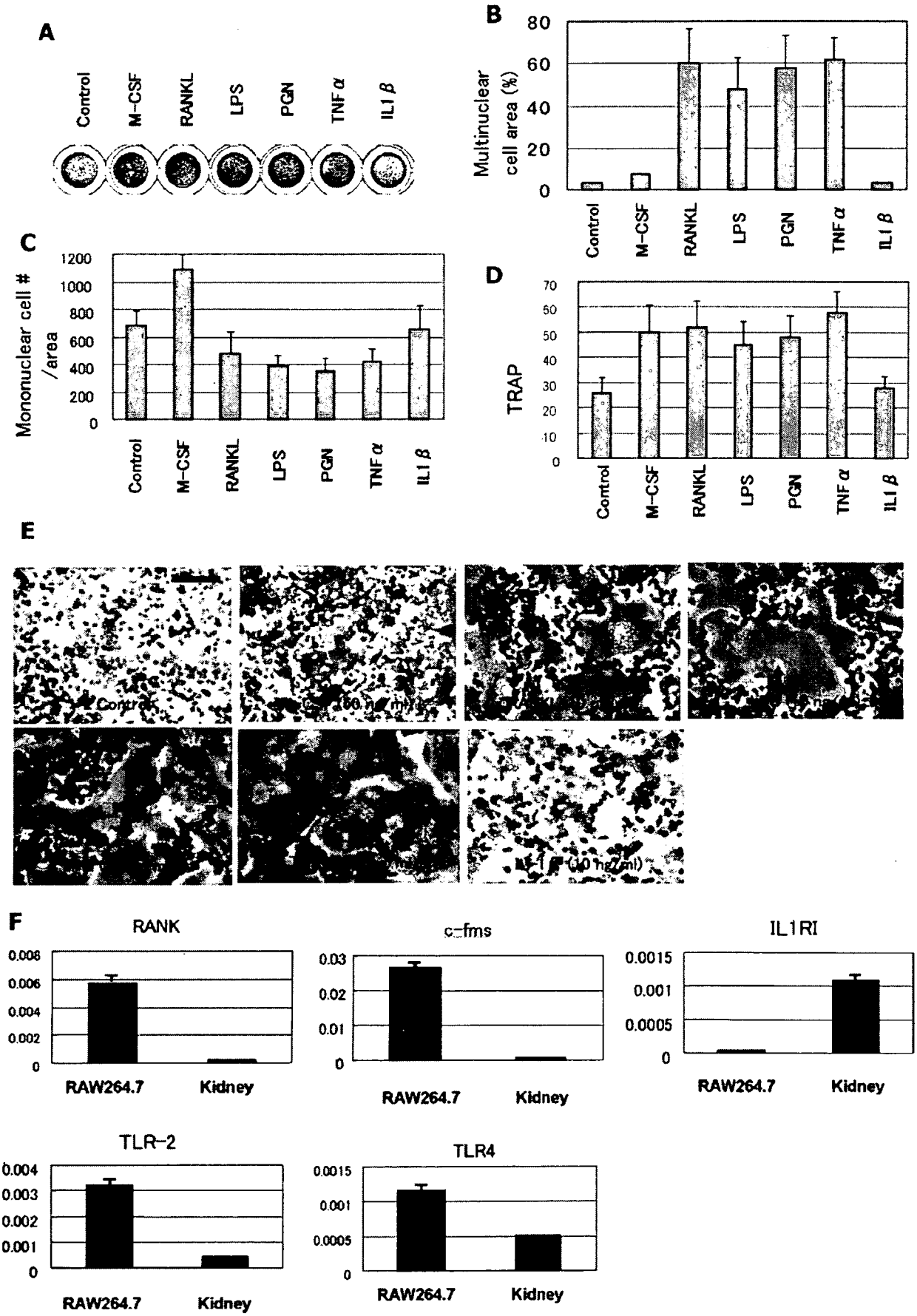
It is known that IL-1β-induced cell signaling is similar to that of LPS and PGN with respect to involvement of MyD88, an adaptor molecule of IL1R family members [Subramaniam et al., 2004]. We investigated whether the IL-1β receptor IL1RI was expressed in RAW264.7 cells. Real-time RT-PCR analysis revealed that IL1RI was not expressed in RAW264.7 cells, whereas RANK, c-fms, TLR2, and TLR4, receptors for RANKL, M-CSF, PGN, and LPS, respectively, were expressed in the cells (Fig. 2F). Absence of the IL-1β receptor in the cell line may account for the disability of IL-1β to induce cell fusion.

### Ability of LPS and PGN to Induce TNF-α-Independent Cell Fusion

LPS and PGN are known to induce TNF-α in RAW264.7 and other cells [Remick et al., 1988; Gupta et al., 1995]. Furthermore, TNF-α was reported to induce osteoclastogenesis in macrophages exposed to permissive levels of RANKL [Lam et al., 2000]. In order to determine whether cytokines or TLR ligands directly or indirectly induced cell fusion, OPG—a neutralizing antibody against TNF-α (anti-TNF-α), and polymyxin B were used to block RANKL, TNF-α, and LPS, respectively (Fig. 3). OPG, anti-TNF-α, and polymyxin B (each 0.3 μg/ml) specifically inhibited the osteoclast cell fusion induced by RANKL, TNF-α, and LPS, respectively. Anti-TNF-α strongly inhibited TNF-α-induced cell fusion and also had some inhibitory effects at 1 μg/ml on LPS- and PGN-induced cell fusion (Fig. 3A). Anti-TNF-α (1 μg/ml) completely suppressed cell fusion induced by TNF-α (10 ng/ml) and the secretion of TNF-α induced by LPS or PGN was less than 1.2 ng/ml, suggesting that LPS and PGN had the ability to induce TNF-α-independent cell fusion (Fig. 3B).

