insulin-like growth factor binding protein 5, and α-defensin.[6–13] MMP-7 is produced mainly by glandular epithelial cells and macrophages in various diseased tissues[14,15] and overexpressed by carcinoma cells in human cancers with correlation to metastases.[16] In addition, tumorigenesis is suppressed in MMP-7–deficient mice.[17] Thus, MMP-7 is considered to play roles in tumorigenesis and tumor progression.[18] However, information is still limited about activation of proMMP-7 within tissues and pathobiologic roles of MMP-7 in inflammatory conditions such as IPF. In the current study, we examined the production levels and activation of proMMP-7 and tissue localization of MMP-7 in the lung tissues of IPF. The results demonstrate that proMMP-7 is overproduced in hyperplastic alveolar epithelial cells and alveolar macrophages and activated in the lung tissues of IPF. The possible functions of MMP-7 in IPF lung tissues are discussed.

MATERIALS AND METHODS

Study Population

Idiopathic pulmonary fibrosis was diagnosed according to the criteria of the American Thoracic Society/European Respiratory Society international multidisciplinary consensus classification of IPF.[19] Briefly, 23 patients satisfied the following major criteria: (1) exclusion of other known causes of interstitial lung diseases such as certain drug toxicities; no history of exposure to asbestos, silica, and any other inorganic dusts; and no evidence of arthritis, myalgia, skin eruption, or serum autoantibodies suggestive of collagen vascular diseases; (2) abnormal pulmonary function studies that include evidence of restriction; (3) bibasilar reticular abnormalities with minimal ground glass opacities on high-resolution computed tomography scans; and (4) transbronchial lung biopsy or bronchoalveolar lavage (BAL) showing no features to support an alternative diagnosis. They also had all of the minor criteria: (1) age older than 50 years; (2) insidious onset of otherwise unexplained dyspnea on exertion; (3) duration of illness more than 3 months; and (4) bibasilar, inspiratory crackles. In addition, the following patients were eliminated from the IPF group to avoid the effect of acute inflammation and drugs: patients with a possible respiratory tract infection as a complication and those with present or past history of treatment with glucocorticoid or immunosuppressive agents. Seventeen patients were enrolled for BAL analysis and 23 for blood analysis. We also collected blood from 20 healthy volunteers and performed BAL in 6 healthy volunteers.[20] Informed consent was obtained from all patients and healthy volunteers. The patients were men and older than 52 years. On high-resolution computed tomography, all patients had patchy, peripheral-dominant, reticular lesions and did not show any findings suggestive of other interstitial lung diseases. No patients showed any findings of malignancy on BAL and transbronchial lung biopsy.

Peripheral Blood and Bronchoalveolar Lavage Samples

Blood was drawn from peripheral veins into a small glass tube, and serum was obtained by centrifugation at 3000 rpm for 5 minutes. Bronchoalveolar lavage was performed according to the standard protocol as described previously.[21] Briefly, a bronchoscope was wedged in a segment of the right middle lobe or lingual under local anesthesia. Sterile normal saline at room temperature was instilled through the bronchoscope. The recovered BAL fluids (total 150 mL) were filtered through sterile gauze and the cells were counted. Cytospin preparations were made for cell differential counts. The fluids were centrifuged at 200*g* for 5 minutes and the supernatants were used for the assays.

Quantification of proMMP-7 in BAL Fluids and Serum Samples

proMMP-7 levels in the BAL fluids were measured by a sandwich enzyme immunoassay (Daiichi Fine Chemicals, Co Ltd, Takaoka, Japan) as described previously.[22] This assay system detects solely proMMP-7 and the detection limit is 0.63 ng/mL. Serum samples from the patients and healthy volunteers were also measured by the system.

Histopathologic and Immunohistochemical Examination of Human Lungs

Lung tissues of IPF (n = 6) and control normal lung tissues (n = 6), the latter of which were from patients without underling pathologic conditions in the lungs, were obtained at autopsy and fixed with 10% formalin. In addition, we obtained an autopsied lung tissue from 1 patient with interstitial pneumonia associated with rheumatoid arthritis and biopsied lung tissues from 3 patients with nonspecific interstitial pneumonia. Paraffin sections (4 μm thick) were made and stained with hematoxylin-eosin, elastica van Gieson, and silver impregnation. For immunohistochemistry, the sections were reacted with monoclonal antibodies specific to proMMP-7 (141-7B2; 4 μg/mL), active MMP-7 (176-5F12; 10 μg/mL), or CD151 (11G5a; 5 μg/mL) for approximately 18 hours at 4°C after blocking endogenous peroxidase and nonspecific binding according to our methods.[5] They were incubated with biotinylated horse immunoglobulin G (IgG) to mouse IgG (1:200; Vector Laboratories, Inc, Burlingame, California) for 30 minutes and then with an avidin-biotin peroxidase complex (Dako, Glostrup, Denmark) for 30 minutes at room temperature. Color was developed with 0.03% 3,3′-diaminobenzidine tetrahydrochloride in 50mM Tris-HCl buffer, pH 7.6 containing 0.006% H_2O_2 for Figure 2 and diaminobenzidine containing 10mM imidazole for Figure 3. Counterstaining was performed with hematoxylin. For a control, the primary antibodies were replaced with nonimmune mouse IgG (4 μg/mL) followed by the procedures described previously.

Immunoblotting

Bronchoalveolar lavage fluids were concentrated by trichloroacetic acid and then electrophoresed on 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions. Proteins in the gels were electrotransferred onto polyvinylidene difluoride membranes (ATTO Corporation, Tokyo, Japan). The membranes were reacted with monoclonal anti–MMP-7 antibody (10 μg/mL; 125-20H11), which recognizes both proMMP-7 and active MMP-7,[22] at 4°C for 12 hours after blocking nonspecific reaction with 3% bovine serum albumin, and incubated with horseradish peroxidase–labeled secondary antibody (Vector). Immunoreactive bands were detected with ECL Western blot reagents (Amersham Bioscience Co) using Lumivision HSII (Taitec Co Ltd, Tokyo, Japan).

Detection of MMP-7 Activity in BAL Fluids in a Cross-Linked Carboxymethylated Transferrin Film Assay

Bronchoalveolar lavage fluids were placed on cross-linked carboxymethylated transferrin (CCm-Tf) films, which were prepared by coating with cross-linked CCm-Tf solution on polyethylene terephthalate support films as described previously.[23] Immediately after placing BAL fluid specimens on the films, they were incubated at 37°C for 8 to 16 hours in a moist chamber and then stained with staining solution containing 0.3% Biebrich Scarlet. The samples were also subjected to the CCm-Tf films impregnated with a mixture of aprotinin and elastinal or a mixture of aprotinin, elastinal, and 1,10-phenanthroline.

Statistical Analysis

All data are presented as mean ± standard deviation. Unpaired Student *t* tests or 1-factor analysis of variance were used to compare mean values and χ^2 test was used to compare detection rate. *P* values less than .05 were considered significant.

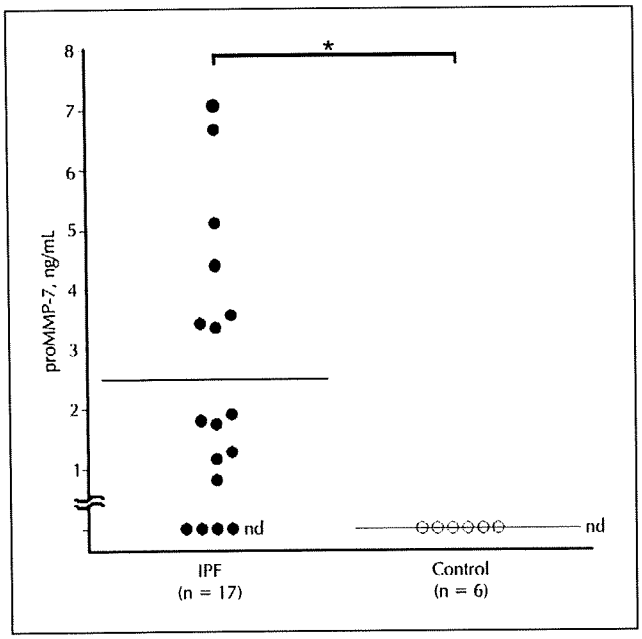

Figure 1. *Pro matrix metalloproteinase 7 (proMMP-7) levels in the bronchoalveolar lavage fluids. The bronchoalveolar lavage fluids were subjected to the enzyme immunoassay for proMMP-7 as described in "Materials and Methods." Abbreviations: IPF, idiopathic pulmonary fibrosis; nd, not detectable. *, P < .001.*

RESULTS

proMMP-7 Levels in BAL Fluids and Serum Samples of IPF Patients

proMMP-7 in BAL fluids was assayed by the enzyme immunoassay system in 17 patients with IPF. Total cell number in the BAL fluids was 5.14 ± 3.61 × 10^5/mL (mean ± standard deviation). Cell differential percentages in the BAL fluids were as follows: macrophages, 75.7 ± 19.4%; lymphocytes, 10.8 ± 8.2%; neutrophils, 11.0 ± 18.3%; and eosinophils, 6.3 ± 9.4%. As shown in Figure 1, 13 of 17 samples from the patients showed detectable levels of proMMP-7 (2.49 ± 2.29 ng/mL), but negligible levels of proMMP-7 were observed in the control samples from 6 healthy volunteers (<0.63 ng/mL). The levels were significantly higher in the patient samples than in the control samples ($P < .001$). On the other hand, although proMMP-7 was detected in the serum samples from IPF patients (3.47 ± 2.15 ng/ml) and healthy volunteers (3.77 ± 1.61 ng/mL), there was no significant difference between the 2 groups.

Immunohistochemical Localization of proMMP-7, Active MMP-7, and CD151

Histologic analysis of the lung tissues of IPF showed septal fibrosis and inflammatory cell infiltration in the alveolar walls (Figure 2, A). By immunohistochemistry, proMMP-7 was localized mainly to the hyperplastic alveolar epithelial cells in the lung tissue of IPF (Figure 2, B), whereas active form of MMP-7 was detected mainly on the cell surfaces of alveolar macrophages and epithelial cells (Figure 2, C). We observed similar findings in all 6 patients with IPF. On the other hand, in patients with nonspecific interstitial pneumonia (Figure 2, D and G) and interstitial pneumonia associated with rheumatoid arthritis (Figure 2, E and H), proMMP-7 was positive in the regenerative epithelial cells. However, no definite signal of active MMP-7 was detected (data not shown). Control normal lung tissue (n = 6) showed no staining with antibody against proMMP-7 or active MMP-7 (Figure 2, F and I, and data not shown for active MMP-7 immunostaining). Because our previous studies have demonstrated that proMMP-7 is captured and activated by CD151 expressed on the cell surfaces of lung adenocarcinoma cells[5] and osteoarthritis chondrocytes,[24] we further examined colocalization of MMP-7 and CD151 in the mirror sections of the lung tissue of IPF. As shown in Figure 3, CD151 immunostaining was prominent in hyperplastic alveolar and metaplastic bronchiolar epithelial cells and alveolar macrophages, and some of these cells also showed positive staining of MMP-7.

Immunoblotting Analysis of MMP-7

When BAL fluids were immunoblotted with anti–MMP-7 antibody, both proMMP-7 of 28 kDa and its processed forms of active MMP-7 were detected (Figure 4, lanes 1–3). In contrast, neither proMMP-7 nor active MMP-7 was observed in the control samples (Figure 4, lanes 4–6).

Detection of MMP-7 Activity in a CCm-Tf Film Assay

Bronchoalveolar lavage fluids were incubated on the CCm-Tf coated films without or with proteinase inhibitors. As shown in Figure 5, A, the digestion of CCm-Tf on the film without inhibitors was observed in 3 of 7 IPF samples (lanes 1–7), but no activity was detected in 3 samples from healthy volunteers (lanes 8–10). The CCm-Tf–degrading activity in the samples from IPF patients was not altered by incubation on the CCm-Tf film coated with a mixture of aprotinin and elastinal (serine proteinase inhibitors) (Figure 5, B), but it was completely abolished in the film coated with a mixture of the serine proteinase inhibitors and 1,10-phenanthroline (a metalloproteinase inhibitor) (Figure 5, C).

