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Human parotid saliva contains soluble toll-like receptor (TLR) 2 and modulates TLR2-mediated interleukin-8 production by monocytic cells

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Abstract

Toll-like receptor (TLR) family members are pattern-recognition receptors and very important molecules in innate immunity. Although TLRs are originally type I transmembrane receptors, soluble forms of TLRs are detected in human plasma and milk. This study showed that soluble TLR2 (sTLR2) is detected in human parotid saliva. Western blotting with anti-TLR2 antibodies (Abs) showed that three polypeptides are detected as sTLR2 with molecular weights of 55, 40 and 27 kDa, respectively. Parotid saliva neutralized the binding of anti-TLR2 polyclonal Ab to cell-surface TLR2 on THP-1, a human monocytic cell line. Immunohistochemical analysis revealed that TLR2 is expressed in serous and interlobular ductal cells of human salivary gland. Human salivary gland cell lines, AZA3 and HSY, constitutively expressed TLR2. Parotid saliva augmented IL-8 production of THP-1 cells stimulated with a synthetic TLR2 ligand, Pam_3Cys-Ser-$(Lys)_4$ (Pam_3CSK_4). Depletion of sCD14 from parotid saliva by immunoprecipitation eliminated the augmentation of IL-8 production, indicating that the augmentable effects depended on sCD14 in parotid saliva. On the other hand, preincubation of Pam_3CSK_4 with parotid saliva abrogated the augmentation of IL-8 production, indicating that sTLR2 in saliva bound to Pam_3CSK_4 and neutralized its function. These results suggest that parotid saliva modulates the TLR2-mediated immune responses with binary mechanisms via sTLR2 and sCD14 in the oral cavity.

Keywords: Parotid saliva; Salivary gland; TLR2; Soluble form; Monocyte

1. Introduction

Toll-like receptors (TLRs) are type I transmembrane receptors that recognize conserved microbial structures, called pathogen-associated molecular patterns (PAMPs) (Takeda et al., 2003). The binding of specific ligands to TLRs induces many immunological responses (Takeda et al., 2003). TLRs are expressed on various types of cells, including antigen-presenting cells, epithelial cells and endothelial cells, and are considered very important molecules in innate immune systems (Takeda et al., 2003).

In 10 identified human TLRs, TLR2 functions as a heterodimer with TLR1 or TLR6 (Wetzler, 2003). TLR2/TLR1 complex recognizes bacterial triacyl lipopeptide and synthetic lipopeptide structure Pam_3Cys-Ser-$(Lys)_4$ (Pam_3CSK_4) (Takeuchi et al., 2000; Wyllie et al., 2000), whereas TLR2/TLR6 complex recognizes mycoplasmal diacyl lipopeptide, peptideglycan and zymosan (Chu et al., 2005; Nakao et al., 2005; Underhill et al., 1999). It was reported that TLR2-deficient mice produce fewer inflammatory cytokines in response to live and heat-killed *Staphylococcus aureus*, and are highly susceptible to *S. aureus* infection (Knuefermann et al., 2004; Takeuchi et al., 2002). In *Mycoplasma pneumoniae* airway infection, TLR2 plays a critical role in excessive airway mucin production (Chu

Abbreviations: FITC, fluorescein isothiocyanate; Ig, immunoblobulin; IL, interleukin; LPS, lipopolysaccharide; MAb, monoclonal antibody; Pam_3CSK_4, Pam_3Cys-Ser-$(Lys)_4$; PAMPs, pathogen-associated molecular patterns; PBS, phosphate-buffered saline; RT-PCR, reverse transcriptional PCR; TLR, toll-like receptor

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et al., 2005). Moreover, TLR2 deteriorates zymosan-induced arthritis and is necessary for the development of acquired immune responses to zymosan (Fransnelli et al., 2005). These reports indicate that TLR2 is a key molecule in host defense against gram-positive bacteria, mycoplasma and fungus.

CD14, a 55-kDa glycosylphosphatidylinositol-anchored glycoprotein, is a well-established pattern recognition receptor for lipopolysaccharide (LPS) (Haziot et al., 1988). CD14 is mainly expressed on monocytes and macrophages, and is secreted as a soluble form (sCD14) from a wide variety of cells (Tapping and Tobias, 2000). Recently, it was reported that CD14 is involved in the recognition of lipopeptides (Manukyan et al., 2005; Schröder et al., 2004).

Saliva, a complex mix of fluids from major (parotid, submandibular and sublingual) and minor salivary glands is a valuable oral fluid critical for the preservation and maintenance of oral health. Saliva contains a number of antimicrobial and immunofunctional agents, secretory immunoglobulin (Ig) A, proteins (glycoproteins, statherins, agglutinins, histidine-rich proteins and proline-rich proteins), mucins, enzymes (lysozyme, peroxidase and proteases), lactoferrin and cytokines (Humphrey and Williamson, 2001; Tenovuo, 1998). The concerted action of these agents is thought to provide multifunctional and immunological networks in the oral cavity.

Our previous reports revealed that human major salivary glands constitutively express and secrete sCD14, which mediates the activation of CD14-lacking epithelial cells by LPS and *Actinobacillus actinomycetemcomitans*, a gram-negative periodontopathic bacterium (Uehara et al., 2003; Takayama et al., 2003). It was reported that soluble-form TLR2 (sTLR2) is detected in human plasma and milk, and down-regulates Pam_3CSK_4-induced cell activation (LeBouder et al., 2003). These observations led us to investigate whether sTLR2 exists in human saliva, and if so, whether saliva shows biological activity to TLR2-mediated immune responses. Since whole saliva contains serum-derived gingival crevicular fluids, it possibly contaminates sTLR2 derived from serum. We therefore analyzed parotid saliva, which contributes more than 50% of stimulated saliva (Humphrey and Williamson, 2001). In this study, we showed that sTLR2 is detected in human parotid saliva, which modulates TLR2 ligand-induced IL-8 production by monocytic cells with binary mechanisms via sCD14 and sTLR2.

2. Materials and methods

2.1. Reagents

Anti-human TLR2 monoclonal antibody (MAb) TL2.1 (mouse IgG2a) was purchased from HyCult Biotechnology (Uden, The Netherlands). Anti-human CD14 MAb UCH-M1 (mouse IgG2a), anti-human TLR2 polyclonal antibody (Ab) (sc-8689, goat IgG) and its blocking peptide used for immunization (sc-8689p), and Protein G PLUS-Agarose (PGA) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Horseradish peroxidase (HRP)-conjugated anti-mouse IgG (goat) was purchased from Pierce Biotechnology Inc. (Rockford, IL, USA). Fluorescein isothiocyanate (FITC)-conjugated affinity purified anti-mouse IgG (goat) was purchased from Jackson Immuno Research Laboratories Inc. (West Grove, PA, USA). Pam_3CSK_4 hydrochloride was purchased from CALBIOCHEM (Darmstadt, Germany). All other reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA), unless otherwise indicated.

2.2. Collection of parotid saliva

Parotid saliva was collected from five healthy adult donors, aged 22–31 years with the aid of Schaefer cups placed over the Stensen's duct (Schaeffer et al., 1977). The saliva samples were immediately clarified by centrifugation at 14,000 × *g* for 10 min at 4 °C. The clarified samples were sterilized with filtration (0.22-μm pore size) and stored at −80 °C until use. The experimental procedures were approved by the Ethical Review Board of Tohoku University Graduate School of Dentistry (Sendai, Japan).

2.3. Conjugation of HRP and FITC to Ab

Conjugation of HRP to anti-human TLR2 polyclonal Ab was carried out with glutaraldehyde. Briefly, HRP was reacted with 1% glutaraldehyde solution overnight at room temperature, and then Ab was added to the gltaraldehyde-HRP solution, and incubated overnight at 4 °C. After blocking with lysine, the mixture was dialyzed against phosphate-buffered saline (PBS) and used in experiments.

To conjugate FITC, Ab dialyzed against carbonate buffer (pH 9.5) was mixed with FITC dissolved in dimethyl sulfoxide. After overnight incubation at 4 °C with rotation, the mixture was passed through PD-10 gel filtration (Amersham Bioscience Corp., Piscataway, NJ, USA) to remove the free FITC.

2.4. Western blotting

Parotid saliva samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions originally described by Laemmli (1970). After electrophoresis, gel proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA). The blot was blocked for 1 h with 3% (w/v) ovalbumin and 0.05% Tween 20 in PBS, and incubated with HRP-conjugated or non-labeled Ab. HRP-conjugated anti-mouse IgG was used as the second Ab. HRP-conjugated anti-TLR2 polyclonal Ab (20 ng) was incubated with its blocking peptide (5 μg/100 μl) or PBS at 37 °C for 1 h. To prevent nonspecific binding, the second Ab was incubated with parotid saliva at 37 °C for 1 h before use. After washing, the blot was analyzed with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology Inc.) and a Chemi Imager (Alpha Innotech Corp., San Leadron, CA, USA).

