2-dimensional gel electrophoresis analysis in simultaneous influenza pneumonia and bacterial infection in mice

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ABSTRACT

Severe pneumonia is found in simultaneous influenza pneumonia and bacterial infection, and suggested the relations with immunological mechanisms.

Here, we performed two-dimensional gel electrophoresis to detect immunological molecules related to the fulminant pneumonia caused by influenza virus and *Streptococcus pneumoniae* co-infection in mice.

We found two spots that were strongly expressed in co-infected mouse lungs, compared with *S. pneumoniae* or influenza virus singly-infected mouse lungs. The spots were analyzed by mass spectrometry, and identified as alpha-1 antitrypsin (A1AT), known as an antiprotease for neutrophil-derived proteolytic enzymes, and creatine kinase, which reflects greater degree of lung damage and cell death. A1AT expression was significantly increased, and proteolytic enzymes from neutrophils, such as neutrophil elastase, myeloperoxidase, and lysozyme, were also secreted abundantly in influenza virus and *S. pneumoniae* co-infected lungs, compared with *S. pneumoniae* or influenza virus singly-infected lungs.

These data suggest that A1AT may play a central role as a molecule with broad anti-inflammatory properties, and regulation of the neutrophil-mediated severe lung

inflammation is important in the pathogenesis of co-infection with influenza virus and

bacteria.

INTRODUCTION

Influenza virus infection is a major respiratory infectious disease all over the world, and pneumonia is one of the most important complications associated with influenza [1-3]. Influenza infection-associated pneumonia is classified into two categories: primary viral pneumonia and secondary/mixed bacterial pneumonia [2, 4-6].

The pneumonia following influenza virus infection can be a secondary/mixed bacterial pneumonia, which is more common than primary viral pneumonia and usually late in the course of the diseases[5, 6]. The symptoms and signs are similar to those of typical bacterial pneumonia, and the organisms, such as *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus* are involved [2, 4-6]. Infection of epithelial cells by the influenza A virus requires cleavage of the virus hemagglutinin (HA) by proteases, and some *S aureus* produce these proteases [2, 5, 7]. These proteases are also secreted by some host bronchial cells, such as Clara cells [8], suggesting that host proteases are critical to enhance influenza virus-associated pneumonia following bacterial infection

Furthermore, some immunological mechanisms and synergistic effects between influenza virus and bacteria have been implicated in the enhancement of secondary/mixed bacterial pneumonia. In our previous animal study, fulminant pathological changes were

found in the lungs of mice inoculated with *S. pneumoniae* at 2 days after influenza virus

infection, and some critical immune responses, such as the release of cytokines and

chemokines as well as Toll-like receptor (TLR) expressions, were increased [9]. In addition,

chronic *Pseudomonas aeruginosa* infection in mice is exacerbated by the influenza virus,

while decreased neutrophil function due to viral infection may be one inducer of the lethal

pneumococcal pneumonia observed in such mice [10]. These results suggest that the

synergistic effects of co-infection with influenza virus and bacteria are mediated through

immune reactions.

In the present study, we performed two-dimensional gel electrophoresis (2DE) to

detect specific proteases and immunological molecules related to the fulminant pneumonia

caused by influenza virus and *S. pneumoniae* co-infection in mice, compared with *S.*

pneumoniae or influenza virus singly-infected mice.

MATERIALS AND METHODS

Virus, Bacteria, Mice and Sampling

A mouse-adapted A-strain influenza virus (strain A/PR8/34: H1N1 type) and *S. pneumoniae* (penicillin-resistant *S. pneumoniae*-187 serotype 19 isolated at our university) were inoculated into 6-wk-old male CBA/J mice (specific pathogen-free) as previously reported [9]. Briefly, each mouse was inoculated intranasally with 50 μL of influenza virus (1×10^4 plaque-forming units/mL), followed by intranasal inoculation with 50 μL of *S. pneumoniae* (1×10^8 CFU/mL) at 2 d after the influenza virus infection. The lungs were harvested as biological samples at 48 h after the *S. pneumoniae* infection, as previously described [9]. For 2DE analysis, lungs harvested from influenza virus and *S. pneumoniae* co-infected mice (Co-infection mice) were compared with lungs harvested from *S. pneumoniae* , influenza virus, or saline infected mice (Single or Mock infection mice).