COMMENT

In the present study, we have demonstrated that significantly higher levels of proMMP-7 are detected in the BAL fluids of IPF patients. Immunohistochemistry further indicated that proMMP-7 and CD151 are localized to the hyperplastic alveolar and metaplastic bronchiolar epithelial cells. Furthermore, active form of MMP-7 was detected by immunohistochemical and immunoblotting analyses, and the activity of MMP-7 was confirmed in the CCm-Tf film assay. Based on these data, we propose that proMMP-7 is excessively produced and efficiently activated in the lung tissues of IPF probably through interaction with CD151.

Our enzyme immunoassay system has indicated significant elevation of proMMP-7 in BAL fluids from IPF patients compared with normal subjects. Because proMMP-7 was immunolocalized mainly in the hyperplastic alveolar and metaplastic bronchiolar epithelial cells and macrophages, these cells are considered to be responsible for the production. During preparation of our manuscript, other groups reported that MMP-7 levels in BAL fluids from patients with IPF or other interstitial lung diseases including nonspecific interstitial pneumonia, cryptogenic organizing pneumonia, and sarcoidosis are higher than those from normal subjects and that the levels are not different between IPF and other interstitial lung

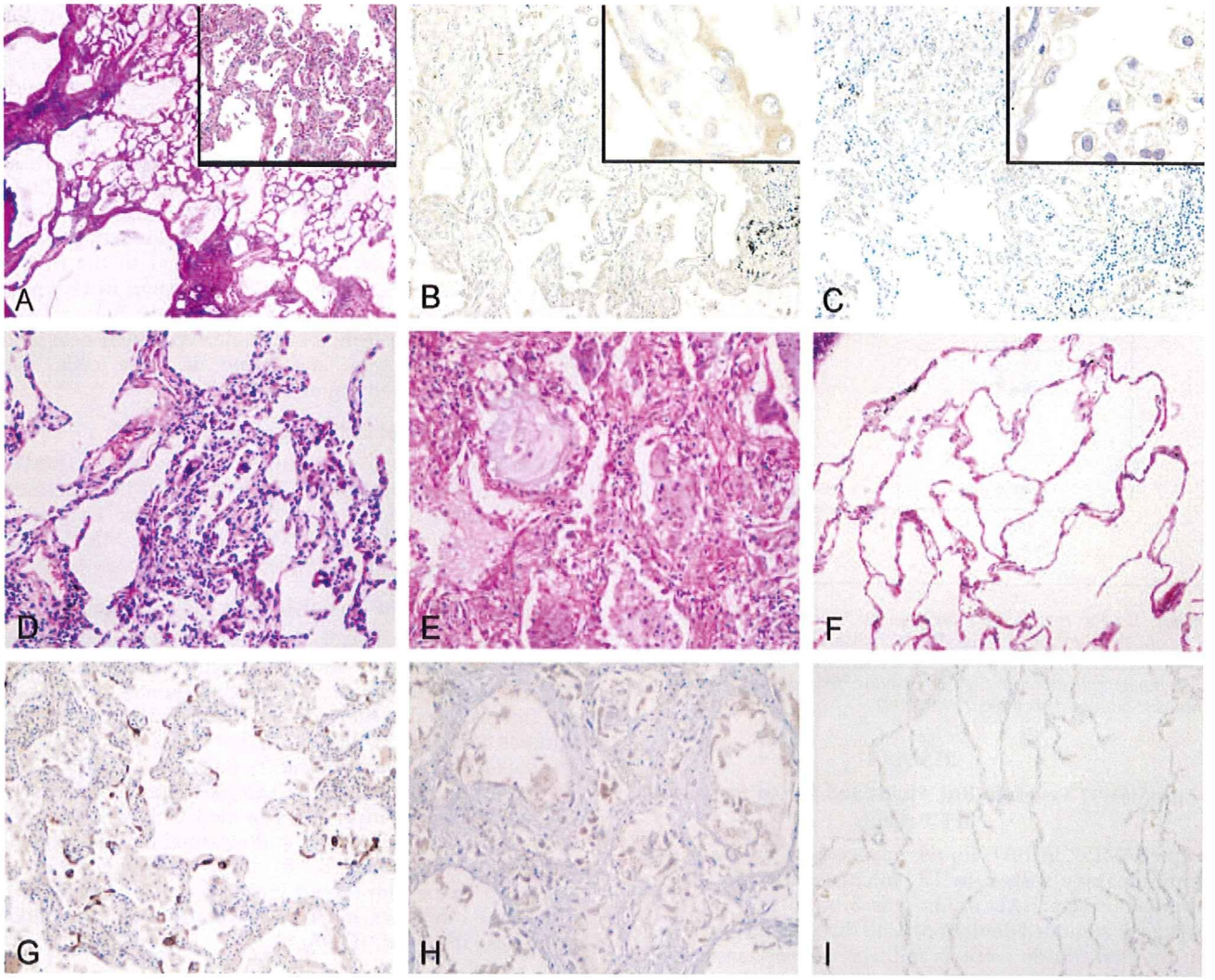

Figure 2. *Histology and immunohistochemistry for pro matrix metalloproteinase 7 (proMMP-7) and active MMP-7 in the lung tissues. A, Lung tissue of idiopathic pulmonary fibrosis (IPF) showing septal inflammation and fibrosis (hematoxylin-eosin, original magnifications ×2 [A] and ×20 [inset]). B, Immunohistochemistry for proMMP-7 in the lung tissue of interstitial pneumonia. Note that proMMP-7 is predominantly immunostained in the hyperplastic alveolar epithelial cells and alveolar macrophages. (original magnifications ×20 [B] and ×40 [inset]). C, Immunohistochemistry for MMP-7 in the lung tissue of IPF using antibody specific to active MMP-7 (original magnifications ×20 [C] and ×40 [inset]). Note that active MMP-7 is localized on alveolar macrophages and alveolar epithelial cells. D and G, Histology and immunohistochemistry, respectively, for proMMP-7 in the lung tissue of nonspecific interstitial pneumonia (original magnifications ×20). E and H, Histology and immunohistochemistry, respectively, for proMMP-7 in the lung tissue of interstitial pneumonia associated with rheumatoid arthritis (original magnifications ×20). Note that proMMP-7 is positive in the regenerative epithelial cells in nonspecific interstitial pneumonia and interstitial pneumonia associated with rheumatoid arthritis. F and I, Histology and immunohistochemistry, respectively, for proMMP-7 in the control normal lung tissue (original magnifications ×20). No positive immunostaining is observed in the sample.*

diseases.[25,26] Although we did not find differences in the serum levels of proMMP-7 between IPF and control groups, a recent article showed significantly higher levels of plasma MMP-7 in IPF[27] in an assay that detects only proMMP-7. The discrepancy may be derived from the different characteristics of the enzyme immunoassay kits between R&D Systems, Inc (Minneapolis, Minnesota) and ours.[27] Previous studies have shown that MMP-7 is preferentially secreted to the apical surface of normal mucosal and gland epithelium[14,28] and localized mainly to the luminal epithelial surface of the uterine and mammary glands.[29] Therefore, our data showing no changes in serum proMMP-7 levels may be because proMMP-7 produced by the alveolar epithelial cells is secreted mostly to the alveolar spaces but not into blood circulation. This may also be explained by the fact that the concentrations of proMMP-7 are too low to be detected by our assay system because of dilution after being drained into blood circulation. However, another plausible explanation might be that proMMP-7 is locally activated and consumed by acting as a proteinase in the extracellular milieu without drainage into blood circulation.

One of the most interesting findings in the present study is that proMMP-7 is activated within the lung tissue of IPF. This was demonstrated by immunohistochemistry using anti–MMP-7 antibody specific to active MMP-7, immunoblotting, and CCm-TF film assay. Our immunohistochemical study also showed that no definite active MMP-7 is produced in the lungs from interstitial pneumonia associated with rheumatoid arthritis and nonspecific

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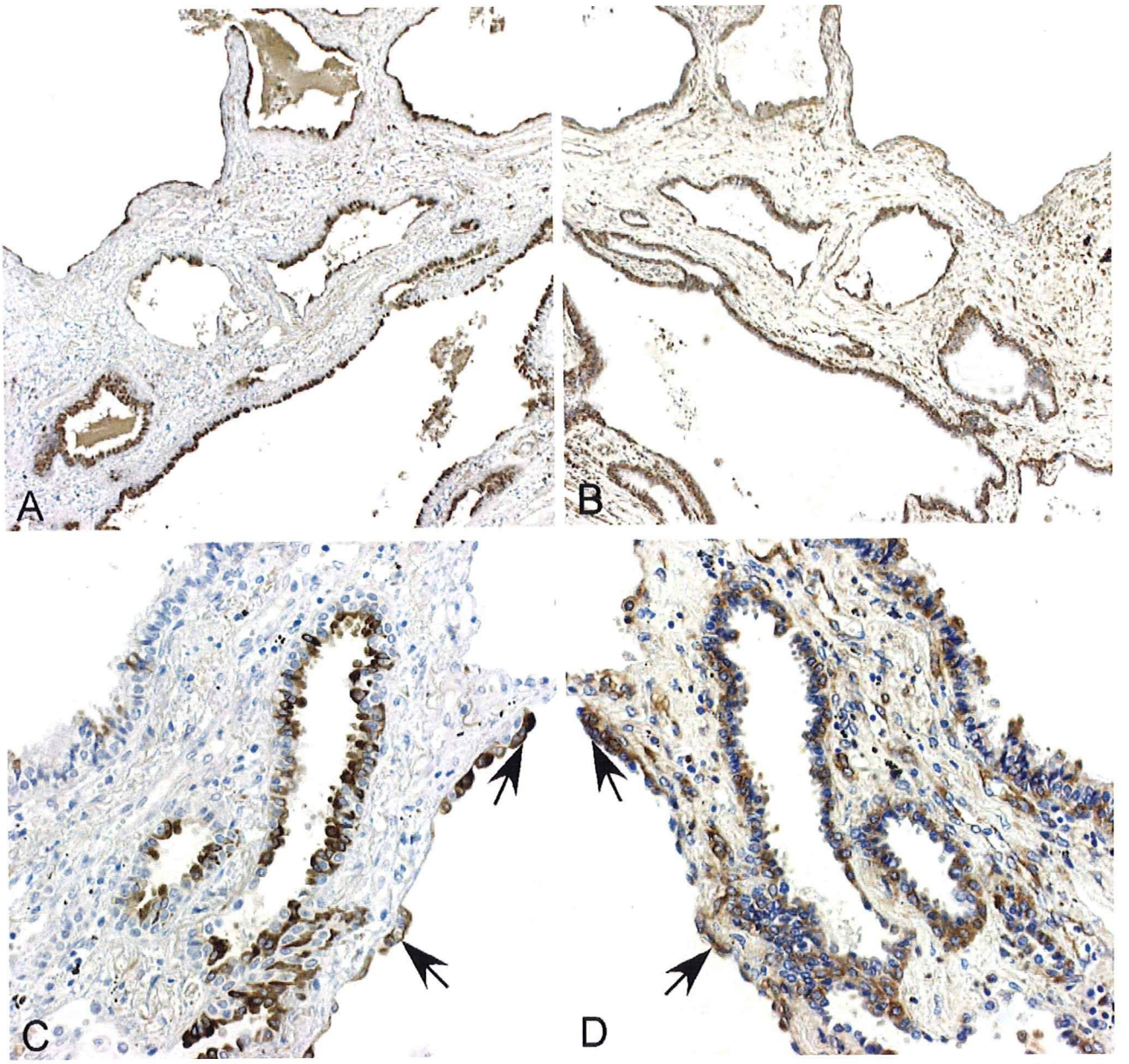