2.5. Cell and cell culture

The parotid gland intercalated ductal cell line HSY (Yanagawa et al., 1986) and the human salivary gland acinar cell

line AZA3 (Sato et al., 1987) were donated by Sato (Tokushima University, Tokushima, Japan). THP-1, a human monocytic leukemia cell line was obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). HSY and THP-1 cells were grown in RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) with 10% fetal calf serum (FCS, Tissue Culture Biologicals, Tulare, CA, USA). AZA3 cells were grown in Dulbecco's modified Eagle's minimum medium (Nissui Pharmaceutical) with 10% FCS.

2.6. Flow cytometry

Flow cytometric analyses were performed using FACSCalibur flow cytometer and CELLQuest software (BD Biosciences, San Diego, CA, USA). Adherent cell lines were collected by nonenzymatic cell dissociation solution (Sigma–Aldrich) and washed with PBS. Cells were incubated with FITC-conjugated anti-TLR2 polyclonal Ab, anti-TLR2 MAb TL2.1 or control IgG on ice for 30 min. Cells stained with TL2.1 or control IgG were further incubated with FITC-conjugated goat anti-mouse IgG on ice for 30 min.

2.7. Reverse transcriptional PCR (RT-PCR) assay

Cells were lysed in 1 ml of ISOGEN (Nippon Gene, Toyama, Japan), and total RNA was extracted as described in the instruction manual. Total RNA was dissolved in 30 μl of diethyl pyrocarbonate-treated water (Nippon Gene) and incubated at 65 °C for 10 min. cDNA synthesis was carried out with a first-strand cDNA synthesis kit (Amersham Bioscience Corp.). PCR mixtures contained 0.25 μl of cDNA mixture, 2.5 μl of 10× PCR buffer (Applied Biosystems, Foster City, CA, USA), 200 μM of deoxynucleotide triphosphates (Applied Biosystems), 25 pmol of each primer and 0.625 units of AmpliTaq DNA polymerase (Applied Biosystems) in a total volume of 25 μl. Amplification was performed using a GeneAmp PCR System 9700 (Applied Biosystems). The primers used for PCR were as follows: TLR2, forward 5′-GCCAAAGTCTTGATTGATTGG-3′ and reverse 5′-TTGAAGTTCTCCAGCTCCTG-3′ (Zhang et al., 1999); β-actin, forward 5′-GTGGGGCGCCCCAGGCACCCA-3′ and reverse 5′-CTCCTTAATGTCACGCACGATTTC-3′ (Butch et al., 1993). The PCR conditions were as follows: with TLR2, 38 cycles at 94 °C for 30 s, 54 °C for 30 s and 72 °C for 30 s for amplifying a 347-bp product; β-actin, 32 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s for amplifying a 548-bp product. PCR products were electrophoresed using 3% agarose (Nusieve 3:1 agarose; BMA, Rockland, ME, USA). After staining with ethidium bromide, amplified DNA bands were analyzed with a Chemi Imager.

2.8. Immunohistochemistry

Human labial salivary glands tissues were obtained with informed consent from healthy volunteers ($n = 3$). Tissues were fixed in perodate-lysine-4% paraformaldehyde for 4 h at 4 °C. After washing in PBS containing sucrose, fixed tissues were embedded in OCT compound (Sakura, Tokyo, Japan), and immediately frozen. Six-micrometer frozen tissue sections were incubated with anti-TLR2 polyclonal Ab overnight at 4 °C. Subsequently, sections were treated with rabbit anti-goat Simple stain MAX PO (Nichirei, Tokyo, Japan) overnight at 4 °C. The chromogen used was 3′,3-diaminobenzidine tetrahydrochloride (Dako Cytomation, Kyoto, Japan). The sections were counterstained with hematoxylin. As a negative control, normal goat serum (ZYMED, San Francisco, CA, USA) and blocking peptide were used. The Ethical Review of Tohoku University Graduate School of Dentistry approved the experimental procedures.

2.9. Measurement of IL-8 in culture supernatant of THP-1 cells

THP-1 cells were suspended in RPMI 1640 with 1% of FCS and seeded in a 96-well flat-bottomed plate (Nunc; Nalge Nunc International, Rochester, NY, USA) at 5×10^4 cells/200 μl/well. The cells were stimulated with Pam_3CSK_4 in the presence or absence of parotid saliva at 10% (v/v) for 6 h at 37 °C. The amounts of IL-8 in culture supernatant were measured with an OptEIA Human IL-8 ELISA set (BD Biosciences).

2.10. Depletion of sTLR2 and sCD14 from parotid saliva

sTLR2 and sCD14 was depleted with immunoprecipitaion. Briefly, parotid saliva samples were precleared by incubation with PGA for 1 h at 4 °C with rotation. The precleared samples were incubated with anti-human TLR2 polyclonal Ab or anti-human CD14 MAb on ice for 2 h, and then further incubated with PGA overnight at 4 °C with rotation. After centrifugation to remove PGA, the supernatants were sterilized with filtration (0.22-μm pore size).

2.11. Data analysis

All of the experiments in this study were performed at least three times to confirm the reproducibility of the results. The data shown are representative results. Experimental values are given as the mean ± S.D. of triplicate assays. Statistical analysis was performed with one-way analysis of variance using the Dunnett's or Bonferroni's method, and $P < 0.05$ was considered significant.

3. Results

3.1. sTLR2 polypeptides were detected in parotid saliva

We first examined whether sTLR2 exists in saliva by Western blotting. Four polypeptides of sTLR2 were detected in parotid saliva with anti-TLR2 polyclonal Ab (Fig. 1A). The molecular weights of these polypeptides were 55, 40, 27 and 26 kDa, respectively. The intensity of each band varied among donors. Preincubation of anti-TLR2 polyclonal Ab with blocking peptides abrogated the three polypeptides of 55, 40 and 27 kDa. Only a weak signal of 26 kDa polypeptide was detected with anti-TLR2 polyclonal Ab and blocking peptides, indicating that part of the 26 kDa signal was nonspecific or that some other polypep-

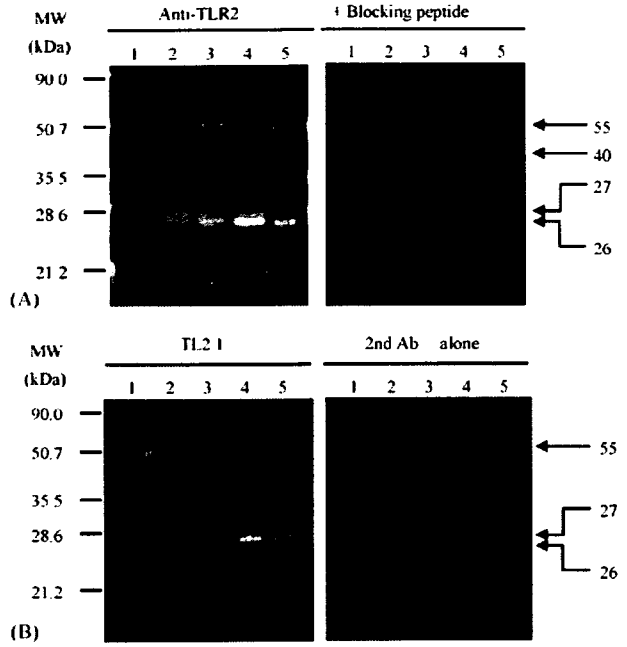

Fig. 1. Detection of sTLR2 in human parotid saliva. (A) Parotid saliva (10 μl each) was subjected to Western blotting with HRP-conjugated anti-TLR2 polyclonal Ab. To confirm the specificity of anti-TLR2, Ab and its blocking peptides were incubated at 37 °C for 1 h before Western blotting analysis. (B) Western blotting with anti-TLR2 MAb TL2.1 and HRP-conjugated anti-mouse IgG as second Ab. To prevent the nonspecific binding of second Ab, Ab was incubated with parotid saliva at 37 °C for 1 h before use. Each lane was applied with parotid saliva collected from five donors, respectively.

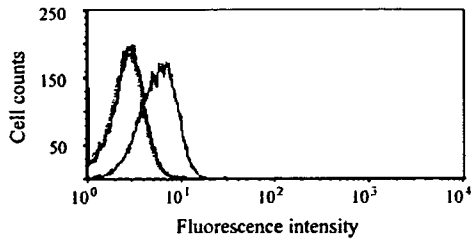

Fig. 2. Neutralization of anti-TLR2 polyclonal Ab reactivity with parotid saliva. Human monocytic THP-1 cells were stained with FITC-conjugated anti-TLR2 polyclonal Ab preincubated with PBS (thin line) or parotid saliva (bold line), and analyzed by flow cytometry. Dotted line shows unstained THP-1 cells (control).

tide(s) reacting nonspecifically with anti-TLR2 polyclonal Ab migrated at the same position. Anti-TLR2 MAb TL2.1 was also used for Western blotting. As shown in Fig. 1B, polypeptides of 55, 27 and 26 kDa were detected with TL2.1. A weak signal of two polypeptides (55 and 26 kDa) was also detected with second Ab alone. The 40 kDa polypeptide was not detected probably due to a lack of epitope to TL2.1. These results from two anti-TLR2 Abs indicated that the three polypeptides (55, 40 and 27 kDa) are sTLR2 in parotid saliva.

