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Presence of Neutrophil Extracellular Traps and Citrullinated Histone H3 in the Bloodstream of Critically Ill Patients

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Abstract

Neutrophil extracellular traps (NETs), a newly identified immune mechanism, are induced by inflammatory stimuli. Modification by citrullination of histone H3 is thought to be involved in the in vitro formation of NETs. The purposes of this study were to evaluate whether NETs and citrullinated histone H3 (Cit-H3) are present in the bloodstream of critically ill patients and to identify correlations with clinical and biological parameters. Blood samples were collected from intubated patients at the time of ICU admission from April to June 2011. To identify NETs, DNA and histone H3 were visualized simultaneously by immunofluorescence in blood smears. Cit-H3 was detected using a specific antibody. We assessed relationships of the presence of NETs and Cit-H3 with the existence of bacteria in tracheal aspirate, SIRS, diagnosis, WBC count, and concentrations of IL-8, TNF-α, cf-DNA, lactate, and HMGB1. Forty-nine patients were included. The median of age was 66.0 (IQR: 52.5–76.0) years. The diagnoses included trauma (7, 14.3%), infection (14, 28.6%), resuscitation from cardiopulmonary arrest (8, 16.3%), acute poisoning (4, 8.1%), heart disease (4, 8.1%), brain stroke (8, 16.3%), heat stroke (2, 4.1%), and others (2, 4.1%). We identified NETs in 5 patients and Cit-H3 in 11 patients. NETs and/or Cit-H3 were observed more frequently in "the presence of bacteria in tracheal aspirate" group (11/22, 50.0%) than in "the absence of bacteria in tracheal aspirate" group (4/27, 14.8%) ($p<.01$). Multiple logistic regression analysis showed that only the presence of bacteria in tracheal aspirate was significantly associated with the presence of NETs and/or Cit-H3. The presence of bacteria in tracheal aspirate may be one important factor associated with NET formation. NETs may play a pivotal role in the biological defense against the dissemination of pathogens from the respiratory tract to the bloodstream in potentially infected patients.

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Introduction

Neutrophils play an important role as the first line of innate immune defense [1]. One function of neutrophils, called "neutrophil extracellular traps" (NETs), has been discovered recently. NETs are fibrous structures that are released extracellularly from activated neutrophils in response to infection and also the sterile inflammatory process [2–5]. This distinctive phenomenon was first reported by Brinkmann et al in 2004 [6]. The main components of NETs are deoxyribonucleic acid (DNA) and histones H1, H2A, H2B, H3, and H4; other components such as neutrophil elastase, myeloperoxidase, bactericidal/permeability-increasing protein, cathepsin G, lactoferrin, matrix metalloproteinase-9, peptidoglycan recognition proteins, pentraxin, and LL-37 have also been reported [5–11]. The type of active cell death involving the release of NETs is called NETosis [12], which differs from apoptosis and necrosis. Because formation of NETs does not require caspases and is not accompanied by DNA fragmentation, it is believed that this process is independent of apoptosis [12]. Despite several in vitro and animal experiments that have clearly shown the biological importance of NETs, little is known about the function of NETs in the human body [13,14].

Before the discovery of NETs, several studies reported on an increase in the concentration of circulating free DNA (cf-DNA) in

the blood in various diseases including sepsis, trauma, stroke, autoimmune disorders, and several cancers [15–20]. This cf-DNA is thought to be derived from necrotic and/or apoptotic cells [21]. Recent articles have suggested that NETs and cf-DNA are related [15,16]. In these reports, cf-DNA was quantified directly in plasma, and the cf-DNA in plasma was treated the same as NETs in blood. However, it remains unknown whether cf-DNA is derived from NETs.

Citrullination of histone H3 is considered to be involved in NET formation in vitro. Neutrophils show highly decondensed nuclear chromatin structures during NETosis, and hypercitrullination of histone H3 by peptidylarginine deiminase 4 (PAD4) plays an important role in chromatin decondensation [14,22,23]. Inhibition of PAD4 prevents citrullination of H3 and NET formation [23]. Thus, measuring the presence of citrullinated histone H3 (Cit-H3) in conjunction with the presence of NETs may help clarify the kinetics of the response of NETs to systemic stress.

In preliminary studies, we recently identified NETs immunocytochemically in sputum and blood smear samples from intensive care unit (ICU) patients [24,25], whereas NETs could not be detected in blood smears from healthy volunteers [25].

In the present study, we used immunofluorescence to prospectively explore the existence of NETs and Cit-H3 in the blood of critically ill patients hospitalized in an ICU.

The respiratory tract is considered one of the most vulnerable places for bacterial invasion of the body, and NETs might start to be produced in response to pathogens before infection is completely apparent. Therefore, in this study we evaluated the presence of bacteria by Gram staining in tracheal aspirate as the preclinical stage of manifested infection to highlight its relationship with the induction of NETs in blood. The purpose of this study was to evaluate the relationships between NET or Cit-H3 and various clinical and biological parameters.

Materials and Methods

Patients and Setting

This study was a prospective observational study and was approved by the Ethics Committee of Osaka University Graduate School of Medicine. The institutional review board waived the need for informed consent. From April to June 2011, we examined blood samples collected from all patients who required intubation at the time of admission into the ICU of the Trauma and Acute Critical Care Center at the Osaka University Hospital (Osaka, Japan).

Evaluation of Clinical Background and Severity of Illness

Age, sex, Acute Physiological And Chronic Health Evaluation (APACHE) II score, and Sequential Organ Failure Assessment (SOFA) score were recorded at the time of admission. Systemic inflammatory response syndrome (SIRS) was diagnosed at the time of admission on the basis of the criteria for SIRS defined by the American College of Chest Physicians/Society of Critical Care Medicine Consensus [26]. At admission, the blood samples were analyzed to obtain the following laboratory data: white blood cell (WBC) count and concentrations of lactate, IL-8, TNF-α, HMGB1, and cf-DNA. WBC count was measured by an automated hematology analyzer (KX-21N; Sysmex, Hyogo, Japan). Lactate concentration was measured by a blood gas analyzer (ABL 835 Flex; Radiometer, Brønshøj, Denmark). The serum levels of IL-8 (R&D Systems, Minneapolis, MN, USA), TNF-α (R&D Systems), and HMGB1 (Shino-Test Corporation, Tokyo, Japan) were measured by enzyme-linked immunosorbent assay (ELISA) kits, and cf-DNA concentration was quantified using the Quant-iT PicoGreen dsDNA Assay kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions.

Immunofluorescence Analysis to Identify the Presence of NETs and Cit-H3

For histological analysis, each blood sample collected at the time of admission to the ICU was immediately smeared in a thin layer on a glass slide. After drying, the specimens were stored at −80°C until immunostaining was performed. We confirmed that this sample preparation method did not induce additional generation of NETs or citrullination of histone H3 using neutrophils isolated from healthy donors on the smear (Fig. S1). To identify NETs, DNA and histone H3, the main components in NETs, were visualized simultaneously by immunofluorescence, and Cit-H3 was also detected using a specific antibody as follows. The sample on the glass slide was fixed with 4% paraformaldehyde for 30 min, washed with phosphate-buffered saline (PBS) (pH 7.4), and then blocked with a solution containing 20% Block-Ace (Dainippon-Sumitomo Seiyaku, Osaka, Japan) and 0.005% saponin in PBS for 10 min. The samples were then incubated for 60 min with the primary antibody as follows: anti-human histone H3 mouse monoclonal antibody (diluted 1:100) (MABI0001; MAB Institute, Inc., Hokkaido, Japan) and anti-human Cit-H3 rabbit polyclonal antibody (1:100) (ab5103; Abcam, Cambridge, UK). After washing in PBS, each primary antibody was visualized using secondary antibodies coupled to 1:500 Alexa Fluor 546 goat anti-mouse IgG (Invitrogen) and 1:500 Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen). The primary and secondary antibodies were diluted with 5% Block-Ace and 0.005% saponin in PBS. After incubation for 60 min with the secondary antibodies, the specimens were washed with PBS, and the DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) in PBS for 5 min. All procedures were performed at room temperature. The specimens were analyzed using a confocal laser-scanning microscope (BZ-9000; Keyence Corporation; Osaka, Japan).

The validity of immunostaining was ensured by the negative results of control experiments in which whole mouse or rabbit IgG (Abcam) was used instead of primary antibodies or primary antibodies were omitted in the procedure (Fig. S2). In addition, neutrophils stimulated with phorbol myristate acetate from healthy donors were used as a positive control for immunostaining (Fig. S3).

In the preliminary experiments, string-like structure extending from the cell body, which was positive for DNA and histone, was exclusively also positive for neutrophil elastase (Fig. S4). Hence, we considered the extracellular component that is double-positive for DNA and H3 to be a NET. The production of NETs and the specific expression of the citrullination of histone H3 in neutrophils were confirmed using anti-CD66b antibody (Fig. 1). Diff-Quik staining revealed the presence of a variety of blood cells in the smears (Fig. S5).

For the purpose of estimating the presence of NETs and the occurrence of citrullination of histone H3 concurrently, triple staining for DNA, H3, and citrullinated H3 was performed in this study. Samples were considered negative for the presence of NETs or Cit-H3 if cells harboring NETs or Cit-H3 were not identified in 300 neutrophils by immunostaining. If at least one of NETs and Cit-H3 was positive in the smear according to the definition mentioned above, the corresponding patient was classified into the "NET- and/or Cit-H3-positive" group.

Detection of the presence of bacteria in tracheal aspirate

Aspiration is defined as the inhalation of oropharyngeal or gastric contents into the larynx and lower respiratory tract, and

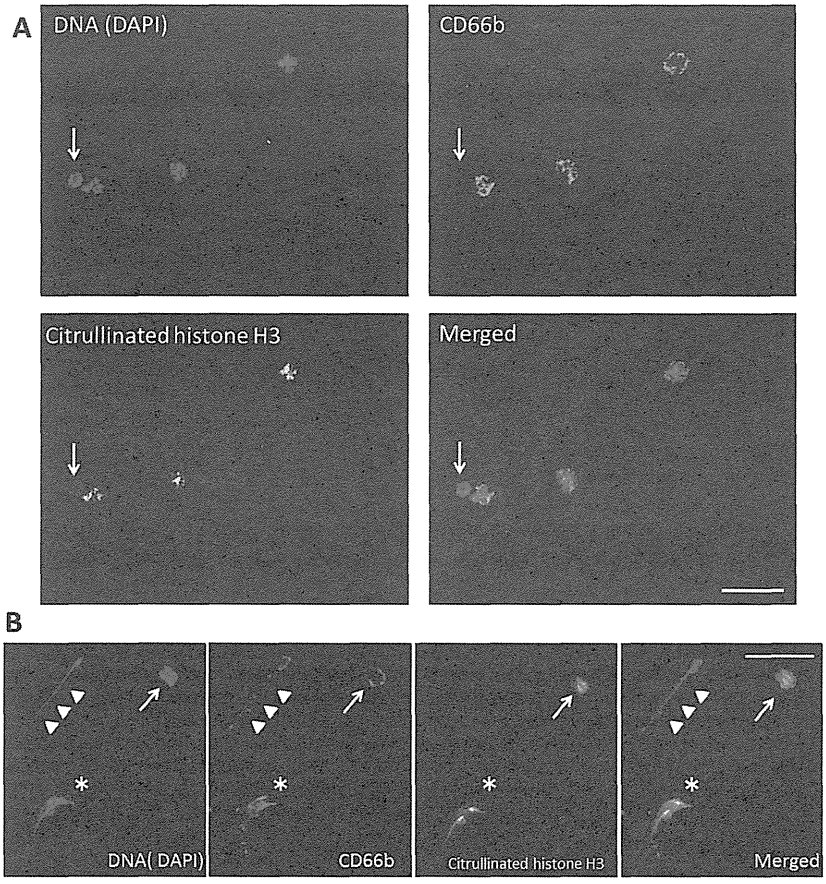

Figure 1. Representative images of immunostaining using anti-CD66b antibody in the blood smear sample from a critically ill patient. Triple staining by DAPI, anti-CD66b antibody, and anti-citrullinated histone H3 was performed using the blood smear sample obtained from a critically ill patient. A. The CD66b-positive cells were subjected to citrullination of histone H3 in their nuclei. Citrullination of histone H3 was not detected in the CD66b-negative cell (arrow). B. Arrow indicates the occurrence of citrullination of histone H3 in a neutrophil that had immunoreactivity against CD66b. Arrowheads indicate NETs stained with CD66b, whose appearance was of a string-like structure extending from the cell body. Asterisk indicates a neutrophil that was beginning to release NETs from its ruptured cell body. Interestingly, freshly produced NETs (asterisk) held immunoreactivity against citrullination of histone H3. In contrast, elongated NETs (arrowheads) were not stained with anti- citrullinated histone H3 antibody. Blue, DAPI; Red, CD66b; Green, citrullinated histone H3. (Magnification ×400). Scale bar; 50 μm.
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aspiration pneumonia is an infectious process caused by the inhalation of oropharyngeal secretions that are colonized by pathogenic bacteria [27]. The presence of bacteria in tracheal aspirate by Gram staining is regarded as part of aspiration that favors the development of infection. In this study, we evaluated the presence of bacteria in tracheal aspirate as the preclinical stage of manifested infection. To screen for the presence of bacteria in tracheal aspirate, an aspirated sputum smear was also prepared independently from immunostaining at the time of each patient's admission to the ICU. For Gram staining, the smear was dried, stained with crystal violet (Merck KGaA, Darmstadt, Germany) followed by iodine (Merck KGaA), washed with 99.5% ethanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and stained with Safranin (Merck KGaA). Images were captured on an optical microscope system (ECLIPSE 50i; Nikon Instruments Inc., Tokyo, Japan).

Statistical Analysis

Continuous variables are presented as the median and interquartile range (IQR). The Wilcoxon rank-sum test and Pearson's chi-square test were used to compare two patient groups. Single and multiple logistic regression analyses were used to identify associations between the presence of NETs and/or Cit-H3 and the clinical and biological parameters studied. A p-value of $< .05$ was considered significant. All statistical analyses were performed using JMP 9.0.2 (SAS Institute Inc., Cary, NC, USA) and reviewed by a statistician.