### Immunoblot Analysis During the Cell Fusion Process

An intracellular domain of RANK interacts with TNF receptor-associated factor (TRAF) 2 and TRAF 6 [Galibert et al., 1998; Wong et al., 1998, 1999; Darnay et al., 1999; Kim et al., 1999], which appear to be involved in the activation of downstream signaling molecules such as nuclear factor kappa B (NF-κB), Src [Horne et al., 1992; Lowe et al., 1993], phosphatidylinositol 3 (PI3), protein kinase B (PKB/Akt), and mitogen-activated protein kinases (MAPKs) including p38, ERK, and JNK [Xia et al., 1995; Wong et al., 1998, 1999; Matsumoto et al., 2000; Chang and Karin, 2001; Lee et al., 2002; Wei et al., 2002]. In order to study cell signaling during the cell fusion process, we determined the amounts of several


A
Control
M-CSF
RANKL
LPS
PGN
TNF α
IL1 β
B
Multinuclear cell area (%)
80
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20
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Control
M-CSF
RANKL
LPS
PGN
TNF α
IL1 β
C
Mononuclear cell # /area
1200
1000
800
600
400
200
0
Control
M-CSF
RANKL
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Control
M-CSF
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IL1 β
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0.03
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RAW264.7
Kidney
IL1RI
0.0015
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RAW264.7
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TLR-2
0.004
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Kidney
TLR4
0.0015
0.001
0.0005
0
RAW264.7
Kidney

Fig. 2.

transcription factors and phosphorylation of various signaling molecules by immunoblot analysis. In our culture system, TRAP-positive mononuclear cells began to fuse at 8 h, and they were fully fused at 16 h after stimulation. As shown in Figure 2, RANKL, TNF-α, and LPS equally induced fusion of TRAP-positive mononuclear cells, but M-CSF did not. Phosphorylation of Akt, Src, ERK, JNK, and p38 was observed at 480 or 960 min in cells stimulated with RANKL, TNF-α, and LPS (Fig. 4). p38 showed the most prominent phosphorylation by stimulation with RANKL, TNF-α, and LPS throughout the incubation period. Continuous phosphorylation of Akt was observed in cells treated with M-CSF, in contrast to the transient phosphorylation in cells treated with RANKL, TNF-α, or LPS at 8 and 16 h. The continuous or transient phosphorylation of Akt may be involved in cell fate, growth, or fusion. Since phosphorylation of signaling molecules is thought to be involved in the subsequent cellular responses, we determined the importance of the signaling molecules in the cell fusion process by using specific inhibitory compounds. As expected, LY294002 (PI3K inhibitor), PP2 (Src inhibitor), U0126 (ERK inhibitor), and SP600125 (JNK inhibitor) efficiently inhibited the RANKL-induced cell fusion, but SB203580 (p38 inhibitor) had no inhibitory effect (Fig. 5). Another p38 inhibitor, PD169316, also had no effect (data not shown). In order to study whether p38 is involved in an activity of osteoclast, resorption pit formation was measured. However, resorption pit formation induced by RANKL, TNF-α, or LPS was not inhibited by the p38 inhibitor SB203580. The role of p38 during the cell fusion process in osteoclastogenesis remains unclear.

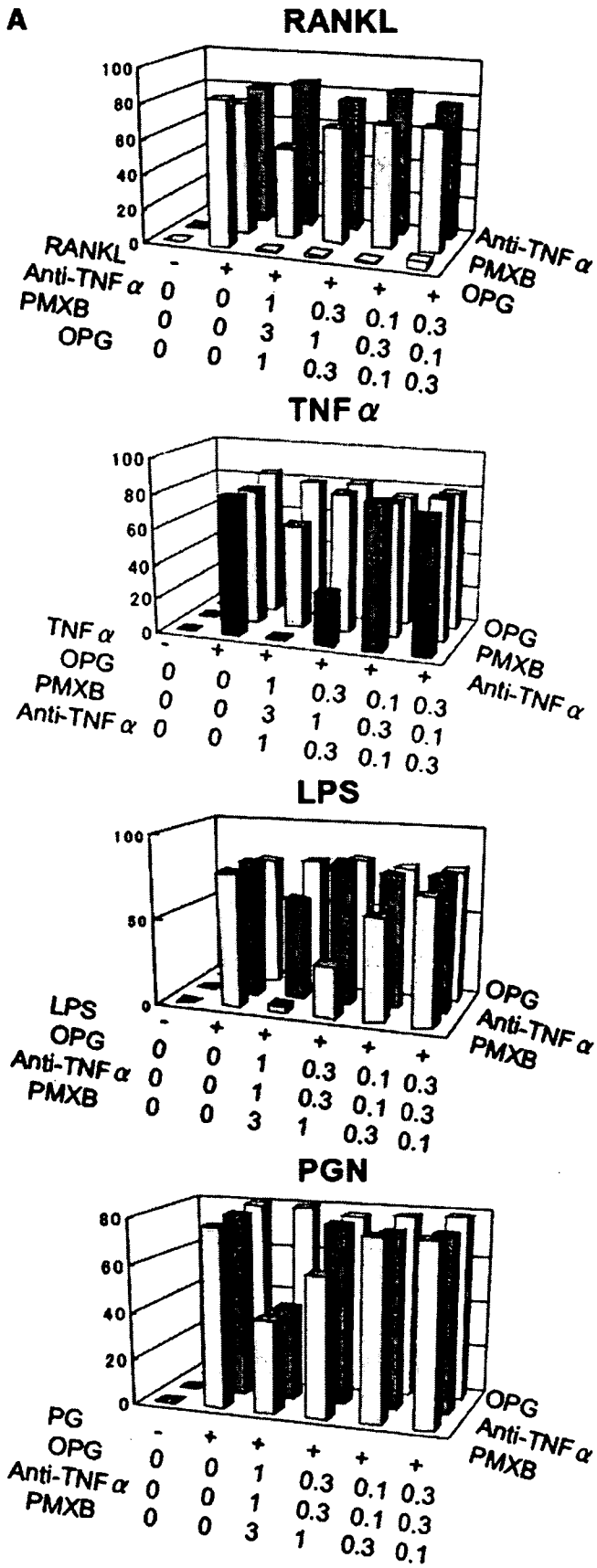
### Real-Time PCR Analysis of Expression of NFATc1, DC-STAMP, and TRAP

It was recently found that DC-STAMP participates in the cell fusion of osteoclasts [Kukita et al., 2004; Yagi et al., 2005]. First, we determined levels of mRNA of NFATc1, DC-STAMP, and TRAP in cells treated with RANKL at several time points. The mRNA level of NAFTc1 increased at an earlier time point than did the mRNA levels of DC-STAMP and TRAP. DC-STAMP and TRAP mRNAs increased immediately before cell fusion (Fig. 6). We then investigated the expression of NFATc1, DC-STAMP, and TRAP in the latest step of osteoclastogenesis. RAW264.7 cells that had been treated with RANKL for 36 h were then treated with RANKL, M-CSF, LPS, or TNF-α for 6 h. At this time point, the cells began to fuse. Then the expression of NFATc1, DC-STAMP, and TRAP in the cells was determined by real-time PCR analysis. Expression of DC-STAMP in RANKL- or LPS-treated cells was not significantly different from that in M-CSF-treated cells or non-treated cells. The M-CSF-treated cells and non-treated cells showed no cell fusion, whereas RANKL-, LPS-, and TNF-α-treated cells showed cell fusion. We could not find any significant difference in expression of NFATc1, DC-STAMP, and TRAP between the fused cells and non-fused cells. These results suggest that an essential factor other than DC-STAMP may determine whether TRAP-positive mononuclear cells are fused or not.