Figure 3. *Immunohistochemistry for pro matrix metalloproteinase 7 (proMMP-7) and CD151 in the mirror sections of the lung tissue of idiopathic pulmonary fibrosis. A and C, Low- and high-power views of proMMP-7 immunostaining showing that alveolar epithelial cells are positively stained (original magnifications ×20 in all). B and D, Low-power and high-power views of CD151 immunostaining. Note that proMMP-7 and CD151 are colocalized in some epithelial cells (arrows) (original magnifications ×20 in all).*

interstitial pneumonia, although proMMP-7 is expressed. Active MMP-7 has been detected in several pathophysiologic tissues including physiologic duodenal mucosa,[10] endometrial adenocarcinomas,[30] and lung adenocarcinomas,[5] but to our knowledge our observation is the first to demonstrate the proMMP-7 activation within the lung tissues of IPF. Although in vitro biochemical studies have shown that proMMP-7 is fully activated by trypsin or MMP-3, and partially by plasmin or leukocyte elastase,[31] the activation mechanism of proMMP-7 in tissues remains unknown. We have recently reported that CD151 acts as a docking molecule for proMMP-7 through binding to the propeptide of proMMP-7 on cell surfaces, leading to its activation.[5] The findings in the current study that both proMMP-7 and CD151 are localized in the hyperplastic alveolar epithelial cells and macrophages and that active MMP-7 is stained on these cells suggest activation of proMMP-7 through the interaction with CD151. We have recently shown similar CD151-associated activation of proMMP-7 in the human lung adenocarcinomas[5] and in cultured osteoarthritic chondrocytes.[24] Thus, we speculate that the activation of MMP-7 may be characteristic to IPF and contribute to the pathology of IPF. Although the expression of other MMPs (MMP-1, 2, 9, and 13 and MT1, 2, 3, 4, and 5-MMP) and TIMPs (TIMPs-1, 2, 3, 4) has been previously reported in IPF and other interstitial lung diseases, their local and specific activation in IPF has not been detected or clarified.[32–35]

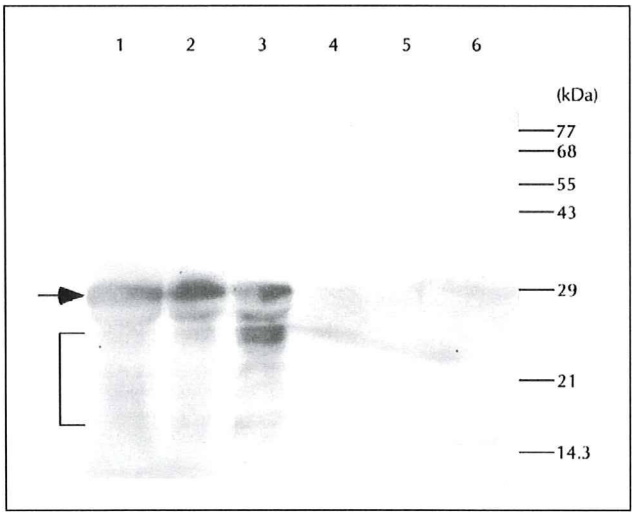

Figure 4. *Immunoblotting analysis of matrix metalloproteinase 7 (MMP-7) in bronchoalveolar lavage fluids. Bronchoalveolar lavage fluids from patients with idiopathic pulmonary fibrosis (lanes 1–3) and healthy volunteers (lanes 4–6) were subjected to immunoblotting for MMP-7 as described in "Materials and Methods." Note the immunoreactive bands corresponding to 28-kDa proMMP-7 (arrow) and lower-molecular-weight forms of active MMP-7 in the samples of idiopathic pulmonary fibrosis patients.*

In normal lung tissue, the expression of MMP-7 is restricted to the epithelial cells of peribronchial glands and conducting airways, and the activation is suspected to contribute to exocrine function.[36] In injured lungs such as in cystic fibrosis, MMP-7 is overexpressed by alveolar type II cells.[14] MMP-7 has strong activity against various ECM components including basement membrane collagen IV, elastin, proteoglycans, and other cell adhesion molecules, all of which are implicated in cell motility.[31,36] Experimental study using MMP-7–deficient mice has demonstrated that MMP-7 is essential to epithelial cell migration over and reepithelialization of the damaged airways.[14] In addition, MMP-7 is known to shed E-cadherin, which is necessary for the epithelial repair.[8] Thus, it seems very likely that MMP-7 contributes to the formation of hyperplastic epithelial foci and tissue repair in IPF. However, because MMP-7 is a multifunctional proteinase by digesting various non-ECM bioactive molecules, we can speculate other functions in addition to repair in the lung tissues of IPF: (1) MMP-7 is known to cleave Fas-L, which could trigger epithelial cell apoptosis.[11,37] Previous studies have demonstrated soluble Fas-L in the BAL fluids and apoptosis of the epithelial cells increases in IPF. Thus, MMP-7 may be involved in the epithelial cell apoptosis through cleavage of the membrane-bound Fas-L.[38,39] (2) MMP-7 could facilitate bioavailability of insulin-like growth factor binding protein 3 and insulin-like growth factor binding protein 5, which are autocrine growth factors for myofibroblasts in the colon.[6,7] Because myofibroblasts are thought to actively produce ECM molecules in IPF, it is reasonable to speculate that MMP-7 may play a role in the growth of myofibroblasts in the lungs. (3) Osteopontin is expressed in IPF[40] and particle-induced lung diseases[41] and upregulated in the bleomycin-induced lung fibrosis model.[42] Because MMP-7 is known to process osteopontin into an active form,[43] MMP-7 might act as a positive modulator for fibrosis signaling.[44] (4) We have

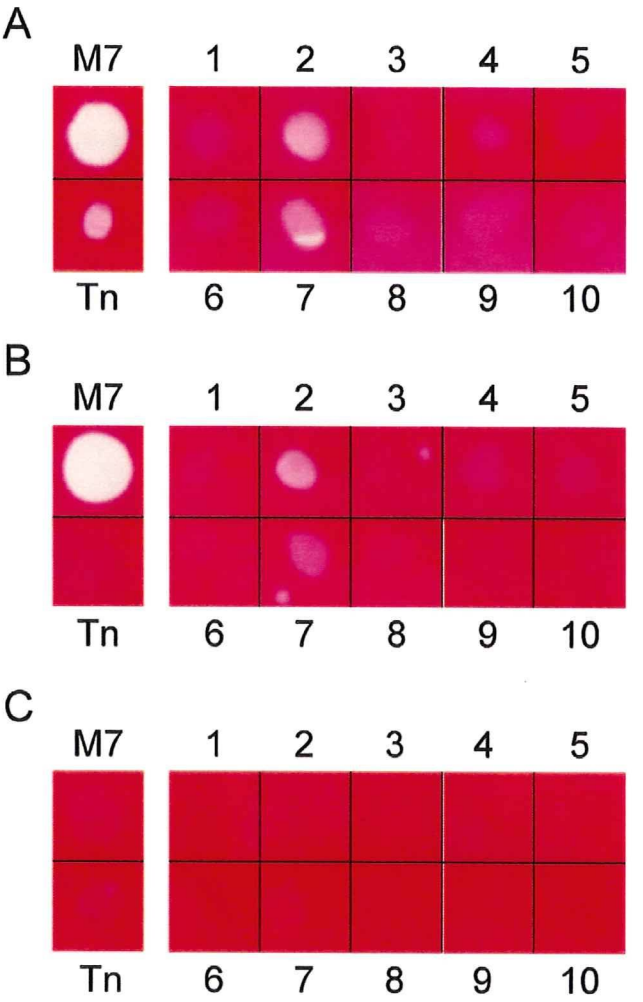

Figure 5. *Detection of cross-linked carboxymethylated transferrin (CCm-Tf)–degrading activity in bronchoalveolar lavage fluids. A, Bronchoalveolar lavage fluid specimens (3 μL) from 7 patients with idiopathic pulmonary fibrosis (lanes 1–7) and 3 healthy volunteers (lanes 8–10) were spotted and incubated on the CCm-Tf film as described in "Materials and Methods." Note the digestion of CCm-Tf substrate in lanes 2, 4, and 7 as well as positive controls. B, Inhibition study using CCm-Tf film impregnated with a mixture of aprotinin and elastinal. The same samples as in A were subjected to the film with the inhibitors. Note that no inhibition of the activity in the samples is seen, but trypsin activity is completely blocked. C, Inhibition study using CCm-Tf film impregnated with a mixture of 1,10-phenathroline, aprotinin. and elastinal. Note that activity of all the samples including positive controls is inhibited. Abbreviations: M7, positive control of matrix metalloproteinase 7; Tn, positive control of trypsin.*

previously shown that alveolar macrophages are primed for interleukin 8 production in IPF patients during its chronic phase, and high levels of interleukin 8 are detected during the phase of acute exacerbation.[21] Because MMP-7 is reported to promote neutrophil transmigration to alveolar spaces through shedding of chemokine-binding syndecan 1,[9] it might be possible to speculate that excessive MMP-7 production and activation may contribute to the induction of neutrophilic infiltration into alveolar walls in IPF, especially during the phase of acute exacerbation.[9]

In summary, our study showed that proMMP-7 is overproduced by hyperplastic epithelial cells and activated in the lung tissue of IPF. The data suggest that excessive

production and macrophages and activation of proMMP-7 may contribute to the pathology of IPF through a variety of pathways such as epithelial cell migration and apoptosis, myofibroblast autocrine growth, and neutrophil accumulation. Further studies are mandatory for establishing these pathologic roles.

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《シンポジウム2》

EBMに基づくALI/ARDS診療のUp-To-Date

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藤　島　清太郎

急性呼吸促迫症候群(ARDS, acute respiratory distress syndrome)は病態生理学的に肺微小血管の透過性亢進，過剰な炎症反応を特徴とする症候群であるが，臨床的に$PaO_2/FIO_2 < 200$と画像上の両側浸潤影というAmerican-European Consensus Conferenceの簡便な診断基準を満たす症例には，発症機序の異なる多彩な病態が包含されている．一方，$PaO_2/FIO_2 < 300$で定義される急性肺損傷(ALI, acute lung injury)は，より軽症，初期病態を含めたさらに広い疾患概念といえる．

ALI/ARDSの病態は現在でも未解明な部分が多いが，我々はこれまで，重症傷病に罹患した患者が，経過中感染などを契機にALI/ARDSを発症し，病態が急激に重症化する現象(two hit phenomenon)をしばしば経験し，その病態解明を行ってきた(図1)．マウスの熱傷後敗血症モデルを用いた検討では，熱傷後に細菌内毒素を投与した群で，炎症性サイトカインの産生が増大かつ遷延することを認めている[1]．最近では，少量IL-18投与により，生存率が改善し，炎症性サイトカインの産生が抑制されることを見いだした(図2)[2]．

一方，臨床面では近年各領域でEBMが急速に導入されているが，救急・集中治療領域ではRCT(randomized controlled trial)の実施が困難なこともあり，エビデンスの集積が未だ不十分である．ALIやARDSの診療に関する記載のあるガイドラインとして，我が国の"日本呼吸器学会ALI/ARDS診療のためのガイドライン2005年版"，国際的には"Surviving Sepsis Champaign Guidelines 2008年改訂版"があるが，いずれも必ずしも十分なエビデンスに基づかない事項を列記せざるを得ないのが現状である[3,4]．