Protein Extraction and IEF for 2DE Analysis

After measurement of the lung tissue, a 5-fold volume of T-PER Tissue Protein Extraction Reagent (Pierce, Rockford, IL) supplemented with Protease Inhibitor Cocktail (Sigma Aldrich Japan, Tokyo, Japan) and 1 mM PMSF was added to the tissue. Following

addition of 0.15 g of zirconium beads (2 mm), the mixture was homogenized (4,000 rpm for

60 s, 4°C) in a Bead Homogenizer (Micro Smash MS-100R; Tomy, Tokyo, Japan) and then

centrifuged at 20,000 g for 20 min. The protein concentration of the supernatant (protein

extraction solution) was determined by the Bradford method (Sigma Aldrich Japan), with

reference to a standard curve for BSA. The protein extraction solution (200 µg protein) was

precipitated in -30°C cold acetone for 3 h and the precipitated proteins were collected by

centrifugation at 20,000 g for 15 min. The acetone was discarded and the pellet was dried for

a few minutes at room temperature.

The protein precipitate was resuspended in 250 µL of IEF lysis buffer and vortexed

gently for 45 min at room temperature. The IEF lysis buffer comprised 6 M urea, 2 M

thiourea, 3% CHAPS, 1% Triton X-100 and DeStreak reagent (GE Healthcare Biosciences,

Tokyo, Japan). After lysis of the proteins in the IEF lysis buffer, the solution was centrifuged

at 20,000 g for 20 min).

The sample was rehydrated in an Immobiline DryStrip gel (pH 3-10 non-linear, 13

cm; GE Healthcare Biosciences) for 12 h, before being subjected to IEF at 150 V for 1 h,

5,000 V ramping for 2.5 h and IEF at 5,000 V for 15 h.

2DE Conditions

Before the 2DE, the gel strip was equilibrated in strip equilibration buffer (6 M urea, 20% glycerol, 2% DTT, 2% SDS, 375 mM Tris-HCl pH 8.8) for 45 min. 2DE was carried out at a constant current of 25 mA for about 4 h (10-18% polyacrylamide gradient gel; 14 x 14 cm). The gel was placed in 0.02% Coomassie Brilliant Blue G-250 staining solution overnight, and then destained in 5% acetic acid. Images were acquired with a GS-800 Calibrated Imaging Densitometer (Bio-Rad Japan, Tokyo, Japan).

Peptide Mass Fingerprinting (PMF) Analysis

The gel pieces were washed with water while vortexing and dehydrated in acetonitrile. The acetonitrile was removed and the gel pieces were dried in a vacuum centrifuge. Next, 10 mM DTT in 100 mM ammonium bicarbonate was added and incubated for 1 h at 56°C. The solution was replaced by the same volume of 55 mM iodoacetamide in 100 mM ammonium bicarbonate and incubated for 45 min at room temperature in the dark with vortexing. The gel pieces were washed with 100 mM ammonium bicarbonate while vortexing, dehydrated with acetonitrile, rehydrated again with 100 mM ammonium bicarbonate and dehydrated again with acetonitrile. The acetonitrile was removed and the gel

pieces were dried in a vacuum centrifuge. Trypsin solution (50 mM ammonium bicarbonate,

5 mM calcium chloride, 10 μg/mL trypsin) was added and incubated for 45 min at 4°C,

followed by incubation at 37°C for 12 h. The resulting peptides were extracted by sequential

incubations in 20 mM ammonium bicarbonate, followed by 5% formic acid in 50%

acetonitrile. The extract was dried in a vacuum centrifuge, rehydrated in 0.1% trifluoroacetic

acid and desalted using a ZipTip C18 column (Millipore, Bedford, MA) according to the

manufacturer's protocol. The peptides were eluted with 0.1% trifluoroacetic acid in 50%

acetonitrile and applied to a MALDI plate.

Mass Spectrometry (MS) and Database Analysis

MS of the peptides was performed using a MALDI-TOF MS AXIMA-CFR

(Shimadzu Corporation, Tsukuba, Japan). Protein identification was performed by Mascot

searches (Matrix Science, http://www.matrixscience.com/).

RT-PCR Analysis for Alpha-1 Antitrypsin (A1AT) Expression

Total RNA was extracted from lung homogenates using ISOGEN (Nippon Gene,

Tokyo, Japan). In order to prepare lung homogenates, lungs were homogenized in 1 mL of

PBS for 60 s using a glass tissue homogenizer (Takashima, Tokyo, Japan) in an ice-cold

water bath. RT-PCR was performed using a First Strand cDNA Synthesis and Detection Kit

(Life Technology, Gaithersburg, MD). Mouse A1AT and hypoxanthine

phosphoribosyltransferase (HPRT; internal control) cDNAs were amplified using the

following specific primers: A1AT: forward, 5'-AGGGTCATCATGGGCACCTT-3', reverse,

5'-AGGGTCATCATGGGCACCTT-3', product size, 416 bp; HPRT[9], forward,

5'-AAGCAGTACAGCCCCAAAAT-3', reverse, 5'-CATAGTGCAAATCAAAAGTC-3',

product size, 392 bp. PCR was performed for 30 cycles for each molecule, with one PCR

cycle consisting of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C. The signal intensity of

each band was analyzed using an Alpha Imager (Alpha Innotech Co., San Leandro, CA).