## DISCUSSION

In an in vitro culture system using osteoclast precursor cells purified from various tissues such as bone marrow, spleen, and liver, it is difficult to avoid contamination with other cell lineages such as T-cells and mesenchymal stromal cells. In this study, we used RAW264.7—a murine macrophage cell line that can differentiate into osteoclast-like cells in the presence of RANKL [Hsu et al., 1999]. There are some characteristic differences between RAW264.7 cells and macrophages; for example, RAW264.7 cells do not respond to IL-1β, and the osteoclast-like cells differentiated from RAW264.7 cells form smaller and shallower

**Fig. 2.** Effects of factors on cell fusion of osteoclasts. RAW264.7 cells ($1.6 \times 10^5$) were cultured for 36 h in 250 μl of α-MEM containing 10% FCS, 25 ng/ml RANKL, and 2 μM U0126 in a 96-well tissue culture plate. Then the medium was replaced with 250 μl of α-MEM containing 10% FCS and indicated factors. Concentrations used were 50 ng/ml for M-CSF, 10 ng/ml RANKL, 10 ng/ml LPS, 100 ng/ml PGN, 10 ng/ml TNF-α, and 50 ng/ml IL-1β. **A:** scanned culture plate. **B:** area % of multinuclear cells. **C:** number of mononuclear cells. **D:** TRAP intensity. **E:** cell shapes under an optical microscope. **F:** mRNA quantified by real-time RT-PCR. Total RNAs were prepared from RAW264.7 cells treated with RANKL and U0126 for 36 h and kidney cells of mice, and they were subjected to real-time RT-PCR using primers for RANK, c-fms, TLR4, and IL-1R type 1. Bar indicates 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]


A
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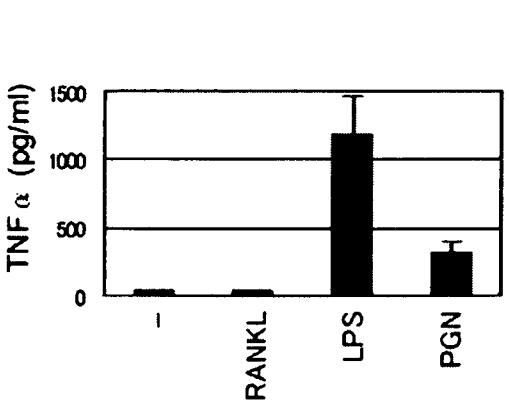
B
TNF α (pg/ml)
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Fig. 3.

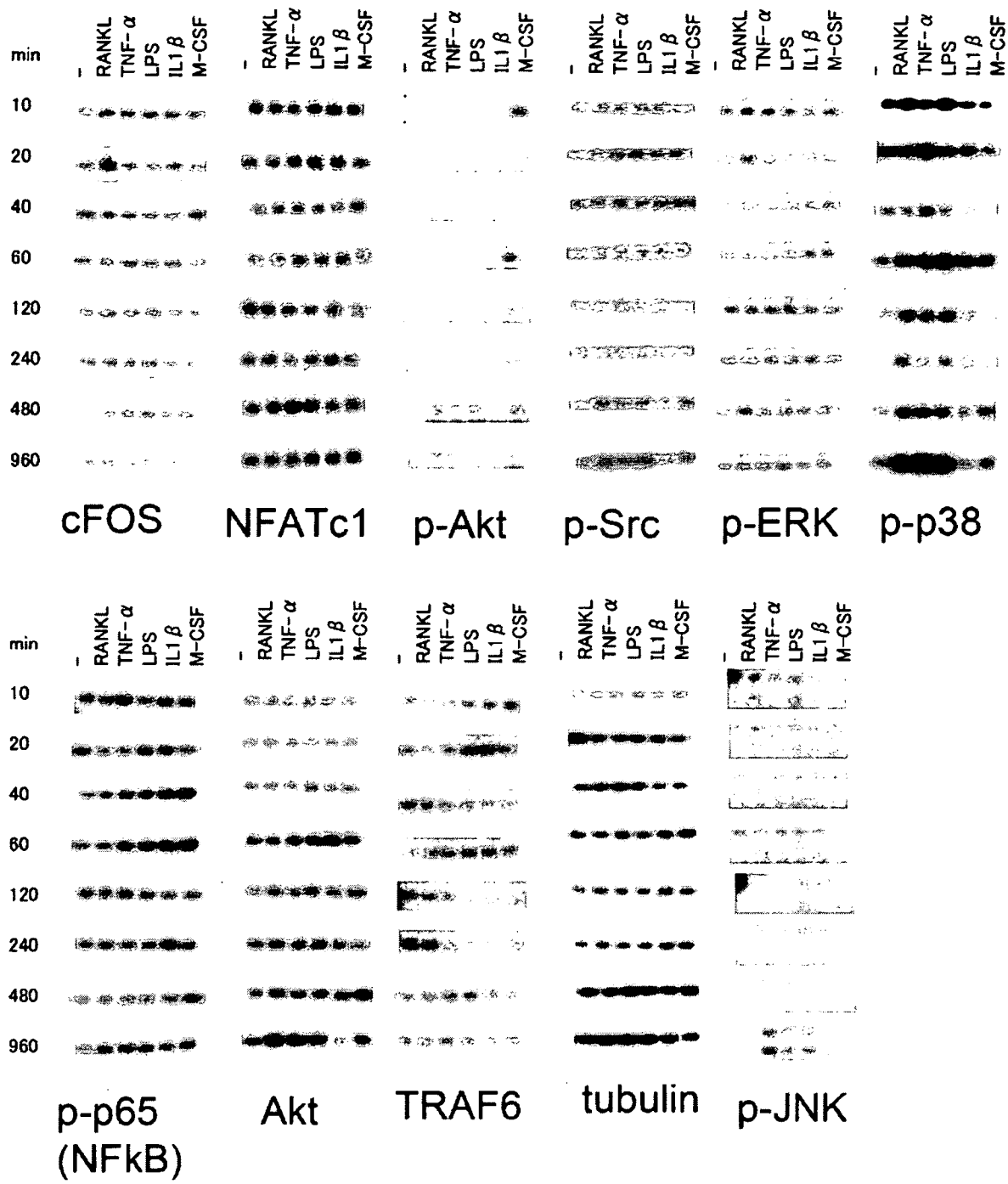
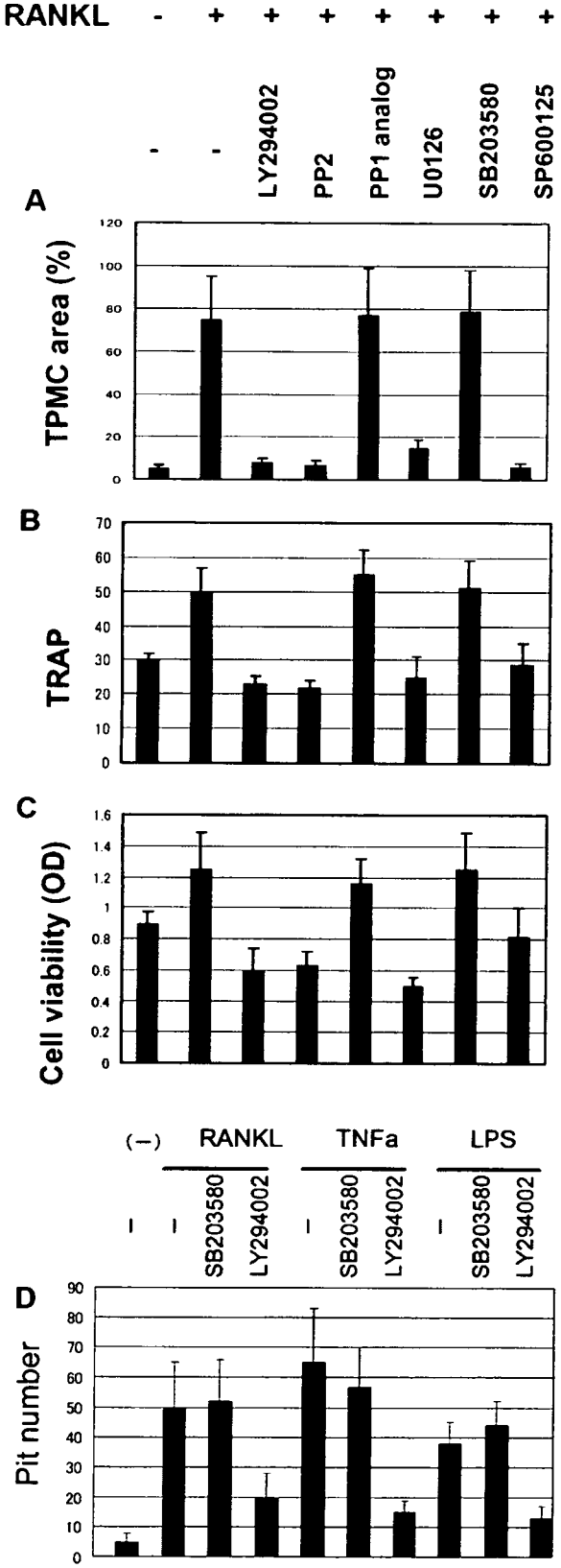