3.2. *Parotid saliva neutralized the binding of anti-TLR2 Ab to THP-1 cells*

To further confirm the existence of sTLR2 in parotid saliva, the neutralizing effects on anti-TLR2 Ab binding were examined by flow cytometric analysis. FITC-conjugated anti-TLR2

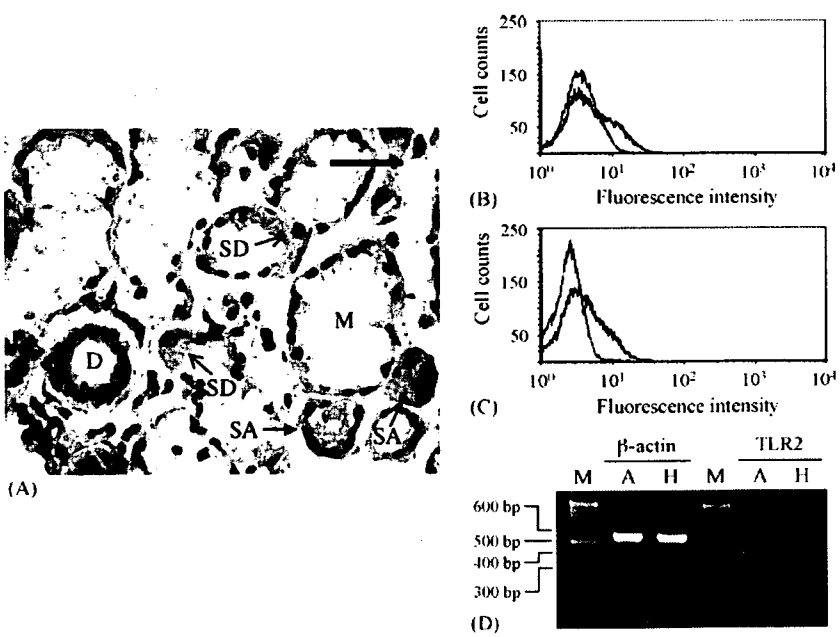

Fig. 3. Expression of TLR2 in human salivary gland. (A) Cryosections of human minor salivary gland from healthy volunteers were stained with anti-TLR2 polyclonal Ab (brown). The sections were counterstained with hematoxylin in blue. SA, serous acinus; SD, serous demilune; M, mucous acinus; D, interlobular duct. Scale bar: 25 μm. The results presented are representative of three volunteers with similar results. (B and C) Human salivary gland cell lines, AZA3 (B) and HSY (C) were stained with anti-TLR2 MAb TL2.1 (bold line) or isotype control Ab (thin line), and analyzed by flow cytometry. (D) mRNA expressions of β-actin (518-bp) and TLR2 (347-bp) were examined by RT-PCR methods. Lane M, molecular weight marker; lane A, AZA3; lane H, HSY. The results presented are representative of three independent experiments with similar results.

polyclonal Ab bound to the surface of THP-1 cells (Fig. 2). However, when Ab was preincubated with parotid saliva, no Ab binding was observed. These results indicated that sTLR2 in parotid saliva neutralizes the reactivity of anti-TLR2 polyclonal Ab.

3.3. Salivary gland cells constitutively expressed TLR2

We analyzed TLR2 expression in human salivary gland with immunohistochemical method. As shown in Fig. 3A, TLR2 protein was expressed in serous (acinus and demilune) cells and interlobular ductal cells but not mucous acinar cells of human salivary gland. We also examined TLR2 expression in human salivary gland cell lines, AZA3 and HSY. Both cell lines constitutively expressed TLR2 on the cell surface (Fig. 3B and C). Moreover, RT-PCR assay revealed that TLR2 mRNA was constitutively expressed in both cell lines (Fig. 3D). These results indicated that TLR2 is constitutively expressed in salivary gland cells.

3.4. Parotid saliva augmented IL-8 production by THP-1 cells stimulated with TLR2 ligand

Next, we analyzed the effects of parotid saliva on TLR2-mediated monocytic cell activation. Pam_3CSK_4 induced IL-8 production by THP-1 cells in a dose-dependent manner (Fig. 4). In the presence of 10% parotid saliva, basal levels of IL-8 production from unstimulated THP-1 cells were significantly ($P < 0.01$) augmented, and stimulation of THP-1 cells with Pam_3CSK_4 induced the marked production of IL-8, especially at 1 μg/ml, as compared with the control (no parotid saliva). The IL-8 levels in 10% parotid saliva were 11.4 ± 6.5 pg/ml, indicating that the increase in basal IL-8 levels in the presence of 10% parotid saliva was derived from saliva IL-8.

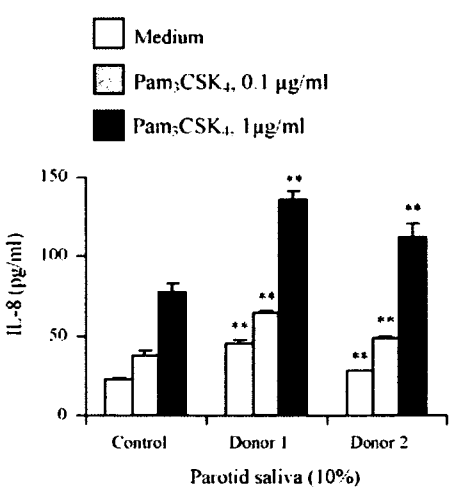

Fig. 4. Effects of parotid saliva on Pam_3CSK_4-induced IL-8 production by THP-1 cells. Cells were stimulated with Pam_3CSK_4 in the presence or absence of parotid saliva (10%) for 6 h at 37 °C. Concentrations of IL-8 in culture supernatants were measured by ELISA. The results are presented as the mean ± S.D. of results of triplicate cultures. $^{**}P < 0.01$ compared with control (without parotid saliva).

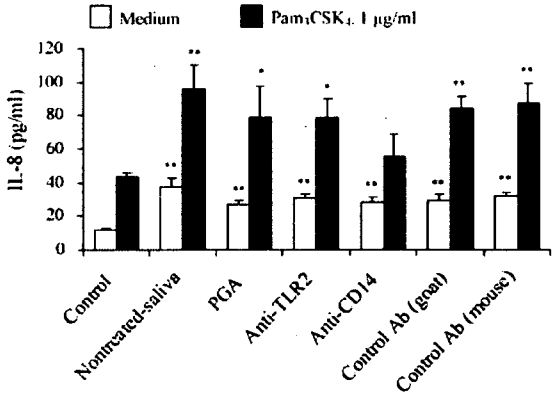

Fig. 5. Effects of sTLR2 and sCD14 depletion from parotid saliva on the augmenting effects of parotid saliva. Parotid saliva is precleaned with PGA, and immunoprecipitated with anti-TLR2 polyclonal Ab (goat IgG), anti-CD14 MAb (mouse IgG) or each control Ab. Cells were stimulated with 1 μg/ml of Pam_3CSK_4 in the presence or absence of parotid saliva (10%) for 6 h at 37 °C. Concentrations of IL-8 in culture supernatants were measured by ELISA. The results are presented as the mean ± S.D. of results of triplicate cultures. $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with the control (without parotid saliva); $^{\#\#}P < 0.01$ compared with untreated saliva.

3.5. sCD14 depletion eliminated augmentable effects of parotid saliva

Recently, it was reported that CD14 is involved in cell activation via TLR2 (Manukyan et al., 2005; Schröder et al., 2004). We previously reported the existence of sCD14 in human parotid saliva (Uehara et al., 2003). Therefore, we examined whether sCD14 in parotid saliva is involved in augmenting IL-8 production. The results showed that the depletion of sCD14 from parotid saliva by immunoprecipitation with anti-CD14 MAb significantly eliminated the augmentation of Pam_3CSK_4-induced IL-8 production with parotid saliva (Fig. 5). The use of control MAb was comparable to untreated or PGA-treated parotid saliva. These results indicated that sCD14 in parotid saliva contributes to the augmentation of Pam_3CSK_4-induced IL-8 production. Depletion of sTLR2 from parotid saliva resulted in the same IL-8 levels as untreated parotid saliva.

3.6. Preincubation of a TLR2 ligand with parotid saliva impaired the function of TLR2 ligand

We further examined the possible role of sTLR2 in parotid saliva. When Pam_3CSK_4 was preincubated with parotid saliva for 1 h at 37 °C, the induction of IL-8 with Pam_3CSK_4 at 1 μg/ml was significantly ($P < 0.001$) impaired compared to without preincubation (Fig. 6). These results indicated that sTLR2 in parotid saliva plays a role in neutralizing and clearing TLR2 ligands in the oral cavity.