Results

Patient Characteristics

During the study period, 263 patients were admitted to the ICU; 49 of these 263 patients were intubated patients and were included in this study. We excluded patients with cardiopulmonary arrest (CPA) who could not be resuscitated on admission. The patients' characteristics are shown in Table 1. The study group comprised 29 men and 20 women with a median age of 66.0 (IQR, 52.5–76.0) years. The median APACHE II score was 18.0 (IQR, 12.5–21.5), and the median SOFA score was 5.0 (IQR, 4.0–8.0). Thirty-eight patients (77.6%) were diagnosed as having SIRS, and 22 patients (44.9%) were judged as positive for "the presence of bacteria in tracheal aspirate". Thirty-six patients (73.5%)

survived and 13 patients died. The ICU mortality rate of intubated patients during this study period was 26.5%. The median WBC count was 10,900/μL (IQR, 8215–14,915/μL). The diagnoses included trauma (n = 7, 14.3%), infection (n = 14, 28.6%), resuscitation from CPA (n = 8, 16.3%), acute poisoning (n = 4, 8.1%), heart disease (n = 4, 8.1%), brain stroke (n = 8, 16.3%), heat stroke (n = 2, 4.1%), and others (n = 2, 4.1%) (Table 2).

Presence of NETs and Cit-H3 in the Bloodstream

NETs were identified as extracellular string-like structures that were simultaneously immunoreactive for DNA and histone H3 (Fig. 2). Cit-H3 was detected by a specific antibody, and its presence was confirmed to be located inside lobulated nuclei and histone H3 (Fig. 3). In the blood smears surveyed in this study, we identified NETs in 5 patients and Cit-H3 in 11 patients (Table 2). Both NETs and Cit-H3 were identified concurrently in one patient with infection. We detected the presence of circulating NETs and/or Cit-H3-positive cells in samples from patients with infection (4/14, 28.6%), resuscitation from CPA (5/8, 62.5%), acute poisoning (1/4, 25.0%), brain stroke (3/8, 37.5%), and heat stroke (1/2, 50.0%). We found no NETs or Cit-H3-positive cells in samples from patients with trauma (0/7) or heart disease (0/4).

Identification of Factors Related to the Presence of NETs and Cit-H3 in the Bloodstream

We tried to identify the factors that are related to the presence of NETs or Cit-H3 in the bloodstream. We first examined clinical parameters recorded at the time of admission including age, APACHE II and SOFA scores, number of patients who presented with SIRS or with the presence of bacteria in tracheal aspirate, and biological parameters such as the total WBC count and concentrations of lactate, IL-8, TNF-α, HMGB1, and cf-DNA. We also recorded the number of survivors. We compared these variables between the patients positive or negative for NETs and/or Cit-H3. The results are shown in Table 3. Among the factors evaluated in this research, only "the presence of bacteria in tracheal aspirate" differed significantly between the NET- and/or Cit-H3-positive and -negative groups ($p<.01$, Wilcoxon rank-sum test and Pearson's chi-square test). The other factors were not significantly related to the presence of NETs and/or Cit-H3. In patients classified into two groups based on the presence or absence of bacteria in tracheal aspirate, the occurrence rate of NETs and/or Cit-H3 was significantly higher in "the presence of bacteria in tracheal aspirate" (BTA (+)) group (11/22, 50.0%) than in "the absence of bacteria in tracheal aspirate" (BTA (−)) group (4/27, 14.8%) ($p<.01$) (Table S1). In patients with SIRS on admission, there was a trend toward greater expression of NETs and/or Cit-H3 ($p = .079$) (Table S2).

Logistic regression analysis was performed to identify the factors related to the presence of NETs and Cit-H3 in the bloodstream. The results of single logistic regression analysis of factors associated with the presence of NETs and Cit-H3 are shown in Table 4. Only BTA (+) at the time of intubation was a significant factor associated with the presence of NETs and Cit-H3 ($p = .0112$). Although there were indications of a trend toward an association between the presence of circulating NETs and/or Cit-H3 and the comorbid conditions of SIRS or elevated cf-DNA concentration ($p = .1093$ and.3003, respectively), these were not statistically significant. Table 5 shows the results of multiple logistic regression analysis of factors associated with the presence of NETs and/or Cit-H3 and model selection. Two methods of multiple regression analysis, backward and forward regression, yielded similar models. Again, "the presence of bacteria in tracheal aspirate" was the only factor that was significantly related to the presence of NETs and/or Cit-H3 in the bloodstream; the odds ratio for aspiration was 5.750.

Discussion

A series of in vitro and animal experiments have uncovered a suppressive function of NETs against the dissemination of microorganisms in blood by mechanical trapping and by exploiting coagulant function to segregate these microorganisms within the circulation [28,29]. However, direct evidence remains scarce in living human systems. In this clinical study of blood smears, we attempted to identify morphologically the presence of NETs and Cit-H3 in the bloodstream of critically ill patients at the time of admission to the ICU and to characterize the factors associated with the presence of NETs and Cit-H3.

Among the 49 enrolled patients, immunofluorescence analysis revealed blood-borne NETs in five patients (10.2%), Cit-H3 in 11 patients (22.4%), and NETs and/or Cit-H3 in 15 patients (30.6%) (Table 2). These data replicate the results of our previous preliminary study in which NETs were present in patients in a critical condition [25] and show for the first time, to our knowledge, the presence of Cit-H3 in circulating blood cells. Cit-H3-positive cells possessed a multi-segmented nucleus, and most were immunoreactive for CD66b (Fig. 1), suggesting that citrullination of histone H3 occurred exclusively in neutrophils.

Table 1. Patient characteristics.

Variable	Value
No. of patients (M/F)	49 (29/20)
Age (years, median, IQR)	66.0 (52.5–76.0)
APACHE II score (median, IQR)	18.0 (12.5–21.5)
SOFA score (median, IQR)	5 (4–8)
No. of patients with SIRS	38 (77.6%)
The presence of bacteria in tracheal aspirate	22 (44.9%)
No. of survivors	36 (73.5%)
WBC (median, IQR)	10,900 (8215–14,915)

During the study period, 263 patients were admitted to the ICU of whom 49 were intubated and were included in this study. We excluded patients with cardiopulmonary arrest who could not be resuscitated on admission. IQR: interquartile range, APACHE: Acute Physiological And Chronic Health Evaluation, SOFA: Sequential Organ Failure Assessment, SIRS: systemic inflammatory response syndrome, WBC: white blood cell.
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Table 2. Diagnoses and the number of patients exhibiting neutrophil extracellular traps and citrullinated histone H3 in each diagnostic group.

Diagnosis	NET positive (n)	Cit-H3 positive (n)	NET and/or Cit-H3 positive (%)
Trauma (n = 7)	0	0	0/7 (0)
Infection (n = 14)	3	2	4/14 (28.6)
Resuscitated from cardiopulmonary arrest (n = 8)	2	3	5/8 (62.5)
Acute poisoning (n = 4)	0	1	1/4 (25.0)
Heart disease (n = 4)	0	0	0/4 (0)
Brain stroke (n = 8)	0	3	3/8 (37.5)
Heat stroke (n = 2)	0	1	1/2 (50.0)
Others (n = 2)	0	1	1/2 (50.0)
Total (n = 49)	5	11	15/49 (30.6)

In the blood smears surveyed in this study, we identified NETs in 5 patients and Cit-H3 in 11 patients. Both NETs and Cit-H3 were identified concurrently in one patient with infection. We found no NETs or Cit-H3-positive cells in samples from patients with trauma (0/7) or heart disease (0/4). NETs: neutrophil extracellular trap, Cit-H3: citrullinated histone H3.

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Citrullination of histone H3 is considered an important process in the release of NETs through decondensation of chromatin [14,22,23]. Interestingly, the occurrence ratio of Cit-H3 was twice that of NETs. In vitro experiments imply that a substantial period of time is necessary to expel NETs extracellularly after the initiation of cell death by a stress stimulus [12,30,31]. However, it is still not clear how much time is required in vivo for NETs to appear intravascularly. The number of patients who exhibited circulating NETs in this study was lower than anticipated. We collected blood samples on admission to the ICU, and the timing might have been too early to detect NETs after the onset of a critical illness. The 11 Cit-H3-positive patients could be considered to have been in an early stage of NET formation. The change in the appearance of NETs and Cit-H3 during the course of hospitalization should be studied. If it can be shown clinically that Cit-H3 expression is followed by NET formation, it might be important to evaluate Cit-H3 expression in the blood upon admission to an ICU.

Table 2 shows that NETs and Cit-H3 were detected in patients with infection, resuscitation from CPA, acute poisoning, brain stroke, or heat stroke; surprisingly, we could not detect NETs or Cit-H3 in patients with trauma or heart disease. NETs are formed

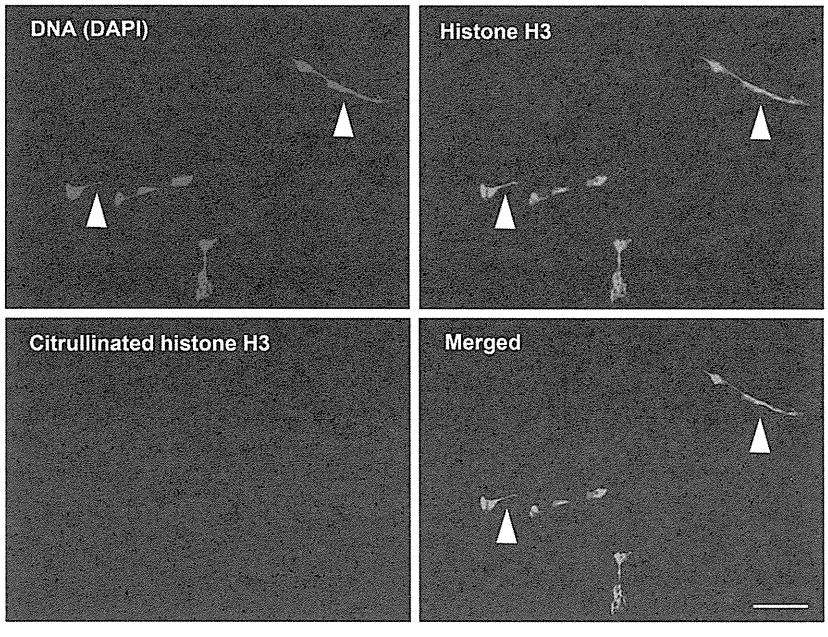

Figure 2. Representative images of immunofluorescence staining to detect neutrophil extracellular traps (NETs). NETs were visualized in the blood smear samples by immunocytochemistry and identified as extracellular string-like structures composed of chromatin (DNA and histone H3). NETs were present in the bloodstream of critically ill patients. Citrullination of histone H3 was not recognized in these images. In the blood smears surveyed in this study, we identified NETs in five patients (5/49, 10.2%). Blue, 4′,6-diamidino-2-phenylindole (DAPI); red, histone H3; green, citrullinated histone H3. Arrowheads indicate the double-stained areas containing NETs (Magnification ×400). Scale bar; 50 μm.

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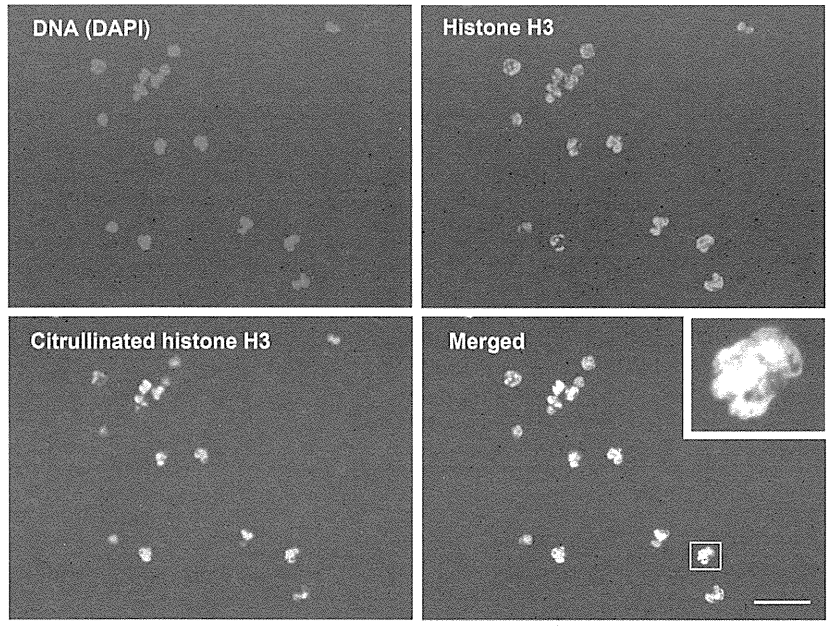

Figure 3. Representative images of immunofluorescence staining to detect citrullinated histone H3 (Cit-H3). Citrullination of histone H3, which is a critical enzymatic process to produce NETs through decondensation of chromatin, was visualized in the blood smear samples using anti-citrullinated histone H3 antibody by immunohistochemistry. Cit-H3 was present in the bloodstream of critically ill patients. The inset in the merged image is the magnified image of a representative cell (white rectangle) expressing citrullinated histone H3 in the nucleus. Neutrophil extracellular traps are not recognized here. In the blood smears surveyed in this study, we identified Cit-H3 in 11 patients (11/49, 22.4%). Blue, 4′,6-diamidino-2-phenylindole (DAPI); red, histone H3; green, citrullinated histone H3 (Magnification ×400). Scale bar; 50 μm.
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in response to various microorganisms and pathogens [14]. McDonald et al reported that NETs ensnare circulating bacteria and provide intravascular immunity that protects against bacterial dissemination during septic infection [29]. In this context, the presence of NETs and/or Cit-H3 in infected patients is to be expected. By contrast, trauma or heart disease patients were

Table 3. Comparison between patients positive and negative for neutrophil extracellular traps and/or citrullinated histone H3.