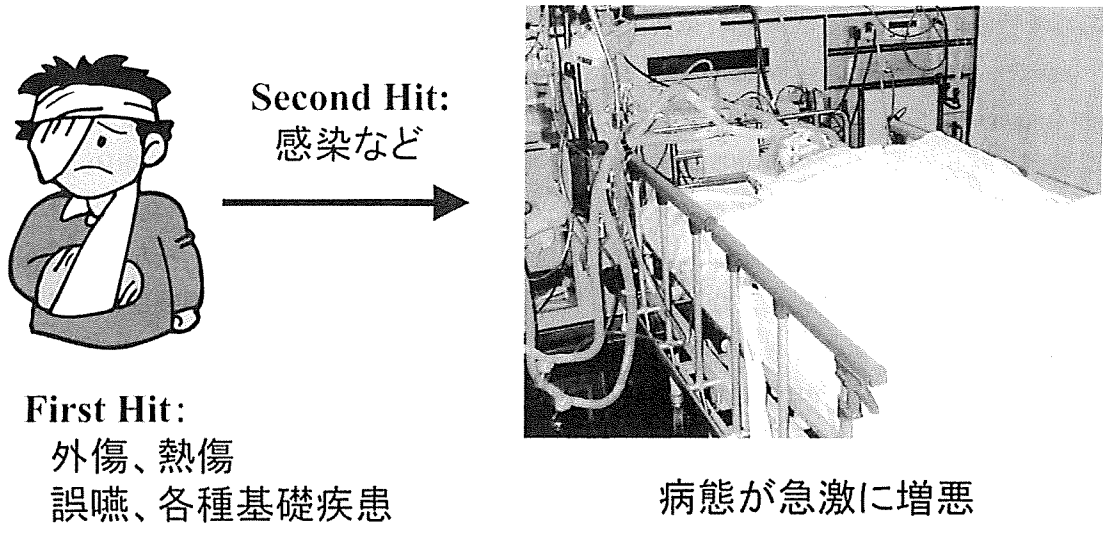

図1　Two Hit Phenomenon

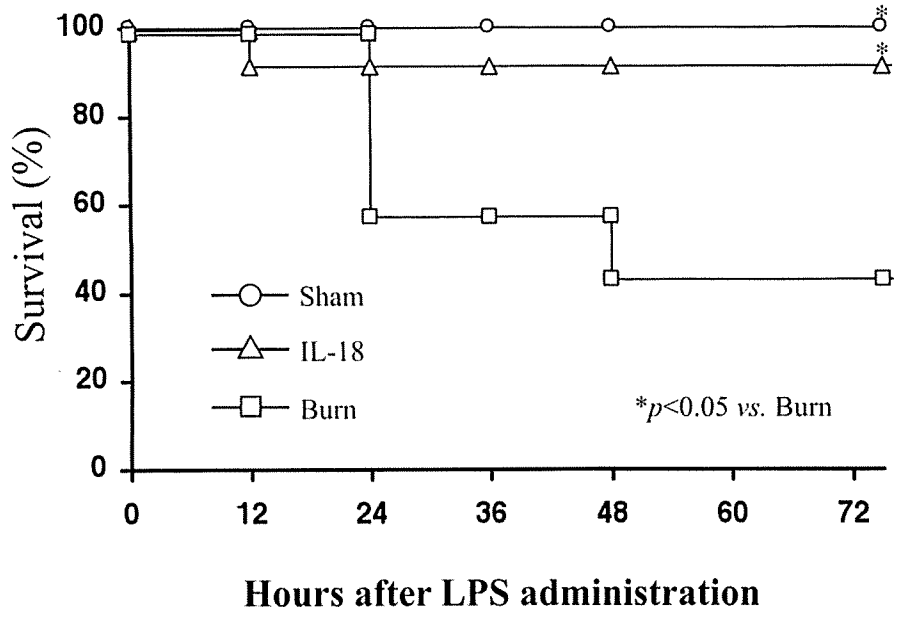

図2 Cumulative survival after LPS administration in the sham, burn and IL-18 groups
（文献2より）

表1 Surfactant療法：今後のアプローチ

1. 新たなsurfactant開発：
   リコンビナントSP-A, SP-D（親水性、生体防御機能）生体防御作用の付加
2. 対象の選定：
   直接傷害によるALI/ARDS小児・若年者
3. 投与法の改良：
   気管支鏡下instillation一時的気管挿管
4. 投与時期：
   早期投与（second hit前）

(Lewis JF, et al. Sem Respir Crit Care Med 2006; 27(4): 377-88)
(Stevens TP, et al. Chest 2007; 131(5): 1577-82)

様々な治療法のうち，これまでにALI/ARDSの長期生存率改善が示されている治療法は，低容量換気による呼吸管理のみであり，高圧の呼気終末陽圧（PEEP, positive end-expiratory pressure）負荷，recruitment maneuver（バッグマスク換気などによる一時的な高気道内圧負荷）などの有効性は明確でない．一方，薬物療法としては，副腎皮質ステロイド，好中球エラスターゼ阻害薬（sivelestat，エラスポール®），サーファクタント補充療法などがあるが，いずれの有効性も確立されておらず，新たに報告されるRCTの成績により，その位置づけが刻々と変化する状況にある．

副腎皮質ステロイドに関しては，ARDSNetによる大規模RCTの成績では，methylprednisolone早期投与により人工呼吸器からの離脱が早まるものの，神経筋合併症が増し，さらに発症14日以降の投与では死亡率が増加したことが報告されている[5]．一方我が国における臨床治験で肺機能の改善と，人工呼吸器装着期間，ICU在室期間の短縮を認めたsivelestatは，その後の海外臨床試験では有効性が示されず，現在我が国と韓国でのみ市販されている．海外試験の対象患者の多くが重症例であり，我が国と同程度の重症度例に絞った再解析で改善傾向を認めたこと，我が国の臨床治験の層別解析で，発症後3日以内投与群での有効率が高いこと，市販後調査で臨床試験と同様の有効性が示されたことから，現状で同薬を使用する場合，発症早期で肺以外の臓器障害が少ない患者への使用が推奨されている．

サーファクタント補充療法に関しては，過去に行われた3つのRCTにより成人ARDSに対する有効性がいずれも否定されており，上述の2ガイドラインでも推奨されていない．一方，小児ARDSに対しては2005年に有効性が示唆されて以降，様々なエビデンスが集積されており，大規模RCTによる成績が待ち望まれている[6]．最後に，サーファクタント療法のALI/ARDSに対する今後のアプローチの可能性を**表1**にまとめた．

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Clonal dissemination of human isolates of *Streptococcus suis* serotype 14 in Thailand

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Most cases of *Streptococcus suis* infection in humans are caused by serotype 2 strains, and only a few cases caused by other serotypes have been reported. Among 177 human isolates of *S. suis* in Thailand, 12 (6.8 %) were identified as being of serotype 14, and an occurrence of sporadic *S. suis* serotype 14 infection was noted during 2006–2008, particularly in northern Thailand. Clinical presentations of the 12 patients (median age 62.9 years) included meningitis (58.3 %), septic arthritis (25 %) and sepsis (16.7 %). These clinical features were similar to those previously reported for *S. suis* infections, except that there were no fatal cases. All of the 12 serotype 14 strains belonged to the multilocus sequence types (ST) 105 (n=11) and the novel ST127 (n=1). Molecular typing by PFGE revealed four different pulsotypes, including an identical pattern for nine ST105 strains and three closely related patterns for two ST105 strains and one ST127 strain. Our PFGE data suggested clonal dissemination of ST105 strains in Thailand. Because serotype 14 is becoming a more common cause of *S. suis* infections in humans, diagnostic tests for serotype 14 should be performed in South-East Asian countries.

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INTRODUCTION

Streptococcus suis, an important zoonotic pathogen, causes meningitis and sepsis including streptococcal toxic shock syndrome in humans who are in close contact with infected pigs or contaminated pork-derived products (Lun *et al.*, 2007). Based on capsular polysaccharides, 33 serotypes of *S. suis* have been identified. Serotype 2 is the most common cause of human disease (Lun *et al.*, 2007; Wertheim *et al.*, 2009). Currently, only seven human cases worldwide have been attributed to serotype 14 (Gottschalk *et al.*, 1989; Haleis *et al.*, 2009; Mai *et al.*, 2008; Poggenborg *et al.*, 2008; Takamatsu *et al.*, 2008; Watkins *et al.*, 2001; Ye *et al.*, 2008).

In 1987, two human cases of *S. suis* infection were first reported in Thailand (Phuapradit *et al.*, 1987). Since the occurrence of a large outbreak of *S. suis* serotype 2 infection in Sichuan Province, China, in 2005 (Ye *et al.*, 2006), this disease has been increasingly recognized worldwide. During the past decade, the number of reported human cases has increased, particularly in South-East Asia, with more than 700 *S. suis* infections reported worldwide (Wertheim *et al.*, 2009). To date, the number of *S. suis* infections in humans reported in previous studies from Thailand (Wertheim *et al.*, 2009; Wangkaew *et al.*, 2006; Rusmeechan & Sribusara, 2008; Wangsomboonsiri *et al.*, 2008; Khadthasrima *et al.*, 2009) and on the website of the Bureau of Epidemiology, Ministry of Public Health, Thailand, exceeds 300 cases. Therefore, *S. suis* is an emerging human pathogen in Thailand.

Because of the variable biochemical characteristics (Lun *et al.*, 2007; Ma *et al.*, 2008), *S. suis* infection is often either undiagnosed or misdiagnosed by the local hospital laboratories in South-East Asian countries, including

Abbreviations: MLST, multilocus sequence typing; NIAH, National Institute of Animal Health; 89K PAI, an ~89 kb candidate pathogenicity island; ST, sequence type.

Thailand. In 2006, therefore, the Miscellaneous Bacteriology Laboratory, National Institute of Health (NIH), initiated a microbiological service for the identification of *S. suis* in clinical isolates from laboratories of local hospitals in Thailand. Through this microbiological service, 177 clinical isolates were confirmed as *S. suis* and 12 were determined to be of serotype 14.

Herein, we report on both the clinical features of 12 human cases of *S. suis* serotype 14 infection that occurred in 2006–2008 in Thailand and the clonal dissemination of the serotype 14 isolates.

METHODS

Bacterial isolates and identification. A total of 1154 unidentified streptococcal isolates from blood or cerebrospinal fluid were collected from hospitals in all 76 provinces of Thailand by the Miscellaneous Bacteriology Laboratory, NIH, between January 2006 and September 2008 for species identification. Biochemical tests including API Strep (bioMérieux), and specific PCR amplification of the *S. suis* 16S rRNA gene generating a 294 bp PCR product, confirmed 177 isolates of *S. suis* from 34 hospitals in 25 provinces of Thailand (Marois *et al.*, 2004). A previous study reported that four genes of *S. suis* serotype 1 specifically hybridized with serotype 1 and 14 strains only, while five genes of *S. suis* serotype 2 specifically hybridized with serotype 2 and 1/2 strains only (Smith *et al.*, 1999). Based on these findings, the 177 isolates of *S. suis* were designed to be serotyped for 1 or 14 and 2 or 1/2 using duplex PCR. The following primers were used for duplex PCR: SS-cps1 J-F, 5′-gatagagttagttatttgactga-3′; SS-cps2 J-F, 5′-gttgagtccttatacacctg-3′; and SS-cpsJ-R, 5′-aacattaRtaagctataataaa-3′. The PCR products detected for reference strains of serotype 1 (NIAH 10227) or 14 (NIAH 13730) were 217 bp in size, while those for serotype 2 (CCUG 7984, provided by the US Centers for Disease Control) or 1/2 (NIAH 11318) were 515 bp (Fig. 1). NIAH 10227, 13730 and 11318 were kindly provided by the National Institute of Animal Health (NIAH), Japan. No PCR products were detected for the reference strains of other serotypes such as 3, 4, 5, 6, 7, 8, 9 or 16. Of the 177 strains, 165 (93.2 %) had PCR products of 515 bp, while 12 strains (6.8 %) had 217 bp PCR products. Coagglutination tests using rabbit antisera (Statens Serum Institute, Copenhagen, Denmark) showed that the strains that gave 515 bp PCR products were of serotype 2 and the strains that gave 217 bp PCR products belonged to serotype 14. These results were confirmed by Dr M. Gottschalk at the International Reference Laboratory, Université de Montréal, Canada.

Molecular characterization of isolates. The 12 serotype 14 strains were examined using multilocus sequence typing (MLST) as previously described with some modifications (King *et al.*, 2002). eBURST was used to identify clonal complexes in the MLST database, which can be accessed at http://ssuis.mlst.net (Feil *et al.*, 2004). The 12 serotype 14 strains were subjected to PFGE using the restriction enzyme *Sma*I (Luey *et al.*, 2007). PCR was used to test the serotype 14 strains for the virulence-associated gene profile, including the extracellular factor gene (*epf*), the suilysin gene (*sly*) and the muramidase-released protein gene (*mrp*) (Silva *et al.*, 2006), as well as for an ~89 kb candidate pathogenicity island (89K PAI), which were identified in serotype 2 isolates obtained from a previous outbreak of infections in Sichuan, China (Chen *et al.*, 2007).

Patients. The medical records of the 12 patients whose cultures were positive for *S. suis* serotype 14 were retrospectively reviewed by attending physicians at local hospitals in Thailand. Meningitis was defined as a presentation of nuchal rigidity of acute onset and a positive culture from either cerebrospinal fluid or blood. Sepsis was defined as a presentation of systemic inflammatory response syndrome without localized infection and a positive blood culture (Muckart & Bhagwanjee, 1997). Septic arthritis was defined as a presentation of acute arthritis and a positive blood culture.