**Fig. 4.** Western blotting analysis during cell fusion process. The cells were treated with 25 ng/ml RANKL, 25 ng/ml TNF-α, 25 ng/ml LPS, or 100 ng/ml PGN for the indicated time and then subjected to Western blotting analysis probing with antibodies against c-Fos, NFATc1, phosphorylated (P-) Akt, P-Src, P-ERK, P-p38, P-p65 (RelA, 65 kD subunit of NF-κB), Akt, TRAF6, tubulin, and P-JNK.

**Fig. 3.** RANKL, TNF-α, LPS, and PGN induced cell fusion specifically through their receptors. RAW264.7 cells (1.6 × 105) were cultured for 36 h in 250 μl of α-MEM containing 10% FCS, 25 ng/ml RANKL, and 2 μM U0126 in a 96-well tissue culture plate. Then the medium was replaced with 250 μl of α-MEM containing 10% FCS, indicated stimulation factors (RANKL, TNF-α, LPS, and PGN), and blocking reagents (OPG, PMXB, and Anti-TNF-α; indicated number μg/ml were used). The fused cell area was measured as percent of total plate area. Concentrations used were 10 ng/ml RANKL, 10 ng/ml TNF-α, 10 ng/ml LPS, and 100 ng/ml PGN (**A**). Amounts of TNF-α in the culture media stimulated with RANKL, LPS, and PGN were measured by ELISA (**B**). The mean of three different determinations was plotted.




resorption pits on dentin slices than do osteoclasts derived from bone marrow macrophages. However, the cell line is useful to analyze the detailed mechanisms of osteoclast differentiation.

In this study, we focused on the cell fusion step of osteoclastogenesis. Several investigators reported the later stage of osteoclastogenesis [Lam et al., 2000; Zou and Bar-Shavit, 2002; Zou et al., 2002]. However, this process is not characterized well since it is not easy to isolate cells at each step during the process of osteoclast differentiation. In this study, we used an enhanced culture condition for osteoclastogenesis of RAW264.7 cells. By culturing the cell line in the presence of RANKL and the MEK/ERK inhibitor U0126, the culture period for osteoclastogenesis was shortened to 48 h, less than half of the standard culture period, and almost all the cells are fused to one another at the end of the culture period. Therefore, the TRAP-positive mononuclear cells (preosteoclasts) prepared shortly before cell fusion in the enhanced culture condition appear to be almost homogeneous in terms of differentiation.

In the present study, LPS suppressed RANKL-induced osteoclast formation at the initial step; in contrast, at the latest step it induced cell fusion without RANKL, resulting in osteoclast formation. The suppressive effect of LPS on osteoclastogenesis, which was observed in the present study when LPS and RANKL were simultaneously added at the initial step, has previously been reported for bone marrow macrophages by Takami et al. [2002]. Moreover, it has been reported that LPS promotes the survival of mature osteoclasts via TLR4 [Itoh et al., 2003]. Taken together, the results indicate that the effects of LPS on osteoclastogenesis vary among steps of osteoclast differentiation. In this context, it is notable

**Fig. 5.** Effects of chemical inhibitors of signal transduction on cell fusion and pit formation. RAW264.7 cells ($1.6 \times 10^5$) were cultured for 36 h in 250 μl of α-MEM containing 10% FCS, 25 ng/ml RANKL, and 2 μM U0126 in a 96-well tissue culture plate. Then the medium was replaced with 250 μl of α-MEM containing 10% FCS, 25 ng/ml RANKL, TNF-α, and 10 of chemical inhibitors, and the cells were cultured for a further 12 h. The fused cell area was measured as percent of total plate area (**A**). The TRAP intensity and cell viability were also measured (**B** and **C**). **D**: Pit formation was measured on a plate coated with calcium phosphate (BD BioCoat Osteologic Bone Cell Culture System, Nippon BD, Tokyo, Japan) stimulated with 25 ng/ml RANKL, TNF-α, or LPS in the presence or absence of 10 μM SB203580 or LY294002.