4. Discussions

In this study, three polypeptides, 55, 40 and 27 kDa were specifically detected as sTLR2 polypeptides in human parotid saliva by Western blotting. Moreover, flow cytometric analysis

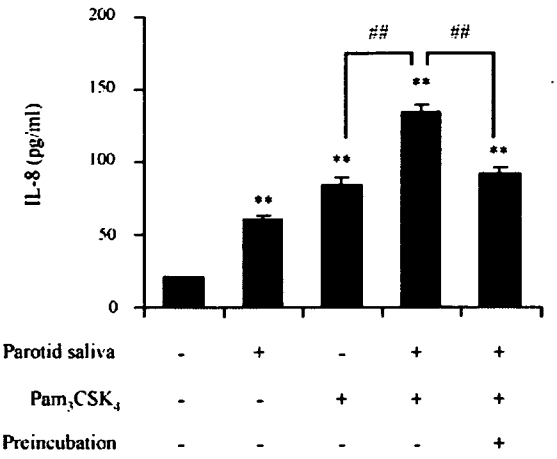

Fig. 6. Effects of preincubation of Pam_3CSK_4 with parotid saliva on augmenting effects of parotid saliva. Cells were stimulated with Pam_3CSK_4 in the presence or absence of parotid saliva, or Pam_3CSK_4 preincubated with parotid saliva for 1 h at 37 °C. Final concentrations of Pam_3CSK_4 and parotid saliva were 1 μg/ml and 10%, respectively. After stimulation for 6 h at 37 °C, culture supernatants were collected and concentrations of IL-8 were measured by ELISA. The results are presented as the mean ± S.D. of results of triplicate cultures. $^{**}P < 0.01$ compared with the control; $^{\#\#}P < 0.01$ compared with parotid saliva and Pam_3CSK_4 (without preincubation).

showed that parotid saliva neutralized the binding of anti-TLR2 polyclonal Ab to cell-surface TLR2 on THP-1 cells. These results clearly indicated that sTLR2 existed in human parotid saliva. Although 27 kDa polypeptide was detected with both polyclonal and monoclonal Ab, 55 and 40 kDa polypeptides were not detected specifically with MAb. This difference was probably caused by epitopes recognized by polyclonal and monoclonal Ab.

The molecular weight of unprocessed form of TLR2 is approximately 90 kDa (Swiss-Prot protein database). The extracellular domain of human TLR2 is approximately 64 kDa, and increases to 84 kDa with N-linked glycosylation (Weber et al., 2004). Therefore, it is suggested that sTLR2 polypeptides in parotid saliva are processed fragments from the entire TLR2 molecule or extracellular domain. LeBouder et al. reported that cell-surface TLR2 is endocytosed and processed in an internal acidic compartment and secreted as sTLR2 (LeBouder et al., 2003). The possible origins of sTLR2 in parotid saliva may be either (i) the bloodstream, similar to other saliva proteins, (ii) cells infiltrated in the parotid gland, or (iii) salivary gland cells. Immunohistochemical analysis in this study clearly showed that TLR2 is constitutively expressed on serous and interlobular ductal cells in the human salivary gland. The parotid gland is a serous gland. Moreover, two salivary gland cell lines, AZA3 and HSY constitutively expressed TLR2. Acinar and ductal cells of salivary gland produce various biological and immunological mediators (Pammer et al., 1998; Sahasrabudhe et al., 2000; Uehara et al., 2003). These observations suggest that the origin of sTLR2 in saliva is salivary gland cells.

It was reported that alternative-spliced TLR4 mRNA encoded 20 kDa of soluble-form TLR4 in mice (Iwami et al., 2001). In fish, membrane- and soluble-form TLR5 are encoded in two separate genes (Tsoi et al., 2006; Tsujita et al., 2004; Tsukada et al., 2005); therefore, it is also possible that sTLR2 polypeptides in parotid saliva are other gene products, but not processed fragments.

In this study, we demonstrated that parotid saliva augmented Pam_3CSK_4-induced IL-8 production by THP-1 cells (Fig. 4). On the other hand, preincubation of Pam_3CSK_4 with parotid saliva impaired the function of Pam_3CSK_4 (Fig. 6), indicating that sTLR2 in parotid saliva bound to Pam_3CSK_4 and neutralized its function. The extracellular domain of TLRs contributes to ligand recognition but not signal transduction (Takeda et al., 2003), and possibly functions as a soluble decoy receptor. It was reported that recombinant extracellular domains of TLRs attenuate TLR-mediated cellular activation (Hyakushima et al., 2004; Iwami et al., 2001; LeBouder et al., 2003). Oral bacterial flora consist mainly of gram-positive bacteria and some pathogenic organisms. Therefore, we considered that sTLR2 in parotid saliva binds and neutralizes to its specific ligands, and actively contributes to the maintenance of oral health by inhibiting excessive cell activation. On the other hand, it cannot be ruled out that other proteins in saliva contribute to neutralizing effects. It is also possible that immunoprecipitation did not completely deplete sTLR2 in parotid saliva. Further investigations are needed to clarify this point.

On the other hand, the depletion of sTLR2 from parotid saliva showed no effects on IL-8 production (Fig. 5). As mentioned above, sTLR2 in saliva is probably processed fragments of the entire molecule or extracellular domain of TLR2, suggesting that the affinity to Pam_3CSK_4 of sTLR2 is weaker than that of intact TLR2 molecules. This study showed that neutralization was detected with the preincubation of parotid saliva and Pam_3CSK_4, but not simultaneous stimulation; therefore, we considered that differences of affinity to Pam_3CSK_4 are the reason why depletion of sTLR2 did not affect IL-8 production.

We previously reported that sCD14 was detected in human parotid saliva (Uehara et al., 2003), and the present study clearly demonstrated that the depletion of sCD14 from parotid saliva eliminated the augmentation of IL-8 production (Fig. 5). It was reported that CD14 bound to lipopeptides and enhanced cell activation through TLR2 (Manukyan et al., 2005; Schröder et al., 2004). Therefore, we considered that sCD14 in parotid saliva is involved in the augmentation of IL-8 production by THP-1 cells induced by Pam_3CSK_4. Human oral epithelial HSC-2 cells constitutively express TLR2 but not CD14, and are refractory to peptidoglycans (Uehara et al., 2002), suggesting that sCD14 in parotid saliva up-regulates the activation of oral epithelial cells through TLR2.

We showed two contrary effects of parotid saliva; the impairment of Pam_3CSK_4 function by sTLR2, and the augmentation of Pam_3CSK_4-induced IL-8 production by sCD14. We consider that these binary mechanisms are important for the maintenance of oral health. sTLR2 captures and excludes its ligands from the oral cavity, and contributes to the prevention of excessive inflammatory responses. In the case of infections, excessive amounts of TLR2 ligands bind to sCD14, and the TLR2-mediated immune responses are augmented.

Although the augmentable effects of parotid saliva varied among donors, no clear correlation was detected between the

effects on IL-8 production and the levels of sTLR2. As shown in Fig. 1, sTLR2 in parotid saliva varied among donors. We previously reported that the levels of sCD14 in parotid saliva also varied among donors (Uehara et al., 2003). Therefore, we considered that the augmentable effects were based on the balance between sTLR2 and sCD14.

We previously reported that saliva modulates bacteria and LPS-induced immune responses by epithelial cells (Takayama et al., 2003; Uehara et al., 2003). In this study, we revealed that parotid saliva modulates TLR2-mediated immune responses with binary mechanisms via sTLR2 and sCD14. Our series of studies reemphasized the important roles of saliva in the maintenance of oral health. Further investigations are needed to elucidate the mechanisms of sTLR2 production in parotid saliva and the contributions of soluble forms of other TLRs to immune surveillance in the oral cavity.

Acknowledgments

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Histidine decarboxylase-stimulating and inflammatory effects of alendronate in mice: Involvement of mevalonate pathway, TNFα, macrophages, and T-cells

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Abstract

Nitrogen-containing bisphosphonates (NBPs) are powerful anti-bone-resorptive drugs, but they frequently induce various inflammatory side effects. Recent clinical applications have disclosed an unexpected new side effect, jaw-bone necrosis and exposure. *In vitro* studies suggest that the inflammatory effects of NBPs are due to Vγ2Vδ2 T-cells, stimulated directly and/or indirectly [the latter via isopentenylpyrophosphate (IPP) in the mevalonate pathway]. Rats and mice, however, lack Vγ2Vδ2 T-cells, yet NBPs still induce necrotic and inflammatory reactions. In mice, NBPs induce IL-1-dependent inflammatory reactions, such as inductions of histidine decarboxylase (HDC, the histamine-forming enzyme) in the liver, lung, spleen, and bone marrow, an increase in granulocytic cells in the peritoneal cavity, pleural exudation, and splenomegaly. Here, we examined the involvement of IPP, TNF, macrophages, and T-cells in the inflammatory actions of alendronate (a typical NBP) in mice. Various statins (mevalonate-synthesis inhibitors) suppressed the alendronate-induced HDC inductions, while mevalonate itself augmented such inductions. IPP injection also induced HDC. Like IL-1-deficient mice, TNF-deficient mice were resistant to alendronate-stimulated HDC induction. Alendronate-stimulated HDC inductions were significantly weaker in macrophage-depleted mice and in nude mice than in control mice. Similar, though generally less clear-cut, results were obtained when other alendronate-induced inflammatory reactions were examined. These results suggest that (i) inhibition of the mevalonate pathway causes and/or modifies at least some inflammatory actions of alendronate in mice, (ii) in addition to IL-1, TNF is also involved in the inflammatory actions of alendronate, and (iii) alendronate may act on a variety of cells, including macrophages and T-cells.