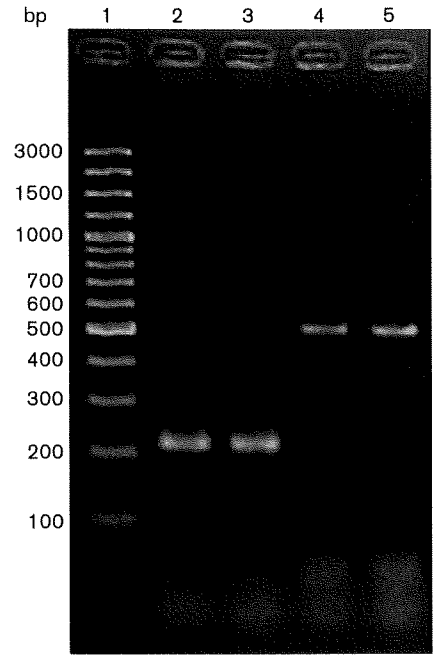


Fig. 1. Differentiation of *S. suis* serotype 1 or 14 and serotype 2 or 1/2 by duplex PCR serotyping. The 217 bp amplification products for serotype 1 and 14 are shown in lanes 2 and 3 and the 515 bp amplification products for serotype 2 and 1/2 are shown in lanes 4 and 5. Lanes: 1, molecular markers; 2, serotype 1 strain (NIAH 10227); 3, serotype 14 strain (NIAH 13730); 4, serotype 2 strain (CCUG 7984); 5, serotype 1/2 strain (NIAH 11318).

RESULTS AND DISCUSSION

Patients

Of the 12 serotype 14 strains, 11 were isolated on different occasions in northern Thailand (Table 1). Another strain was isolated in central Thailand. *S. suis* serotype 14 infections that occurred sporadically were noted, especially in northern Thailand. The median age (range) of the 12 patients was 62.9 (40–79) years and 58.3 % were male. Two patients (16.7 %) had a history of eating raw pork or blood. No patients had occupational contact with raw pork. Clinical presentations included meningitis (58.3 %), septic arthritis (25.0 %) and sepsis (16.7 %). Hearing loss was a complication in five cases (41.7 %) and four of these cases were associated with meningitis. Acute respiratory distress syndrome and extradural and subdural abscesses were also found as a complication for each patient with meningitis.

Table 1. Clinical and microbiological features of 12 cases of *S. suis* serotype 14 infection in Thailand

M, Male; F, female; CSF, cerebrospinal fluid; ST, sequence type; VAG, virulence-associated gene; 89K PAI, a candidate pathogenicity island ~89 kb in length.

Case no.	Age	Sex	Diagnosis	Complication	Outcome	Duration of admission (days)	Strain no.*	Isolation site	Province/region†	ST	VAG profile	89K PAI
1	48	F	Meningitis	Hearing loss	Survived	18	21928	CSF	Lampang/North	105	$epf^+/sly^+/mrp^+$	−
2	79	M	Septic arthritis	None	Survived	8	27964	Blood	Sukhothai/North	105	$epf^+/sly^+/mrp^+$	−
3	63	F	Meningitis	Hearing loss	Survived	31	27578	CSF	Phechabul/North	105	$epf^+/sly^+/mrp^+$	−
4	68	M	Meningitis	Acute respiratory distress syndrome	Survived	5	27071	Blood	Lampang/North	105	$epf^+/sly^+/mrp^+$	−
5	78	F	Septic arthritis	None	Survived	12	26012	Blood	Phechabul/North	105	$epf^+/sly^+/mrp^+$	−
6	48	M	Sepsis	None	Survived	16	25780	Blood	Tak/North	105	$epf^+/sly^+/mrp^+$	−
7	78	F	Sepsis	None	Survived	14	24524	Blood	Phayao/North	105	$epf^+/sly^+/mrp^+$	−
8	70	M	Meningitis	Hearing loss	Survived	28	24451	CSF	Lampang/North	105	$epf^+/sly^+/mrp^+$	−
9	62	F	Septic arthritis	Hearing loss	Survived	37	22695	Blood	Phechabul/North	105	$epf^+/sly^+/mrp^+$	−
10	48	M	Meningitis	None	Survived	1	22692	Blood	Lampang/North	105	$epf^+/sly^+/mrp^+$	−
11	71	M	Meningitis	Extradural and subdural abscess	Survived	45	26390	Blood	Bangkok/Central	105	$epf^+/sly^+/mrp^+$	−
12	40	M	Meningitis	Hearing loss	Survived	15	27024	CSF	Lampang/North	127	$epf^+/sly^+/mrp^+$	+

*DMST (Department of Medical Science, Thailand) number.

†Province and region of Thailand where the serotype 14 strain was isolated.

The median duration (range) of admission was 15.5 (1–45) days and no fatalities were reported.

Patients with serotype 14 infection in the present study were approximately 10 years older than those with *S. suis* infections in previous reports (Wertheim *et al.*, 2009; Mai *et al.*, 2008; Wangkaew *et al.*, 2006; Wangsomboonsiri *et al.*, 2008). However, the clinical manifestations of patients with *S. suis* serotype 14 infection, including hearing loss, were similar to previously reported findings of *S. suis* infections that were caused mostly by serotype 2, with the noted exception of a lack of fatalities (Wertheim *et al.*, 2009; Mai *et al.*, 2008; Wangkaew *et al.*, 2006; Wangsomboonsiri *et al.*, 2008). Complications of subdural abscess and acute respiratory distress syndrome found in this study were also reported in previous studies in Thailand (Wangsomboonsiri *et al.*, 2008) and China (Tang *et al.*, 2006), respectively.

A recent outbreak of *S. suis* infection, including 29 laboratory confirmed cases, occurred in Phayao Province during May 2007 (Khadthasrima *et al.*, 2009). A major route of transmission during this outbreak was consumption of raw blood from infected pigs. A retrospective study of 66 cases of *S. suis* infection in humans living in northern Thailand also found that the majority (59 %) had a history of eating undercooked pork (Wangsomboonsiri *et al.*, 2008). The general public should be made aware of the risks associated with the traditional Thai custom of consuming uncooked pork products. In the present study, only two (16.7 %) of the 12 patients with *S. suis* serotype 14 infection reported a history of eating raw pork or blood and no occupational exposure to raw pork was found. However, these results might be due to inadequate evaluation of traditional dietary practice at the time of admission. Although previous studies reported low proportions of female patients with *S. suis* infections (13.3–28.1 %) (Wangkaew *et al.*, 2006; Wangsomboonsiri *et al.*, 2008; Ma *et al.*, 2008), a relatively high proportion of female patients (41.7 %) was found in this study. Collectively, these findings indicate that local residents, including housewives, may have unintentional exposure during cooking to contaminated pork products that are sold at markets in northern Thailand, because a recent study from Hong Kong demonstrated a dense contamination of *S. suis* in raw pork meats available in local supermarkets or at wet markets (Cheung *et al.*, 2008).

Molecular characterization of isolates

MLST analysis using seven selected housekeeping genes (*aroA*, *cpn60*, *dpr*, *gki*, *mutS*, *recA* and *thrA*) confirmed that 11 of the 12 strains had identical sequence type (ST) profiles (1,1,1,52,1,1,1) (King *et al.*, 2002). The ST profile of the strain that differed from the others was 1,1,1,1,1,1,18. These strains were assigned to ST105 and the novel ST127 (Table 1). Snapshots of all isolates of *S. suis* generated by eBURST, including those of our 12 serotype 14 isolates and all 408 isolates available in the MLST dataset (Feil *et al.*, 2004), suggested six major clonal complexes: ST1, ST17, ST27, ST29, ST87 and ST94 (Fig. 2). The ST105 and ST127 strains in this study were derived from ST1, which is the primary strain of the ST1 complex.

All 12 serotype 14 strains were positive for three virulence-associated genes (Table 1). However, the 11 ST105 serotype 14 strains were negative for the 89K PAI, while the ST127 strain was positive for the 89K PAI since three sets of PCR for the 89K PAI were positive (Chen *et al.*, 2007). Because all of the ST105 serotype 14 strains were positive for the three virulence-associated genes, but negative for the 89K PAI, the ST105 human isolates may have another PAI. However, the role of the 89K PAI remains to be determined. PFGE typing revealed four different DNA profiles among the 12 serotype 14 isolates (Fig. 3). An identical pattern, type A, was found for nine of the ST105 strains. Closely related patterns – A1 and A2 – were also noted for each of the ST105 serotype 14 isolates. The pulsotypes of ST105 strains were assigned to clusters of isolates with >70 % similarity in the dendrogram. Other closely related patterns – A3 and A4 – were found for the ST127 strain of serotype 14 as well as a serotype 14 reference strain (NIAH 13730).

Of the 177 *S. suis* isolates identified in the present study, 12 strains (6.8 %) were serotype 14 with STs 105 and 127. By contrast, in southern Vietnam, only one (1.1 %) of 92 human isolates of *S. suis* was of serotype 14; all other strains belonged to serotype 2 (Mai *et al.*, 2008). Our data on PFGE suggest that clonal dissemination of serotype 14 with ST105 occurred in Thailand, and an involvement of three pulsotypes in 11 ST105 strains indicates that PFGE is more discriminatory than MLST, which corresponds to a previous report (Ma *et al.*, 2008). Of the seven serotype 14 isolates previously reported, the STs of five strains were determined as follows: ST1 from China (Ye *et al.*, 2008) and England (http://ssuis.mlst.net), ST6 from the Netherlands (Ye *et al.*, 2008; King *et al.*, 2002), ST11 from Thailand (Takamatsu *et al.*, 2008) and ST105 from Vietnam (Mai *et al.*, 2008). Collectively, all of the serotype 14 strains reported in our and previous studies belonged to the ST1 complex (Fig. 2). Interestingly, the pulsotype of the serotype 14 strain with ST105 isolated from Vietnam in 2004 appears to be identical or closely related to the type A of serotype 14 strains with ST105 in the present study (Mai *et al.*, 2008).

The results of the present study and earlier reports (Lun *et al.*, 2007; Ma *et al.*, 2008) indicate that this pathogen is often either undiagnosed or misdiagnosed by the laboratories of local hospitals in South-East Asian countries, including Thailand. Given the increased awareness of this disease on the part of clinicians and the availability of diagnostic tests for this pathogen at local hospitals, the true prevalence of this disease in this region will be determined.

In conclusion, over the past 3 years, 12 cases of *S. suis* serotype 14 infection have occurred sporadically in Thailand. The clinical features of these patients were