Bronchoalveolar Lavage (BAL) for Neutrophil Elastase (NE) Measurement

BAL was performed at 2 d after *S. pneumoniae* infection, as described previously [9,

10]. Briefly, mice were sacrificed and their chests were opened to expose the lungs. Next, a

disposable sterile plastic cut-down intravenous catheter was inserted into the trachea. BAL

was performed in situ four times sequentially using 1 mL of saline each time and the

recovered fluid fractions were pooled for each mouse. For differential cell counts, cells were

centrifuged onto a slide in a tabletop centrifuge at 1000x g for 1 min and the slides were

stained with May-Giemsa stain, and differential cell counts were performed by counting 100

cells[9, 10].

The NE activity in each BAL fluid (BALF) was determined using the synthetic

substrate Suc-Ala-Ala-Pro-Val pNA, which is highly specific for NE, as previously described

[11, 12]. Briefly, samples were incubated in 0.1 M NaCl and 1 mM substrate for 24 h at 37°C,

and the amount of pNA released was measured spectrophotometrically at 405 nm and

considered to represent the NE activity.

Analysis of Myeloperoxidase (MPO), Lysozyme and MIP-2

MPO activity in the cells from BAL was detected by the method reported previously

[10]. Antigenic lysozyme in the cells from BAL was also detected by western blotting

analysis using a rabbit anti-lysozyme antibody (1:10000 dilution; Rockland Inc., Gilbertsville,

PA) as previously described [10]. Horseradish peroxidase-conjugated anti-rabbit IgG

(1:20000 dilution; Santa Cruz Biotechnology) was used as a secondary antibody and the

positive signals were developed with ECL-plus (GE Healthcare Biosciences). The

concentrations of macrophage inflammatory protein-2 (MIP-2) in the cells from BAL were

assayed by mouse Quantikine Kit (R & D Systems, Minneapolis, MN, USA).

Statistical Analysis

All data were expressed as means ± SD and analyzed using the StatView software

(Abacus Concepts, Cary, NC). Differences between groups were examined for significance

using an unpaired *t*-test. Values of $P<0.05$ were considered to indicate a statistically

significant difference.

RESULTS

2DE Analysis

Figure 1 shows the patterns of protein spots visualized by Coomassie Brilliant Blue

staining and the differences between co-infected lungs (Figure 1A) and singly-infected lungs

(Figure 1B; *S, pneumoniae* alone, and 1C: influenza virus alone) / mock-infected lungs

(Figure 1D: saline, respectively). Computer-assisted image analyses detected 290 spots in the

co-infected samples (Figure 1A) and 304 spots in the *S. pneumoniae* singly-infected samples

(Figure 1B). A comparison of the patterns in the two gels revealed a total of 10 spots that

were more strongly detected in the co-infected samples (Table 1). Among these 10 spots, two

spots, designated SSP 1610 and SSP 8501, were strongly induced, with ratios of 18.09 and

14.38 relative to the *S. pneumoniae* singly-infected samples, respectively. The density ratio of

these two spots were also high, compared with either in influenza virus singly-infected

samples (Figure 1C) or in mock-infected samples (Figure 1D).

Identification of Proteins

Gel digests of the two strongly expressed spots were further analyzed by MS and

PMF. Analysis by MALDI-TOF MS and Mascot searches provided significant scores for

these spots, revealing that SSP 1610 was significantly matched with the A1AT precursor and

its related proteases, while SSP 8501 was matched with creatine kinase (CK) and citrate

synthase (Table 2).

Expression of A1AT in Co-infected Lungs

The detection of CK may just be a reflection of lung tissue damage due to the severe

pneumonia, since increased CK has been reported to result from severe myositis [13, 14] and

rhabdomyolysis [15]. However, A1AT was reported as a key molecule in the resolution of

severe lung inflammation, including that associated with pneumonia and acute respiratory

distress syndrome [16-20]. Therefore, we further analyzed the A1AT expression in

co-infected lungs, compared with influenza virus or bacteria singly-infected lungs.

The levels of A1AT mRNA expression in co-infected lungs were significantly

up-regulated, compared with influenza virus or bacteria singly-infected lungs as well as

non-infected control lungs (Figure 2A and B).

NE, MPO, Lysozyme and MIP-2 Secretions in Co-infected Lungs

Since A1AT is known to be an antiprotease that inhibits neutrophil-derived enzymes

[16-19], we next analyzed neutrophil-secreted proteins, such as NE, MPO and lysozyme.