	NET and/or citrullinated histone H3		
	Positive	**Negative**	***p***
Number	15	34	
Age (years)	67.0 (49.0–78.0)	65.5 (56.8–75.3)	.8197
APACHE II score	20.0 (16.0–23.0)	17.5 (11.8–21.3)	.3171
SOFA score	6.0 (5.0–10.0)	5.0 (4.0–8.0)	.4062
Survivors (n)	10 (66.7%)	26 (76.5%)	.4737
SIRS patients (n)	14 (93.3%)	24 (70.6%)	.0786
The presence of bacteria in tracheal aspirate (n)	11 (73.3%)	11 (32.3%)	.0079
WBC count (/μl)	12,430 (8310.0–16510.0)	10,835 (8032.5–14307.5)	.5654
IL-8 (pg/mL)	57.6 (19.9–143.0)	65.3 (23.3–229.5)	.9136
TNF-α (pg/mL)	8.2 (6.2–21.6)	9.0 (4.8–16.3)	.9740
cf-DNA (ng/mL)	1038.3 (744.9–1329.7)	1072.7 (828.6–1770.7)	.6025
Lactate (mg/mL)	39 (11.0–71.0)	17.5 (12.0–56.3)	.5010
HMGB1 (ng/mL)	11.0 (6.8–21.5)	9.7 (5.9–16.3)	.5151

Among the factors evaluated to highlight the relation to the presence of NETs or Cit-H3 in the bloodstream, only "the presence of bacteria in tracheal aspirate" differed significantly between the NET- and/or Cit-H3-positive and -negative groups ($p<.01$). The other factors were not significantly related to the presence of NETs and/or Cit-H3. Continuous variables are presented as the median and IQR unless otherwise noted. The Wilcoxon rank-sum test and Pearson's chi-square test were used to compare two patient groups. NETs: neutrophil extracellular traps, Cit-H3: citrullinated histone H3, IQR: interquartile range, APACHE: Acute Physiological And Chronic Health Evaluation, SOFA: Sequential Organ Failure Assessment, SIRS: systemic inflammatory response syndrome, WBC: white blood cell, IL: interleukin, TNF: tumor necrosis factor, cf-DNA: circulating free DNA, HMGB1: high mobility group box-1.
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Table 4. Results of single logistic regression analysis.

Variable	*p*
The presence of bacteria in tracheal aspirate	.0112
SIRS	.1093
cf-DNA	.3003
Lactate	.5476
WBC count	.7862
IL-8	.7875
TNF-α	.8321
HMGB1	.9439

Logistic regression analysis was performed to identify the factors related to the presence of NET and Cit-H3 in the bloodstream. Only "the presence of bacteria in tracheal aspirate" (+) at the time of intubation was a significant factor associated with the presence of NET and Cit-H3 ($p = .0112$). NETs: neutrophil extracellular traps, Cit-H3: citrullinated histone H3, SIRS: systemic inflammatory response syndrome, cf-DNA: circulating free DNA, WBC: white blood cell, IL: interleukin, TNF: tumor necrosis factor, HMGB1: high mobility group box-1.
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transported to the hospital immediately after the onset of the condition, and there was no potential risk of infection on admission; this may explain why NETs and Cit-H3 were not detected in these patients.

Intriguingly, a high percentage (62.5%) of patients with CPA exhibited circulating NETs and/or Cit-H3. Acute poisoning, brain stroke, and heat stroke are clinical conditions that can cause disturbance of consciousness, which may induce aspiration. Adnet and Baud demonstrated that the risk of aspiration increases with the degree of unconsciousness (as measured by the Glasgow Coma Scale [GCS]) [32]. In the present study population, the GCS score on admission was significantly lower in the BTA (+) group than in the BTA (−) group (4 [IQR, 3–10.75] vs 13 [IQR, 7–14]; $p<.01$). Except for the infected patient group, the patients who exhibited NETs and/or Cit-H3 in their blood had a significantly lower GCS score on admission ($p = .0418$). We therefore investigated whether "the presence of bacteria in tracheal aspirate", which was represented as part of aspiration and as the presumable preclinical stage of manifested infection, was associated with the presence of NETs and/or Cit-H3, and found a significant association (odds ratio for aspiration, 5.750) (Tables 3–5). Bacteria drawn into the respiratory tract can induce epithelial injury, which provides an opportunity for bacterial translocation as well as leukocyte transmigration until completion of epithelial repair [33,34]. Concomitance of acid aspiration under impaired consciousness additionally enhances bacterial adherence to the epithelium [35]. Injured airway epithelium produces cytokines including IL-8 and alarmins such as HMGB1, both of which are representative inducers for NETs [36–39]. Next, bacteria and inflammatory mediators infiltrating into the interstitial space secondary to epithelial injury will affect the endothelial integrity [40]. The presence of NETs in sputum following aspiration, a phenomenon that we reported previously [24], suggests breakdown of the epithelial barrier that is induced by local inflammation through direct contact between aspirated bacteria and epithelium or through activation of resident immune cells such as macrophages in the respiratory tract [41]. Such epithelial breakdown would allow influx of pathogens, pathogen-associated molecular patterns, cytokines, chemokines, and alarmins from the lumen of the respiratory tract into the circulation. These materials might stimulate the production of NETs intravenously to inhibit systemic invasion of bacteria. We assumed that NETs are induced in the respiratory tract to suppress bacterial dissemination leading to pneumonia and in the vessels to inhibit bacteremia against the invasion of bacteria into the blood and that even such colonization of bacteria in the respiratory tract could trigger citrullination of histone H3 to produce NETs in blood. Single logistic regression analyses of whether infection and/or BTA (+) were associated with the presence of NETs and/or Cit-H3 produced an odds ratio of 7.312 (Table S3). These results suggest that induction of NETs systemically through the citrullination of histone H3 in blood maybe an initial response for protection against bacterial dissemination from latent respiratory infection.

Some researchers consider cf-DNA to be equivalent to NETs in the blood [15,16]. However, our results showed that the occurrence rate of NETs and/or Cit-H3 was not significantly associated with cf-DNA concentration ($p = .6025$) (Table 3). Although the number of patients was different due to sample limitations, additional analysis by MPO-DNA ELISA (Data S1) was also performed. As a result, there was no difference in the values between the group positive for (0.076 [IQR, 0.067–0.100]; n = 8) and the group negative for NET and/or citrullinated histone H3 (0.078 [IQR, 0.070–0.111]; n = 26). We reported recently that in patients with an acute respiratory infection, NETs became fragmented during recovery from infection [24], suggesting that NETs should also be digested in the blood with time. Our method using blood smear samples cannot detect NETs that harbor inside vessels or that are already degraded, whereas the method based on MPO-DNA ELISA might also measure neutrophil DNA fragments derived from necrosis or apoptosis and cannot detect NETs that are not truncated from the cell body. We consider that at the early phase of critical illness, i.e., when the production of NETs is just starting, the morphological approach has an advantage in being able to detect NETs that are still anchored to the cell body, in conjunction with the merit that identification of citrullination of histone H3 is possible at a stage prior to the release of NETs.

HMGB1 is a nuclear protein present in the nucleus of all nucleated cells. HMGB1 binds to DNA and acts as an inflammatory mediator once it is released extracellularly [42,43]. In this study, HMGB1 was significantly higher in SIRS patients

Table 5. Results of multiple logistic regression analysis of factors associated with the presence of neutrophil extracellular traps and/or citrullinated histone H3.

	Coeff (β)	*p*	OR	Lower	Upper
"the presence of bacteria in tracheal aspirate"	0.875	0.011	5.750	1.583	24.755

Two methods of multiple regression analysis, backward and forward regression, yielded similar models. "The presence of bacteria in tracheal aspirate" was the only factor that was significantly related to the presence of neutrophil extracellular traps and/or citrullinated histone H3 in the bloodstream. The odds ratio for aspiration was 5.750. Coeff (β): coefficient; OR: odds ratio, Lower: lower level of 95% confidence interval, Upper: upper level of 95% confidence interval.
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than in non-SIRS patients (Table S2). Unexpectedly, however, HMGB1 was not a significant factor associated with the presence of NETs and/or Cit-H3 (Tables 3–5). NETs contain HMGB1 [44], and one possibility is that HMGB1 binding to NETs is not reflected in the amount of circulating HMGB1 measured by ELISA.

Although IL-8 and TNF-α are considered stimulatory factors that induce NET formation [14,39,45], they were not associated with the presence of NETs and/or Cit-H3 in this study (Tables 3–5). This negative result suggest the presence of an unknown complex regulatory mechanism for the production of NETs in vivo.

As limitations of this study, first, the sample size was small, and the patients were very heterogeneous. Second, we evaluated the presence of NETs and Cit-H3 and the associated factors in the bloodstream of critically ill patients only at admission. It should be investigated in the future how NETs are processed after the induction of NETosis in the circulation. It is presumable that NETs could be degraded by DNase, and the fragments would contribute partially to the formation of cf-DNA. Third, we did not rigorously quantify the amount of NETs and Cit-H3. The possibility of the degradation of NETs and the difficulty in detecting NETs, which are anchored in the vessels, might lead to underestimation of the presence of NETs in our method using blood smear samples. Further study is required to establish finer methods of quantification. We hope that future elucidation of the biological significance of NETs will lead to new strategies to treat critical illness by monitoring NET formation in blood.

Conclusions

The presence of NETs and Cit-H3 were identified immunocytochemically in the bloodstream of a subset of critically ill patients. "The presence of bacteria in tracheal aspirate" may be one important factor related to the presence of circulating NETs. NETs may play a pivotal role in biological defense in the bloodstream of infected and potentially infected patients.

Supporting Information

Figure S1 Representative images of immunostaining of isolated neutrophils that underwent drying and freezing steps before fixation. We tried to evaluate the influence of drying and freezing steps preceding paraformaldehyde fixation on the induction of NETs or citrullination of histone H3 in smear samples. For this, neutrophils separated by density gradient centrifugation from whole blood of a healthy donor were smeared on glass slides, dried, and frozen before fixation. At least through this method, the presence of NETs or citrullinated histone H3 was not identified in immunostaining. Blue, Hoechst 33342; Red, histone H3; Green, citrullinated histone H3 (left panels) or neutrophil elastase (right panels) (Magnification ×400). Scale bar; 50 μm.
(TIF)

Figure S2 Representative images of immunostaining for the negative control study using isotype control antibodies. To ensure accuracy for the immunoreactivity of primary antibodies against blood smear samples, whole mouse and rabbit IgG were used instead of primary antibodies in the immunostaining procedure. This control study resulted in negative signals for histone H3 and citrullinated histone H3. Blue, 4′,6-diamidino-2-phenylindole (DAPI); Red, histone H3; Green, citrullinated histone H3. (Magnification ×200). Scale bar; 50 μm.
(TIF)

Figure S3 Representative images of immunostaining to detect citrullinated histone H3 (left panels) and neutrophil extracellular traps (NETs) (right panels) in the neutrophils from a healthy donor stimulated by phorbol myristate acetate. Neutrophils were isolated by density gradient centrifugation from the whole blood of a healthy donor and stimulated by phorbol myristate acetate. Citrullinated histone H3 and NETs were detected by immunohistochemistry using the same antibodies that were used against the smear samples collected from the critically ill patients. Blue, Hoechst 33342; Red, histone H3; Green, citrullinated histone H3 (left panels) or neutrophil elastase (right panels). (Magnification ×400). Scale bar; 50 μm.
(TIF)

Figure S4 Representative images of immunostaining to detect neutrophil extracellular traps (NETs) in the blood smear from a critically ill patient. The presence of circulating NETs was confirmed by immunohistochemistry using anti-neutrophil elastase antibody. String-like structures extending from the cell body (arrowheads) were composed of DNA and histone, and they contained neutrophil elastase. Blue, 4′,6-diamidino-2-phenylindole (DAPI); Red, histone H1; Green, Neutrophil elastase. (Magnification ×400). Scale bar; 50 μm.
(TIF)

Figure S5 Diff-Quik staining of a blood smear sample from the critically ill patient. Diff-Quik staining confirmed a subpopulation of cells other than neutrophils. (Magnification ×400). Scale bar; 50 μm.
(TIF)

Table S1 Comparison between patients presenting with and without "the presence of bacteria in tracheal aspirate". In patients classified into two groups based on the presence or absence of bacteria in tracheal aspirate, the rate of occurrence of NETs and/or Cit-H3 was significantly higher in "the presence of bacteria in tracheal aspirate" group (11/22, 50.0%) than in "the absence of bacteria in tracheal aspirate" group (4/27, 14.8%) ($p<.01$). Continuous variables are presented as the median and IQR unless otherwise noted. The Wilcoxon rank-sum test and Pearson's chi-square test were used to compare the two patient groups. NETs: neutrophil extracellular traps, Cit-H3: citrullinated histone H3, IQR: interquartile range, APACHE: Acute Physiological And Chronic Health Evaluation, SOFA: Sequential Organ Failure Assessment, SIRS: systemic inflammatory response syndrome, WBC: white blood cell, IL: interleukin, TNF: tumor necrosis factor, cf-DNA: circulating free DNA, HMGB1: high mobility group box-1.
(DOCX)

Table S2 Comparison between patients with and without systemic inflammatory response syndrome. In patients with SIRS on admission, there was a trend toward greater expression of NETs and/or Cit-H3 ($p = .079$). Continuous variables are presented as the median and IQR unless otherwise noted. The Wilcoxon rank-sum test and Pearson's chi-square test were used to compare the two patient groups. NETs: neutrophil extracellular traps, Cit-H3: citrullinated histone H3, IQR: interquartile range, APACHE: Acute Physiological And Chronic Health Evaluation, SOFA: Sequential Organ Failure Assessment, SIRS: systemic inflammatory response syndrome,

WBC: white blood cell, IL: interleukin, TNF: tumor necrosis factor, cf-DNA: circulating free DNA, HMGB1: high mobility group box-1.
(DOCX)

Table S3 Results of single logistic regression analysis of factors associated with the presence of neutrophil extracellular traps and/or citrullinated histone H3 according to the presence of infection and/or "the presence of bacteria in tracheal aspirate". Single logistic regression analyses of whether infection and/or "the presence of bacteria in tracheal aspirate" were associated with the presence of NETs and/or Cit-H3 produced an odds ratio of 7.312. Coeff (β): coefficient, OR: odds ratio, Lower: lower level of 95% confidence interval, Upper: upper level of 95% confidence interval.
(DOCX)

Data S1 MPO-DNA ELISA.
(DOCX)

Author Contributions

Conceived and designed the experiments: TH SH NM TI. Performed the experiments: TH SH NM TI HH NY. Analyzed the data: TH SH NM TI MS OT NY KY YA KO TS KT. Contributed reagents/materials/analysis tools: TH SH HH NY. Wrote the paper: TH SH NM.