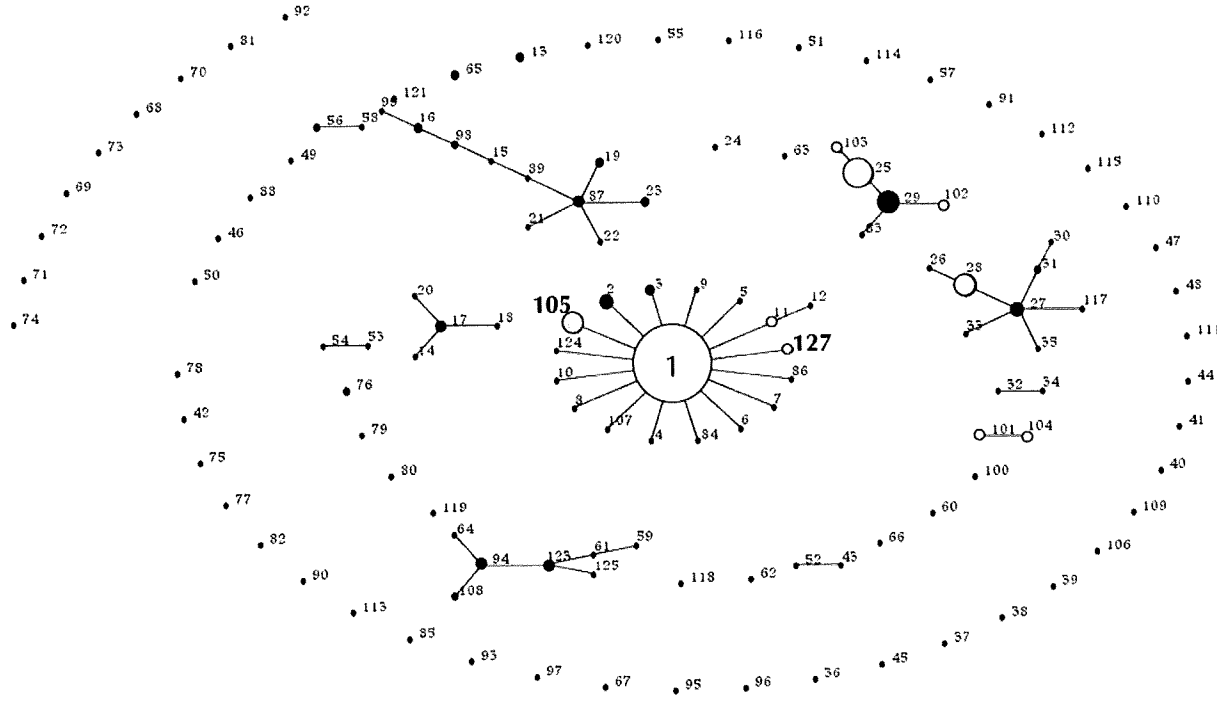
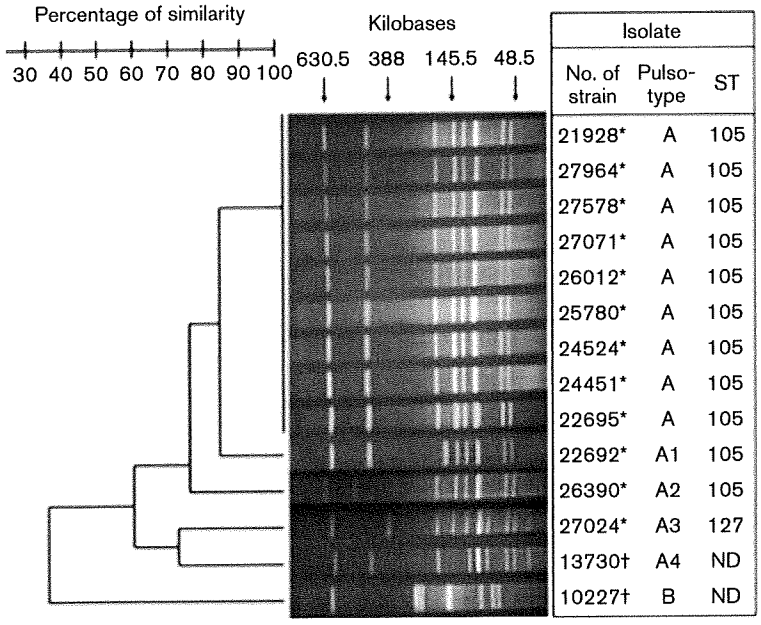


Fig. 2. Entire *S. suis* MLST database displayed as a single eBURST diagram. The primary founders of ST1 are located in the centre of the cluster, and subgroup founders are shown in closed circles, except for STs 1, 11, 25, 28, 101, 103, 104, 105 and 127. ST105 for 11 serotype 14 strains in this study and one strain of serotype 14 from Vietnam (Mai *et al.*, 2008) and ST127 for one strain of serotype 14 in this study are shown in grey circles. STs 1, 11, 25, 28, 101, 103 and 104 for human isolates of *S. suis* serotypes 2 and 14 in Thailand previously reported by Takamatsu *et al.* (2008) are shown in open circles. The size of each circle in the diagram corresponds to the abundance of the isolates of the ST in the input data.



Isolate		
No. of strain	Pulso-type	ST
21928*	A	105
27964*	A	105
27578*	A	105
27071*	A	105
26012*	A	105
25780*	A	105
24524*	A	105
24451*	A	105
22695*	A	105
22692*	A1	105
26390*	A2	105
27024*	A3	127
13730†	A4	ND
10227†	B	ND

Fig. 3. Dendrogram generated from PFGE profiles after *Sma*I digestion of 12 human isolates of *S. suis* serotype 14 obtained from humans living in Thailand and reference strains of serotype 14 (NIAH 13730) and serotype 1 (NIAH 10227). The numbers in the dendrogram indicate the percentage similarity. Arrows indicate molecular size; * indicates DMST (Department of Medical Science, Thailand) strain number; † indicates NIAH (National Institute of Animal Health) strain number.

similar to those previously reported for *S. suis* infections, except that there were no fatal cases. Clonal dissemination of serotype 14 with ST105 was demonstrated in 11 of the 12 isolates. To our knowledge, this is the first report of clonal dissemination of serotype 14 in humans. Because this serotype is becoming more common in human infections, continuous surveillance of this disease using the diagnostic tests, which include serotyping PCR and a coagglutination test, for both serotypes 2 and 14 should be required at hospital laboratories in South-East Asian countries.

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Intranasal immunization with a mixture of PspA and a Toll-like receptor agonist induces specific antibodies and enhances bacterial clearance in the airways of mice

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ABSTRACT

To develop an effective nasal vaccine for *Streptococcus pneumoniae*, the effects of a panel of Toll-like receptor (TLR) agonists in combination with pneumococcal surface protein A (PspA) on induction of PspA-specific antibodies and bacterial clearance were compared in mice. Mice were nasally immunized with 10 μg of TLR agonist (TLR 2–4 and 9) and 2.5 μg of PspA once per week for 3 weeks. Significantly increased levels of PspA-specific immunoglobulin G (IgG) and IgA in the airways and PspA-specific IgG in plasma were found in mice administered PspA plus each TLR agonist, compared with mice administered PspA alone. In a sub-lethal pneumonia model using a serotype 3 pneumococcal strain, bacterial density in the lungs of mice was significantly reduced in mice administered PspA plus each TLR agonist, compared with mice administered either PspA alone or phosphate-buffered saline alone 3 h after bacterial challenge. Similarly, enhanced bacterial clearance was found in the nasopharynx of mice administered PspA plus each TLR agonist 1 day after infection with a serotype 19F strain. Our data suggest that PspA-specific antibody induced by nasal immunization with PspA plus TLR agonist is capable of reducing the bacterial load in both the nasopharynx and lungs after challenge with pneumococci with different serotypes. Despite the skewed Th1/Th2 immune responses, the effects of nasal immunization with PspA plus each TLR agonist on bacterial clearances from the lungs 3 h after infection and from nasopharynx 1 day after infection in mice were equivalent.

1. Introduction

Streptococcus pneumoniae (*S. pneumoniae*) is a leading human pathogen causing diseases ranging from otitis media to pneumonia, bacteremia, and meningitis in children and adults. Although pneumococcal conjugate vaccine provides protective immunity against pneumonia as well as invasive disease in infants [1,2], polysaccharide-based vaccines are not ideal because they must include multiple polysaccharide serotypes and do not protect against strains with non-vaccine serotypes [3]. Previous investigators have examined several pneumococcal proteins as potential vaccine candidates with promising results [4–7]. One of these candidates, pneumococcal surface protein A (PspA) is a choline-binding protein tethered to the cell surface through its C-terminal choline-binding repeat region [4]. PspA is present on all pneumococcal strains, and anti-PspA antibody enhances bacterial clearance and induces cross-protection against infection from strains with different serotypes [8]. According to the mapping studies of the major cross-protective epitopes that reside in the ~100 amino acids of the α-helical region, PspAs have been divided into seven clades that constitute three families [9]. PspAs of families 1 and 2 are expressed by >98% of strains. Anti-PspA antibodies overcome the anti-complementary effect of PspA, allowing for increased complement activation and C3 deposition on PspA-bearing bacteria [10,11].

Nasal immunization is the most effective way to induce both mucosal secretory-IgA responses and systemic IgG responses [12]. An appropriate mucosal adjuvant is required to elicit an antigen-specific immune response in both mucosal and systemic compartments [13]. The Toll-like receptor (TLR) family is the best-studied family of pattern recognition receptors, and it recognizes

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a broad spectrum of pathogen-associated molecular patterns from different classes of microbes [14]. TLR ligands may stimulate dendritic cells (DC), thereby acting as an effective adjuvant to allow a DC-targeted protein to induce protective CD4 T cell responses at mucosal surfaces [13,14]. The balance of Th1/Th2 immune responses appears to be dependent on each TLR ligand [15]. Th1 immune responses augment IgG2a production, while Th2 immune responses enhance IgG1 and IgE production by B cells [16–18]. The pattern of IgG subclass response may affect the bacterial clearance afforded by such humoral immunity during infections. Two recent studies employing a PspA DNA vaccine [19] and a nasal lactococcal vaccine producing PspA [20] have suggested that the induction of a balanced IgG1/IgG2a response to PspA correlates with an increased protection against pneumococcal infections. Therefore, in this study, we examined the relationship between the Th1- or Th2-associated IgG isotype response and the enhanced bacterial clearance of *S. pneumoniae* from the airways after intranasal immunization using a mixture of PspA plus each TLR 2–4 or 9 agonist in mice.

2. Material and method

2.1. Mice

Female C57BL/6 mice (6–8-week-old) were purchased from Charles River Japan, Kanagawa, Japan. Mice were transferred to microisolators and maintained in horizontal laminar flow cabinets. They were provided sterile food and water in a specific pathogen-free facility. All mice used in these experiments were free of bacterial and viral pathogens.

2.2. Bacterial strains

S. pneumoniae WU2 strain with serotype 3, expressing PspA belongs to family 1, clade 2 and is virulent in mice [21]. *S. pneumoniae* EF3030 strain with serotype 19F is a clinical isolate, expressing PspA belongs to family 1, clade 1, and is relatively avirulent in mice [22]. These strains were kindly provided by Dr. D.E. Briles, University of Alabama at Birmingham.

2.3. Recombinant PspA

PspA used for nasal immunization in this study was recombinant PspA/Rx1 (pUAB055) [5]. The recombinant plasmid pUAB055 containing the 0.9 kb *pspA* gene fragment inserted between the *pelB* leader sequence and the His-tag site in vector pET20b (a gift from Dr. S.K. Hollingshead, University of Alabama at Birmingham) was transformed into *E. coli* strain BL21 (DE3) for protein production. Rx1/PspA is of PspA family 1 (clade 2), which is the same family as both the WU2 strain and EF3030 strains. Induction with isopropyl-thio-β-D-galactopyranoside (Sigma, St. Louis, MO) resulted in production of 6× His-tagged recombinant PspA. The recombinant PspAs were purified by chromatography chelating-sepharose 4B pre-loaded with Ni^+ (GE Healthcare, Buckinghamshire, England) according to the manufacturer's instruction. The fraction containing PspA was loaded onto a gel filtration superdex-75 5/30 GL column (GE Healthcare) to further purify the PspA. Contaminated endotoxin was removed from the PspA preparation by using EndoTrap^R (Profos AG, Rosenberg, Germany). The purified PspA preparation was analyzed for the presence of endotoxin using a chromogenic *Limulus* lysate endopoint assay, QCL-1000^R (Cambrex, Walkersville, MD), and it contained 1.30 ng of LPS per 1 μg of PspA. To remove LPS extensively from the PspA preparations, we used another LPS removal column, ProteoSpin^R (Norgen, Thorold, Canada) and prepared the PspA with a lower concentration of LPS (0.048 ng of LPS per 1 μg of PspA).

2.4. Adjuvant

Pam3CSK4 is a synthetic tripalmitoylated lipopeptide that mimics bacterial peptides [23], and is recognized by the TLR2/TLR1 heterodimer. Poly(I:C) is a synthetic analog of double-stranded RNA, a TLR3 agonist [24]. Pam3CSK4, Poly(I:C), and Ultra Pure *Escherichia coli* K12 LPS, a TLR4 agonist, were purchased from InvivoGen (San Diego, CA). CpG DNA ODN1826 (TLR9 ligand, 5′-TCCATGACGTTCCTGACGTT-3′) was purchased from Hokkaido System Science (Sapporo, Japan) [25]. Each of these adjuvants was used in a dose of 10 μg for nasal immunization, because these TLR agonists demonstrated potent adjuvant effects at this dose in mouse experiments [24–26].