Keywords: Bisphosphonates; Alendronate; Side effects; Statins; Histidine decarboxylase; Mevalonate; TNF

1. Introduction

Nitrogen-containing bisphosphonates (NBPs) have powerful bone-resorption-inhibitory activities [1]. However, most NBPs have inflammatory side effects (such

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as fever, increase in acute-phase proteins, gastrointestinal disturbance, ophthalmic inflammation, and a serious influenza-like reaction in children with osteogenesis imperfecta) [2–8]. Unexpectedly, clinical applications have recently disclosed an additional serious side effect, jaw-bone necrosis and exposure, with about 400 patients having been reported to exhibit this effect in the space of a few years [9,10]. Oral bacteria and/or stimulation by dental treatments have been suggested as triggers and/or promoters of this serious side effect [11]. Recently, the Novartis company reported via the internet about 3000 cases of jaw necrosis caused by their NBP products.

In 1986, Schenk et al. [12] reported that NBPs induce necrosis at the injection site in rats, and that in terms of potency, these necrotic effects roughly parallel the anti-bone-resorption activities of the same NBPs. Further, a single intraperitoneal injection of an NBP into mice induces a variety of inflammatory reactions, such as a long-lasting induction of the histamine-forming enzyme (histidine decarboxylase, HDC) in various tissues, an accumulation of granulocytic cells in the peritoneal cavity, an increase in the pleural exudate, splenomegaly, exacerbation of collagen-induced arthritis, and augmentation of LPS-induced IL-1 production [13–15]. NBPs also induce profound changes in hematopoiesis [16], and their inflammatory actions in mice are IL-1-dependent [17]. In the context of jaw necrosis, it seems very important to note the existence of mutual augmentation of HDC inductions between alendronate (a typical NBP) and such immunostimulants as IL-1, TNF, and lipopolysaccharide (LPS) [15–19]. In response to these inflammatory stimuli, HDC is induced in a variety of immunocompetent cells (macrophages, vascular endothelial cells, granulocytic precursor cells, neutrophils, mast cells, etc.) [20–23].

Kunzmann et al. [24,25], who found that NBPs stimulate Vγ9Vδ2 T-cells to produce inflammatory cytokines (such as TNF), suggested that these cells might be responsible for the inflammatory actions of NBPs. Reportedly, NBPs may stimulate Vγ9Vδ2 either directly [24,25] or indirectly via isopentenylpyrophosphate (IPP), which accumulates as a result of the inhibition of farnesylpyrophosphate synthase in the mevalonate pathway by NBPs [26,27]. Importantly, however, it should be noted that γδT-cells corresponding to human Vγ9Vδ2 T-cells have not been identified in mice or other non-primates [28,29], and hence effects of NBPs due to stimulation of γδT-cells are not seen in non-primates [30,31]. Despite this, NBPs do induce inflammatory and necrotic reactions in mice and rats (see above). The mevalonate pathway exists widely in eukaryotic cells, and (like LPS, IL-1, and TNF) NBPs induce HDC in various tissues in mice (liver, lung, spleen, and bone marrow) [13]. In addition, NBPs reportedly inhibit cholesterol biosynthesis in rat liver in vitro [32]. The observations described above suggested to us that NBPs [by themselves or in combination with immunostimulants (LPS, IL-1, and/or TNF)] may induce inflammatory reactions or necrosis through their actions or influences on a variety of cell-types. In other words, Vγ9Vδ2 T-cells may not be the only cell-type responsible for the inflammatory and necrotic actions of NBPs.

Against the background described above, the present study was designed to examine the contributions made by the mevalonate pathway, TNF, macrophages, and T-cells to the inflammatory actions of alendronate in mice.

2. Materials and methods

2.1. Mice

BALB/c mice (6–7 weeks of age) were bred in our laboratory, with females being selected for the present study. Female BALB/c nude mice were purchased from SLC (Shizuoka, Japan). BALB/c IL-1KO mice (deficient in both IL-1α and IL-1β), TNFαKO mice, and IL-1/TNFαKO mice (deficient in IL-1α, IL-1β, and TNFα) were established from the original IL-1αKO, IL-1βKO, and TNFαKO mice [33,34] by back-crossing to BALB/c mice. All experiments complied with the Guidelines for Care and Use of Laboratory Animals in Tohoku University.

2.2. Reagents

Alendronate (synthesized by ourselves) [35], mevalonate, IPP, and clodronate (Sigma, St. Louis, MO, USA), LPS from *Escherichia coli* O55:B5 (prepared by Westphal's method; Difco Laboratories, Detroit, MI, USA), and other reagents (Wako Pure Chemical Ind., Osaka, Japan) were used. The element analysis of the alendronate synthesized by us was found to coincide with the chemical structure of alendronate, and its biological effects were essentially the same as those reported previously [13] and those of the alendronate commercially available from Sigma (St. Louis, MO, USA). Alendronate, mevalonate, IPP, LPS, and pravastatin were dissolved in sterile saline (S), and in each case the pH was adjusted to 7 with NaOH or HCl, if necessary. Atrovastatin and simvastatin were dissolved in dimethylsulfoxide (DMSO) to a concentration of 20 μmol/ml. Injections were given intraperitoneally (i.p.) or intravenously (i.v.). Experimental protocols and doses are described in the text or in the legend to the figure relating to the relevant experiment. Previous studies have shown that the inflammatory effects of NBPs are dose-dependent, and that alendronate induces an almost maximal HDC induction at 40 μmol/kg [13]. Consequently, we chose this dose for the present study.

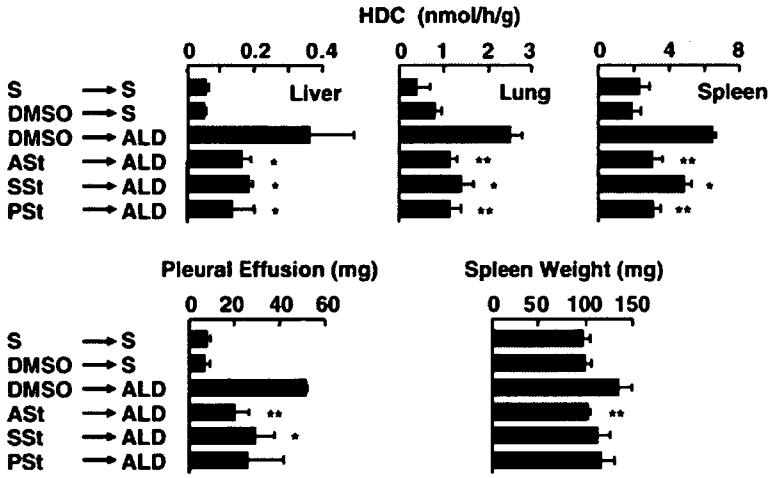

Fig. 1. Inhibition by statins of the inflammatory actions of alendronate (ALD). Saline (S), DMSO, atrovastatin (ASt) (20 μmol/kg), simvastatin (SSt) (50 μmol/kg), or pravastatin (PSt) (50 μmol/kg) was injected (i.p.) into mice. Five minutes later, the mice were injected with S or ALD (40 μmol/kg, i.p.). The mice were decapitated at 3 days after the second injection, and inflammatory markers were measured. Each value is the mean ± SD from four mice. * $P<0.05$ and ** $P<0.01$ vs DMSO → ALD.

2.3. Assay of HDC activity

HDC activities were measured as previously described [19,36]. Briefly, mice were decapitated. Then, before removal of tissues, the number of cells in the peritoneal cavity and the amount of pleural effusion were measured as described below. Next, the required tissues were rapidly removed and stored in a jar with dry-ice until use. Each tissue sample, having been put into a cooled Teflon tube with phosphorylated cellulose and ice-cold 0.02 M phosphate buffer (pH 6.2) containing pyridoxal 5′-phosphate and dithiothreitol, was homogenized. The supernatant obtained after centrifugation of the homogenate was used as the enzyme solution. The histamine in the tissues was bound to the phosphorylated cellulose, and was removed almost completely from the enzyme solution by the centrifugation. Reaction mixture containing the enzyme solution was incubated at 37 °C for 3 h with histidine. After the enzyme reaction had been terminated by adding $HClO_4$, the histamine formed during the incubation was separated by chromatography on a small phosphorylated cellulose column, then quantified fluorometrically. HDC activity was expressed as nmol of histamine formed during a 1 h period of incubation by the enzyme contained in 1 g (wet weight) of each tissue (nmol/h/g).

2.4. Measurement of cell number in peritoneal cavity (PEC)

Briefly, saline (10 ml) was injected into the PEC of ether-anesthetized mice, and the cavity was massaged. Then, the suspension of cells in this saline (5 ml) was recovered using a syringe, and the number of cells in the suspension was counted.

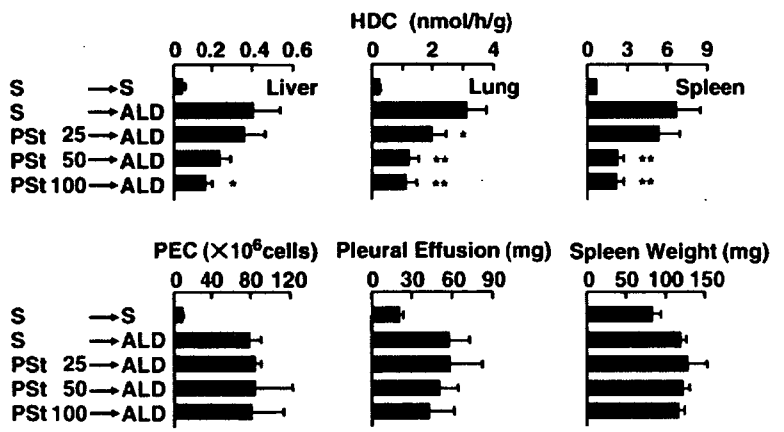

Fig. 2. Dose-related inhibition by pravastatin (PSt) of the inflammatory actions of alendronate (ALD). Saline (S) or PSt (at the indicated dose) was injected (i.p.) into mice. Five minutes later, the mice were injected with S or ALD (40 μmol/kg, i.p.). The mice were decapitated at 3 days after the second injection, and inflammatory markers were measured. Each value is the mean ± SD from four mice. * $P<0.05$ and ** $P<0.01$ vs S → ALD.