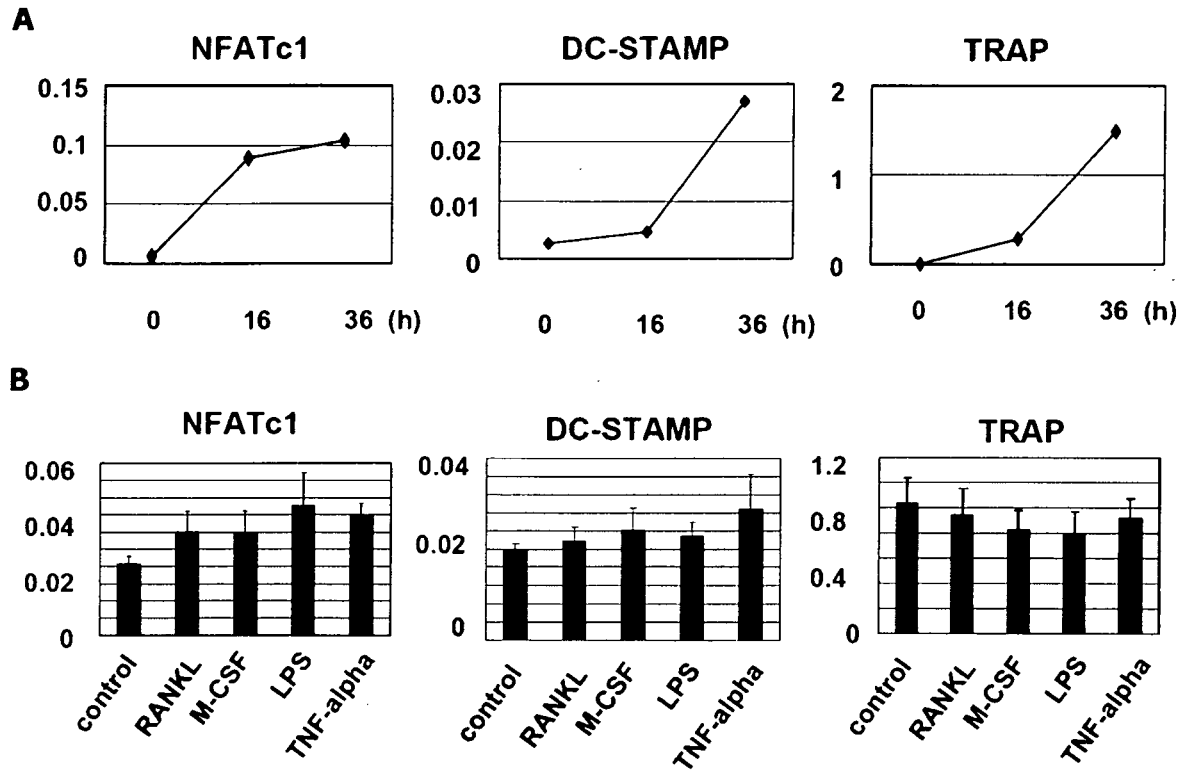



**Fig. 6.** Real-time PCR for NFATc1, DC-STAMP, and TRAP. RAW264.7 cells ($1.6 \times 10^5$) were cultured for 36 h in 250 μl of α-MEM containing 10% FCS, 25 ng/ml RANKL, and 2 μM U0126 in a 96-well tissue culture plate (**A**). Then the medium was replaced with 250 μl of α-MEM containing 10% FCS, 25 ng/ml RANKL, or TNF-α, and the cells were cultured for further 6 h (**B**), followed by RNA preparation and real-time RT-PCR. Each value was normalized by that of GAPDH. The mean of three different determinations was plotted.

that LPS induces bone resorption when injected into bone surfaces of mice [Umezu et al., 1989]. Since osteoclast precursor cells of various steps of differentiation exist together in an in vivo condition, osteoclast precursor cells of the pre-fusion step around bones might differentiate into mature osteoclasts in response to LPS, resulting in bone resorption.

Cell fusion is seen in muscle, nerves, bone in their development, and in the liver in its repair and regeneration [Ogle et al., 2005]. TRAP-positive multinuclear osteoclasts appear in bone. TRAP-positive mononuclear cells fuse to one another because the cell fusion increases cell size and enables the cells to resorb bone to a larger extent [Vignery, 2005]. This explanation is supported by the finding that a DC-STAMP knockout mouse in which TRAP-positive mononuclear cells do not fuse to one another shows an increase in bone density due to a decrease in bone resorption by osteoclasts [Yagi et al., 2005]. Osteoclasts seem to have the same origin in cell lineage as that of macrophages. Macrophages sometimes fuse with one another during infection and tissue repair. The resulting multinucleated macrophages effectively phagocytise pathogens and repair tissues. The mechanism by which macrophages repair tissues seems to be similar to bone resorption of osteoclasts. In this study, inflammatory factors such as TNF-α, LPS, and PGN induced cell fusion, which may be one of mechanisms to repair the inflammatory circumstance of bone, although the inflammation results in an unfavorable bone loss.

Cell fusion-inducing factors include several groups. One is known as fusogens that can directly induce cell fusion. And other groups that include receptors, signaling proteins, transcription factors, and proteins organizing cytoskeleton and membrane, indirectly induce cell fusion [Ogle et al., 2005]. Although EFF-1 of *Caenorhabditis elegans* might be the only one fusogen that have been found in higher eukaryotes so far as we know [Kontani and Rothman, 2005], no fusogens have been found in mammalian cells. Some molecules such as meltrin-α [Harris et al., 1997; Inoue et al.,

1998], CD47, MFR (also reported as SIRP, SHPS-1, BIT, and MyD-1) [Vignery, 2005], and DC-STAMP have been suggested as cell fusion-inducing factors in osteoclasts. In the bones of mice lacking DC-STAMP multinuclear osteoclasts were completely absent, although development of mononuclear osteoclasts was normal. The DC-STAMP-deficient mice suffer from mild osteopetrosis probably because mononuclear osteoclasts can still resorb bones [Yagi et al., 2005]. The expression of DC-STAMP was not significantly different between fused and non-fused cells in this study, suggesting the presence of other cell fusion-inducing factor(s) that directly function at the latest stage of osteoclastogenesis. Further study is needed to understand the molecular mechanisms of cell fusion of osteoclasts.

