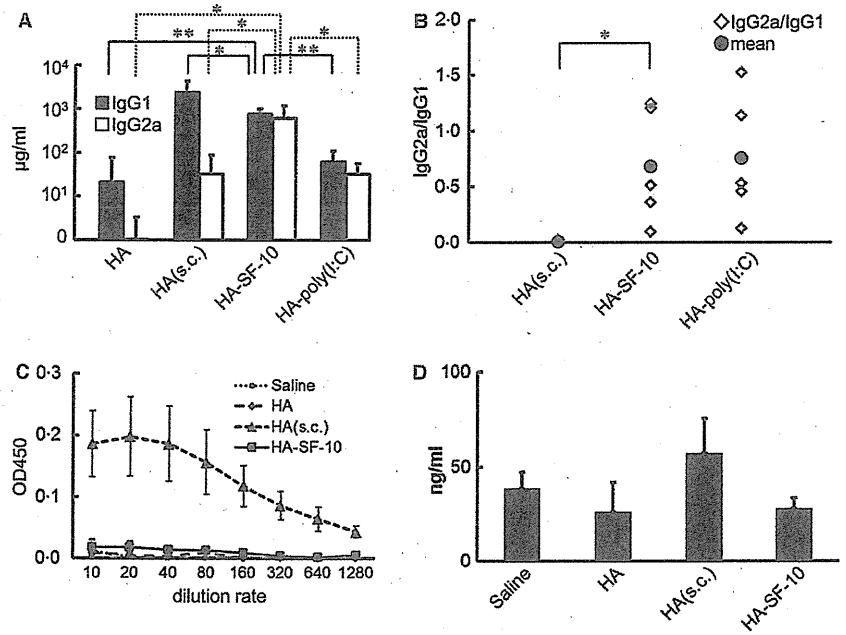


Figure 4. Induction of HA-specific Th1- and Th2-type immune responses by intranasal administration of HA–SF–10. Mice (n = 5) were immunized with intranasal HA (0·2 μg) combined with or without SF-10 (2 μg) or poly(I:C) (2 μg) three times every 2 weeks. Another group of mice received subcutaneous (s.c.) HA (0·2 μg) twice every 2 weeks. Two weeks after the last immunization, sera were collected, and anti-HA-specific IgG1, IgG2a, IgE, and total IgE levels were measured by ELISA. (A) Data are mean ± SD of IgG1 and IgG2a in sera. *P < 0·05, **P < 0·01. (B) Anti-HA-specific IgG2a/IgG1 ratio in sera of individual mice and their mean values. *P < 0·05. (C) Induced anti-HA-specific IgE levels in each animal group. Data (OD 450) are mean ± SD of each serum dilution rate. (D) Data are mean ± SD of total IgE concentrations in each animal group.

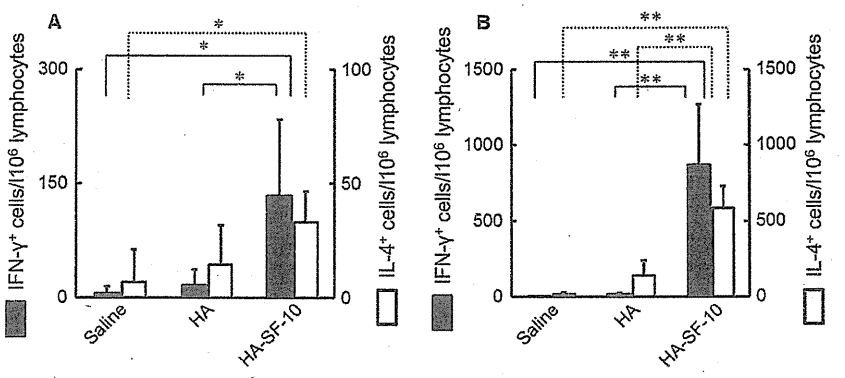


Figure 5. Induction of Th1- and Th2-type cytokines in airway lymphocytes by intranasal administration of HA–SF–10. Mice (n = 5) were immunized with intranasal HA (0·2 μg) combined with or without SF-10 (2 μg) or saline three times every 2 weeks. Two weeks after the last immunization, lymphocytes of NALT (A) and nasopharynx (B) were isolated and incubated with HA (5 μg/ml) for 16 hour. IFN-γ- and IL-4-producing lymphocytes were measured by ELISPOT. Data are mean ± SD of cytokine-producing lymphocytes per 10^6 lymphocytes in 4–5 independent experiments. *P < 0·05, **P < 0·01.

of SSF clinically: (i) Although SP-C(1–35) is an essential part of SSF, this hydrophobic peptide is insoluble in common organic solvents for large-scale manufacturing. In the present study, we identified a peptide from the SP-C-related peptides with stable adjuvanticity and solubility in methanol as a substitute for human SP-C(1–35). (ii) To further increase the antigen delivery efficacy of SSF, we increased HA binding to SSF at ≥92% by lyophilization. (iii) As HA and SSF mixing in a sonic oscillator is difficult to apply in industrial processing, we designed a processing method instead of sonication for large-scale manufacturing. (iv) As mucosal adjuvanticity of natural Surfacten or SSF was less potent than that of poly(I:C) (Table 2 and Figure 1), we increased the efficacy of SSF adjuvanticity by further modification.