NE activity in BALF (in whole lung) was significantly higher in co-infected lungs

(146.0±102.9 x 10 nmol/mL/24 h) than in *S. pneumoniae* singly-infected lungs (40.0±38.4 x

10 nmol/mL/24 h, *P*<0.01). The NE activities in influenza virus singly-infected lungs and

non-infected lungs were very low (2.0±4.0 and 0.1±0.3 x 10 nmol/mL/24 h, respectively),

and differed significantly from the activity in co-infected lungs (Figure 3A). Furthermore, we

measured NE activities divided by neutrophil counts (Co-infection:150.5±10.5, *S.*

pneumoniae: 50.5±7.0, Influenza: 20.5±1.5, and Mock: 0.1±0.0 x10^3 cells/ mL, respectively,

as same as previously reported [9]) in BALF, and also found significant increase of NE

activities in co-infected lungs, compared with singly-infected lungs and non-infected lungs

(Figure 3B).

The secretion of MPO was barely detected in non-infected and influenza virus

infected-lungs, but increased in bacteria singly-infected lungs. However, the MPO activity

was significant higher in co-infected lungs than in influenza virus or bacteria singly-infected

lungs (Figure 4A). These results are very similar to the A1AT expression data.

Neutrophil-derived lysozyme plays a major role in the intracellular destruction of ingested bacteria, through the formation of phagolysosomes with primary (or azurophilic) and secondary granules [10, 21, 22]. The secretion of lysozyme in the cells from BAL was also significantly increased in co-infected lungs compared to influenza virus or bacteria singly-infected lungs (Figure 4B), similar to the findings for A1AT expression and MPO secretion.

Furthermore, MIP-2 is the chemokine similar to IL-8 in human, and known as the roles related with neutrophil activations and accumulations [9, 10]. We measured the MIP-2 in the cells from BAL, and found it increased significantly in co-infected lungs compared with either single-infected lungs or non-infected lungs (Figure 4C), as previously reported [9].

DISCUSSION

2DE and MALDI-TOF MS analysis are useful methods for rapid identification of proteins from various tissues, including pleural effusions from severe pneumonia patients [23]. However, there are few reports of lung diseases being analyzed by these methods, and systemic experiments linking influenza pneumonia with protein expression have not previously been reported to the best of our knowledge. Since the proteome composition of pneumonia may alter with its severity, we performed a comparative proteome analysis of severe pneumonia caused by influenza virus and bacteria co-infection with mild pneumonia due to virus or bacteria infection alone in the present study.

We found two spots that were strongly expressed in 2DE gels of co-infected samples, and identified the proteins as A1AT and CK by PMF analysis. The increase in CK may reflect the results of severe damage of the lung tissues, similar to myocardial muscles and skeletal muscles [13-15]. However, A1AT was considered to be a critical protein, since trypsin and trypsin-like proteases, such as tryptase Clara, are known to activate the infectivity of influenza virus [7, 8]. In addition, A1AT is known to have potent anti-inflammatory properties and its major function is thought to be inhibition of the deleterious effects of neutrophil proteases, including NE [16-20]. A1AT forms complexes with NE, and

significantly elevated levels of these complexes were reported in BALF from severe

pneumonia patients [18].

As shown in Figure 2, A1AT expression was upregulated in severe pneumonia in the

co-infected lungs compared with mild pneumonia due to influenza virus or bacteria infection

alone. The ability of A1AT to act as an antiprotease and inhibit NE may be impaired in

severe pneumonia, possibly due to cleavage, oxidation or complex formation with NE [18].

Our previous study demonstrated that neutrophils and interleukin-8 secretion were

significantly increased in co-infected lungs, compared with influenza virus or bacteria

singly-infected lungs [9]. A1AT was reported to try to block the interleukin-8 production by

epithelial cells, as well as the cytotoxicity of NE and neutrophil defensins [19, 24, 25]. These

results suggest that the balance of A1AT and neutrophil-derived proteins/enzymes may be

one of the most important factors that induce severe tissue damage in influenza virus and

bacteria co-infected lungs.

NE activities were also significantly increased in co-infected lungs, compared with

either bacteria or virus infected lungs and non-infected lungs. In addition, increases of NE

activites were observed not only in whole cells, but also in each neutrophil of BALF from the

lungs of co-infected mice. These results reflected the significant activation of each neutrophil,

not just accumulations of neutrophils in the lungs of co-infected mice, compared with

singly-infected mice. These results were similar when we used the cells from BAL (data not

shown). Furthermore, we performed small kinetic study of NE at 8, 24, and 48h, and found

increased significantly and peaked at 48h (8hr: 0±0, 24h: 2.644±3.508, and 48h:

12.581±7.780 x10nmol/ml/24h, respectively, $p < 0.001$) .