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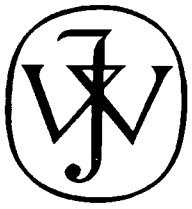

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# Identification of two subpopulations of Bacillus Calmette-Guérin (BCG) Tokyo172 substrain with different RD16 regions

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**Abstract**

Two types of colonies with different morphologies (smooth: S and rough: R) formed when Bacillus Calmette-Guérin (BCG) Tokyo172 substrain was cultured on Middlebrook 7H10 agar medium, and their genotypes were analyzed by multiplex PCR on five RD regions and SenX3-RegX3. In most cases these two colony types had different genotypes, i.e., S colonies showed a characteristic 22 bp deletion in *Rv3405c* of the RD16 region (type I), and R colonies did not have this deletion (type II) similar to many other BCG substrains. Thus, there was a strong relationship between colony morphology and genotype. Both genotypes were found in every Tokyo172 preparation tested, including the seed lot for production, the origin of seed lot from the 1960s and ATCC BCG Japan. Type I was always in the majority. It was suggested that types I and II constituted independent subpopulations within the Tokyo172 substrain. Type I was shown to have a growth advantage over type II both on culture media and in mice organs.


*Keywords:* BCG Tokyo172; RD16 genotype; Subpopulations

## 1. Introduction

Shortly after Bacillus Calmette-Guérin (BCG) started to be used as a prophylactic vaccine for tuberculosis, dissociations of BCG into several colony morphologies were reported and their relevance to virulence discussed [1–3]. This was also suggested after BCG was distributed to many countries and various substrains (or daughter strains, such as Pasteur, Danish, Russia or Japan) were established, as Osborn have reported the presence of minor populations showing different colony morphologies, in the Japanese and Danish substrains [4]. We have also found differences of colony morphology when the Tokyo172 substrain is cultured on Middlebrook 7H10 agar medium. As for genetic differences, Bedwell et al. recently reported that there were two genotypes in the commercial Japan (Tokyo172) preparation, which differed in the presence or absence of 22 bp deletion in RD16, in a study to differentiate BCG substrains by using multiplex PCR on RD regions and SenX3-RegX3 [5]. RD16 is one of the several deletions found in BCG compared with *Mycobacterium tuberculosis* H37Rv, of 7608 bp size and containing six ORFs (*Rv3400–Rv3405c*) [6]. RD16 is totally absent from BCG Moreau [6], while present in all other BCG substrains. Bedwell et al. could not find a genotype without 22 bp deletion of RD16 in ATCC BCG Japan, so they implied that the presence of this genotype in commercial preparation might have been caused by the inadvertent contamination of another BCG substrain. This genotype was shown to have a multiplex PCR pattern identical to that of BCG Birkhaug. The presence of two genotypes in a BCG substrain was also suggested by them for the Copenhagen

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(Danish) substrain, with differences in SenX3-RegX3 [5]. Another reported mutation concerning the presence of multiple genotypes in a BCG substrain is tandem duplications, i.e., DU1 is specific to BCG Pasteur and DU2 is considered to exist in all BCG substrains with different sizes, while both double and triple DU2 are suggested to exist in the Pasteur substrain [7,8].

In the present study we investigated the possible relationship between differences of colony morphology and the genotype of the Tokyo172 substrain, as well as whether these different genotypes were originally present in the Tokyo172 substrain or not.

## 2. Materials and methods

### 2.1. Bacteria

Lyophilized preparations of BCG vaccine final products made from Tokyo172 substrain (Japan BCG Laboratory Inc., Tokyo, Japan), the seed lot used for production, stock of Tokyo172 from the 1960s which was used to establish the seed lot (origin of seed lot), ATCC BCG Japan (ATCC35737), BCG Birkhaug (ATCC35731), and BCG Pasteur (ATCC35734), were used. *M. tuberculosis* Aoyama B strain was obtained from the Japanese National Institute of Health as a control for PCR analysis.

### 2.2. Culture of BCG

Middlebrook 7H10 agar medium supplemented with OADC, 7H9 liquid medium (containing 0.05% Tween80) supplemented with ADC (Japan Becton Dickinson Inc., Tokyo, Japan), Sauton's medium (prepared in our laboratory), and Kudoh PD (egg slant) medium (Japan BCG Laboratory Inc., Tokyo, Japan) were used for culture. For serial passaging, the bacteria grown for 2 weeks on each medium were all harvested and mixed, after which a portion was inoculated into the new medium. All cultures were done at 37 °C.

### 2.3. Multiplex and RD16 PCR analysis

DNA was extracted using a DNA extraction kit (ISOPLANT, Nippon Gene Co. Ltd., Tokyo, Japan) according to the manufacturer's instructions. Multiplex PCR was done to amplify five RDs, which were RD1, RD2, RD14 (RD8 according to the nomenclature of Bedwell et al. [5]), RD15 and RD16, as well as SenX3-RegX3. The sequences of the primers, the cycling profiles, and other conditions were the same as reported by Bedwell et al. The primer set for RD1 was devised to reveal deletion by yielding a PCR product with a different size [5]. For amplification of RD16 alone, a different primer pair (ACATTGGGAAATCGCTGCTGTTG and GGCTGGTGTTTCGTCACTTC) to that used by Bedwell et al. [9] was employed to amplify a more restricted region of RD16 and increase the difference in size relative to the RD16 PCR product. PCR products were subjected to electrophoresis on 10–20% polyacrylamide gel and stained with ethydium bromide.

### 2.4. Sequencing of RD16 PCR products

The bands of the RD16 PCR products were cut out of the gel and sequenced with a DNA sequencer (ABI PRISM 310, Applied Biosystems Japan Ltd., Tokyo, Japan).

### 2.5. Tandem duplication PCR analysis

Junction regions resulting from tandem duplications (JDU) were analyzed by PCR using primers for JDU2A and JDU2B [7]. Because we could not obtain a clear PCR product for JDU2A using the primers described by Brosch et al. under our PCR conditions, we chose a modified primer pair for JDU2A (GGTCCACGGTCAGGTAATTG and GATGTCCAGCAGATCACCAA). The primer pair for JDU2B was TB3608.OF (GAACAGGGTCGCGGAGTCT) and TB3671.7R (GGGTTCATGAGGTGCTAGGG), as described by Brosch et al. [7].

### 2.6. Growth in mice organs

Six-week-old female C57BL/6 mice (Japan Charles River Inc, Yokohama, Japan) were intravenously injected with 10 μg (wet weight) of BCG grown to mid-log phase on Sauton's medium. Then the spleen, lungs, and liver were removed at various times, homogenized, and plated onto Middlebrook 7H10 agar medium. After 2–3 weeks, colonies were counted.

### 2.7. Statistical analysis

Statistical analyses were done using a Statview software (SAS Institute, Cary, NC, USA).

## 3. Results

### 3.1. Two types of colonies in Tokyo172

When BCG Tokyo172 preparations were cultured on Middlebrook 7H10 agar medium, two types of colonies with different shapes were observed (Fig. 1A). One was smooth, round, and dome-shaped in appearance (S-colony), while the other had a rough granular surface, and an irregular shape (R-colony). Both types were found in every Tokyo172 preparation examined, but S-colonies were always in the majority (over 90%). After several selections of each colony type, they were clearly separated into distinct types (Fig. 1B and C), implying that the bacilli forming S- and R-colonies were genetically different.