In the present study, we solved four major problems of SSF stated above and developed a potent synthetic adjuvant,

SF-10. Among the active SP-C-related peptides (Tables 1 and 2), we selected K6L16 as a substitute for human SP-C(1–35), because K6L16 is soluble in methanol and expresses high adjuvanticity. To achieve more efficient interaction between HA and SSF and more efficient antigen delivery of SSF, they were mixed at 42°C, the critical temperature for surfactant lipids, for 10 minute, followed by lyophilization to remove water molecules between them, as a substitute for sonication. As shown in Table 3, lyophilization increased the binding of HA to SSF by ≥92% and markedly increased mucosal and systemic immunity. Lyophilization also seems to have two other benefits: protection against sonication-related loss of heat-labile HA antigenicity and large-scale manufacturing.

We added CVP to HA-SSF mixture at 0·5% to increase viscosity. The results showed the HA-SSF mixture prepared by lyophilization had higher adjuvanticity than that prepared by sonication (Table 3). The addition of CVP to the HA-SSF resulted in further increase in nasal-wash s-IgA production, probably due to the prolonged antigen presentation in the nasal cavity. Taken these improvements together, HA–SF–10 increased the induction of anti-HA-specific s-IgA in nasal washes and anti-HA-specific IgG in sera compared with intranasal HA-poly(I:C) and subcutaneous administration of HA (Figure 1). These data were supported by the neutralization activities in nasal washes, HI titers in serum, protective immunity, and high survival rates of animals immunized with intranasal HA–SF–10 (Figures 2 and 3, and Figure S1).

It has been reported that intranasal administration of the most potent toxin-based mucosal adjuvant of cholera toxin (CT) or *Escherichia coli* heat-labile enterotoxin (HLT) induces both nasal-wash s-IgA and serum IgG at about 3·8-fold of those by poly (I:C).[21] The data suggest that the efficacy of mucosal adjuvanticity of SF-10 is similar to that of CT and HLT, because nasal-wash s-IgA and serum IgG induced by HA–SF–10 were about 4-fold those induced by HA-poly (I:C) (Figure 1). Importantly, application of virosomal influenza vaccine adjuvanted with HLT resulted in high incidence of Bell's palsy[8] and CT-induced IgE against antigen and CT.[22] In contrast, SF-10 did not result in such adverse reactions in animal experiments. Intranasal mucosal live attenuated virus vaccines, FluMist® and FLUENZ, are currently available on the market. In a related issue, concern has been raised regarding the safety of FluMist® in young children aged<2 years with previous asthma or with recurrent wheezing.[11,12] Although not tested yet, HA–SF–10 influenza vaccine could be potentially useful in young children, because Surfacten® has been used in premature babies without significant adverse effects[13] and is known to enhance systemic and mucosal immunity in minipigs even just after weaning.[15]

For the development of effective and safe mucosal vaccine, it is important that the mucosal adjuvant induces a balanced Th1- and Th2-type cytokine response to support antigen-specific antibody production (Figure 4) without inflammatory or allergic side effects.[23,24] Figure 5 shows that intranasal immunization with HA–SF–10 elicited anti-HA-specific Th1 (IFN-γ)- and Th2(IL-4)-type cytokine responses in the nasopharynx and NALT, compared with HA and saline. HA–SF–10 also induced a balanced Th1- and Th2-type cytokine response in the airway mucosa. Of note, there was no detectable anti-HA-specific IgE and total IgE response in the sera of animals immunized intranasally with HA–SF–10. These results confirm that intranasal HA–SF–10 induces a balanced Th1- and Th2-type cytokine response without the risk of allergy.

IAV-specific $CD8^+$ cytotoxic T lymphocytes and IFN-γ-producing $CD4^+$ T lymphocytes promote clearance of IAV and recovery from infection.[5] Intranasal HA–SF–10 activated IFN-γ-producing lymphocytes in the nasopharynx and NALT and probably stimulated cellular immunity against IAV. Considered together, our results indicate that intranasal immunization with HA–SF–10 provided efficient protection against IAV infection and markedly increased survival rates even in mice with fulminant viral infection. Administration of antigen-SF-10 by other mucosal routes should be evaluated in future studies.

Acknowledgements

This study was supported in part by grants-in-aid (21249061, 22790433 and 21249061) of the Ministry of Education, Culture, Sports, and Science.

Conflict of interest

HK and DM are inventors of an applied patent related to the technology described in this study (Preparation methods of SF-10 adjuvant; application number WO 2011/108521 A1), which is owned by the University of Tokushima. All remaining authors declare no conflict of interest.

References

1 Demicheli V, Jefferson T, Rivetti D, Deeks J. Prevention and early treatment of influenza in healthy adults. Vaccine 2000; 18:957–1030.
2 Jefferson T, Rivetti D, Rivetti A, Rudin M, Di Pietrantonj C, Demicheli V. Efficacy and effectiveness of influenza vaccines in elderly people: a systematic review. Lancet 2005; 366:1165–1174.
3 Clements ML, Betts RF, Tierney EL, Murphy BR. Serum and nasal wash antibodies associated with resistance to experimental challenge with influenza A wild type virus. J Clin Microbiol 1986; 24:157–160.
4 Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. Nat Med 2005; 11:45–53.
5 Doherty PC, Topham DJ, Tripp RA, Cardin RD, Brooks JW, Stevenson PG. Effector $CD4^+$ and $CD8^+$ T-cell mechanisms in the control of respiratory virus infections. Immunol Rev 1997; 159:105–117.