References

1. Lekstrom-Himes JA, Gallin JI (2000) Immunodeficiency diseases caused by defects in phagocytes. New Engl J Med 343: 1703–1714.
2. Savchenko AS, Inoue A, Ohashi R, Jiang S, Hasegawa G, et al. (2011) Long pentraxin 3 (PTX3) expression and release by neutrophils in vitro and in ulcerative colitis. Pathol Int 61: 290–297.
3. Vitkov L, Klappacher M, Hannig M, Krautgartner WD (2009) Extracellular neutrophil traps in periodontitis. J Periodontal Res 44: 664–672.
4. Garcia-Romo GS, Caielli S, Vega B, Connolly J, Allantaz F, et al. (2011) Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. Sci Transl Med 3: 73ra20.
5. Kessenbrock K, Krumbholz M, Schonermarck U, Back W, Gross WL, et al. (2009) Netting neutrophils in autoimmune small-vessel vasculitis. Nat Med 15: 623–625.
6. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, et al. (2004) Neutrophil extracellular traps kill bacteria. Science 303: 1532–1535.
7. Jaillon S, Peri G, Delneste Y, Fremaux I, Doni A, et al. (2007) The humoral pattern recognition receptor PTX3 is stored in neutrophil granules and localizes in extracellular traps. J Exp Med 204: 793–804.
8. Curran CS, Demick KP, Mansfield JM (2006) Lactoferrin activates macrophages via TLR4-dependent and -independent signaling pathways. Cell Immunol 242: 23–30.
9. Zhang LT, Yao YM, Lu JQ, Yan XJ, Yu Y, et al. (2008) Recombinant bactericidal/permeability-increasing protein inhibits endotoxin-induced high-mobility group box 1 protein gene expression in sepsis. Shock 29: 278–284.
10. Urban CF, Ermert D, Schmid M, Abu-Abed U, Goosmann C, et al. (2009) Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against Candida albicans. PLoS Pathog 5: e1000639.
11. Cho JH, Fraser IP, Fukase K, Kusumoto S, Fujimoto Y, et al. (2005) Human peptidoglycan recognition protein S is an effector of neutrophil-mediated innate immunity. Blood 106: 2551–2558.
12. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, et al. (2007) Novel cell death program leads to neutrophil extracellular traps. J Cell Biol 176: 231–241.
13. Logters T, Margraf S, Altrichter J, Cinatl J, Mitzner S, et al. (2009) The clinical value of neutrophil extracellular traps. Med Microbiol Immunol 198: 211–219.
14. Remijsen Q, Kuijpers TW, Wirawan E, Lippens S, Vandenabeele P, et al. (2011) Dying for a cause: NETosis, mechanisms behind an antimicrobial cell death modality. Cell Death Differ 18: 581–588.
15. Margraf S, Logters T, Reipen J, Altrichter J, Scholz M, et al. (2008) Neutrophil-derived circulating free DNA (cf-DNA/NETs): A potential prognostic marker for posttraumatic development of inflammatory second hit and sepsis. Shock 30: 352–358.
16. Logters T, Paunel-Gorgulu A, Zilkens C, Altrichter J, Scholz M, et al. (2009) Diagnostic accuracy of neutrophil-derived circulating free DNA (cf-DNA/NETs) for septic arthritis. J Orthop Res 27: 1401–1407.
17. Thijssen MA, Swinkels DW, Ruers TJ, de Kok JB (2002) Difference between free circulating plasma and serum DNA in patients with colorectal liver metastases. Anticancer Res 22: 421–425.
18. Sozzi G, Conte D, Leon M, Ciricione R, Roz L, Ratcliffe C, et al. (2003) Quantification of free circulating DNA as a diagnostic marker in lung cancer. J Clin Oncol 21: 3902–3908.
19. Kamat AA, Bischoff FZ, Dang D, Baldwin MF, Han LY, et al. (2006) Circulating cell-free DNA: A novel biomarker for response to therapy in ovarian carcinoma. Cancer Biol Ther 5: 1369–1374.
20. Swarup V, Rajeswari MR (2007) Circulating (cell-free) nucleic acids—a promising, non-invasive tool for early detection of several human diseases. FEBS Lett 581: 795–799.
21. van der Vaart M, Pretorius PJ (2007) The origin of circulating free DNA. Clin Chem 53: 2215.
22. Neeli I, Khan SN, Radic M (2008) Histone deimination as a response to inflammatory stimuli in neutrophils. J Immunol 180: 1895–1902.
23. Wang Y, Li M, Stadler S, Correll S, Li P, et al. (2009) Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation. J Cell Biol 184: 205–213.
24. Hirose T, Hamaguchi S, Matsumoto N, Irisawa T, Seki M, et al. (2012) Dynamic changes in the expression of neutrophil extracellular traps in acute respiratory infections. Am J Respir Crit Care Med 185: 1130–1131.
25. Hamaguchi S, Hirose T, Akeda Y, Matsumoto N, Irisawa T, et al. (2013) Identification of neutrophil extracellular traps in blood of patients with systemic inflammatory response syndrome. J Int Med Res 41: 162–168.
26. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, et al. (1992) Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. Chest 101: 1644–1655.
27. Marik PE (2001) Aspiration pneumonitis and aspiration pneumonia. N Engl J Med 344: 665–671.
28. Massberg S, Grahl L, von Bruehl ML, Manukyan D, Pfeiler S, et al. (2010) Reciprocal coupling of coagulation and innate immunity via neutrophil serine proteases. Nat Med 16: 887–896.
29. McDonald B, Urrutia R, Yipp BG, Jenne CN, Kubes P (2012) Intravascular neutrophil extracellular traps capture bacteria from the bloodstream during sepsis. Cell Host Microbe 12: 324–333.
30. Yipp BG, Petri B, Salina D, Jenne CN, Scott BN, et al. (2012) Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. Nat Med 18: 1386–1393.
31. Brinkmann V, Zychlinsky A (2007) Beneficial suicide: Why neutrophils die to make NETs. Nat Rev Microbiol 5: 577–582.
32. Adnet F, Baud F (1996) Relation between Glasgow Coma Scale and aspiration pneumonia. Lancet 348: 123–124.
33. Evans SE, Xu Y, Tuvim MJ, Dickey BF (2010) Inducible innate resistance of lung epithelium to infection. Annu Rev Physiol 72: 413–435.
34. Sousa S, Lecuit M, Cossart P (2005) Microbial strategies to target, cross or disrupt epithelia. Curr Opin Cell Biol 17: 489–498.
35. Mitsushima H, Oishi K, Nagao T, Ichinose A, Senba M, et al. (2002) Acid aspiration induces bacterial pneumonia by enhanced bacterial adherence in mice. Microb Pathog 33: 203–210.
36. Hippenstiel S, Opitz B, Schmeck B, Suttorp N (2006) Lung epithelium as a sentinel and effector system in pneumonia–molecular mechanisms of pathogen recognition and signal transduction. Respir Res 7: 97.
37. Pittet JF, Koh H, Fang X, Iles K, Christiaans S, et al. (2013) HMGB1 accelerates alveolar epithelial repair via an IL-1beta- and alphavbeta6 integrin-dependent activation of TGF-beta1. PLoS One 8: e63907.
38. Tadie JM, Bae HB, Jiang S, Park DW, Bell CP, et al. (2013) HMGB1 promotes neutrophil extracellular trap formation through interactions with Toll-like receptor 4. Am J Physiol Lung Cell Mol Physiol 304: L342–L349.
39. Gupta AK, Hasler P, Holzgreve W, Gebhardt S, Hahn S (2005) Induction of neutrophil extracellular DNA lattices by placental microparticles and IL-8 and their presence in preeclampsia. Hum Immunol 66: 1146–1154.
40. Hiraiwa K, Van Eeden SF (2014) Nature and consequences of the systemic inflammatory response induced by lung inflammation. Lung Inflammation. Available: http://www.intechopen.com/books/lung-inflammation/nature-and-consequences-of-the-systemic-inflammatory-response-induced-by-lung-inflammation. Accessed 2014 Jul 4.
41. Hussell T, Bell TJ (2014) Alveolar macrophages: plasticity in a tissue-specific context. Nat Rev Immunol 14: 81–93.
42. Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, et al. (1999) HMG-1 as a late mediator of endotoxin lethality in mice. Science 285: 248–251.
43. Wang H, Yang H, Tracey KJ (2004) Extracellular role of HMGB1 in inflammation and sepsis. J Intern Med 255: 320–331.
44. Mitroulis I, Kambas K, Chrysanthopoulou A, Skendros P, Apostolidou E, et al. (2011) Neutrophil extracellular trap formation is associated with IL-1beta and autophagy-related signaling in gout. PLoS One 6: e29318.
45. Gupta AK, Joshi MB, Philippova M, Erne P, Hasler P, et al. (2010) Activated endothelial cells induce neutrophil extracellular traps and are susceptible to NETosis-mediated cell death. FEBS Lett 584: 3193–3197.

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Protective properties of a fusion pneumococcal surface protein A (PspA) vaccine against pneumococcal challenge by five different PspA clades in mice

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ABSTRACT

An increase in the appearance of nonvaccine serotypes in both children and adults with invasive pneumococcal disease (IPD) after introduction of pneumococcal conjugate vaccine represents a limitation of this vaccine. In this study, we generated three recombinant pneumococcal surface protein A (PspA) proteins comprising PspA families 1 and 2, and we examined the reactivity of antisera raised in mice immunized with a PspA fusion protein in combination with CpG oligonucleotides plus aluminum hydroxide gel. The protective effects of immunization with PspA fusion proteins against pneumococcal challenge by strains with five different PspA clades were also examined in mice. Flow cytometry demonstrated that PspA3+2-induced antiserum showed the greatest binding of PspA-specific IgG to all five challenge strains with different clades. PspA2+4- or PspA2+5-induced antiserum showed the lowest binding of PspA-specific IgG to clade 3. Immunization with PspA3+2 afforded significant protection against pneumococcal challenge by five strains with different clades in mice, but immunization with PspA2+4 or PspA2+5 failed to protect mice from pneumococcal challenge by strains with clades 1 and 3. The binding of PspA-specific IgG in antisera raised by three PspA fusion proteins was examined in 68 clinical isolates from adult patients with IPD. Immunization of mice with PspA3+2-induced antiserum with a high binding capacity for clinical isolates expressing clades 1–4, but not clade 5. Our results suggest that the PspA3+2 vaccine has an advantage over the PspA2+4 or PspA2+5 vaccine in terms of a broad range of cross-reactivity with clinical isolates and cross-protection against pneumococcal challenge in mice.

1. Introduction

Streptococcus pneumoniae is a major cause of morbidity and mortality caused by pneumonia, bacteremia, and meningitis worldwide [1]. After introduction of the seven-valent pneumococcal conjugate vaccine (PCV7) in children, significant declines in the incidence of invasive pneumococcal disease (IPD) caused by vaccine serotypes were reported in children and adults [2,3]. However, an increase in the incidence of IPD caused by non-PCV7 serotypes has been also observed in children and adults [3–5]. In addition, after introduction of a 13-valent pneumococcal conjugate vaccine (PCV13) in children, serotypes not included in PCV13 have been isolated with increasing frequency in pediatric and adult patients with IPD [6,7]. Because there are >90 different pneumococcal capsular serotypes, continuous supplementation of pneumococcal conjugate vaccines with new serotypes for serotype replacement may not be a practical strategy.

Previous studies have demonstrated that several pneumococcal proteins are potential vaccine candidates [8–11]. One candidate protein antigen is pneumococcal surface protein A (PspA), which is

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an exposed virulence factor found in virtually all pneumococcal strains [12,13]. Anti-PspA antibodies overcome the anticomplement effect of PspA, allowing for increased complement activation and C3 deposition on PspA-bearing bacteria [14,15]. Serum from humans immunized with PspA can passively protect mice against challenge with various pneumococcal strains [16]. Importantly, a recent study confirmed that the rabbit antibodies to PspA could mediate killing in the modified opsonophagocytosis killing assay [17].

PspA is composed of five domains: (i) a signal peptide, (ii) an α-helical highly charged (N-terminal) domain, (iii) a proline-rich region domain, (iv) a choline-binding domain, and (v) a short hydrophobic tail [18,19]. The α-helical domain of PspA has an antiparallel coiled-coil motif and is considered to be the most exposed part of the molecule [20]. The α-helical domain binds to protective monoclonal antibodies and inhibits killing of pneumococci by at least two host cationic peptides [21,22]. The proline-rich domain is composed of many repetitive sequences shared by other proline-rich domains making its inclusion important for achieving broad protection [23].

PspA proteins have been grouped into three families encompassing six different clades based on the C-terminal 100 amino acids of the α-helical region [24]. Family 1 comprises clades 1 and 2; family 2 comprises clades 3, 4 and 5; and family 3 comprises clade 6 [22,24]. Pneumococcal strains expressing family 1 or 2 PspA proteins constitute >96% of clinical isolates from patients with IPD [6,13,25]. Although different PspA proteins induce antibodies with different degrees of cross-reactivity in vitro and cross-protection of mice [26,27], our previous studies demonstrated that no single PspA construct can elicit complete protection against challenge by strains with all PspA clades and families [28]. To accommodate this variability, it was proposed that a combination of two PspA antigens, one from PspA family 1 and one from PspA family 2, should elicit protection against the vast majority of pneumococcal strains [29–31]. Thus, it is important to determine which PspA fragments show the broadest cross-reactivity. In this study, we prepared fusion proteins of three pairs of PspA molecules, and determined which provided the broadest cross-reaction with clinical isolates of *S. pneumoniae*.

2. Materials and methods

2.1. Pneumococcal strains

Six laboratory strains (all originally from patients), including BG9739 (serotype 4, PspA clade 1), D39 (serotype 2, PspA clade 2), WU2 (serotype 3, PspA clade 2), TIGR4 (serotype 3, PspA clade 3), EF5668 (serotype 4, PspA clade 4), and ATCC 6303 (serotype 3, PspA clade 5) were used to construct the fusion PspA proteins. These laboratory strains and a recent clinical isolate, KK1162 (serotype 3, PspA clade 4), were used for bacterial challenge. Sixty-eight clinical isolates, including KK1162 strain, from Japanese adult patients with IPD were also used [32]. These isolates were serotyped using agglutination assay, and their PspA clades were determined using a method published previously [32,33].