Similar to NE, MPO is one of the critical molecules involved in the tissue infiltration

of neutrophils in pneumonia [26], and its secretion was significantly increased in co-infected

mouse lungs, compared with singly- or non-infected mice lungs in our previous study [10].

Lysozyme, which is secreted by neutrophils, was also increased in the lungs of co-infected

mice. We and Pang *et al*. previously reported that impaired neutrophil function with

decreased lysozyme secretion and bactericidal activity may foster bacterial colonization in

the respiratory tract after influenza infection (17, 19). MIP-2 was reported the strong relations

with neutrophil activations and accumulations [9, 10]. We found MIP-2 was also increased

significantly in incubated BAL cells from co-infected lungs compared with either

single-infected lungs or non-infected lungs as previously reported [9]. These results also

suggested the important roles of these cytokines/chemokines and neutrophils in the

pathogenesis of severe pneumonia due to influenza virus and bacteria co-infection [27, 28].

Braun *et al*. reported elevated A1AT concentrations despite decreases of the NE and

MPO concentrations in BALF from acute pneumonia patients, and suggested the importance

of the balance between aggressive factors (NE, MPO, lysozyme and MIP-2) and protective

factors (e.g. A1AT) [29]. The increases in these aggressive factors after influenza virus

infection followed by *S. pneumoniae* infection in the present study may also reflect more

significant activation of neutrophils and increased tissue damage due to the synergistic effects

of co-infection with influenza virus and *S. pneumoniae* in severe pneumonia, compared with

either influenza virus or bacterial infection alone. Aggressive factors may be more

significantly upregulated than protective factors in severe pneumonia, while the imbalance

between aggressive factors and protective factors may be less marked in either mild

pneumonia or healthy controls.

In relation with neutrophil activations and bacterial number/virus titers, proliferation

of *S pneumoniae* may be influenced more directly, because bacterial numbers, but not viral

titers, were increased [9]. However, viral infection may be more critical because former viral

infection injures lung epithelial cells and accelerate the latter bacterial proliferation. In

addition, it was reported anti-virus drugs are more effective than antibiotics to prevent

secondary bacterial pneumonia after influenza virus infection in mice[30]. Further study

about relationship synergic effects by virus and bacteria co-infection with neutrophil

activations will be necessary.

In conclusion, we performed 2DE analysis, thought to be the useful tool for

accelerating the discovery of new drug targets and protein disease markers, to detect key

molecules related to the severe pneumonia caused by co-infection with influenza virus and

bacteria, and found a significant increase in A1AT, compared with influenza virus or bacteria

singly-infected lungs. A1AT and its related proteins secreted by activated neutrophils,

including NE, MPO, lysozyme, and MIP-2, may play important roles in the pathogenesis of

severe pneumonia due to influenza virus and bacteria co-infection. These data suggest that

the regulation of neutrophil activation may be critical for understanding the severe lung

damage, and therapy which targeted neutrophil and its related enzymes may be one of the

useful medications for damaged lungs observed in co-infected with influenza virus and

bacteria [20].

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FIGURE LEGENDS

Figure 1: Proteome maps of pneumonia in influenza virus and bacteria co-infected mice (A), bacteria (B) or influenza virus (C) singly-infected mice. Each numbered spot indicates the spots listed in (Table1). pH 3.0-10.0 non-linear 10-18% gradient gels were stained with Coomassie Brilliant Blue G-250. The circled spots are significantly stronger in (A) than in (B) and (C).

Figure 2: A1AT expression levels in the lungs of mice infected with influenza virus and/or bacteria. Samples were analyzed by RT-PCR with HPRT as an internal control (A). Lane 1: mock-infected lungs; lane 2: influenza virus singly-infected lungs; lane 3: bacteria singly-infected lungs; 4: influenza virus and bacteria co-infected lungs. The relative densities are shown in (B).

Figure 3: Neutrophil elastase (NE) secretion from whole BALF cells (A) and neutrophils (B) in the lungs of mice infected with influenza virus and/or bacteria. Lane 1: mock-infected lungs; lane 2: influenza virus singly-infected lungs; lane 3: bacteria singly-infected lungs; 4:

influenza virus and bacteria co-infected lungs. Values of $P<0.05$ are considered to indicate a

statistically significant difference.

Figure 4: (A) MPO activity, (B) lysozyme, and (C) MIP-2 secretion from the BAL cells of

the mice. (A) MPO activity in 6 mice in each group, Data are mean ± SEM. (B) Lysozyme

secretions were analysed by western blotting and representative data from 6 mice are

presented. (C) MIP-2 were measured at 48h by ELISA. Lane 1: mock-infected lungs; lane 2:

influenza virus singly-infected lungs; lane 3: bacteria singly-infected lungs; lane 4: influenza

virus and bacteria co-infected lungs.