6 Briscoe JH. Intranasal immunization with inactivated influenza virus vaccine in a boys' boarding school. Practitioner 1975; 214:821–826.
7 Davis SS. Nasal vaccines. Adv Drug Deliv Rev 2001; 51:21–42.
8 Mutsch M, Zhou W, Rhodes P *et al.* Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. N Engl J Med 2004; 350:896–903.
9 Mizuno D, Kimoto T, Takei T *et al.* Surfactant protein C is an essential constituent for mucosal adjuvanticity of Surfacten, acting as an antigen delivery vehicle and inducing both local and systemic immunity. Vaccine 2011; 29:5368–5378.
10 Belshe RB, Nichol KL, Black SB *et al.* Safety, efficacy, and effectiveness of live, attenuated, cold-adapted influenza vaccine in an indicated population age 5–49 years. Clin Infect Dis 2004; 39:920–927.
11 Belshe RB, Edwards KM, Vesikari T *et al.* Live attenuated versus inactivated influenza vaccine in infants and young children. N Engl J Med 2007; 356:685–696.
12 Esposito S, Montinaro V, Groppali E, Tenconi R, Semino M, Principi N. Live attenuated intranasal influenza vaccine. Hum Vaccin Immunother 2012; 8:17–20.
13 Konishi M, Chida S, Shimada S *et al.* Surfactant replacement therapy in premature babies with respiratory distress syndrome: factors affecting the response to surfactant and comparison of outcome from 1982–86 and 1987–91. Acta Paediatr Jpn 1992; 34:617–630.
14 Mizuno D, Ide-Kurihara M, Ichinomiya T, Kubo I, Kido H. Modified pulmonary surfactant is a potent adjuvant that stimulates the mucosal IgA production in response to the influenza virus antigen. J Immunol 2006; 176:1122–1130.
15 Nishino M, Mizuno D, Kimoto T *et al.* Influenza vaccine with Surfacten, a modified pulmonary surfactant, induces systemic and mucosal immune responses without side effects in minipigs. Vaccine 2009; 27:5620–5627.
16 Wright JR, Clements JA. Metabolism and turnover of lung surfactant. Am Rev Respir Dis 1987; 136:426–444.
17 Baritussio A, Pettenazzo A, Benevento M, Alberti A, Gamba P. Surfactant protein C is recycled from the alveoli to the lamellar bodies. Am J Physiol 1992; 263:607–611.
18 Takahashi E, Kataoka K, Fujii K *et al.* Attenuation of inducible respiratory immune responses by oseltamivir treatment in mice infected with influenza A virus. Microbes Infect 2010; 12:778–783.
19 Asanuma H, Inaba Y, Aizawa C, Kurata T, Tamura S. Characterization of mouse nasal lymphocytes isolated by enzymatic extraction with collagenase. J Immunol Methods 1995; 187:41–51.
20 Ichinohe T, Watanabe I, Ito A *et al.* Synthetic double-strand RNA poly (I :C) combined with mucosal vaccine protects against influenza virus infection. J Virol 2005; 79:2910–2919.
21 Asahi-Ozaki Y, Itamura S, Ichinohe T *et al.* Intranasal administration of adjuvant-combined recombinant influenza virus HA vaccine protects mice from the lethal H5N1 virus infection. Microbes Infect 2006; 8:2706–2714.
22 Tamura S, Shoji Y, Hasiguchi K, Aizawa C, Kurata T. Effects of cholera toxin adjuvant on IgE antibody response to orally or nasally administered ovalbumin. Vaccine 1994; 12:1238–1240.
23 Trumpfheller C, Caskey M, Nchinda G *et al.* The microbial mimic poly IC induces durable and protective $CD4^+$ T cell immunity together with a dendritic cell targeted vaccine. Proc Natl Acad Sci USA 2008; 105:2574–2579.
24 Tantilipikorn P, Auewarakul P. Airway allergy and viral infection. Asian Pac J Allergy Immunol 2011; 29:113–119.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. HI activity in sera of mice immunized with intranasal administration of HA–SF–10, HA and saline and with subcutaneous administration of HA.

OPEN ACCESS Freely available online

PLOS | ONE

Blood Lactate/ATP Ratio, as an Alarm Index and Real-Time Biomarker in Critical Illness

Junji Chida[1], Rie Ono[2], Kazuhiko Yamane[1], Mineyoshi Hiyoshi[1], Masaji Nishimura[2], Mutsuo Onodera[2], Emiko Nakataki[2], Koichi Shichijo[3], Masatami Matushita[3], Hiroshi Kido[1]*

1 Division of Enzyme Chemistry, Institute for Enzyme Research, The University of Tokushima, Tokushima, Japan, **2** Emergency and Critical Care Unit, Tokushima University Hospital, The University of Tokushima, Tokushima, Japan, **3** Department of Pediatrics, Tokushima Red Cross Hospital, Tokushima, Japan

Abstract

Objective: The acute physiology, age and chronic health evaluation (APACHE) II score and other related scores have been used for evaluation of illness severity in the intensive care unit (ICU), but there is still a need for real-time and sensitive prognostic biomarkers. Recently, alarmins from damaged tissues have been reported as alarm-signaling molecules. Although ATP is a member of the alarmins and its depletion in tissues closely correlates with multiple-organ failure, blood ATP level has not been evaluated in critical illness. To identify real-time prognostic biomarker of critical illness, we measured blood ATP levels and the lactate/ATP ratio (ATP-lactate energy risk score, A-LES) in critically ill patients.