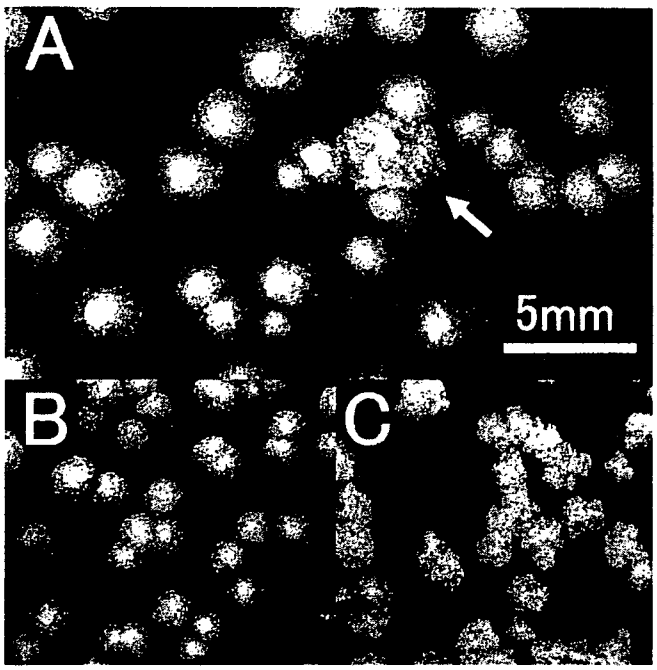


Fig. 1. Two types of colonies in BCG Tokyo172 substrain: (A) Tokyo172 preparation cultured on Middlebrook 7H10 agar medium for 4 weeks. Arrow indicates a R-colony surrounded by S-colonies; (B) isolated S-colonies; and (C) isolated R-colonies, each after single colony selections.

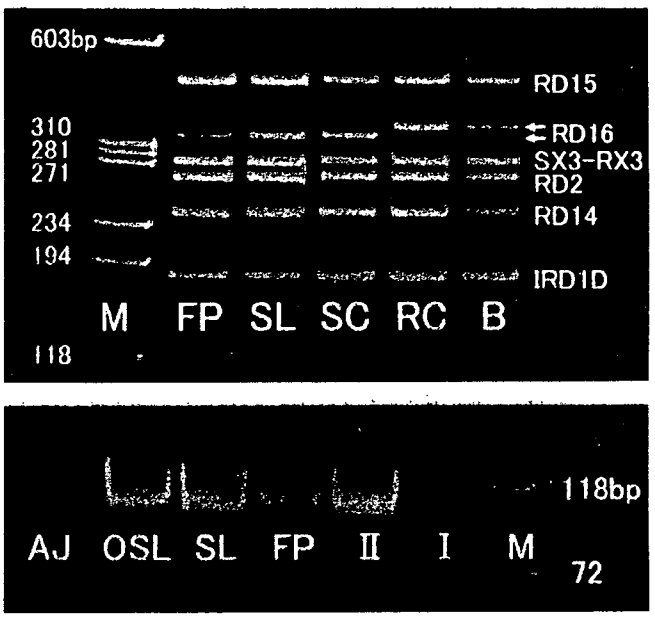


Fig. 2. (Top) Multiplex PCR analysis of the various BCG Tokyo172 specimens and BCG Birkhaug. FP, final product; SL, seed lot; SC, S-colony; RC, R-colony of BCG Tokyo172, respectively, and B, BCG Birkhaug. M, size marker. SX3-RX3, SenX3-RegX3. IRD1D, indicator of RD1 deletion. Arrows at RD16 indicate two types of PCR products differing in size; and (bottom) PCR analysis for the RD16 region showing the presence of type II genotype in BCG Tokyo172 preparations. The 22 bp sequence deleted from type I RD16 was used as one of the primer pair for RD16. AJ, ATCC BCG Japan; OSL, origin of seed lot; SL, seed lot; FP, final product; II, type II; I, type I; and M, size marker.

### 3.2. *Two RD16 genotypes and relationship with colony morphology*

When multiplex PCR was conducted on the Tokyo172 final product, seed lot, separate S- and R-colonies, and BCG Birkhaug, the patterns were almost indistinguishable from one another, except for slight differences of RD16 PCR product size-the R-colony and Birkhaug products were somewhat larger than the others on electrophoresis (Fig. 2 top). The genotype of Tokyo172 producing the smaller RD16 PCR product was defined as type I, and that producing the larger product was defined as type II. When PCR was done on each Tokyo172 colony formed on 7H10 medium, a strong relationship between colony morphology and genotype was shown, since 98.7% of S-colonies had the type I genotype and 95.9% of R-colonies had the type II genotype (Table 1). From these results, it was suggested that the Tokyo172 preparation mainly consisted of organisms with the type I genotype.

Sequencing of the type I and II RD16 PCR products showed a 22 bp deletion (GTGCAACAGTCTGGTCAGCTTC) in type I compared with type II. This sequence is a part of *Rv3405c* in RD16 (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). These results were exactly the same as Bedwell et al. reported for two genotypes found in a commercial Japanese preparation [5].

Table 1
Relationships between the colony morphology on Middlebrook 7H10 agar medium (S- or R-colony) and the RD16 genotype (type I or II) in BCG Tokyo172 preparation

| | S-colonies ($n$ = 153) | R-colonies ($n$ = 97) |
|---|---|---|
| RD16 genotype | | |
| I | 151 (98.7%) | 4 (4.1%) |
| II | 2 (1.3%) | 93 (95.9%) |

To specifically detect type II genotype, PCR analysis was performed using the 22 bp sequence (GTGCAACAGTCTGGTCAGCTTC) deleted from the RD16 of type I, instead of one of the RD16 primer pair, to test ATCC BCG Japan, the origin of seed lot, the seed lot for production, the Tokyo172 final product, type I, and type II. In all cases except for type I, a band of the expected size (110 bp) was detected (Fig. 2 bottom), which indicated the presence of type II in all of the Tokyo172 preparations tested. However, the band for ATCC BCG Japan had a low density compared with others, suggesting a low content of type II in this preparation.