Table 1. Density ratios of spots differentially expressed between co-infected and singly/mock-infected lungs

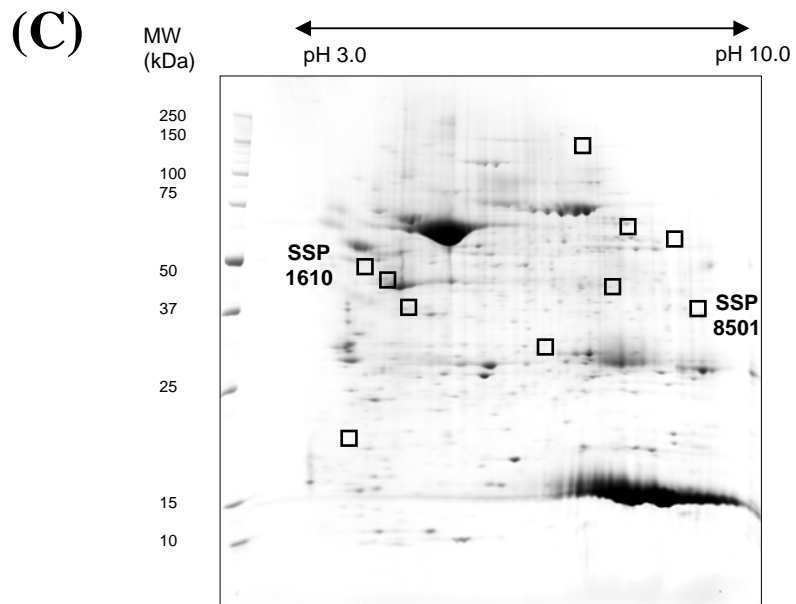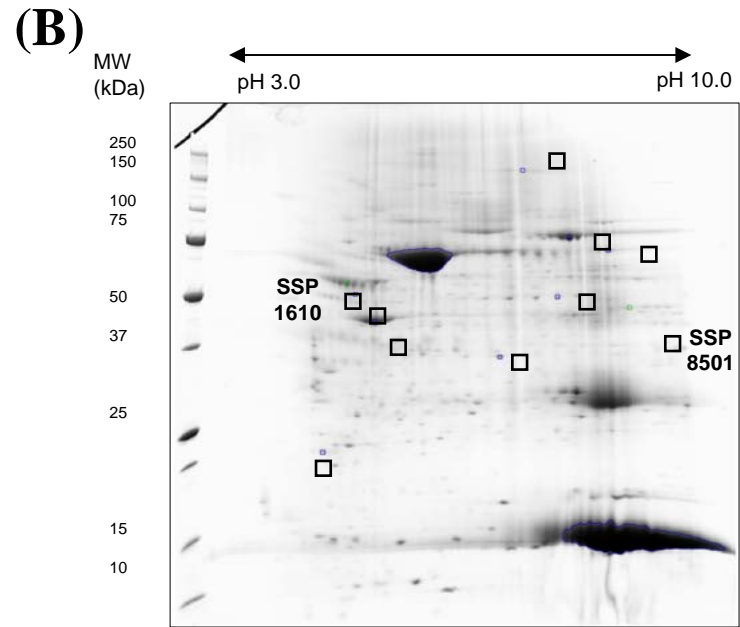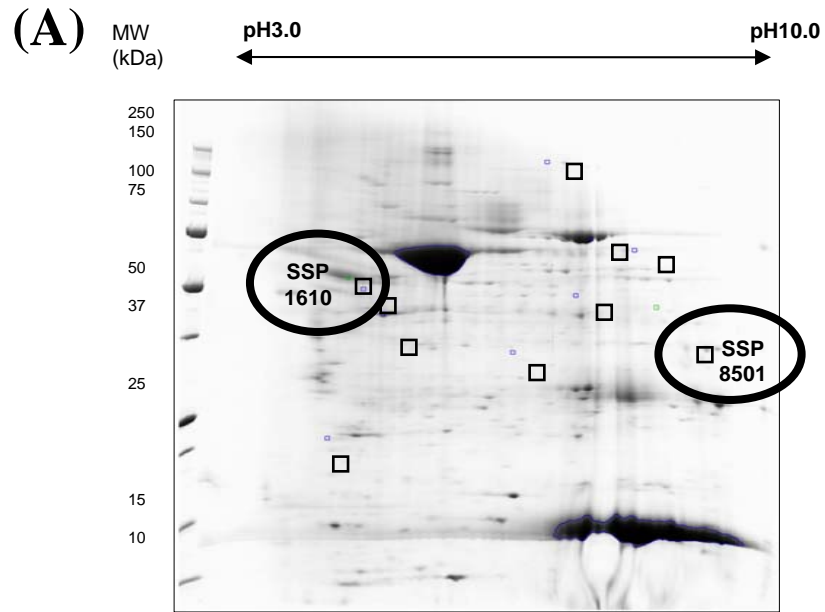| Spot	Mean intensity		Ratio (A/)	Spot	Mean intensity		Ratio (A/)
SSP 1201	A	466.8		SSP 6902	A	679.8	
	B	149.5	3.12		B	70.6	9.63
	C	179.9	2.59		C	82.5	8.24
	D	303.2	1.54		D	152.9	4.47
SSP 1610	A	2105.7		SSP 7601	A	390.7	
	B	116.4	18.09		B	129.8	3.10
	C	169.4	12.43		C	95.4	3.01
	D	87.7	24.01		D	42.5	9.19
SSP 2607	A	219.7		SSP 7705	A	4809.6	
	B	55.2	3.98		B	770.4	6.24
	C	1100.4	0.20		C	8402.2	0.57
	D	498.8	0.44		D	1211.9	3.97
SSP 3501	A	9352.9		SSP 8501	A	1226.4	
	B	1674.4	5.59		B	86.5	14.18
	C	6041.9	1.54		C	193.8	6.32
	D	6741.9	1.39		D	122.5	10.01
SSP 5403	A	473.4		SSP 8702	A	1084.0	
	B	67.5	7.01		B	256.8	4.22
	C	1258.8	0.38		C	1130.6	0.96
	D	368.9	1.28		D	852.8	1.27