Methods and Results: Blood samples were collected from 42 consecutive critically ill ICU patients and 155 healthy subjects. The prognostic values of blood ATP levels and A-LES were compared with APACHE II score. The mean ATP level (SD) in healthy subjects was 0.62 (0.19) mM with no significant age or gender differences. The median ATP level in severely ill patients at ICU admission was significantly low at 0.31 mM (interquartile range 0.25 to 0.44) than the level in moderately ill patient at 0.56 mM (0.38 to 0.70) ($P<0.01$). Assessment with ATP was further corrected by lactate and expressed as A-LES. The median A-LES was 2.7 (2.1 to 3.3) in patients with satisfactory outcome at discharge but was significantly higher in non-survivors at 38.9 (21.0 to 67.9) ($P<0.01$). Receiver operating characteristic analysis indicated that measurement of blood ATP and A-LES at ICU admission are as useful as APACHE II score for prediction of mortality.

Conclusion: Blood ATP levels and A-LES are sensitive prognostic biomarkers of mortality at ICU admission. In addition, A-LES provided further real-time evaluation score of illness severity during ICU stay particularly for critically ill patients with APACHE II scores of ≥20.0.

Citation: Chida J, Ono R, Yamane K, Hiyoshi M, Nishimura M, et al. (2013) Blood Lactate/ATP Ratio, as an Alarm Index and Real-Time Biomarker in Critical Illness. PLoS ONE 8(4): e60561. doi:10.1371/journal.pone.0060561

Editor: Andrej A. Romanovsky, St. Joseph's Hospital and Medical Center, United States of America

Received January 18, 2013; **Accepted** February 27, 2013; **Published** April 5, 2013

Funding: This research was funded by a Grant-in-Aid for Exploratory Research (No. 23791180) from JSPS and from JST (AS232Z00497F). This work was also funded by a grant from Intellectual Property Office of The University of Tokushima. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: kido@tokushima-u.ac.jp

Introduction

The recent years have witnessed a wide used of the acute physiology, age and chronic health evaluation (APACHE) II score [1], the simplified acute physiology score (SAPS) II [2] and other related scoring systems [3,4] in the evaluation of the severity of illness in the intensive care unit (ICU). These scoring systems have several drawbacks, in particular their time-consuming evaluation, some unavailable data from automatic equipments and inter-examiner discrepancies [5]. Thus, these scores are not always utilized in daily practice of many ICUs [6].

Adenosine 5′-triphosphate (ATP) is the "energy currency" of organisms and plays central roles in bioenergetics, whereby its level is used to evaluate cell viability and proliferation [7–10], cell death [11,12], and energy transmission [13]. Human typically uses about body weight of ATP over the course of the day [14]. In addition, ATP release from damaged cells and tissues has recently attracted attention, and has been reported as an alarm signal compound, alarmin [15,16]. Alarmins were originally categorized as endogenous damage-associated molecular pattern (DAMP) molecules, to separate them from exogenous pathogen-associated molecular pattern (PAMP) molecules [17], associated with overstimulation of the immune system [18]. The released ATP in serum, however, is rapidly degraded within few minutes [19] and the levels are difficult to evaluate correctly. Therefore, we can only monitor ATP levels in blood cells, as the net value of intracellular ATP production and ATP degradation and/or release.

The three main pathways to generate ATP in eukaryotic organisms are glycolysis, the citric acid cycle/oxidative phosphorylation and fatty acid β-oxidation. Once ATP generation in the mitochondria is impaired in various diseases, energy source metabolites, such as carbohydrate metabolites and fatty acid metabolites are converted to and stored as lactate and ketone bodies, respectively. In fact, hyperlactatemia develops in nearly half of patients admitted to the ICU, and presentation with or development of hyperlactatemia is associated with a significant

increase in the incidence of organ failure, metabolic dysfunction and mortality [20]. To find real-time and reliable biomarker(s) for the progression state of critical illness, we evaluated blood ATP levels in combination with serum lactate levels as a new alarm reporter in critical illness and the values were compared with the APACHE II score.

Critically ill patients with multiple organ failure (MOF) and septic non-survivors show a decrease in mitochondrial activity and ATP production, and increase in lactate concentrations in leg muscles [21–23]. In addition, we recently demonstrated that influenza A virus infection triggers MOF and acute myocarditis with ATP depletion in mice as well as impairment of mitochondrial membrane potential in cardiomyoblasts [24]. Blood ATP depletion was also identified in children with influenza-associated acute encephalopathy and in patients with mitochondrial diseases [25].

In the measurement of ATP levels in various tissues and blood, we recently found that the chaotropic ATP extraction reagents recommended in the commercially available assay kits so far (e.g., trichloroacetic acid, perchloric acid and ethylene glycol), are useful only for materials with relatively low protein concentrations, but not suitable for tissues with high protein concentrations. ATP is co-precipitated with insoluble protein during homogenization in high protein concentrations. Accordingly, we improved the ATP extraction efficiency from tissues and cells using a novel phenol-based extraction reagent [26].

The present study was designed to determine the control gender-specific blood ATP levels measured in healthy individuals of various ages, using our highly reliable extraction method. We then used the "normal" blood ATP levels of the control to evaluate the blood ATP levels and lactate (mM)/ATP (mM) ratio (expressed as the ATP-lactate energy risk score, A-LES) in patients admitted to the ICU. The A-LES was used as a real-time prognostic alarm biomarker and the values were compared with the APACHE II scores.