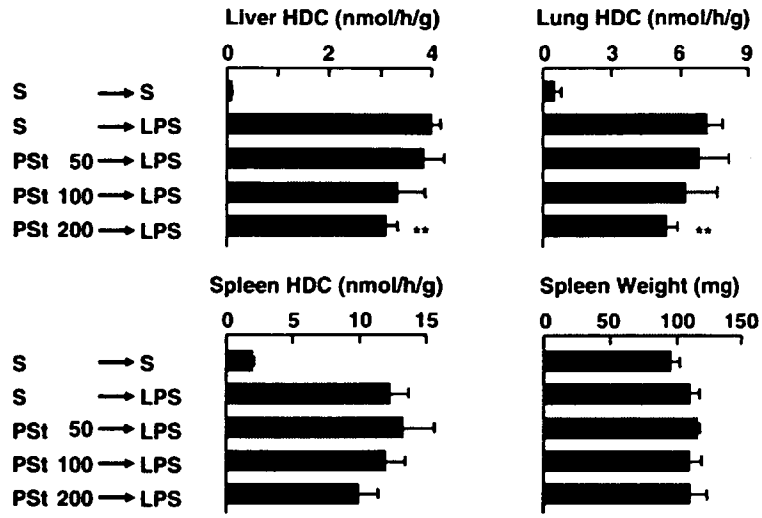

Fig. 3. Dose-related inhibition by pravastatin (PSt) of LPS-stimulated HDC induction. Saline (S) or PSt (at the indicated doses) was injected (i.p.) into mice. Five minutes later, the mice were injected with S or LPS (0.1 mg/kg, intravenously). The mice were decapitated at 3 h after the second injection, and inflammatory markers were measured. Each value is the mean±SD from four mice. $^{**}P<0.01$ vs S → LPS.

2.5. Measurement of exudate in thorax

After the thorax had been opened using scissors, the exudate present in the thoracic cavity was absorbed using small pre-weighed pieces of filter paper. The amount of exudate present was measured as the increase in the weight of the filter paper.

2.6. Depletion and detection of macrophages

Clodronate-encapsulated liposomes (Clo-lip) have been shown to deplete phagocytic macrophages, but not either dendritic cells or neutrophils [37]. A suspension of Clo-lip was prepared as described previously [20,38]. Briefly, 75 mg of phosphatidylcholine and 11 mg of cholesterol were dissolved in chloroform (20 ml) in a round-bottomed flask (1000 ml). The thin film that formed on the walls of the flask after rotary evaporation at 37 °C was dispersed by gentle shaking for 10 min in 10 ml of clodronate solution (200 mg/ml) in 10 mM sodium phosphate buffer (pH 7.4). This suspension was kept for 2 h at room temperature, then sonicated for 3 min (50 Hz) and kept for another 2 h. The resulting liposomes floating on the aqueous phase were collected by Pasteur pipette, then suspended in 10 ml of PBS and centrifuged at 5000 g for 30 min. The precipitated liposomes were finally suspended in 4 ml of PBS and stored at 4 °C. For use, this suspension was diluted five times with saline, and the diluted Clo-lip was injected intravenously (i.v.) at 0.2 ml/mouse. Macrophages were detected by immunohistochemical staining using an anti-F4/80 antibody (Serotec, Kidlington, UK), as described elsewhere [38].

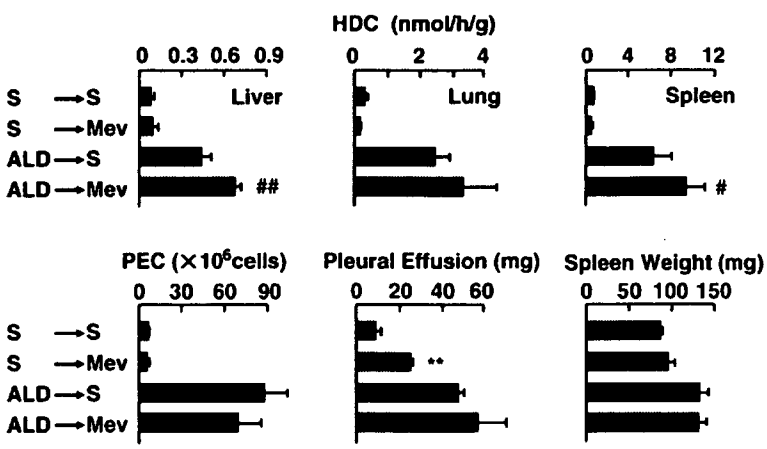

Fig. 4. Augmentation by mevalonate (Mev) of the inflammatory actions of alendronate (ALD). Saline (S) or ALD was injected (i.p.) into mice. One hour later, the mice were injected with S or Mev (7.7 mmol/kg, i.p.). Three days later, the mice were decapitated, and inflammatory markers were measured. Each value is the mean±SD from four mice. $^{**}P<0.01$ vs S → S, $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs ALD → S.

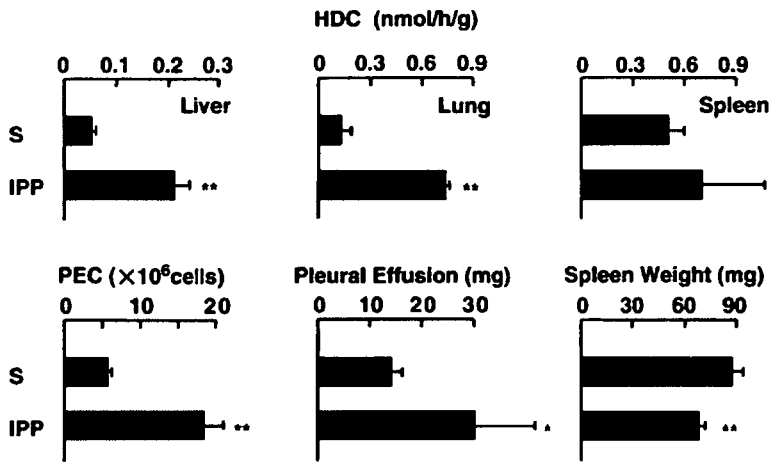

Fig. 5. Inflammatory actions of isopentenylpyrophosphate (IPP). Saline (S) or IPP (40 μmol/kg) was injected (i.p.) into mice, and 8 h later the mice were decapitated. Then, inflammatory markers were measured. Each value is the mean±SD from four mice. $^{*}P<0.05$ and $^{**}P<0.01$ vs S.

2.7. Data analysis

Experimental values are given as mean ± standard deviation (SD). The statistical significance of the difference between two means was evaluated using Dunnett's multiple comparison test. P values less than 0.05 were considered to be significant.

3. Results

3.1. Effects of statins on the inflammatory actions of alendronate

There are several statins. Atrovastatin and simvastatin are lipophilic, while pravastatin is hydrophilic. Hence, the first two were dissolved in DMSO, and the last in saline. All three statins, at the doses tested, suppressed or tended to suppress the inflammatory reactions of mice to alendronate (Fig. 1). The effects of pravastatin on alendronate-induced HDC levels were dose-related (Fig. 2).

3.2. Effects of pravastatin on LPS-stimulated HDC induction

LPS is a potent inducer of HDC in mouse tissues, and it induces HDC in nude mice (lacking matured T-cells) similar to or greater degree than in control mice, although this effect is very weak in C3H/HeJ mice [39] (which are LPS-resistant mice with a mutation in Toll-like receptor 4 [40,41]). The inhibitory effect of pravastatin on LPS-induced HDC induction was weak.

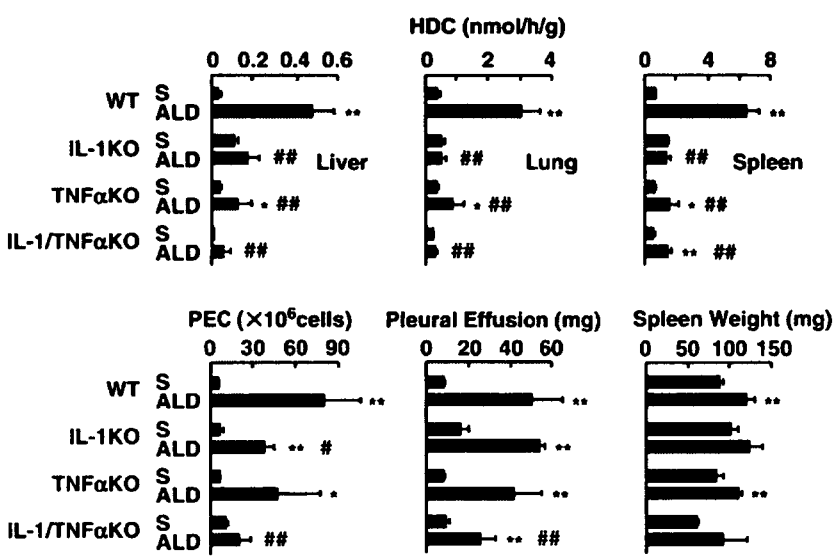

Fig. 6. Involvement of IL-1 and TNFα in the inflammatory actions of alendronate. Saline (S) or alendronate (ALD) (40 μmol/kg) was injected (i.p.) into control wild-type (WT), IL-1KO, TNFαKO, or IL-1/TNFαKO mice. Three days later, tissues were removed and inflammatory markers measured. Each value is the mean±SD from four mice. $^{*}P<0.05$ and $^{**}P<0.01$ vs S in corresponding mice. #$P<0.05$ and ##$P<0.01$ vs ALD of WT.

being significant only at the highest dose used (Fig. 3, compare with Fig. 2).