2.5. Nasal immunization

Mice were immunized three times at weekly intervals intranasally with 12 μl of phosphate-buffered saline (PBS) containing 10 μg of each TLR agonist and 2.5 μg of PspA, 2.5 μg of PspA alone or 12 μl of PBS alone on day 0, days 7 and days 14. On days 21, mice were euthanized to obtain plasma, bronchoalveolar lavage fluid (BALF) and nasal wash (NW). A dose of 2.5 μg of PspA was employed for nasal immunization in this study, as nasal immunization with this dose of PspA plus 10 μg of each TLR agonist induces PspA-specific antibodies in the airways. A dose of PspA alone for nasal immunization, therefore, contained 3.25 ng of LPS. After removing the mandible, the nasal cavity was gently flushed from the posterior opening of the nose with 1 ml of PBS [27]. The NW flushing out from the anterior openings of the nose was collected. BALF was obtained by irrigation with 1 ml of PBS using of a blunted needle inserted into the trachea after tracheotomy [28].

2.6. PspA-specific antibody assays

PspA-specific antibody titers of IgG, IgG1, IgG2a or IgA in plasma, BALF and NW were determined by ELISA as previously described [28]. The coefficient variation (CV) of the levels of PspA-specific IgG, IgG1, IgG2a or IgA was also determined.

PspA was used as the coating antigen (1 μg/ml). 100 μl of sample was added to each well, followed by incubation at 37 °C for 30 min. The plate was washed, and then reacted with 100 μl of alkaline phosphatase-conjugated goat anti-mouse IgA, IgG, IgG1 or IgG2a (Zymed, San Francisco, CA). The OD at 405 nm was then measured. The end-point titers were expressed as the reciprocal Log_2 of the last dilution giving an OD_{405} of 0.1 OD unit above the OD_{405} of negative control samples obtained from non-immunized mice.

2.7. Pneumonia model

To determine the effects of nasal immunization with PspA plus each TLR agonist, *S. pneumoniae* WU2 strain at a dose of 2.0×10^6 cfu suspended in 30 μl of sterile saline was intranasally administered to both immunized and untreated mice 2 weeks after the last immunization. The 2-week interval between the last immunization and the bacterial challenge was kept to avoid the influence of each TLR agonist on pulmonary defense, as some TLRs are involved in the innate immune response to *S. pneumoniae* [29–31]. The lungs were removed aseptically from mice that had been euthanized with pentobarbital at 3 h, 6 h and 12 h post-bacterial challenge. The lung tissue was homogenized in 9 ml of sterile saline per gram of lung tissue prior to culturing and quantitative bacterial cultures of lung tissue were performed on horse blood agar. The detection limit of bacterial culture of the lung tissue was 10^3 cfu/g. The survival rate after intranasal challenge with 2.0×10^6 cfu of the WU2 strain was 100%.

2.8. Nasal carriage model

S. pneumoniae EF3030 strain at a dose of 3×10^5 cfu in suspended 30 μl of sterile saline was similarly intranasally administered to both immunized and untreated mice 2 weeks after the last immunization. One or 6 days after bacterial challenge, NW was obtained as described above, and a quantitative bacterial culture of the NW was performed.

2.9. Statistics

Statistical analyses were performed using one-way ANOVA and multiple comparison methods by Fisher's LSD. Data were considered to be statistically significant if the *P*-values were less than 0.05. All data were expressed as mean ± S.D.

3. Results

3.1. PspA-specific IgG and IgG isotypes in plasma

Nasal administration of PspA plus Pam3CSK4, Poly(I:C), LPS or CpG1826 significantly increased the levels of PspA-specific IgG in the plasma, compared with administration of PspA alone ($P < 0.05$, Fig. 1A). No differences were found in the levels of PspA-specific IgG among mice nasally administered PspA plus each TLR agonist. The CV of the levels of PspA-specific IgG by PspA plus each TLR agonist was much smaller than that induced by PspA alone.

Since the preparation of PspA after removal of LPS with Endotrap contained LPS (3.25 ng per 2.5 μg of PspA), PspA-specific IgG might be elicited by the adjuvant effect of the residual LPS. We then compared the levels of PspA-specific IgG in between the plasma of mice nasally administered 2.5 μg of PspA preparations containing either 3.25 ng of LPS or 0.12 ng of LPS. No significant differences were found in the levels of PspA-specific IgG in plasma of mice after nasal immunization with two different PspA preparations (data not shown). These data suggest the residual LPS did not contribute to the induction of PspA-specific IgG in plasma as an adjuvant, and PspA itself could induce PspA-specific IgG in plasma.

To assess whether each TLR agonist induces either a Th1- or a Th2-associated IgG isotype response, plasma samples were analyzed for PspA-specific IgG1 and IgG2a isotypes (Fig. 1B). Nasal administration of PspA plus Pam3CSK4, Poly(I:C) or LPS significantly increased the levels of PspA-specific IgG1 in plasma, while PspA-specific IgG1 increased to a lesser extent in plasma of mice nasally administered PspA plus CpG1826. The IgG1 levels differed significantly between mice administered PspA plus either Pam3CSK4, Poly(I:C) or LPS and mice administered PspA plus CpG1826 ($P < 0.05$, Fig. 1B). Furthermore, PspA-specific IgG1 levels were significantly higher in mice administered PspA plus either Pam3CSK4, Poly(I:C), LPS or CpG1826 than in mice administered PspA alone ($P < 0.05$). In contrast, mice nasally administered PspA plus either Poly(I:C) or CpG1826 demonstrated significant increases in the levels of PspA-specific IgG2a in plasma, compared with mice administered PspA plus either Pam3CSK4, LPS or PspA alone ($P < 0.01$). The CV of the levels of PspA-specific IgG1 in plasma of mice nasally administered PspA plus each TLR agonist was much smaller than that of mice nasally administered PspA alone. In contrast, the CV of the levels of PspA-specific IgG2a induced by either PspA plus each TLR agonist, except for Poly(I:C), or PspA alone was large in plasma.

3.2. PspA-specific IgG and IgA in BALF and NW

Although the levels of PspA-specific IgG were negligible in the BALF and NW of mice given PspA alone, the levels of PspA-specific IgG were significantly greater in the BALF (Fig. 2A) and NW (Fig. 3A) of mice nasally administered PspA plus either Pam3CSK4, Poly(I:C), LPS or CpG1826 than in mice nasally administered PspA alone ($P < 0.05$). A PspA-specific IgG1 response was found in the BALF of mice administered PspA plus either Pam 3CSK4, Poly(I:C), LPS or CpG1826 (Fig. 2C). In contrast, significant increases of PspA-specific IgG2a were also found in the BALF of mice administered PspA plus either Poly(I:C) or PspA plus CpG1826, compared with mice administered PspA plus either Pam3CSK4 or LPS or PspA alone ($P < 0.05$, Fig. 2C). However, PspA-specific IgG2a was rarely detected in the BALF of mice administered PspA plus either Pam3CSK4 or

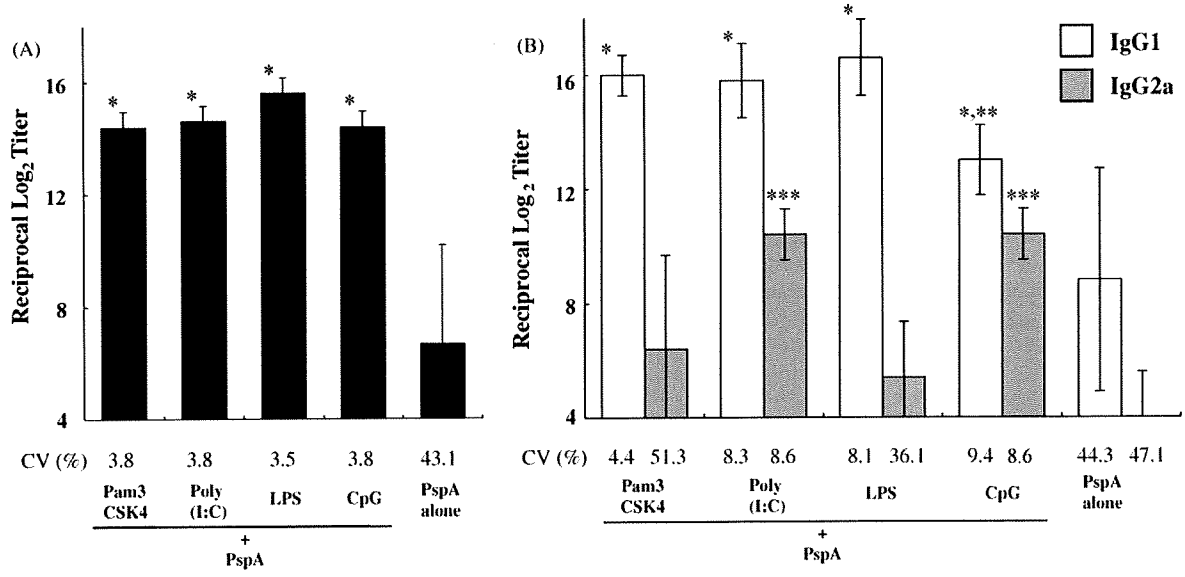

Fig. 1. Induction of PspA-specific IgG (closed bar) (A), PspA-specific IgG1 (open bar) and IgG2a (gray bar) (B) in plasma by intranasal immunization with either PspA plus each TLR agonist or PspA alone. Mice were nasally immunized three times weekly intervals with 10 μg of TLR agonist and 2.5 μg of PspA. One week after the final immunization, mice were euthanized to obtain plasma, and PspA-specific antibody titers were determined using ELISA. The results are expressed as means ± S.D. for six mice per group. CV, coefficient of variation; LPS, *E. coli* K12 LPS; CpG, CpG DNA ODN1826. *$P < 0.05$, when compared with mice nasally administered PspA alone; **$P < 0.05$, when compared with mice nasally administered PspA plus either Pam3CSK4, Poly(I:C) or LPS; ***$P < 0.05$, when compared with mice nasally administered PspA plus either Pam3CSK4, LPS or PspA alone.