Table 1. Whole blood ATP, lactate and A-LES levels in healthy subjects.

Age (years)	Sex	Lactate (mM)	ATP (mM)	A-LES
0 to 19	Males (*n* = 6)	1.43±1.07	0.71±0.11	1.50±1.46
	Females (*n* = 7)	<1.69±1.06	0.64±0.13	<2.88±1.70
20 to 29	Males (*n* = 11)	1.48±0.38	0.79±0.18	1.99±0.61
	Females (*n* = 10)	1.03±0.25	0.71±0.20	1.40±0.48
30 to 39	Males (*n* = 15)	<1.38±0.47	0.68±0.11	<2.05±0.84
	Females (*n* = 8)	1.20±0.25	0.79±0.19	1.60±0.47
40 to 49	Males (*n* = 12)	1.46±0.39	0.83±0.25	1.90±0.73
	Females (*n* = 12)	<1.05±0.35	0.67±0.17	<1.63±0.60
50 to 59	Males (*n* = 9)	<1.38±0.48	0.64±0.19	<2.17±0.45
	Females (*n* = 4)	<0.81±0.03	0.52±0.12	<1.61±0.32
60 to 69	Males (*n* = 8)	<1.05±0.27	0.52±0.10	<2.04±0.54
	Females (*n* = 11)	<0.81±0.03	0.47±0.05	<1.73±0.18
70 to 92	Males (*n* = 13)	<0.99±0.23	0.47±0.06	<2.14±0.62
	Females (*n* = 29)	<1.00±0.36	0.46±0.05	<2.18±0.73
0 to 92	Males (*n* = 74)	<1.31±0.49	0.66±0.20	<2.05±0.73
	Females (*n* = 81)	<1.08±0.49	0.57±0.17	<1.96±0.91
	Total (*n* = 155)	<1.19±0.50	0.62±0.19	<2.00±0.83

Data are mean±SD. *n* = number of healthy subjects. Blood lactate levels below the limit of measurement (<0.8 mM) are reported as <0.8 mM.
doi:10.1371/journal.pone.0060561.t001

Materials and Methods

Ethics Statement

For clinical studies, written informed consent was obtained directly from each study participant or their legal representative before enrolment. Also, all healthy individuals gave assent if able to understand, and their parents or guardians gave written informed consent and permission to participate in this study. Permission to perform scientific studies and ethical approval of the study protocol were granted the Ethics Committee of Tokushima University Hospital (Permit Number: #901). The study was conducted under the supervision of the physicians involved (MN, RO, MO, EN, KS and MM), and patients were advised of risks, benefits and the right to withdraw from further involvement in the study at any point without repercussions. All data, particularly patient identifying data, were physically and electronically secured throughout the study.

Patients

We evaluated 42 consecutive patients admitted to ICU from November 2009 to November 2010. All patients received early goal-directed therapy according to a standard protocol that emphasized adequate volume administration, appropriate therapeutic drug administration, and optimal oxygen delivery. The study also included 155 healthy individuals free from any acute or chronic illness.

Blood Collection

Blood was withdrawn from the antecubital vein of healthy individuals into either Vacutainer tubes (BD vacutainer; Becton Dickinson Diagnostics, Tokyo, Japan) or syringes containing either ethylenediaminetetraacetic acid (EDTA) or sodium heparin. For ICU patients, the blood samples were usually collected from the arterial line into EDTA vacutainers, but in some cases collected from the antecubital vein or central vein. In each patient, blood was sampled at serial time points during the ICU stay. After withdrawal of 5.0 mL of arterial/venous blood, the sample was transferred to a 15.0-mL Falcon tube. Blood gas data, such as PO_2 and PCO_2, and data of total hemoglobin (tHb), blood glucose (BG), and lactate were monitored by a blood gas analyzer (Blood Gas System 860; Bayer Diagnostics, Tokyo, Japan). Blood aliquots (0.1 mL) were added to 1.3 mL of Tris-EDTA-saturated phenol (phenol-TE) ATP extraction reagents (AMERIC-ATP kit; Wako Pure Chemical Industries, Osaka, Japan), thoroughly shaken for 20 seconds and then stored at −20°C until use.

Measurement of Blood ATP

Blood ATP levels were measured by the firefly bioluminescence assay kit (AMERIC-ATP kit; Wako Pure Chemical Industries, Osaka, Japan) according to the protocol supplied by the manufacturer or as described previously [26]. Briefly, the extracted blood sample was shaken and centrifuged (10,000 × *g*, 5 minutes at 4°C) to achieve phase separation; 50 μL of the upper aqueous phases was diluted 10,000-fold with deionized water. Then 10 μL of this diluted extract was injected into 90 μL of luciferin/luciferase mixture, and the bioluminescence product was immediately measured by a luminometer (GloMax-96 Microplate Luminometer; Promega, Tokyo, Japan). Blood ATP level (mM) in each sample was calculated from the calibration curve.

Table 2. Comparison of blood lactate and ATP levels in radial arterial (A), pulmonary arterial (PA), and central venous (CV) blood.