2.2. Construction of fusion PspA fragments

Our previous study demonstrated a significant protection against sepsis caused by WU2 strain (PspA clade 1) by immunization with full-length BG9739 derived PspA (clade 1) but only a weak protection against homologous challenge with BG9739 [28]. Therefore, we excluded PspA clade 1 derived from BG9739 strain from the fusion PspA proteins. In this study, we prepared the fusion proteins from three pairs of PspA clade 2 from family 1 and PspA clades 3, 4 and 5 from family 2. All cloning procedures were performed with *Escherichia coli* DH5α grown in Luria–Bertani medium (Sigma-Aldrich, St. Louis, MO) supplemented with kanamycin (30 μg/ml). DNA fragments encoding portions of the N-terminal regions (containing the α-helix domain and proline-rich region) of PspA clades 2 and 3 were amplified by PCR using strains D39 and TIGR4. The primers used in this procedure are available in Appendix 1. The resulting PCR products were digested with *Nde*I and *Eco*RI, and were ligated to the pET28a (+) vector (Novagen, Madison, WI), and the sequences were confirmed by DNA sequencing. The pET28a–PspA constructs digested with *Eco*RI and *Xho*I, and the resulting fragments, which encoded portions of the N-terminal regions of PspA clades 4, 5, or 2 were amplified by PCR using strains EF5668 (Accession no. U89711), ATCC6303 (Accession no. AF071820), or WU2 (Accession no. AF071814), respectively, and were ligated to the linearized vector. The fusion PspA proteins were obtained with primers that allowed the removal of the signal sequence. The fusion PspA2+4 was constructed by fusing the 3′ terminus of PspA clade 2 of D39 strain (Accession no. AF071814) with the 5′ terminus of PspA clade 4 of EF5668 strain, through the *Eco*RI ligated to pET28a–6 × His. The fusion PspA2+5 was constructed by fusing the 3′ terminus of PspA clade 2 of D39 strain with the 5′ terminus of PspA clade 5 of ATCC6303 strain, through the *Eco*RI ligated to pET28a–6 × His. The fusion PspA3+2 was constructed by fusing the 3′ terminus of PspA clade 3 of TIGR4 strain (Accession no. AE005672.3) with the 5′ terminus of PspA clade 2 of WU2 strain, through the *Eco*RI ligated to pET28a–6 × His.

2.3. PspA expression and purification

Competent *E. coli* BL21 (DE3) cells were transformed with pET28a (+) vectors containing the fusion PspA or the single PspA constructs. The recombinant proteins were purified and stored as described elsewhere [34].

2.4. Immunization of mice

Female C57/BL6j mice (6–8 weeks old) were purchased from CLA Japan. Mice were immunized subcutaneously three times at 7-days intervals with 0.1 μg of recombinant fusion PspA derivatives in lipopolysaccharide-free phosphate-buffered saline (PBS) (Sigma) in combination with 2.5 μg of TLR9 ligand adjuvants K3 CpG oligonucleotides (CpG ODNs) and 5 μg of aluminum hydroxide gel (AHG) (A gift from The Research Foundation for Microbial Diseases of Osaka University) or CpG ODNs alone (final volume of 200 μl per mouse). A subcutaneous route of immunization was chosen because our preliminary study demonstrated the levels of PspA-specific IgG in mice subcutaneously immunized with 0.1 μg of PspA plus 2.5 μg of CpG ODNs were significantly higher than those in mice nasally immunized with 0.1 μg of PspA plus 2.5 μg of CpG ODNs (data not shown). CpG ODNs were prepared as described previously [35]. Because the PspA clade-specific IgG levels tended to be higher in mice immunized with each PspA fusion protein with CpG ODNs plus AHG than in those immunized with PspA fusion protein with CpG ODNs alone (see Appendix 2), we used the CpG ODNs plus AHG (define as the double adjuvants), for the immunization of mice with PspA fusion proteins in this study. These double adjuvants were safe in nonhuman primate models, and were applicable to humans [36]. Serum was collected from mice by retro-orbital bleeding 1 week after the third immunization. All animal experiments were approved by the Animal Care and Use Committee of the Research Institute for Microbial Diseases, Osaka University, Japan (Permit Number: Biken-AP-H23-05-0).

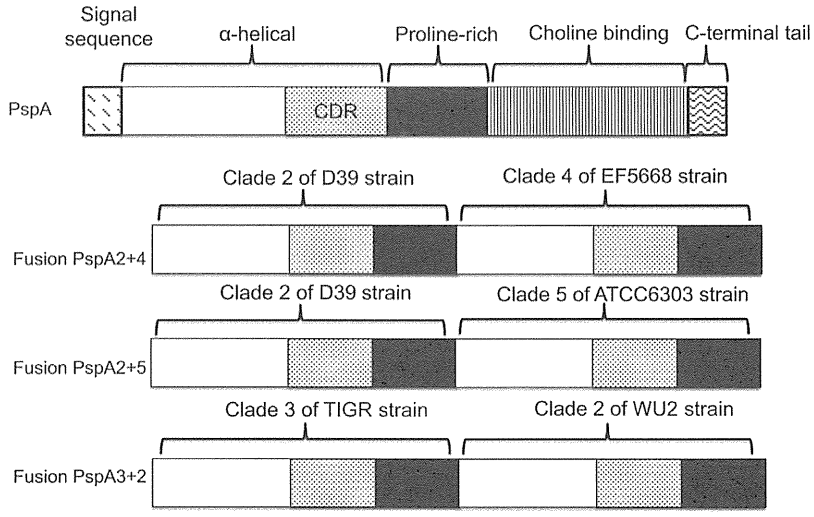

Fig. 1. Schematic diagram of PspA and three fusion PspA proteins. The entire PspA molecule containing the N-terminal α-helical domain, which contains the clade-defining region (CDR), the proline-rich region, the choline-binding domain, and the C-terminal tail (upper column). Each recombinant fusion protein is shown with its different composition (three lower rows).

2.5. Binding of PspA-specific IgG to pneumococcal strains by flow cytometry

Five pneumococcal strains for bacterial challenge and 68 clinical isolates were grown in blood agar plates overnight and then subcultured again on blood agar plates for 4–5 h. The bacteria were collected in PBS, harvested by centrifugation, and washed once with PBS. Ninety microliters of the bacterial suspension at a concentration of 1×10^8 colony-forming units (cfu)/ml in PBS was incubated with 10 μl of mouse antisera for 30 min at 37 °C. After incubation, the suspension was washed once with PBS, resuspended in 100 μl of fluorescein isothiocyanate-conjugated goat anti-mouse IgG (1:100), and incubated for 30 min on ice. After the incubation, the bacterial suspension was washed twice with PBS and suspended in 500 μl of 1% formaldehyde. The samples were kept on ice in the dark until analyzed by flow cytometry using a BD FACSCalibur™ with CellQuest software (BD Sciences, San Jose, CA), and the percentage of fluorescent bacteria (>1 fluorescence intensity unit) in each group was determined. Sera from mice immunized with double adjuvants only were used as the negative controls.

2.6. Protection against pneumococcal challenge

The mice immunized with the PspA fusion protein plus double adjuvants were challenged intranasally with 2×10^7 cfu of strain BG9739 (clade 1), 2×10^7 cfu of strain WU2 (clade 2), 5×10^6 cfu of strain TIGR4, 2×10^7 cfu of strain KK1162 (clade 4), or 5×10^5 cfu of strain ATCC6303 (clade 5). Bacterial challenges were performed 2 weeks after the final immunization. Mortality was monitored for 2 weeks following pneumococcal challenge. The mice immunized with double adjuvants alone were used as a control.

2.7. Statistical analysis

Analysis of variance followed by an unpaired Mann–Whitney *U* test was used to evaluate differences in antibody titer. The percent binding by immune sera to each pneumococcal strain was compared by paired *t*-test. Survival rates were analyzed by the Kaplan–Meier log-rank test. All analyses were performed using GraphPad Prism Software (GraphPad software, La Jolla, CA). *p* values <0.05 were considered significant.

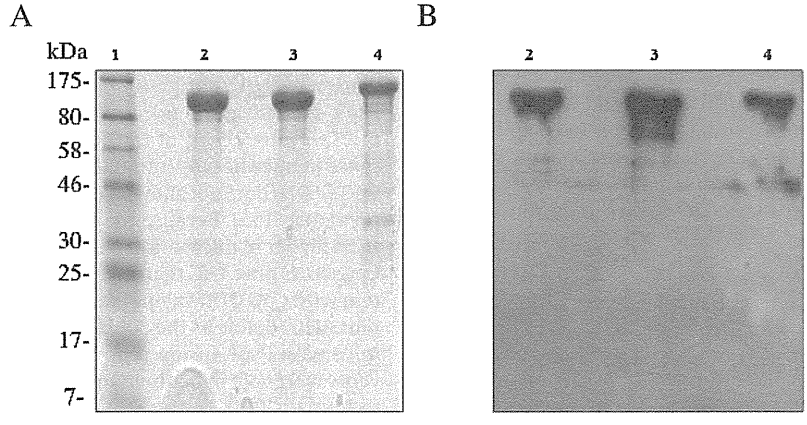

Fig. 2. Characterization of three purified fusion PspA proteins by SDS–PAGE (A) and Western blot analysis (B). The proteins were subjected to SDS–PAGE and detected by direct staining with Coomassie brilliant blue. Lane 1, standard molecular weight markers; lane 2, PspA2+4; lane 3, PspA2+5; 4, lane PspA3+2. The values on the left are molecular sizes in kilodaltons. Mouse antiserum against PspA recombinant protein (clade 2) was used for Western blot analysis. Lane 2, PspA2+4; lane 3, PspA2+5; lane 4, PspA3+2.

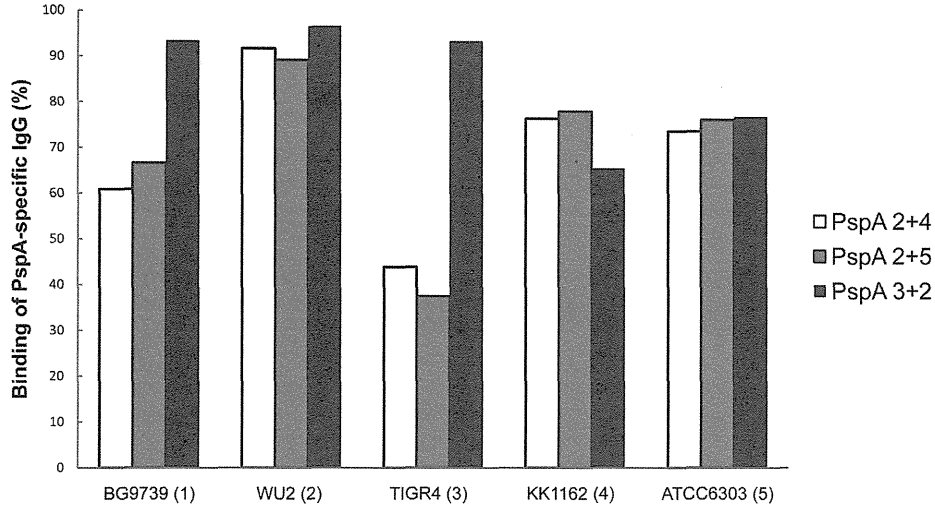

Fig. 3. The binding of PspA-specific IgG by antisera with PspA fusion proteins to the challenge strains with different clades. The mean percentages of fluorescent bacteria positive for IgG binding by antisera from mice immunized with PspA2+4, PspA2+5, or PspA3+2 in combination CpG ODNs plus AHG (double adjuvants) are shown for five pneumococcal strains with PspA clades 1–5 used in the challenge experiments. The numbers in parentheses represents the PspA clade.

3. Results

A schematic diagram of PspA and the three PspA fusion proteins constructed from PspA families 1 and 2 are shown in Fig. 1. The purified recombinant fusion proteins were electrophoresed on sodium dodecyl sulfate–polyacrylamide (SDS–PAGE) gels and evaluated by Coomassie blue staining (Fig. 2A) and by Western blotting using mouse anti-PspA/Rx1 sera (PspA/Rx1 and PspA/D39 are identical clade 2 PspA molecules) (Fig. 2B).

PspA-specific IgG binding >60% was found in antiserum raised by PspA2+4 or PspA2+5 plus double adjuvants for the challenge strains expressing PspA clades 1, 2, 4, and 5, but not for the strain expressing clade 3 (Fig. 3). By contrast, PspA-specific IgG binding > 60% was found for the challenge strains expressing all five PspA clades in antiserum raised by PspA3+2 plus double adjuvants.

For the challenge with the bacterial strain BG9739 with PspA clade 1, the survival rate was greater in mice immunized with PspA3+2 plus double adjuvants ($p < 0.01$) compared with mice

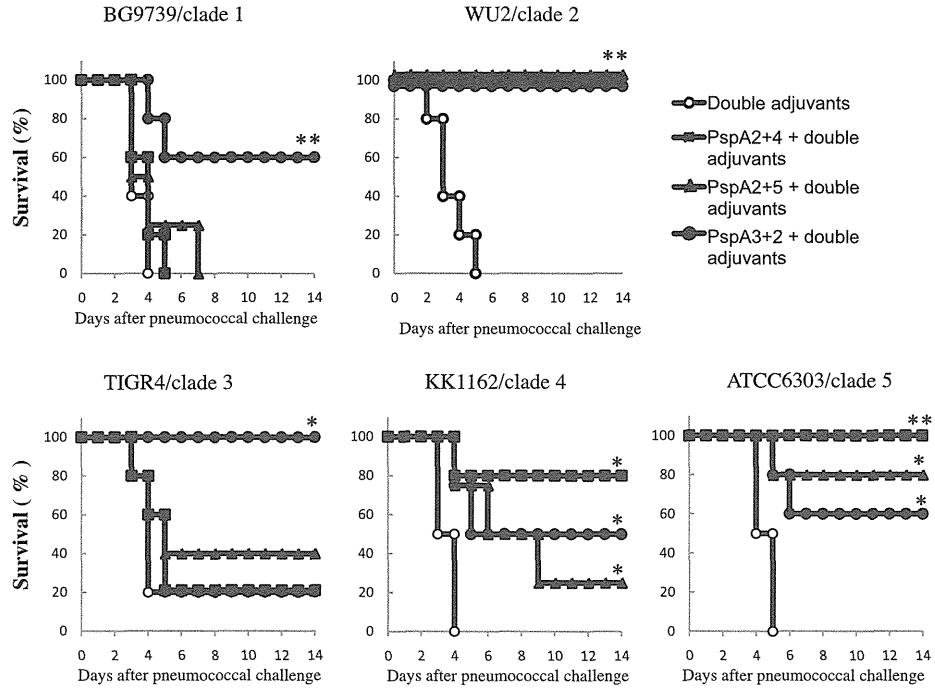

Fig. 4. Protective effects of immunization with fusion PspA proteins against pneumococcal challenge in mice. Mice were immunized subcutaneously with PspA2+4 (closed squares), PspA2+5 (closed triangles), or PspA3+2 (closed circles) in combination with CpG ODNs plus AHG (double adjuvants) or double adjuvants alone (open circles) three times at 1-week intervals. Two weeks after the last immunization, the immunized mice were challenged intranasally with pneumococcal strains with PspA clades 1–5. Mortality was monitored for 2 weeks. Eight to 10 mice per group were examined in each challenge experiment using pneumococcal strain with five different clades. $^{*}p < 0.05$ (vs double adjuvants alone), $^{**}p < 0.01$ (vs double adjuvants alone).