3.3. Effects of mevalonate on the inflammatory actions of alendronate

Injection of a high dose (7.7 mmol/kg, i.p.) of mevalonate, a precursor of IPP in the mevalonate pathway, induced a slight, but significant pleural effusion, but no other inflammatory reactions (Fig. 4). However, this dose of mevalonate augmented (in the liver and spleen) or tended to augment (in the lung) HDC induction by alendronate (Fig. 4). Mevalonate appeared not to be toxic at this dose (no death or signs of adverse reactions among four mice).

3.4. Inflammatory actions of IPP

First, we injected (i.p.) IPP into mice at 8 μmol/kg. This dose of IPP induced HDC slightly, but significantly in the liver, the effect peaking at 12 h after the injection, but there was no evidence of HDC induction in lung or spleen, or of other inflammatory reactions (data not shown). IPP, at 40 μmol/kg (i.p.) (the same dose as that used of alendronate in the present study), induced HDC in the liver and lung, and tended to induce it in the spleen (Fig. 5). IPP also increased the cell number in PEC and the pleural effusion. However, there was a decrease in spleen weight. In this experiment, the mice were killed at 8 h after the IPP injection because the IPP weakened the mice. Finally, we tested the effects of IPP on LPS-stimulated HDC inductions because (as described in Introduction) LPS-stimulated HDC inductions are augmented in mice pretreated with an NBP. LPS was injected (i.p.) into mice 3 h after an injection of IPP (8 μmol/kg, i.p.), and HDC activities were measured in the liver, lung, and spleen (removed 3 h after the LPS injection). There was an augmented HDC induction in the liver, but no significant augmentation in the other tissues (data not shown).

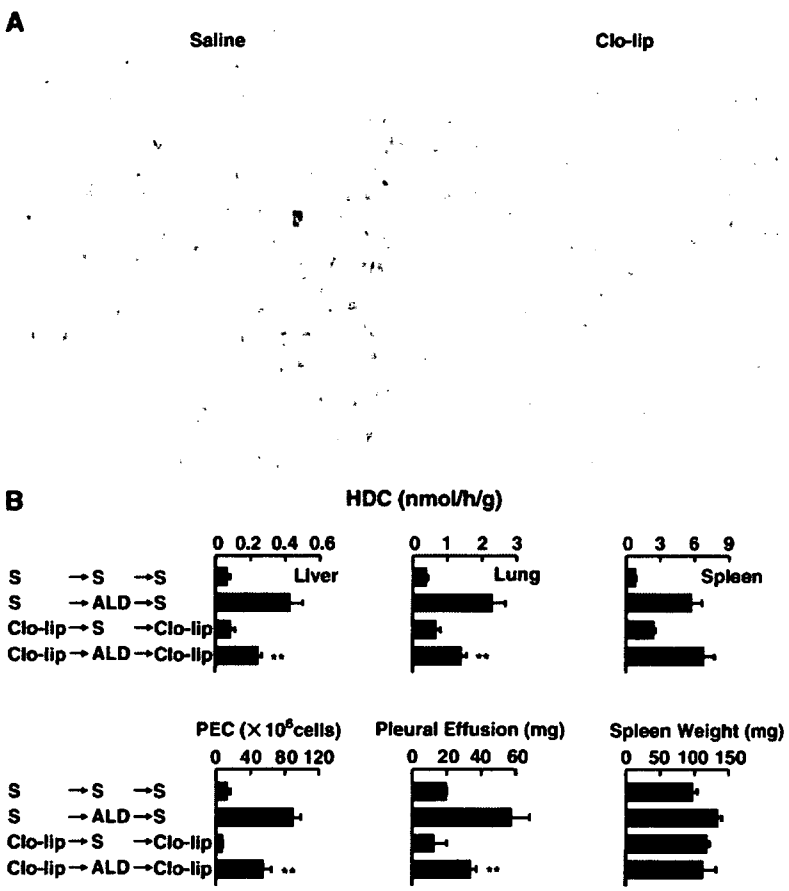

Fig. 7. Effects of macrophage depletion on the inflammatory actions of alendronate (ALD). (A) Saline or Clo-lip was injected (i.v.) into mice. Twenty-four hours later, livers were removed and subjected to immunostaining for the detection of F4/80-positive macrophages. (B) One day after Clo-lip injection, saline (S) or ALD (40 μmol/kg) was injected (i.p.) into mice. Clo-lip was injected again into the mice at 24 h after the second injection. Two days after the second Clo-lip injection, mice were decapitated and inflammatory markers measured. Each value is the mean ± SD from four mice. ** $P<0.01$ vs S→ALD→S.

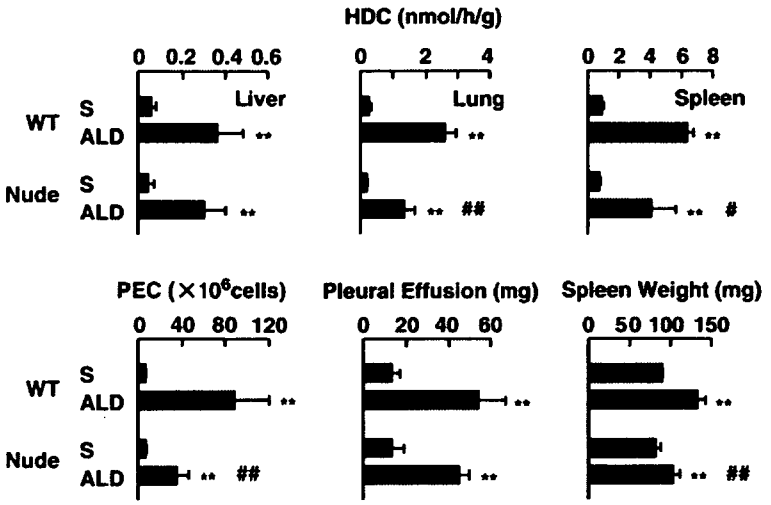

Fig. 8. Inflammatory actions of alendronate (ALD) in nude mice. Saline (S) or ALD (40 μmol/kg) was injected (i.p.) into control wild-type (WT) or nude mice. Three days later, the mice were decapitated and inflammatory markers measured. Each value is the mean±SD from four mice. ** $P<0.01$ vs S. # $P<0.05$ and ## $P<0.01$ vs ALD in WT mice.

3.5. Involvement of TNFα in the inflammatory actions of alendronate

We previously reported that in IL-1KO mice, HDC inductions by NBPs (alendronate, incadronate, and ibandronate) were negligible, and that other inflammatory reactions to these NBPs were very weak [17]. In the present study, HDC inductions by alendronate in all the tissues tested were very small or negligible in IL-1KO, TNFαKO, and IL-1/TNFαKO mice (Fig. 6). In these mice, the other inflammatory reactions were also weaker, or tended to be weaker, than in control wild-type mice, and an absence of both IL-1 and TNFα (i.e., in IL-1/TNFαKO mice) had generally marked effects on these reactions.

3.6. Inflammatory actions of alendronate in macrophage-depleted mice

F4/80 are markers for mature macrophages [42]. Clo-lip injection almost completely depleted F4/80-positive macrophages (or Kupffer cells) from the liver within 24 h of its injection (Fig. 7A). A small number of macrophages appear within 4 days of Clo-lip injection [38]. Thus, to deplete macrophages throughout our experimental period, we injected Clo-lip two times, as described in the legend in Fig. 7B. In such macrophage-depleted mice, alendronate-stimulated HDC induction in the liver and lung (but not in the spleen) and other inflammatory reactions were either significantly reduced or tended to be reduced (Fig. 7B).

3.7. Inflammatory actions of alendronate in nude mice

Nude mice are known to be essentially devoid of mature T-cells ($CD4^+$ T-cells and $CD8^+$ T-cells), and we have confirmed this (data not shown). The HDC inductions and inflammatory reactions induced by alendronate were significantly weaker, or tended to be weaker, in such mice than in control mice (Fig. 8).