Patients no./Time (h)*	Lactate (mM)			ATP (mM)			A-LES		
	A	PA	CV	A	PA	CV	A	PA	CV
07/0	1.79	1.69	1.97	0.70	0.72	0.73	2.56	2.35	2.70
07/3	1.95	1.97	2.16	0.83	0.83	0.80	2.35	2.37	2.70
07/6	2.09	2.01	2.01	0.34	0.35	0.34	6.15	5.74	5.91
07/24	1.68	1.69	1.63	2.88	2.88	2.76	2.47	2.49	2.60
08/3	2.88	2.88	2.76	0.96	0.99	0.98	3.00	2.91	2.82
08/6	2.47	2.49	2.60	0.55	0.53	0.55	4.49	4.70	4.73
09/0	2.29	2.43	2.73	0.36	0.40	0.37	6.36	6.08	7.38
10/3	1.64	–	1.51	0.33	–	0.34	4.97	–	4.44
18/0	6.10	5.48	5.75	0.42	0.41	0.41	14.52	13.37	14.02
19/0	5.39	5.72	5.91	0.35	0.38	0.38	15.40	15.03	15.55
21/0	1.47	1.30	1.37	0.53	0.57	0.53	2.77	2.28	2.58

*Time period (h, hours) after ICU admission.
doi:10.1371/journal.pone.0060561.t002

Calculation of APACHE II Score and A-LES

The severity of illness was evaluated in each patient within the first 24 hours of ICU admission using the APACHE II score [1,27]. The score was also determined every 24 hours during the ICU stay. The A-LES score, representing [serum lactate (mM)/blood ATP (mM)], was determined for each patient during the ICU stay.

Table 3. Patient demographics and clinical findings.

Patient demographics	
n	42
Age, years	68 (34 to 79)
Sex, % male:% female	52:48
First 24-h APACHE II score	18 (13 to 25)
Duration of ICU stay, days	3 (1 to 10)
Diagnosis at admission	No. of patients (%)
Post-cardiovascular surgery	9 (21.4)
Septic shock	7 (16.7)
Acute coronary syndrome	4 (9.5)
Congestive heart failure	4 (9.5)
Infective endocarditis	3 (7.1)
Liver cirrhosis	3 (7.1)
Pneumonia	2 (4.8)
Interstitial pneumonia	2 (4.8)
Stroke	2 (4.8)
Others	6 (14.3)
Blood biochemical tests*	
Glucose, mg/dL	155 (125 to 227)
Lactate, mM	2.72 (1.92 to 5.78)
ATP, mM	0.38 (0.29 to 0.56)
A-LES	8.00 (4.74 to 13.37)
Hemoglobin, g/dL	10.4 (9.1 to 11.1)
Leukocyte count,/μL	12,700 (9,300 to 17,100)
Platelet count, x1,000/μL	114 (46 to 182)

Data are median (interquartile range) and number of patients (%).
*Data represent results of analysis of samples taken at admission to the ICU.
doi:10.1371/journal.pone.0060561.t003

Statistical Analysis

ICU patients were divided into two groups based on the severity of illness on admission: moderately ill patients (APACHE II score <20) and severely ill patients (APACHE II score ≥ 20). The outcome of the patients was divided into two categories: survival and non-survival. Data were analyzed for statistical significance across groups using nonparametric Mann-Whitney's U test. Correlations were calculated by determining Spearman's rank correlation coefficient (r_s). P values less than 0.05 were considered statistically significant. Receiver operating characteristic (ROC) curves were constructed using Microsoft Excel software (Microsoft Corporation, Redmond, WA) add-in Ekuseru-Toukei 2010 version 1.10 (Social Survey, Research Information Co.) to evaluate the accuracy of risk prediction comparing the calculated mortality with the actual deaths.

Results

ATP, Lactate and A-LES Values in Healthy Subjects

At the beginning of the study, we determined the levels of ATP by the new phenol-TE extraction method [26] and lactate in venous blood samples from 155 healthy males and females (age, range 0 to 92 years). The measured levels showed normal distribution pattern, with a mean (SD) value of 0.62 mM (0.19) (Table 1). There was no significant sex difference in ATP level. The ATP levels tended to be slightly lower in subjects aged ≥ 60 years than those in younger subjects, although the difference was not significant. The mean blood lactate level under resting-state condition was <1.19 mM (<0.50) in the healthy group, with no significant age or gender difference. The mean A-LES (SD) was <2.00 (0.83) with no significant age or gender difference in the control subjects.

Similar ATP and Lactate Levels in Arterial and Central Venous Blood

Table 2 shows the data of ATP, lactate and A-LES levels in arterial and central venous blood of 7 representative ICU patients.