Table 1
Serotypes and PspA clades of 68 isolates from adults with invasive pneumococcal disease.

Serotype	No. strain	No. strain				
		Family 1		Family 2		
		Clade 1	Clade 2	Clade 3	Clade 4	Clade 5
1	1	1				
3	10	9		1		
4	4			4		
6A	2		1			1
6B	10	7		3		
6C	1		1			
7F	2			2		
9V	1			1		
10A	3	3				
11A	2				2	
12F	1			1		
14	5	5				
15A	1				1	
15B	1			1		
16	1			1		
18B	1	1				
18C	1	1				
19A	3			3		
19F	3	1		2		
20	1	1				
22F	3	3				
23A	1				1	
23F	5					5
33	1	1				
34	1	1				
35	2				2	
38	1		1			
Total 68 (100%)		34 (50%)	3 (4%)	19 (28%)	6 (9%)	6 (9%)

immunized with double adjuvants alone (Fig. 4). By contrast, the survival rate did not differ between mice immunized with PspA2+4 or PspA2+5 plus double adjuvants compared with mice immunized with double adjuvants alone. For the bacterial challenge with the WU2 strain with PspA clade 2, the survival rate was significantly higher in mice immunized with PspA2+4, PspA2+5, or PspA3+2 plus double adjuvants ($p < 0.01$) compared with mice immunized with double adjuvants alone. For the bacterial challenge with the TIGR4 strain with PspA clade 3, the survival rate was significantly higher in mice immunized with PspA3+2 plus double adjuvants ($p < 0.05$) compared with mice immunized with double adjuvants alone. The survival rate did not differ between mice immunized with PspA2+4 or PspA2+5 plus double adjuvants compared with mice immunized with double adjuvants alone. In the case of challenge with clade 4 and 5 strains, all three PspA fusion vaccines showed significant protection compared with mice immunized with double adjuvants alone ($p < 0.01$ or $p < 0.05$). These data indicate that immunization with the PspA3+2 vaccine conferred significant protection of mice against pneumococcal challenge by all of the strains expressing PspA clades 1–5. The other two PspA fusion proteins failed to elicit protection against two of the challenge strains (PspA clades 1 and 3).

The distribution of serotypes and PspA clades of 68 clinical isolates from adult patients with IPD are shown in Table 1. The major serotypes were serotype 3 (15%) and 6B (15%), followed by serotypes 14 (7%) and 23F (7%). The major PspA clades were clade 1 (50%) and clade 3 (28%), followed by clade 4 (9%), clade 5 (9%), and clade 2 (4%). All the clinical isolates belonged to PspA clades 1–5, which is in agreement with previous studies [6,13,25].

The binding of PspA-specific IgG in antiserum raised by PspA2+4, PspA2+5, or PspA3+2 plus double adjuvants was examined for the 68 clinical isolates (Fig. 5). The binding of PspA-specific IgG for clade 3 strains ($n = 19$) in antiserum raised by PspA3+2 was significantly higher than in that raised by PspA2+4 or PspA2+5 ($p < 0.05$). By contrast, the binding of PspA-specific IgG for clade 5 strains ($n = 6$) in antiserum raised by PspA3+2 was significantly lower than that by PspA2+4 ($p < 0.05$) or PspA2+5 ($p < 0.05$). No significant difference was found in the binding of PspA-specific IgG for 34 clade 1 strains, three PspA clade 2 strains, or six PspA clade 4 strains between the three types of antiserum raised by PspA2+4, PspA2+5, or PspA3+2.

4. Discussion

In this study, we have demonstrated >60% binding of PspA-specific IgG in the antiserum raised in mice by PspA2+4 or PspA2+5 to four challenge strains expressing clades 1, 2, 4, and 5, but low binding of PspA-specific IgG to the strain expressing clade 3 (Fig. 3). By contrast, >60% binding of PspA-specific IgG in antiserum raised in mice by PspA3+2 was found to all five challenge strains expressing PspA clades 1–5. Immunization with PspA3+2 provided significant protection against pneumococcal challenge by these five strains expressing clades 1–5, but PspA2+4 or PspA2+5 protected mice against only three of the strains expressing clades 2, 4 and 5 in this study (Fig. 4). Therefore, it may be speculated that the binding of PspA-specific IgG closely correlates with the protective effects of PspA fusion protein against pneumococcal challenge in mice. These findings are supported by a recent report on the ability of opsonophagocytic killing and protection of mice against pneumococcal infection by human antiserum to PspA [17]. Only one exception for this speculation is that no protection was found against pneumococcal challenge by the clade 1 strain BG9739 (serotype 4) in mice immunized with PspA2+4 or PspA2+5 plus double adjuvants despite of >60% binding of PspA-specific IgG in antiserum raised by PspA2+4 or PspA2+5 for this clade 1 strain. One possible reason for the inefficient immunization with PspA2+4 or PspA2+5 in mice infected with BG9739 strain may be the presence of serotype 4 capsular polysaccharide. Our previous study demonstrated that the difficulty in protecting against serotype 4 strains was eliminated when mice were immunized with a homologous PspA of the same PspA family [37]. However, only weak protection against infection with strain BG9739 was observed by immunization of mice with the homologous PspA clade 1 [28]. Therefore, it remains uncertain whether immunization with PspA2+4 or PspA2+5 plus double adjuvants did not protect against pneumococcal challenge by the clade 1 strain BG9739 in mice.

No differences were found in the binding of PspA-specific IgG to the clinical isolates belonging to the major clade 1 ($n = 34$) and the two minor clades 2 ($n = 3$) and 4 ($n = 6$) between the types of antiserum raised by the three PspA fusion proteins. For the clinical isolates belonging to the second major clade 3 ($n = 19$), antiserum raised by PspA3+2 demonstrated the greatest binding between the three types of antiserum raised by the PspA fusion proteins (Fig. 5). These findings are in agreement with those showing the binding of PspA-specific IgG to the TIGR4 strain expressing clade 3 for the three types of antiserum raised by each PspA fusion protein (Fig. 3). However, antiserum raised by PspA3+2 demonstrated the lowest binding to six clinical isolates belonging to the minor clade 5 between three types of antiserum raised by each PspA fusion protein. Collectively, PspA3+2 appears to be advantageous in terms of its cross-reactivity with clinical isolates and cross-protection against pneumococcal challenge in mice compared with the other two PspA fusion proteins.

Darrieux et al. reported that immunization with fusion proteins containing fragments of PspA from families 1 and 2 provided cross-protection against pneumococcal strains from families 1 and 2 in mice [30]. The fusion proteins containing PspA clade 1 and PspA clade 3 or 4 fragments provided significant protection against the A66.1 strain (PspA clades 1, and 2), but the protection against strains from clades 3 and 4 was of borderline significance. In another

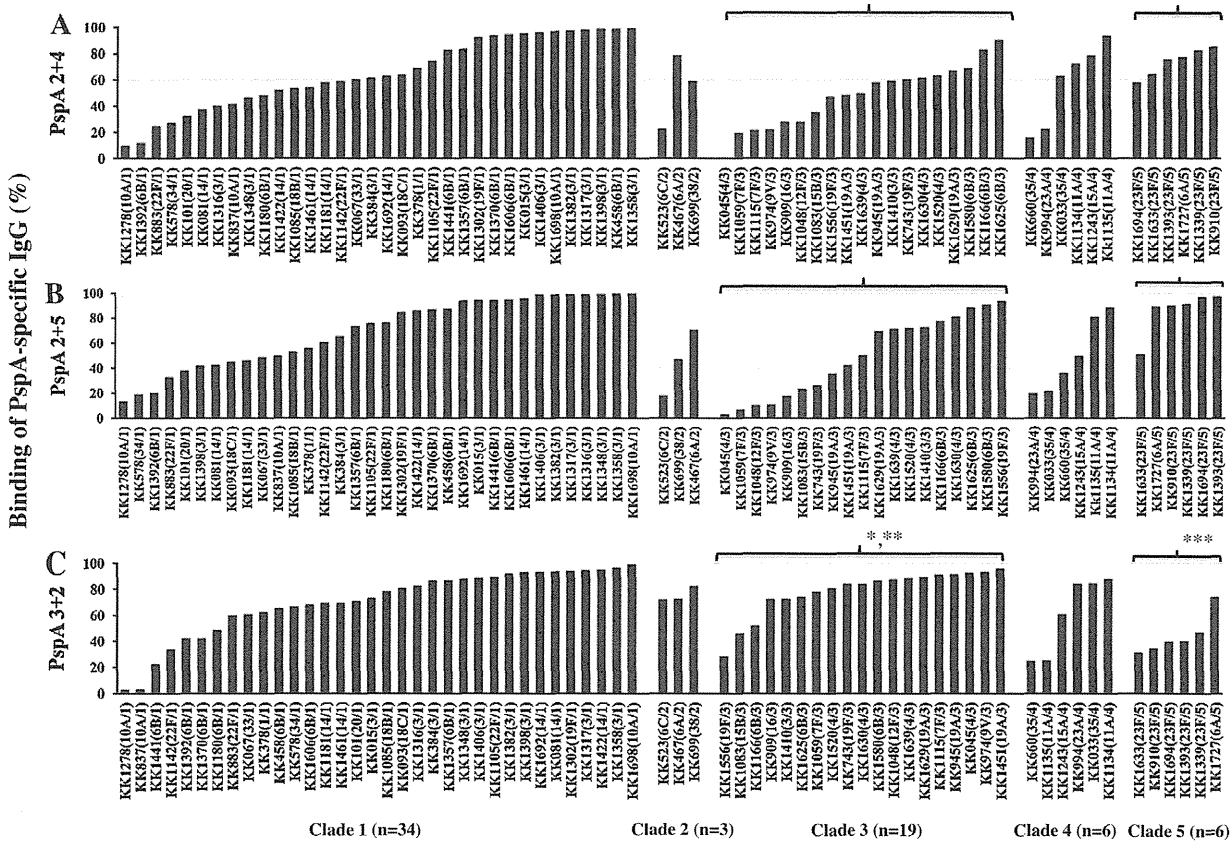

Fig. 5. Comparison of PspA-specific IgG binding by antisera with PspA2+4 (A), PspA2+5 (B), or PspA3+2 (C) in combination with CpG ODNs plus AHG (double adjuvants) to 68 pneumococcal isolates (34 for clade1, three for clade 2, 19 for clade 3, six for clade 4, and six for clade 5). The serotypes and PspA clades are shown in parentheses after the strain names. $^{*}p < 0.01$ (vs PspA2+4), $^{**}p < 0.05$ (vs PspA2+5), $^{***}p < 0.05$ (vs PspA2+4 or PspA2+5).

study, these authors reported that antiserum against fusion protein PspA1+4 demonstrated strong cross-reactivity with PspA clades 1 and 5 but low cross-reactivity with PspA clade 2 or 3 [29]. Consequently, Darrieux et al. failed to demonstrate significant protection against pneumococcal challenge by strains with PspA clades 1–5, although they demonstrated limited cross-protection by immunization with the fusion proteins containing fragments of PspA from families 1 and 2.

A limitation of our study is that we generated and examined only three PspA fusion proteins, which contained one clade each from PspA families 1 and 2. Another limitation is that the binding of PspA-specific IgG was assessed in a small number of clinical isolates from adult patients with IPD.

The antiserum raised by PspA3+2 demonstrated relatively weak binding capacity to the clinical isolates expressing PspA clade 5 in this study. Further studies are required to generate the other types of PspA fusion proteins that can induce PspA-specific IgG with a high affinity to strains expressing PspA clades 5, as well as to strains expressing PspA clade 1–4. In addition, immunization with PspA2+4 or PspA2+5 provided better protection than PspA3+2 against bacterial challenge of clade 4 or clade 5 strain in this study. Therefore, the combined immunization with PspA3+2 with PspA2+4 or PspA2+5 simultaneously or sequentially may have the potential to improve the breadth of immunity against pneumococcal isolates.

In conclusion, immunization of mice with PspA3+2 induced antiserum exhibiting a high binding capacity to the clinical isolates expressing PspA clades 1–4, but not clade 5. Among the three PspA fusion proteins examined in this study, PspA3+2 was found to be advantageous over the other two PspA fusion proteins because PspA3+2 induced a broad range of cross-reactivity with clinical isolates and afforded a cross-protection against pneumococcal challenge in mice.

Author contributions

K.O., Y.A., K.J.I., K.U. and K.T. conceived and designed the experiments. Z.P. and Y.A. performed the experiments. Z.P. and D.T. analyzed the data. K.O., Z.P., Y.A., and D.E.B. wrote the paper.

Conflict of interest statement

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.07.108.

References

[1] O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, McCall N, et al. Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. Lancet 2009;374:893–902.

[2] Pilishvili T, Lexau C, Farley MM, Hadler J, Harrison LH, Bennett NM, et al. Sustained reductions in invasive pneumococcal disease in the era of conjugate vaccine. J Infect Dis 2010;201:32–41.

[3] Singleton RJ, Hennessy TW, Bulkow LR, Hammitt LL, Zulz T, Hurlburt DA, et al. Invasive pneumococcal disease caused by nonvaccine serotypes among Alaska native children with high levels of 7-valent pneumococcal conjugate vaccine coverage. JAMA 2007;297:1784–92.

[4] Ardanuy C, Tubau F, Pallares R, Calatayud L, Domínguez MA, Rolo D, et al. Epidemiology of invasive pneumococcal disease among adult patients in Barcelona before and after pediatric 7-valent pneumococcal conjugate vaccine introduction, 1997–2007. Clin Infect Dis 2009;48:57–64.