### 3.3. *Difference in tandem duplication between Tokyo172 type II and BCG Birkhaug*

The PCR patterns for JDU2A and JDU2B showed differences among BCG substrains, i.e., both the JDU2A and JDU2B bands in Pasteur, only the JDU2B band in Birkhaug, and no band in Tokyo172 (seed lot) or *M. tuberculosis* Aoyama B strain (the negative control). Both the type I and II of Tokyo172 also yielded no band (Fig. 3), which indicated

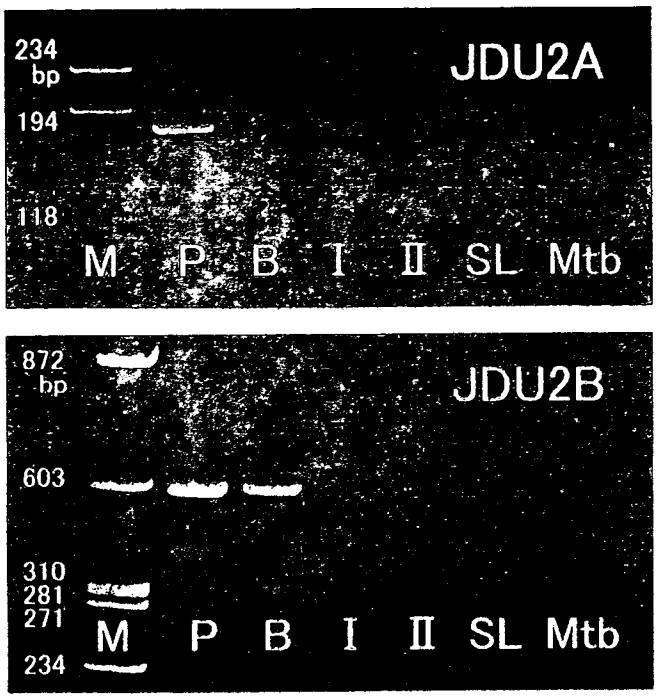


Fig. 3. Tandem duplication analysis by PCR on junction regions, showing distinct patterns between BCG substrains: (top) JDU2A and (bottom) JDU2B. P, BCG Pasteur; B, BCG Birkhaug; I, II and SL: type I, type II and seed lot of BCG Tokyo172, respectively; Mtb, *Mycobacterium tuberculosis* strain Aoyama B and M, size marker.

that DU2 mutations of these might exist in locations different from those of Birkhaug and Pasteur substrains.

### 3.4. Growth in mice organs

After 10 μg wet weight of the type I ($4.6 \times 10^5$ CFU: colony forming units) or type II ($3.7 \times 10^5$ CFU) was intravenously injected into C57BL/6 mice, the recovery of CFU from the spleen, lungs and liver was as shown in Fig. 4. In the spleen, type I increased at 2 weeks after injection to approximately five times the level at 1 day and gradually decreased

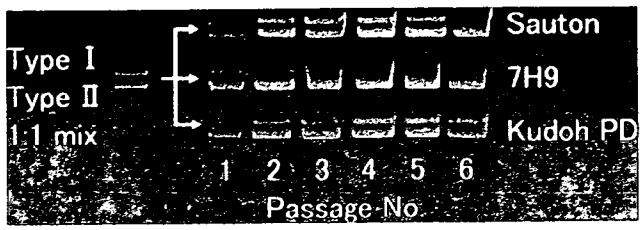


Fig. 5. Competition experiment on culture media showing a growth advantage of type I over type II. A mixture of equal amounts (by wet weight) of type I and type II was inoculated on Sauton's medium, Middlebrook 7H10 medium, or Kudoh PD egg slant medium, and thereafter serially passaged every 2 weeks, and PCR for RD16 was conducted.

thereafter, while type II did not increase much and stayed below type I throughout the experiment. In the lungs, both types did not show any apparent growth except for slight increase in type I at 4 weeks. Although the initial level of type II at 1 day was approximately six-fold higher than that of type I, it decreased rapidly. In the liver the tendency was similar as in the lungs; however, difference in initial level between both types at 1 day was not so apparent, and the increase of type I at 4 weeks was more pronounced, than in the case of the lungs. Difference between type I and type II was statistically significant in the spleen, but not in lungs and liver (Mann-Whitney's *U*-test, $p = 0.001$).

### 3.5. Growth competition studies in vitro and in vivo

When equal amounts (by wet weight) of type I and type II were mixed and inoculated on Sauton's medium, Middlebrook 7H9 medium, or Kudoh PD egg slant medium, and thereafter serially passaged every 2 weeks, the RD16 PCR analysis showed a decrease of type II compared with type I in every case. This occurred most rapidly in 7H9 medium, more slowly in Sauton's medium, and most gradually in Kudoh PD medium (Fig. 5). When 10 μg wet weight of the similar mixture of type I and type II ($3.1 \times 10^5$ CFU) was injected intravenously into C57BL/6 mice, the type II/type I ratio (estimated from the recovered CFU numbers having S- or

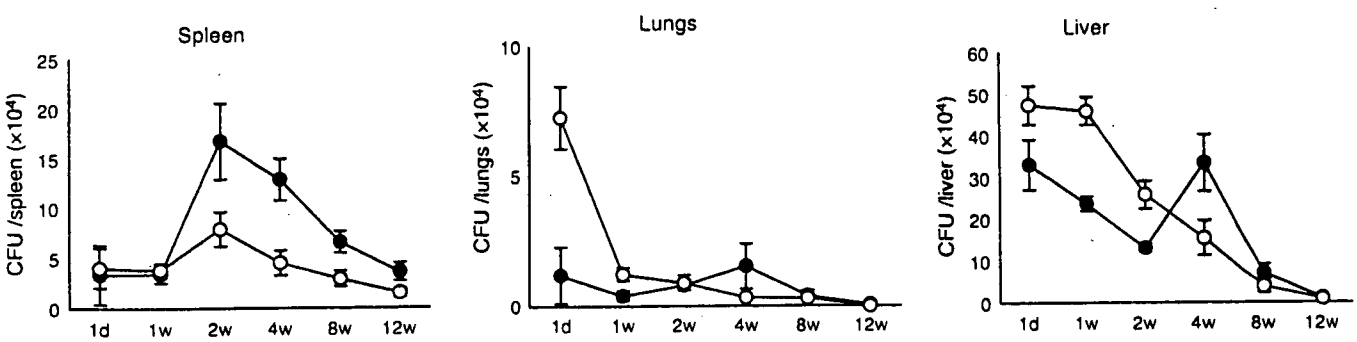


Fig. 4. Growth of type I and type II in mice organs. 10 μg wet weight of type I ($4.6 \times 10^5$ CFU) or type II ($3.7 \times 10^5$ CFU) was intravenously injected into C57BL/6 mice ($n = 3$), and CFU recovered from the spleen, lungs and liver from 1 day to 12 weeks after injection, were counted. Closed circles: type I, open circles: type II. Error bars: standard deviations. Difference between type I and type II was significant in the spleen (Mann-Whitney's *U*-test, $p = 0.001$), but not in lungs and liver.