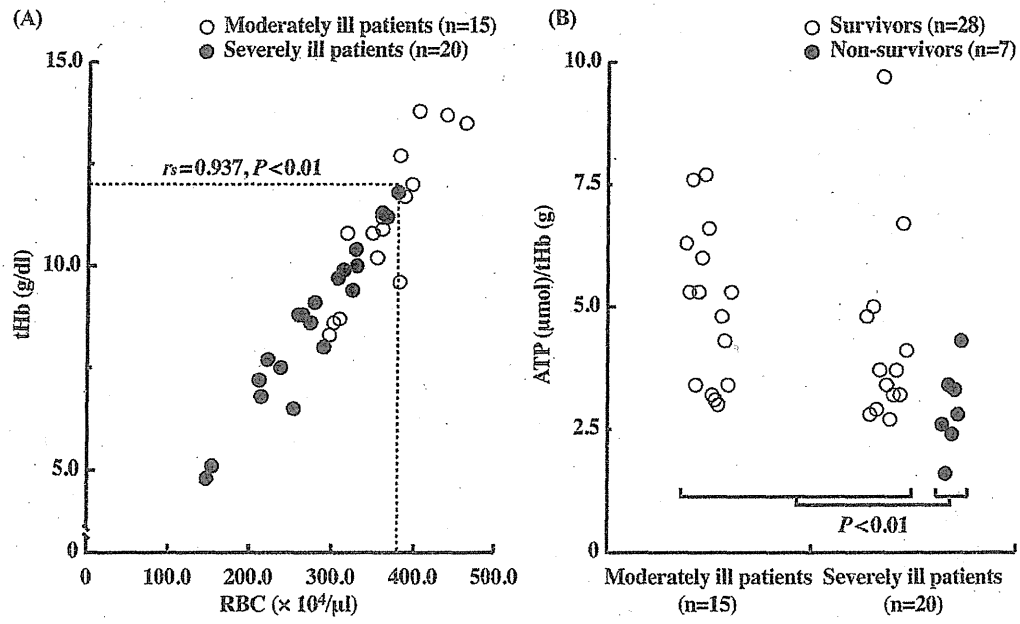


Figure 1. Blood ATP levels correlate with RBC count and ATP levels in ICU patients. (A) Correlation between tHb (total hemoglobin) and RBC count in the available data of moderately (n = 15) and severely ill patients (n = 20) during the ICU stay. The *Dotted lines* represent the lower limits of tHb and RBC count of healthy subjects. (○): moderately ill patients, (●) severely ill patients. (B) ATP concentration in whole blood, expressed as micromoles ATP per gram total hemoglobin (μmol/g tHb) in ICU patients. (○): survivors, (●) non-survivors. The values of ATP/tHb in non-survivors were significantly lower than those in survivors ($P<0.01$).
doi:10.1371/journal.pone.0060561.g001

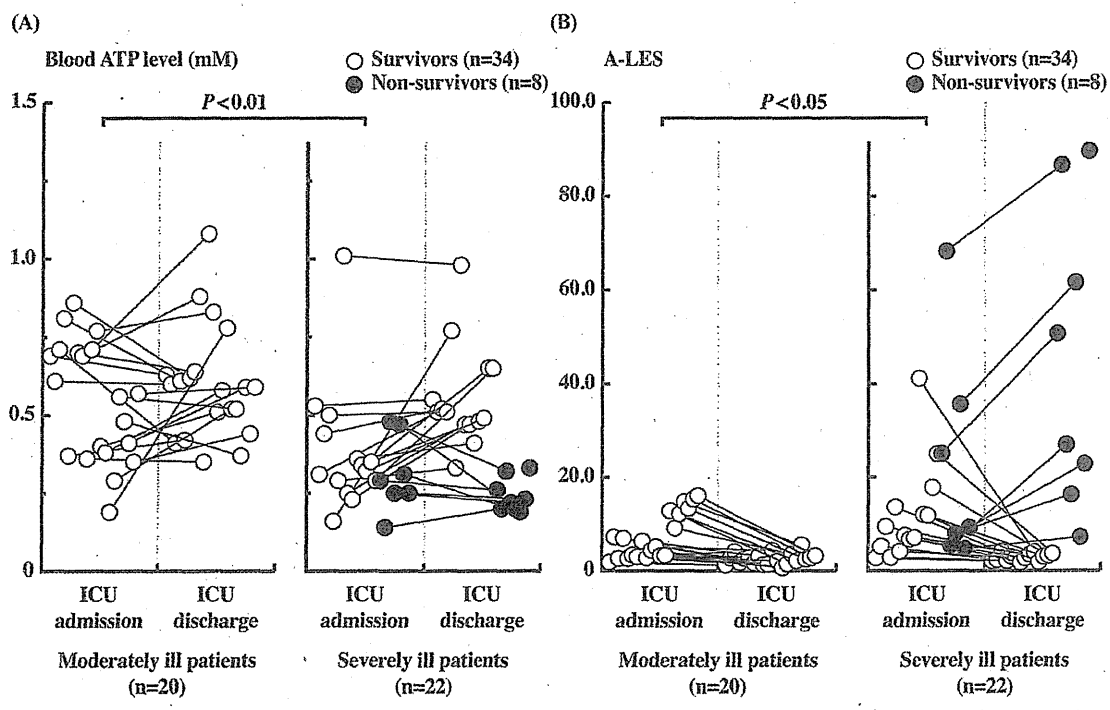


Figure 2. Changes in blood ATP and A-LES levels in moderately and severely ill patients. (A) Blood ATP levels and (B) A-LES values at ICU admission and ICU discharge. Symbols are paired data of individual patients. (○): survivors, (●) non-survivors. Levels of ATP and A-LES at ICU admission in severely ill patients were significantly lower than those in moderately ill patients ($P<0.01$ for ATP and $P<0.05$ for A-LES).
doi:10.1371/journal.pone.0060561.g002

Table 4. Comparison of changes in blood ATP level, A-LES, and APACHE II score during the course of ICU stay.