[5] Miller E, Andrews NJ, Waight PA, Slack MPE, Gorge RC. Herd immunity and serotype replacement 4 years after seven-valent pneumococcal conjugate vaccination in England and Wales: an observation cohort study. Lancet Infect Dis 2011;11:760–8.

[6] Croney CM, Coats MT, Nahm MH, Briles DE, Crain MJ. PspA family distribution, unlike capsular serotype, remains unaltered following introduction of the heptavalent pneumococcal conjugate vaccine. Clin Vaccine Immunol 2012;19:891–6.

[7] Rev-Yochay G, Rahav G, Riesenberg K, Wiener-Well Y, Strahilevitz J, Stein M, et al. Initial effects of the national PCV7 childhood immunization program on adult invasive pneumococcal disease in Israel. PLOS ONE 2014;9:e88406.

[8] Briles DE, Tart RC, Swiatlo E, Dillard JP, Smith P, Benton KA, et al. Pneumococcal diversity: considerations for new vaccine strategies with emphasis on pneumococcal surface protein A (PspA). Clin Microbiol Rev 1998;11:645–57.

[9] Briles DE, Ades E, Paton JC, Sampson JS, Carlone GM, Huebner RC, et al. Intranasal immunization of mice with a mixture of the pneumococcal proteins PsaA and PspA is highly protective against nasopharyngeal carriage of *Streptococcus pneumoniae*. Infect Immun 2000;68:796–800.

[10] Ogunniyi AD, Folland RL, Briles DE, Hollingshead SK, Paton JC. Immunization of mice with combinations of pneumococcal virulence proteins elicits enhanced protection against challenge with *Streptococcus pneumoniae*. Infect Immun 2000;68:3028–33.

[11] Tai SS. *Streptococcus pneumoniae* protein vaccine candidates: properties, activities and animal studies. Crit Rev Microbiol 2006;32:139–53.

[12] Daniels CC, Briles TC, Mirza S, Håkansson AP, Briles DE. Capsule does not block antibody binding PspA, a surface virulence protein of *Streptococcus pneumoniae*. Microb Pathog 2006;40:228–33.

[13] Hollingshead SK, Baril L, Ferro S, King J, Coan P, Briles DE, Pneumococcal Proteins Epi Study Group. Pneumococcal surface protein A (PspA) family distribution among clinical isolates from adults over 50 years of age collected in seven countries. J Med Microbiol 2006;55:215–21.

[14] Tu A-H, Fulgham RL, McCrory MA, Briles DE, Szalai J. Pneumococcal surface protein A inhibits complement activation by *Streptococcus pneumoniae*. Infect Immun 1999;67:4720–4.

[15] Ren B, Szalai AJ, Hollingshead SK, Briles DE. Effects of PspA and antibodies to PspA on activation and deposition of complement on the pneumococcal surface. Infect Immun 2004;72:114–22.

[16] Briles DE, Hollingshead SK, King J, Swift A, Braun PA, Park MK, et al. Immunization of humans with recombinant pneumococcal surface protein A (rPspA) elicits antibodies that passively protect mice from fatal infection with *Streptococcus pneumoniae* bearing heterologous PspA. J Infect Dis 2000;182:1694–701.

[17] Daniels CC, Kim K-H, Burton RL, Mirza S, Walker M, King J, et al. Modified opsonization, phagocytosis, and killing assays to measure potentially protective antibodies against pneumococcal surface protein A. Clin Vaccine Immunol 2013;20:1549–58.

[18] Yother J, Briles DE. Structural properties and evolutionary relationships of PspA, a surface protein of *Streptococcus pneumoniae*, as revealed by sequence analysis. J Bacteriol 1992;174:601–9.

[19] Yother J, White JM. Novel surface attachment mechanism of the *Streptococcus pneumoniae* protein PspA. J Bacteriol 1994;176:2976–85.

[20] Senkovich O, Cook WJ, Mirza S, Hollingshead SK, Protasevich II, Briles DE, et al. Structure of a complex of human lactoferrin N-lobe with pneumococcal surface protein a provides insight into microbial defense mechanism. J Mol Biol 2007;370:701–13.

[21] McDaniel LS, Ralph BA, McDaniel DO, Briles DE. Localization of protection-eliciting epitopes on PspA of *Streptococcus pneumoniae* between amino acid residues 192 and 260. Microb Pathog 1994;17:323–37.

[22] Shaper M, Hollingshead SK, Benjamin Jr WH, Briles DE. PspA protects *Streptococcus pneumoniae* from killing by apolactoferrin, and antibody to PspA enhances killing of pneumococci by apolactoferrin. Infect Immun 2004;72:5031–40.

[23] Daniels CC, Coan P, King J, Hale J, Benton KA, Briles DE, et al. The proline-rich region of pneumococcal surface proteins A and C contains surface-accessible epitopes common to all pneumococci and elicits antibody-mediated protection against sepsis. Infect Immun 2010;78:2163–72.

[24] Hollingshead SK, Becker R, Briles DE. Diversity of PspA: mosaic genes and evidence for past recombination in *Streptococcus pneumoniae*. Infect Immun 2000;68:5889–900.

[25] Qian J, Yao K, Xue L, Xie G, Zheng Y, Wang C, et al. Diversity of pneumococcal surface protein A (PspA) and relation to sequence typing in *Streptococcus pneumonia* causing invasive diseases in Chinese children. Eur J Clin Microbiol Infect Dis 2012;31:217–23.

[26] Oma K, Zhao J, Ezoe H, Akeda Y, Koyama S, Ishi KJ, et al. 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. Vaccine 2009;27:3181–8.

[27] Moreno AT, Oliveira ML, Ferreira DM, Ho PL, Darrieux M, Leite LC, et al. Immunization of mice with single PspA fragments induces antibodies capable of mediating complement deposition on different pneumococcal strains and cross-protection. Clin Vaccine Immunol 2010;17:439–46.

[28] Briles DE, Hollingshead SK, Nabors GS, Paton JC, Brooks-Water A. The potential for using protein vaccines to protect against otitis media caused by *Streptococcus pneumoniae*. Vaccine 2000;19(Suppl. 1):S87–95.

[29] Darrieux M, Moreno AT, Ferreira DM, Pimenta FC, de Andrade AL. Recognition of pneumococcal isolates by antisera raised against PspA fragments from different clades. J Med Microbiol 2008;57:273–8.

[30] Darrieux M, Miyaji EN, Ferreira DM, Lopes LM, Lopes A, Ren B, et al. Fusion proteins containing family 1 and family 2 PspA fragments elicit protection against *Streptococcus pneumoniae* that correlates with antibody-mediated enhancement of complement deposition. Infect Immun 2007;75:5930–8.

[31] Xin W, Li Y, Mo H, Roland KL, Curtiss III R. PspA family fusion proteins delivered by attenuated *Salmonella enterica* serovar Typhmilium extend and enhance protection against *Streptococcus pneumoniae*. Infect Immun 2009;77:4518–28.

[32] Chiba N, Morozumi M, Shouji M, Wajima T, Iwata S, Sunakawa K, et al. Rapid decrease of 7-valent conjugate vaccine coverage for invasive pneumococcal diseases in pediatric patients in Japan. Microb Drug Resist 2013;19:308–15.

[33] Pimenta FC, Ribeiro-Dias F, Brandileone MC, Miyaji EN, Leite LC, Sgambatti de Andrade AL. Genetic diversity of PspA types among nasopharyngeal isolates collected during an ongoing surveillance study of children in Brazil. J Clin Microbiol 2006;44:279–84.

[34] Ezoe H, Akeda Y, Piao Z, Aoshi T, Koyama S, Tanimoto T, et al. Intranasal vaccination with pneumococcal surface protein A plus poly(I:C) protects against secondary pneumococcal pneumonia in mice. Vaccine 2011;29:1754–61.

[35] Takeshita F, Leifer CA, Gursel I, Ishii KJ, Takeshita S, Gursel M, et al. Cutting edge: role of Toll-like receptor 9 in CpG DNA-induced activation of human cells. J Immunol 2001;167:3555–8.

[36] Tougan T, Aoshi T, Coban C, Katakai Y, Kai C, Yasumoti Y, et al. TLR9 adjuvants enhance immunogenicity and protective efficacy of the SE36/AHG malaria vaccine in nonhuman primate models. Hum Vaccine Immunother 2013;9:283–90.

[37] Roche H, Ren B, McDaniel LS, Håkansson A, Briles DE. Relative roles of genetic background and variation in PspA in the ability of antibodies to PspA to protect against capsular type 3 and 4 strains of *Streptococcus pneumoniae*. Infect Immun 2003;71:4498–505.

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Surveillance

Antibiotic susceptibility survey of blood-borne MRSA isolates in Japan from 2008 through 2011

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ABSTRACT

We conducted an antibiotic susceptibility survey of 830 blood-borne methicillin resistant *Staphylococcus aureus* collected from nationwide hospitals in Japan over a three-year period from January 2008 through May 2011. Antibiotic susceptibility was judged according to the criteria recommended by the Clinical Laboratory Standard Institute. Over 99% of the MRSA showed to be susceptible to teicoplanin, linezolid, sulfamethoxazole/trimethoprim and vancomycin, and over 97% of them were susceptible to daptomycin, arbekacin and rifampin. The majority of the MRSA strains showed resistant to minocycline, meropenem, imipenem, clindamycin, ciprofloxacin, cefoxitin, and oxacillin in the rates of 56.6, 72.9, 73.7, 78.7, 89.0, 99.5, and 99.9%, respectively. Among the MRSA strains, 72 showed reduced susceptibility to vancomycin, including 8 strains (0.96%) of vancomycin-intermediate *S. aureus* (VISA), 54 (6.51%) of heterogeneous vancomycin-intermediate *S. aureus* (hVISA), and 55 (5.63%) of β-lactam antibiotics-induced vancomycin resistant *S. aureus* (BIVR). Unexpectedly, among the 54 hVISA and 55 BIVR, 45 isolates (83.3% and 81.8%, respectively) showed both hVISA and BIVR phenotypes. A new trend of vancomycin resistance found in this study was that VISA strains were still prevalent among the bacteremic specimens. The high rates of

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the hVISA/BIVR two-phenotypic vancomycin resistance, and the prevalence of VISA in the bloodborne MRSA call attention in the MRSA epidemiology in Japan.

1. Introduction

Staphylococcus aureus (*S. aureus*) is a leading cause of hospital- and community-*associated* infections. In the hospital, methicillin-resistant *S. aureus* (MRSA) is the highest risk of infection at surgical wounds, the lower respiratory tract and the cardiovascular system, and the second most common cause of health care-associated pneumonia and septicemia [1–3]. Infections caused by MRSA are worse than those caused by other pathogens due to the limited choices of available antibiotics and thus it is difficult to eradicate these strains. Moreover, the worst situation is that blood stream MRSA infection has a higher rate of mortality and must endure longer hospital stays [4].

Vancomycin has been the agent of choice for methicillin-resistant *S. aureus* (MRSA) infections as it provided efficacious and promising therapy [5]. Nevertheless, with the emergence of *S. aureus* strains having intermediate resistance towards vancomycin (vancomycin-intermediate *S. aureus* [VISA]), treatment options for patients infected with these strains have become limited [6]. Heterogeneous vancomycin-intermediate *S. aureus* (*Hetero*-VISA or hVISA) strains are also being reported more frequently worldwide [7]. These strains are interpreted as 'susceptible' to vancomycin using conventional MIC determination tests, but contain a sub-population of cells which can grow in the presence of >2 mg/L vancomycin [8,9]. Another category of MRSA with reduced vancomycin-susceptibility, designated as BIVR, which is resistant to vancomycin only in the presence of β-lactam antibiotics was reported to be prevalent in Japan [10], and the combined phenotype of hVISA and BIVR was associated with a higher probability of mortality in patients with MRSA bacteremia [11]. Therefore, it is important to understand the current state of the prevalence of MRSA with reduced vancomycin-susceptibility from blood stream infections.

Here, we describe a current surveillance study of antibiotic susceptibility on the blood-borne MRSA strains collected from 11 university hospitals and 3 general hospitals covering from Tokyo-Nagoya-Osaka and Kyushyu regions of Japan isolated during a period from 2008 through 2010. The data were compared with the previous surveillance studies with non-bacteremic MRSA isolates studied in 2007 through 2009 by the same laboratory [12–14]. Our study also focused on determining the strains with reduced vancomycin susceptibility. Though information on MRSA epidemiology in Japan is growing [12–15], significant gaps exist on the locality of sampling, availability of central laboratories to serve for characterizing MRSA, and availability of surveillance data. Our data presented here will provide information on the most recent trends in the emergence and dissemination of antibiotic resistant strains among the blood stream MRSA infections in Japan.

2. Materials and methods

2.1. Participating hospitals and collection of bacterial isolate

Blood-borne MRSA strains were isolated during Jan. 2008 through May 2011 at 11 university hospitals and 3 general hospitals in Japan located Kanto, Kinki, Tokai and Kyushyu regions. Only one MRSA sample was collected from the same patient. All the blood-borne MRSA strains were primarily identified at the local participating hospitals. The clinical isolates were suspended in Microbank tubes (Asuka Junyaku, Tokyo, Japan) at the local hospitals, and delivered under frozen states to the surveillance Central Laboratory at Research Center for Infections and Antimicrobials (formerly Research Center for Anti-infectious Drugs) of Kitasato University. A total of 838 isolates were delivered to the Central Laboratory and they were stored at −80 °C until use. All the strains were subjected to the MRSA identification tests again at the Central Laboratory and eventually 830 strains were subjected to the antimicrobial susceptibility tests.

2.2. Antibacterial agents and antimicrobial susceptibility testing

Antibiotic susceptibilities of the MRSA isolates were tested for the following 14 antimicrobial agents: oxacillin (MPIPC; Meiji Seika Pharma Co., Ltd., Tokyo, Japan), cefoxitin (CFX; Banyu Pharmaceutical, Tokyo, Japan), imipenem (IPM; Banyu Pharmaceutical, Tokyo, Japan), meropenem (MEPM; Dainippon Sumitomo Pharma, Tokyo, Japan), arbekacin (ABK; Meiji Seika Pharma Co., Ltd., Tokyo, Japan), clindamycin (CLDM; Dainippon Sumitomo Pharma Co., Ltd.); minocycline (MINO; Wyeth, Madison, NJ, USA/Takeda), vancomycin (VCM; Shionogi & Co., Ltd., Tokyo, Japan), teicoplanin (TEIC; Astellas Phamar Inc., Tokyo, Japan), ciprofloxacin (CPFX; BayerYakuhin, Tokyo, Japan), linezolid (LZD; Pfizer); daptomycin (DAP, Cubist Pharmaceuticals), Sulfamethoxazole-Trimethoprim (ST; Shionogi & Co., Ltd.), and Rifampin (RFP; Daiichi-Sankyo Co., Ltd., Tokyo, Japan). The minimum growth inhibitory concentration of antimicrobial agents (MIC) was determined using the MIC determination kit (Eiken Chemical Co., Ltd., Tokyo, Japan). The microplates in the kit contained serially diluted and lyophilized antimicrobial agents.