4. Discussion

Despite lacking Vγ9Vδ2 T-cells, mice display inflammatory reactions to NBPs. The present results obtained from mice are summarized in Table 1. Like alendronate, IPP injection also induced HDC. Various statins (mevalonate-synthesis inhibitors) suppressed the alendronate-induced HDC inductions, while mevalonate

Table 1
Summary of the present results ALD-IFM, alendronate-induced inflammation; IPP, isopentenylpyrophosphate; ↑, increase; (↑), tendency to increase; ne, not effective; ↓, decrease or suppression; (↓), tendency to decrease or suppress

	HDC induction in			PEC cells	Pleural effusion	Spleen weight
	Liver	Lung	Spleen			
Inflammatory actions of						
ALD	↑	↑	↑	↑	↑	↑
IPP	↑	↑	(↑)	↑	↑	↓
Effects on ALD-IFM						
Statins	↓	↓	↓	ne	↓	↓
Mevalonate	↑	(↑)	↑	ne	ne	ne
Effects of absence on ALD-IFM						
IL-1	↓	↓	↓	↓	ne	(↓)
TNFα	↓	↓	↓	(↓)	ne	ne
IL-1 and TNFα	↓	↓	↓	↓	↓	(↓)
Macrophages	↓	↓	ne	↓	(↓)	↓
T-cells	ne	↓	↓	↓	(↓)	(↓)

itself augmented such inductions. Like IL-1-deficient mice, TNF-deficient mice were resistant to alendronate-stimulated HDC induction. Alendronate-stimulated HDC inductions were significantly weaker in macrophage-depleted mice and in nude mice than in control mice. It should also be noted that HDC is induced in a variety of immunocompetent cells (see Introduction). Similarly, though generally less clear-cut, results were obtained when other alendronate-induced inflammatory reactions were examined. At present, we have no available data to explain these differences. We speculate that the types of cells and/or the sensitivity of the cells responsible for the respective inflammatory reactions may be different among the various reactions. Moreover, we have no available data to explain the reduction in spleen weight induced by IPP. We noted that a lack of both IL-1 and TNFα profoundly affected the ability of alendronate to induce most of the inflammatory reactions. Therefore, these results suggest that (i) an inhibition of the mevalonate pathway causes and/or modifies at least some of the inflammatory actions of alendronate in mice, (ii) in addition to IL-1, TNF is also involved in the inflammatory actions of alendronate, and (iii) alendronate may act on or influence a variety of cells, including macrophages and T-cells.

In contrast to αβT-cells, Vγ9Vδ2 (alternatively termed Vγ2Vδ2) T-cells recognize non-peptide compounds of low molecular weight with phosphate residues [29,43,44]. NBPs share structural homologies with the ligands of Vγ9Vδ2 T-cells, and Kunzmann et al. [24,25], who found that NBPs stimulate Vγ9Vδ2 T-cells, suggested that NBPs may stimulate them directly. However, it has also been shown that activation of primary human γδT-cells by an NBP (unlike IPP, see below) depends strictly on the presence of macrophages (which may present the NBP to the γδT-cells) [45,46]. NBPs inhibit farnesylpyrophosphate (FPP) synthase in the mevalonate pathway [1], and this results in an accumulation of isopentenylpyrophosphate (IPP) [47,48]. IPP is a potent natural stimulant of Vγ9Vδ2 T-cells in the absence of specialized accessory cells, although cell–cell contact among the γδT-cells is necessary for their activation [49]. Thus, in addition to both direct stimulation and presentation via accessory cells (macrophages), NBPs stimulate Vγ9Vδ2T-cells indirectly via the production of IPP [26,50]. Interestingly, acute inflammatory reactions do not occur after repeated administration of NBPs [2], and repeated stimulations of Vγ9Vδ2 T-cells have been shown to inactivate them [51]. These findings may support the idea that Vγ9Vδ2 T-cells are responsible for the acute inflammatory reactions to NBPs in humans.

However, we speculate that NBPs may also act on cell-types other than Vγ9Vδ2 T-cells. It is important to note that in contrast to the acute inflammatory reactions to NBPs, jaw necrosis occurs only after repeated treatments with NBPs over a long period [9,10], and that Vγ9Vδ2 T-cells become inactive after repeated administrations of NBPs, as described above. Hence, it seems unlikely that Vγ9Vδ2 T-cells are responsible for the jaw necrosis. Normally, NBPs bind to bone almost irreversibly [52,53], and thus accumulate in large amounts in bone upon repeated administration. The mevalonate pathway exists widely in eukaryote cells. Therefore, we speculate that a sustained and prolonged release of the accumulated NBPs from the jaw-bone (possibly due to oral bacteria and/or physical stimulation or damage to the bone during dental treatment), and the subsequent inhibition of the mevalonate pathway in a variety of cells in and around the jaw-bone, might be an important cause of the necrosis and exposure of jaw-bones seen following prolonged treatment with NBPs.

We previously reported that the LPS-induced elevations of serum IL-1α and/or IL-1β are augmented in alendronate-treated mice [15,17,19], and that although the LPS-induced elevation of serum TNFα is reduced in such mice [15,19], a significant amount of TNFα (100–500 pg/ml) can still be detected. In those experiments, alendronate itself induced no detectable increase in either IL-1 or TNFα in the serum. Takagi et al. [54] reported that pamidronate (an NBP) augments IFNγ-dependent TNFα production by murine macrophages in vitro, and that co-administration of pamidronate and IFNγ produces a significant amount of TNFα in vivo (approximately 400 pg/ml serum). Further, HDC inductions by IL-1 and TNFα (as well as by LPS) are augmented in mice pretreated with alendronate [19]. In the present study, we found evidence that in addition to IL-1, TNFα is also important in the induction of inflammatory reactions by alendronate (especially HDC inductions). Interestingly, in macrophage-depleted mice the TNFα production seen in alendronate-pretreated mice is enhanced, whereas the augmentation of IL-1 production by the same NBP is reduced [15]. These results suggest that all three of these proinflammatory cytokines (IL-1, TNFα, and IFNγ) are important mediators in NBP-induced inflammatory reactions, and that cells other than macrophages are also involved in the production of TNFα.

Finally, the present study suggests that NBPs act on a variety of cell-types to modify cellular functions via various degrees of mevalonate pathway inhibition. This would result, depending on the organ or tissue examined, in various types and degrees of inflammatory reactions (such as the production of proinflammatory

cytokines, HDC induction, proliferation of cells, pleural exudation, and necrotic reactions). In this study, statins were effective at reducing alendronate-induced HDC induction, but (unfortunately) they were generally less effective at reducing the other inflammatory reactions. We need to examine whether statins are effective at preventing or reducing the necrotic actions of NBPs.

Acknowledgments

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ORIGINAL PAPER

Lipopolysaccharide promotes and augments metal allergies in mice, dependent on innate immunity and histidine decarboxylase

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Summary

Background Few adequate murine models exist for metal allergies, it being especially difficult to induce Ni allergy in mice.

Objective We examined the effect of lipopolysaccharide (LPS) on allergies to Ni and other metals in mice.

Methods Ten days after sensitization with a metal salt and LPS, the ears were challenged with the same metal salt.

Results LPS+$NiCl_2$ (1 mM) was effective at sensitizing mice to Ni, LPS being effective at very low concentrations whether injected intradermally or intraperitoneally. The ear-swelling response to Ni was more severe and more rapid in C57BL/6 mice than in BALB/c mice. In mast-cell-deficient mice, TNF-α-deficient mice, and interestingly even in nude (T cell deficient) mice, $NiCl_2$+LPS induced a Ni allergy similar in degree to that in the respective control mice, but it induced Ni allergy only weakly in TLR4-mutant mice, macrophage-depleted mice, and IL-1-deficient mice. The activity of the histamine-forming enzyme histidine decarboxylase (HDC) in the ears increased in parallel with ear swelling, and HDC-deficient mice were resistant to ear swelling. Challenge with $NiCl_2$+LPS augmented ear swelling (vs. $NiCl_2$ alone). LPS induced effective sensitization to other metals (Cr, Co, Pd, or Ag).

Conclusions These results indicate that in mice, LPS is a very important inducer of metal allergies, and potently promotes them (dependent on both innate immunity and HDC induction in cells other than mast cells). We discussed the idea that the bacterial environment is important for the establishment of metal allergies and for their provocation, and that the current thinking (including the contribution of T cells) should be reappraised in future studies.

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Keywords contact hypersensitivity, histamine, histidine decarboxylase, IL-1, lipopolysaccharide, metal allergies, nickel allergy, TNF, toll-like receptor
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Introduction

Metals are thought to cause various types of allergic reactions (including dermatitis, lichen planus, palmoplantar pustulosis, asthma and rhinitis) [1–6], and even to cause carcinomas [4]. Alloys containing Ni are widely used in our environment and in medicine, exposing us to Ni through a variety of routes. Indeed, Ni is the most frequent contact allergen among the metals [2, 7]. Unlike classical haptens, metal ions form geometrically highly defined, but reversible, coordination complexes with partner molecules. Thus, it is difficult to define the allergenic epitopes of such complexes, and the host recognizes metal ions in complicated ways [8]. The fact that the partner molecules that confer allergenicity to metals are 'intact' self-proteins led us to speculate that metal allergies might belong to the autoimmune diseases.

As described in various reviews [9–11], Ni allergy is thought to involve the maturation of dendritic cells (DCs), their migration in the periphery to the T cell areas of regional lymph nodes (where the matured DCs stimulate naïve T cells to mature or activate them), recruitment of