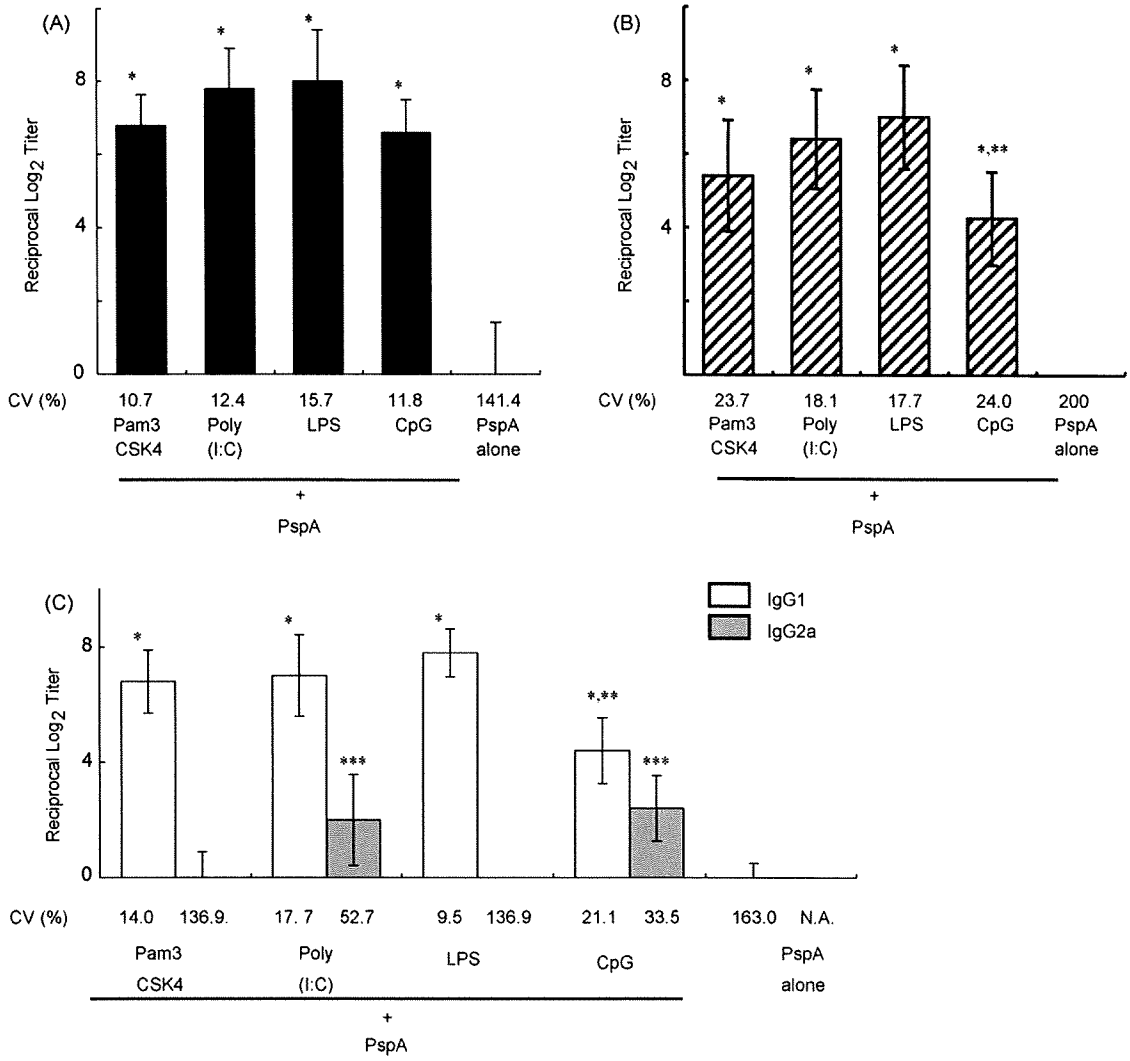

Fig. 2. Induction of PspA-specific IgG (closed bar) (A), IgA (hatched bar) (B) and PspA-specific IgG1 (open bar) and IgG2a (gray bar) (C) in bronchoalveolar lavage fluid (BALF) by intranasal immunization with either PspA plus each TLR agonist or PspA alone. Mice were nasally immunized three times weekly with 10 μg of TLR agonist and 2.5 μg of PspA. One week after the final immunization, mice were euthanized to obtain BALF and NW, and PspA-specific antibody titers were determined using ELISA. The results are expressed as means ± S.D. for six mice per group. CV, coefficient of variation; N.A., not available; LPS, *E. coli* K12 LPS; CpG, CpG DNA ODN1826. *$P < 0.05$, when compared with mice nasally administered PspA alone; **$P < 0.05$, when compared with mice nasally administered PspA plus either Pam3CSK4, Poly(I:C) or LPS; ***$P < 0.05$, when compared with nasally administered PspA plus either Pam3CSK4, LPS or PspA alone.

LPS or PspA alone. Mice nasally administered PspA plus Pam3CSK4, Poly(I:C), LPS or CpG1826 demonstrated significant increases in the levels of PspA-specific IgA in the BALF (Fig. 2B) and NW (Fig. 3B), compared with mice nasally administered PspA alone ($P < 0.05$). The levels of PspA-specific IgA were significantly lower in the BALF of mice administered PspA plus CpG1826 than in mice administered PspA plus either Poly(I:C) or LPS ($P < 0.05$). The CV of the levels of PspA-specific IgG or IgA induced in the BALF by PspA plus each TLR agonist was similarly much smaller than that induced by PspA alone. A similar tendency of the CV was found in NW.

3.3. Bacterial clearance from the lungs

At 3 h post-nasal challenge with a sub-lethal dose of serotype 3 WU2 strain, the bacterial density (mean ± S.D. for Log_{10} cfu/g) in the lungs reached to 6.0 ± 0.4 and 6.0 ± 0.3 in mice nasally administered PspA alone and PBS alone, respectively (Fig. 4A). No significant difference was found between these two groups. In contrast, significant decreases were found in bacterial density in the lungs of mice nasally administered PspA plus Pam3CSK4, Poly(I:C), LPS or CpG1826, compared with mice nasally administered either PspA alone or PBS alone ($P < 0.05$). No significant differences were found in the bacterial density among mice nasally administered PspA plus each TLR agonist. At 6 h post-nasal challenge with the same dose of WU2 strain, the bacterial density (mean ± S.D. for Log_{10} cfu/g) in the lungs remained unchanged at 6.3 ± 0.4 for mice administered PBS alone (Fig. 4B). In contrast, significant decreases were found in the bacterial density in the lungs of mice nasally administered either PspA plus each TLR agonist or PspA alone, compared with mice administered PBS alone ($P < 0.05$). No significant difference was found in the bacterial density among mice nasally administered either PspA plus each TLR agonist or PspA alone. At 12 h post-nasal challenge, the bacterial density (mean ± S.D. for Log_{10} cfu/g) in the lung declined to 4.7 ± 0.7 in mice administered PBS alone (Fig. 4C). In contrast, bacteria were not detected in the lungs of mice nasally administered either PspA plus each TLR agonist or PspA alone. No bacteria were detected in the blood of any mice examined at 3 h, 6 h and 12 h post-nasal challenge.

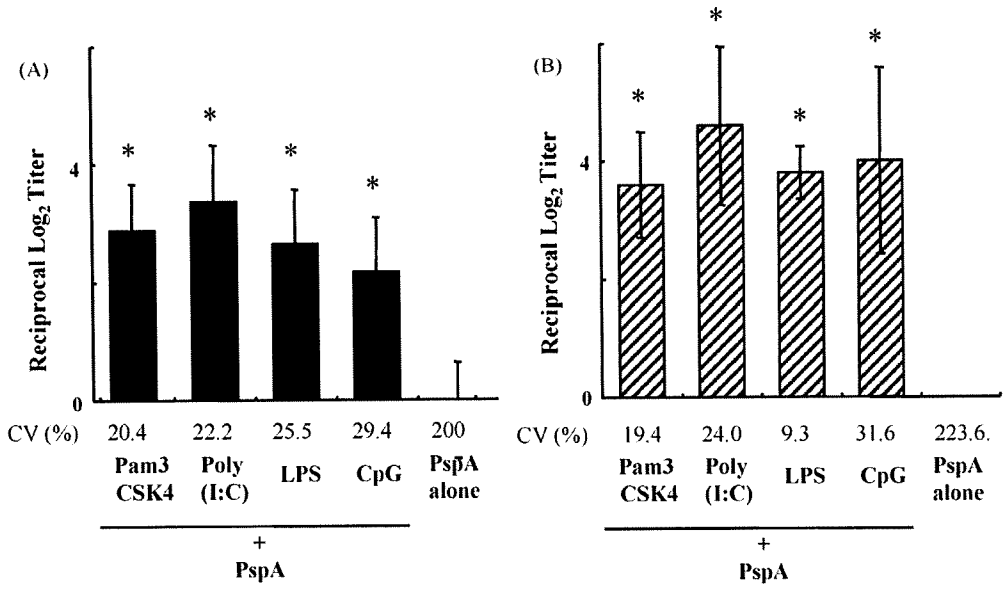

Fig. 3. Induction of PspA-specific IgG (closed bar) (A) and IgA (hatched bar) (B) in nasal wash (NW) by intranasal immunization with either PspA plus each TLR agonist or PspA alone. Mice were nasally immunized three times at weekly intervals with 10 μg of TLR agonist and 2.5 μg of PspA. One week after the final immunization, mice were euthanized to obtain BALF and NW, and PspA-specific antibody titers were determined using ELISA. The results are expressed as means ± S.D. for six mice per group. CV, coefficient of variation; LPS, *E. coli* K12 LPS; CpG, CpG DNA ODN1826. *$P < 0.05$, when compared with mice nasally administered PspA alone.

3.4. Bacterial clearance from the nasopharynx

One day after nasal challenge with 3×10^5 cfu of serotype 19F EF3030 strain, the bacterial density (mean ± S.D. for Log_{10} cfu/ml) in NW reached to 5.21 ± 0.26 and 5.08 ± 0.11 in mice administered both PspA alone and PBS alone, respectively (Fig. 5A). No significant difference was found between these two groups. In contrast, significant decreases were found in the bacterial density of mice nasally administered PspA plus either Pam3CSK4, Poly(I:C), LPS or CpG1826, compared with mice nasally administered PspA alone ($P < 0.05$). No significant differences were found in the bacterial density among mice nasally administered PspA plus each TLR agonist. Six days after challenge with 3×10^5 cfu of the EF3030 strain, the bacterial density (mean ± S.D. for Log_{10} cfu/ml) in NW declined to 4.78 ± 0.29 and 4.69 ± 0.29 for mice administered both PspA and PBS alone, respectively (Fig. 5B). No significant difference was found

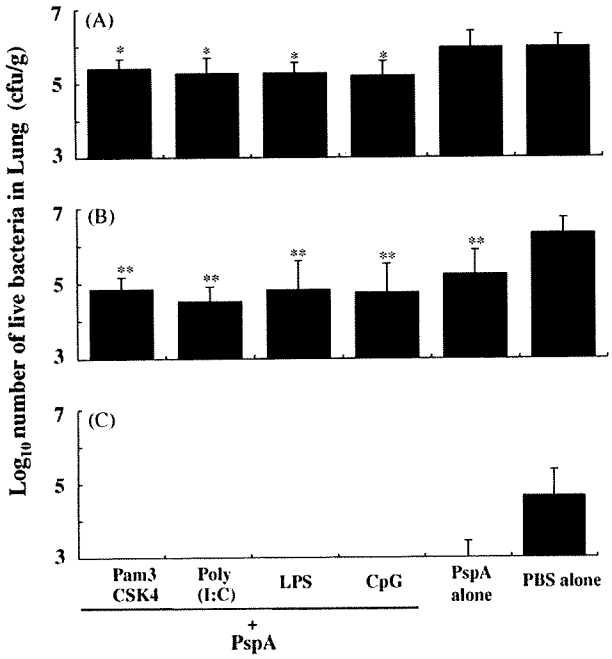

Fig. 4. The effect of intranasal immunization by PspA plus each TLR agonist on the bacterial densities in the lung tissue at 3 h (A), 6 h (B) and 12 h (C) post-challenge with *S. pneumoniae* WU2 strain. A dose of 2×10^6 cfu/mouse was nasally administered to mice previously immunized with either PspA plus each TLR agonist, PspA alone or PBS alone. Mice were euthanized to obtain the lung tissues from infected mice at indicated time-points after bacterial challenge, and quantitative bacterial cultures of lung tissue were performed. Values represent the Log_{10} cfu/g (mean ± S.D.) for six mice per group. CV, coefficient of variation; N.A., not available; LPS, *E. coli* K12 LPS; CpG, CpG DNA ODN1826. *$P < 0.05$, when compared with mice nasally administered either PspA alone or PBS alone; **$P < 0.05$, when compared with mice nasally administered PBS alone.

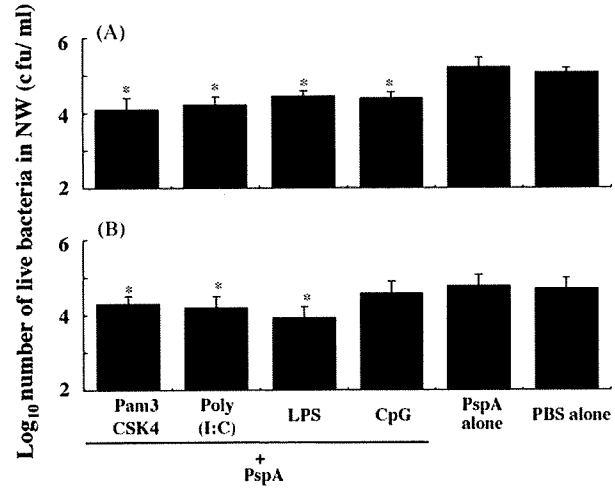

Fig. 5. The effect of intranasal immunization by PspA plus each TLR agonist on the bacterial densities in the nasopharynx 1 day (A) and 6 days (B) after challenge with *S. pneumoniae* EF3030 strain. A dose of 3×10^5 cfu/mouse was nasally administered to mice previously immunized with either PspA plus each TLR agonist, PspA alone or PBS alone. Mice were euthanized to obtain the nasal wash (NW) from infected mice at indicated time-points after bacterial challenge, and a quantitative bacterial culture of NW was performed. Values represent the Log_{10} cfu/ml (mean ± S.D.) of for six mice per group. LPS, *E. coli* K12 LPS; CpG, CpG DNA ODN1826. *$P < 0.05$, when compared with mice nasally administered either PspA alone or PBS alone.