MICs of antibiotics were determined by the micro broth-dilution method at the Central Laboratory according to the method recommended by CLSI (manual M7-A72) as described previously [14,15]. Briefly, cation-adjusted Mueller-Hinton broth (Eiken Chemical Co., Ltd., Tokyo, Japan) was used throughout for bacterial culture and the MIC determination. The fresh bacterial culture was diluted with 0.9% of NaCl saline adjusting cell density to 1×10^8 cfu/ml and that was further diluted by fresh Mueller-Hinton broth adjusting cell density to ~5×10^5 cfu/ml. Then, a 100-μl of this bacterial suspension was inoculated into the MIC-determination microplates with an aid of an MIC-2000 inoculator (Eiken Chemical Co., Ltd., Tokyo, Japan). The final inoculum size was ~10^4 cfu/well. The MIC was defined as the minimum concentration of antibiotics that inhibited bacterial growth macroscopically after 24 h of incubation at 35 °C. The MIC of daptomycin was determined in the cation-adjusted Mueller-Hinton broth supplemented with 50 mg/L of calcium chloride according to the CLSI criterion [16]. *S. aureus* ATCC29213 was used as a quality control reference strain in the MIC determination according to the CLSI recommendations.

2.3. Data collection from previous studies

The Japanese Society of Chemotherapy (JSC) launched Japanese Antimicrobial Surveillance Committee in 2006, and the committee became jointly run by JSC, Japanese Association for Infectious Diseases (JAID) and Japanese Society for Clinical Microbiology

(JSCM) from 2008 (http://www.chemotherapy.or.jp/lang-en/index.html). The committee has been conducing nationwide surveillance on antibiotic resistance twice a year since 2008, and our laboratory (Research Center for Infections and Antimicrobials, Kitasato Institute for Life Science, Kitasato University) has been serving as a Central Laboratory where the all antibiotic-susceptibility tests have been carried out. This committee released antibiotics susceptibility surveillance data on the non-bacteremic MRSA isolates studied in 2007 through 2009 [12–14]. Since the antibiotics susceptibility tests were carried out in our laboratory, we extracted MIC data from the publications for the 14 antibiotics that were used in the current study, and the data were compared with those of the present study.

2.4. Detection of the vancomycin-intermediate S. aureus (VISA) and heterogeneous vancomycin-intermediate S. aureus (hVISA)

The VISA strain was defined as the isolates that showed MIC of VCM 4–8 μg/ml or that of TEIC 16 μg/ml. For screening of hVISA candidates, a combination of vancomycin/teicoplanin macro-Etest described by Walsh et al. [17] and the one-point population analysis described by Yamakawa [18] was employed. The methicillin-susceptible *S. aureus* ATCC29213 and hVISA strain Mu3 served as references. On the macro-Etest screening, the isolates showed the MIC of both VCM and TEIC MICs ≥8 μg/ml or that of VCM <8 μg/ml and TEIC ≥12 μg/ml were defined as hVISA candidates. On the one-point population screening, the strains that generated more than 10 discrete colonies (colony appearance frequency of 1×10^{-6} or greater) were considered as hVISA candidates. The hVISA candidates identified by the two screening methods were then subjected to a simplified population analysis as follow. A bacterial suspension containing ~10^6 cells was inoculated onto the Mueller-Hinton agar impregnated with 2 μg/ml of vancomycin. The isolates, which showed the colony number ≥0.9 compared with that of the Mu3 strain were selected as the hVISA. It was confirmed that selection of the hVISA by this method gave consistent results from that on the brain heart infusion (BHI) agar impregnated with 4 μg/ml of vancomycin without exception so far. Reliability of the above method for the detection of hVISA was verified by taking a few strains, which showed lowest colony numbers on the agar plates, with PAP-AUC method proposed by Wootton et al. [19].

2.5. Detection of the β-lactam antibiotic-induced vancomycin resistant methicillin-resistant S. aureus (BIVR)

The BIVR was detected as described previously [10,20]. Briefly, bacterial cells were grown in MH broth supplemented with 1 μg/ml of ceftizoxime for 16 h at 37 °C under aeration. The cultures were diluted with 0.9% of sodium chloride saline adjusting cell density to $A_{578} = 0.3$, and 0.1 ml of this (about 10^7 cells) was streaked out evenly with a swab on the Mu3 agar plate (Becton–Dickinson, Tokyo, Japan) supplemented with 4 μg/ml of vancomycin. An 8-mm paper disk, impregnated with 0.1, 1.0, or 10 mg/l of ceftizoxime, was placed on the agar plate and the plate was incubated at 35 °C for 24 h. The isolates showing a hollow of growth zone around the disk were defined as BIVR [10,20].

2.6. PCR detection of blaZ and mecA

The *blaZ* gene encoding β-lactamase was amplified by PCR with a forward primer 5′-TCGAAAATAATAAAGGGAAAATCA-3′ and a reverse primer 5′-GCCATTTTGACACCTTCTTTC-3′ [21]. A thermal cycler was set as follow: 98 °C for 30 s for the initial denaturation and then 30 cycles of denaturation, annealing and extension at 98 °C for 5 s, 57 °C for 10 s and 72 °C for 10 s, respectively. The PCR products were analyzed by agarose gel electrophoresis and visualized by staining with GelRed (Biotim Hayward, CA, USA). The *mecA* was amplified by PCR using a primer pair of 5′-GAGCAATGAACTGATTATAC-3′ for forward and a 5′-GTCACTTTCAACATACAATG-3′ for reverse, which covered the whole CDS of *mecA*. PCR condition was as same as that of *blaZ* determination.

2.7. PCR amplification and DNA sequencing of the V domains of 23S rRNA

For the LZD resistant isolates, the V domain of the 23S *rRNAs* gene was sequenced as previously described [22]. Briefly, the 23S *rRNA* genes for 5 individual *rRNA* operons were amplified with 5 sets of primer as described previously [22], and the resulting was sequenced with forward (5′-GCGGTCGCCTCCTAAAAG-3′) and reverse (5′-ATCCCGGTCCTCTCGTACTA-3′) primers correspond to the position at 2280th to 2297th and 2680th to 2699th of the *S. aureus* 23S *rRNA*, respectively. The sequences of the PCR products were analyzed by aligning to the corresponding nucleotide sequences of *S. aureus* N315 (GenBank accession no. BA000018).

3. Results

The overall susceptibility profile of MRSA isolates to 14 antibiotics was shown in Table 1 and Fig. 1. As shown in Table 1, the MIC_{50}, MIC_{90} and MIC ranges of 11 antibiotics including TEIC, VCM, ABK, MINO, MPIPC, IMP, CLDM, CPFX, LZD, CFX and MEPM were comparable with those of in non-bacteremic MRSA isolates studied in 2007 through 2009 by this Central Laboratory [12–14]. Those of the antibiotics that showed high MIC_{90} and MIC_{50} (16–32 μg/ml) were MINO, MPIPC, IPM, CLDM, CPFX, CFX and MEPM. In fact, 56.6, 99.9, 73.4, 78.9, 89.0, 99.6 and 72.9% of MRSA isolates were classified to intermediate to resistant categories against these antibiotics, respectively (Table 1). Therefore, these antibiotics are virtually unusable for the therapy of MRSA infections. In contrast, over 95% of MRSA population appeared susceptible to TEIC, VCM, ABK, RFP, ST, LZD and DAP and therefore these antibiotics may be the choice to be used in the chemotherapy of MRSA infections (Fig. 1 and Table 1). A close look of the data revealed following slight but important differences compared with previous surveillance studies [12–14]. (i) Eight isolates appeared to be glycopeptide intermediate that was not recorded in the earlier studies. (ii) Ten isolates were newly classified to the DAP non-susceptible category, even though DAP has just been approved early 2011 in Japan. (iii) Two isolates were classified to be LZD non-susceptible, increasing the upper range of MIC from 4 μg/ml to ≥16 μg/ml. (iv) The upper ranges of MIC of TEIC, VCM, MINO, and LZD in the isolates studied here increased markedly suggesting that levels of resistance are increasing. However, none of isolates were found to be cross-resistance to major anti-MRSA antibiotics such as VCM, TEIC, LZD, DAP and ABK those have been commonly used in Japan (data not shown).

Among the 830 isolates, one strain showed MPIPC susceptible (MIC 2 μg/ml) but CFX resistant (MIC 8 μg/ml), and *mecA* positive by PCR. Other two strains carried functional *mecA* gene and showed MIC of MPIPC >16 μg/ml and that of CFX 4 μg/ml. Another one strain carried *mecA*, yet was susceptible to both MPIPC and CFX, and yielded microcolonies around the strip by MPIPC E-test suggesting that this was a typical OS-MRSA [23].

We identified a total of 8, 54 and 55 isolates to be VISA, hVISA, and BIVR, respectively, and these were equivalent to 0.96%, 6.51% and 6.63%, respectively, of 830 MRSA isolates (Table 2). The prevalence of the hVISA isolates varied from a hospital to another ranging from 0 to 47.37%. The frequencies of hVISA isolation in 9 out of 14 hospitals was less than 5% in which 3 hospitals had no case of

Table 1
A summary of MICs of 14 antimicrobial agaents for 830 blood-stream MRSA isolates.

	Category	TEIC[a]	VCM	ABK	RFP	ST[b]	MINO	MPIPC	IPM	CLDM	CPFX	LZD	DAP	CFX	MEPM
Number of isolates (%)	Susceptible	829 (99.88)	822 (99.03)	811 (97.72)	809 (97.47)	827 (99.64)	360 (43.37)	1 (0.12)	218 (26.27)	174 (20.96)	91 (10.96)	828 (99.76)	820 (98.80)	3 (0.36)	225 (27.11)
	Intermediate	1 (0.12)	8 (0.96)	–	11 (1.33)	–	120 (14.45)	–	66 (7.95)	1 (0.12)	3 (0.36)	–	–	–	120 (14.46)
	Resistant	0	0	–	10 (1.20)	–	350 (42.17)	829 (99.88)	546 (65.78)	655 (78.54)	736 (88.67)	–	–	827 (99.64)	485 (58.43)
	Nonsusceptible	–	–	19 (2.28)	–	3 (0.36)	–	–	–	–	–	2 (0.24)	10 (1.20)	–	–
Susceptibility ($n = 830$)	MIC range (mg/L)	≤0.5–16	≤0.5–4	0.25–6	≤0.06–≥16	≤0.06–≥16	≤0.125–≥32	2–≥16	≤0.25–≥16	≤0.25–≥16	≤0.25–≥16	0.5–≥16	0.125–2	4–≥32	≤0.125–≥16
	MIC50 (mg/L)	1	1	1	0.06	0.06	8	≥16	≥16	≥16	≥16	2	0.5	≥32	≥16
	MIC90 (mg/L)	4	2	2	0.06	0.125	16	≥16	≥16	≥16	≥16	2	0.5	≥32	≥16
Susceptibility 2007 ($n = 135$)[c]	MIC range (mg/L)	0.125–4	0.5–2	0.25–4	nd	nd	0.125–16	4–≥256	≤0.06–≥128	0.125–≥256	0.25–≥256	1–4	nd	8–≥256	0.25–64
	MIC50 (mg/L)	1	1	0.5	nd	nd	8	≥265	32	≥256	128	2	nd	128	16
	MIC90 (mg/L)	2	2	2	nd	nd	16	≥265	64	≥256	128	2	nd	≥256	32
Susceptibility 2008 ($n = 113$)[d]	MIC range (mg/L)	0.25–8	0.5–2	0.25–8	nd	nd	≤0.06–16	32–≥256	0.25–≥128	0.125–≥256	0.25–≥256	0.5–2	nd	4–≥128	1–128
	MIC50 (mg/L)	0.5	1	0.5	nd	nd	8	≥265	32	≥256	128	1	nd	≥128	16
	MIC90 (mg/L)	2	2	2	nd	nd	16	≥265	64	≥256	≥256	2	nd	≥128	64
Susceptibility 2009 ($n = 54$)[e]	MIC range (mg/L)	0.125–4	0.5–2	0.125–4	nd	nd	0.125–16	16–≥256	≤0.06–≥128	0.125–≥256	0.25–≥256	1–4	nd	8–≥128	0.25–128
	MIC50 (mg/L)	0.5	1	0.5	nd	nd	16	128	16	≥256	128	2	nd	≥128	16
	MIC90 (mg/L)	2	2	1	nd	nd	16	≥265	64	≥256	≥256	2	nd	≥128	32
Susceptibility criteria of CLSI[f]	Susceptible (mg/L)	≤8	≤2	≤2	≤1	≤2	≤4	≤2	≤4	≤0.5	≤1	≤4	≤1	≤4	≤4
	Intermediate (mg/L)	16	4–8	–	2	–	8	–	8	1–2	2	–	–	–	8
	Resistant (mg/L)	≥32	≥16	–	≥4	–	≥16	≥4	≥16	≥4	≥4	–	–	≥8	≥16
	Nonsusceptible (mg/L)	–	–	>2	–	≥4	–	–	–	–	–	>4	>1	–	–

[a] Abbreviation: TEIC, teicoplanin; VCM, vancomycin; ABK, aberkacin; REP, rifampin; ST, sulfamethoxazole-trimethoprim; MINO, minocyline; MPIPC, oxacillin; IPM, imipenem; CLDM, clindamycin; CPFX, ciprofloxacin; LZD, linezolid; DAP, daptomycin; CFX, cefoxitin; MEPM, meropenem.
[b] *Sulfamethoxazole :Trimethoprim is 19:1 mixture, and MIC of Trimethoprim is recored.*
[c] Data were extracted from previous publications [12–14].
[d] Data were extracted from previous publications [12–14].
[e] Data were extracted from previous publications [12–14].
[f] CLSI, Clincal and Laboratory Standards Institute, 2011 [16].