A: Co-infection, B: *S.pneumoniae* alone, C: influenza virus alone, D: saline

Table 2. Mascot search results for the detected proteins

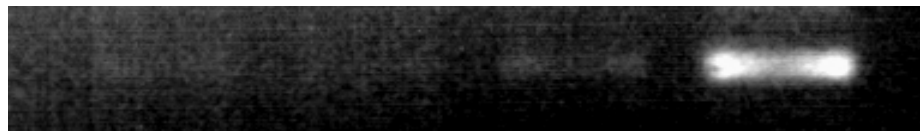| Spot	Accession number (gi)	Protein name	Mass	Score*	Peptide matched	Theoretical MW (Da)	Theoretical p*I*	Sequence coverage (%)
SSP1610	AAA37132(309079)	Alpha-1 antitrypsin precursor	46057	105	16	40957	5.33	43
	AAC28865(191844)	Alpha-1 protease inhibitor 2	44918	85	14	44918	5.33	38
	NP033270(76881807)	Serine (or cysteine) proteinase inhibitor, clade A, member 1b	46117	84	14	46117	5.32	47
SSP8501	NP940807(38259206)	Creatine kinase, mitochondrial 2	47899	161	23	47899	8.64	45
	AAH13554(15488848)	Citrate synthase	51988	82	14	51988	8.72	24

*Protein scores of >74 are significant (P<0.05).

(Figure 1)
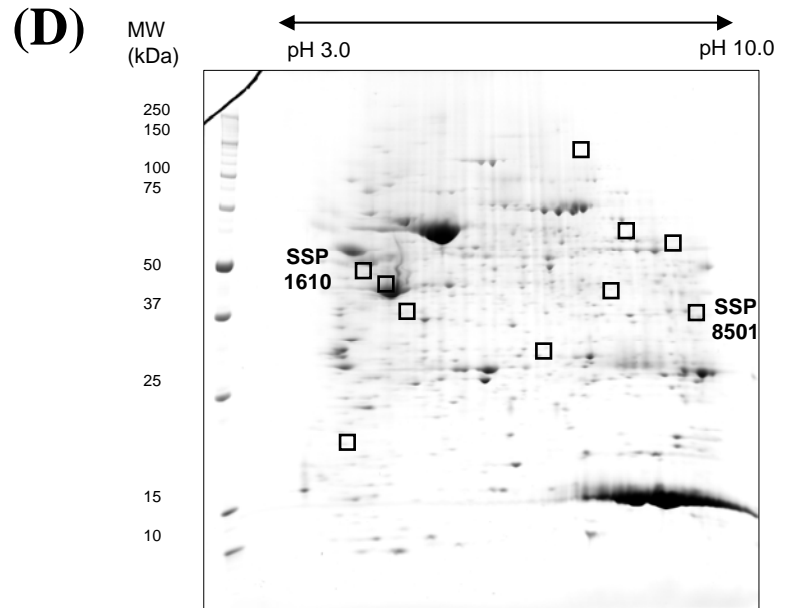
(Figure 2)

(A)

A1AT

HPRT

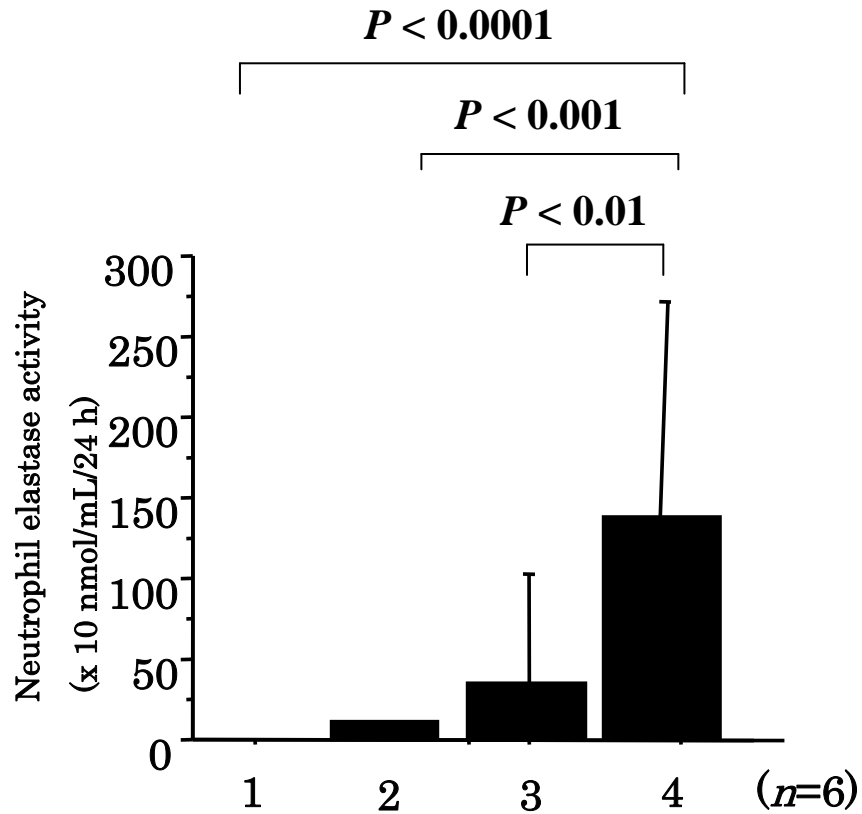1 2 3 4
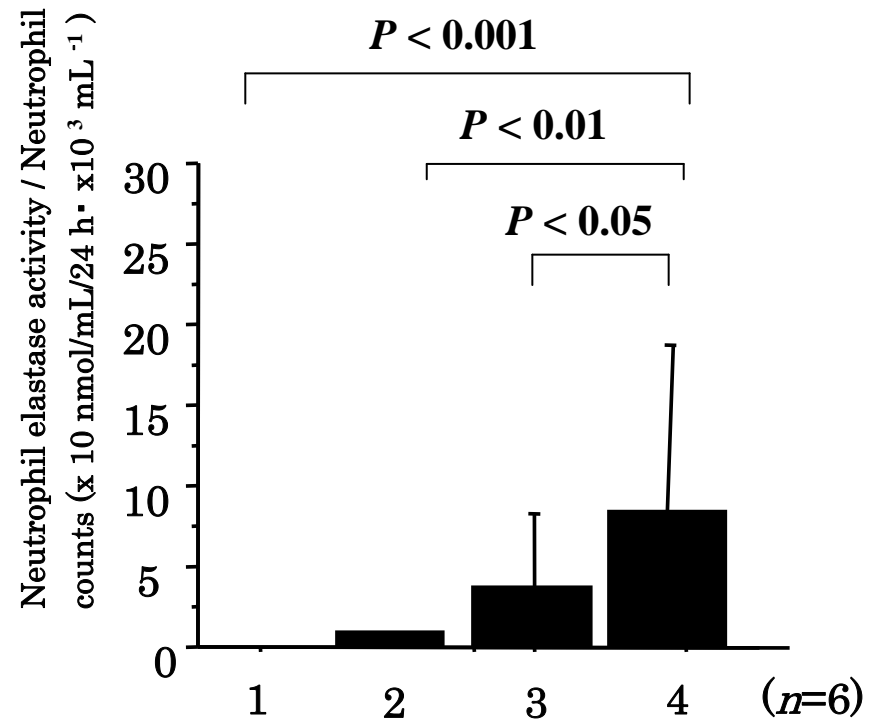
(B)

$P < 0.01$

$P < 0.001$

$P < 0.01$

Density ratio

6
5
4
3
2
1
0

1 2 3 4

($n=6$)

(Figure 3)

(A)

(B)

(Figure 4)

(A)

(B)

$\leftarrow$ 14 kDa

1　2　3　4

(C)