		ICU admission	ICU discharge
Moderately ill patients ($n=20$)	ATP, mM	0.56 (0.38 to 0.70)	0.53 (0.44 to 0.61)
	A-LES	6.7 (3.1 to 12.4)	2.7 (1.8 to 3.2)
	APACHE II score	12.5 (9.0 to14.3)	11.5 (9.0 to 13.3)
Severely ill patients ($n=22$)	ATP, mM	0.31 (0.25 to 0.44)**	0.45 (0.28 to 0.52) *
	A-LES	9.5 (6.8 to 17.9)*	3.6 (2.7 to 21.0) **
	APACHE II score	25.0 (20.0 to 31.0)**	21.0 (16.5 to 25.0)**
Septic shock patients ($n=7$)	ATP, mM	0.29 (0.27 to 0.33)**	0.48 (0.33 to 0.57)
	A-LES	12.1 (10.7 to 19.4)*	3.1 (2.9 to 9.6) **
	APACHE II score	28.0 (22.0 to 36.0)**	15.0 (14.0 to 19.0)**
Survivors ($n=34$)	ATP, mM	0.41 (0.33 to 0.60)	0.52 (0.45 to 0.62)
	A-LES	7.4 (3.7 to 12.7)	2.7 (2.1 to 3.3)
	APACHE II score	15.5 (11.3 to 22.3)	13.5 (11.0 to 16.0)
Non-survivors ($n=8$)	ATP, mM	0.29 (0.25 to 0.39)#	0.23 (0.20 to 0.28)##
	A-LES	9.3 (6.7 to 30.4)#	38.9 (21.0 to 67.9)##
	APACHE II score	25.0 (20.0–31.0)##	21.0 (16.5 to 25.0)##
Total ($n=42$)	ATP, mM	0.38 (0.29 to 0.56)	0.51 (0.36 to 0.59)
	A-LES	8.0 (4.7 to 13.4)	3.0 (2.1 to 4.2)
	APACHE II score	19.0 (13.0 to 25.0)	15.0 (12.3 to 21.5)

Data are median (interquartile range). n = number of healthy subjects.
*$P<0.05$,
**$P<0.01$ versus moderately ill patients: Mann-Whitney's U-test.
#$P<0.05$,
##$P<0.01$ versus survivors: Mann-Whitney's U-test.
doi:10.1371/journal.pone.0060561.t004

Regression analysis to validate the correlation among ATP, lactate and A-LES levels in arterial and central venous blood showed almost perfect correlation with high correlation coefficients: $r_s=1.00$ for ATP, $r_s=0.98$ for lactate and $r_s=1.00$ for A-LES between blood collected from radial and pulmonary arteries ($P<0.001$); and $r_s=1.00$ for ATP, $r_s=0.97$ for lactate and $r_s=0.99$ for A-LES between radial artery and central venous blood ($P<0.001$). The results were consistent with the previous report of equivalent lactate levels in blood samples from peripheral vein, pulmonary artery and central vein [28].

ATP, Lactate Levels and A-LES in ICU Patients

Next, we measured the levels of blood ATP and serum lactate and calculated the A-LES in 42 patients admitted to the ICU. The levels of these parameters showed skewed distribution. Table 3 lists the demographic data while Tables S1, S2 and S3 list the individual data and clinical characteristics. The major diagnosis on admission was post-cardiovascular surgery (21.4%), followed by septic shock (16.7%). The median values (interquartile range) of ATP, lactate and A-LES were 0.38 mM (0.29 to 0.56), 2.72 mM (1.92 to 5.78 mM) and 8.00 (4.74 to 13.37), respectively, and the levels were all within the abnormal range compared to those in healthy subjects (Table 1).

Blood ATP Levels Normalized by Total Hemoglobin in ICU Patients

Blood cells are diluted by transfusion and low red blood cell (RBC) count is usually found in patients with advanced disease, resulting in low blood ATP levels in severely ill patients. Since the major source of ATP in blood is RBC, we determined RBC count and total hemoglobin (tHb) in moderately ill patients (APACHE II score <20) and severely ill patients (APACHE II score ≥20) during ICU admission. The RBC count correlated with tHb level ($r_s=0.937$, $P<0.01$), and was significantly lower in severely ill patients than moderately ill patients ($P<0.01$) (Figure 1A). Blood ATP levels normalized by tHb levels (the ATP/tHb) were significantly lower in non-survivors than those in survivors ($P<0.01$) (Figure 1B).

Changes in Blood ATP and A-LES Levels in Critically Ill Patients

To identify a sensitive and real-time prognostic biomarker of critical illness, we evaluated the levels of ATP and A-LES in 42 patients during critical illness (Figure 2). ATP levels at ICU admission were significantly lower in severely ill patients than in moderately ill patients ($P<0.01$) (Figure 2A): the median ATP level was 0.31 mM (0.25 to 0.44) in severely ill patients and 0.56 mM (0.38 to 0.70) in moderately ill patients at ICU admission (Table 4). Notably, the median ATP level of 7 patients with septic shock on admission was low at 0.29 mM (0.27 to 0.33), which was significantly lower ($P<0.01$) than the level in moderately ill patients (Tables 4 and S2). The ATP levels generally recovered during ICU stay in large numbers of survivors. Furthermore, the median ATP level in non-survivors [0.23 mM (0.20 to 0.28)] was significantly lower than that of survivors [0.52 mM (0.45 to 0.62)] at ICU discharge ($P<0.01$) (Table 4).

In contrast to the changes in blood ATP levels during ICU admission, the change in A-LES was clearer particularly in severely ill patients and non-survivors (Figure 2B). The A-LES decreased in all survivors in both moderately and severely ill patients without exception and the median A-LES of survivors was 2.7 (2.1 to 3.3) at ICU discharge (Table 4). In contrast, the median

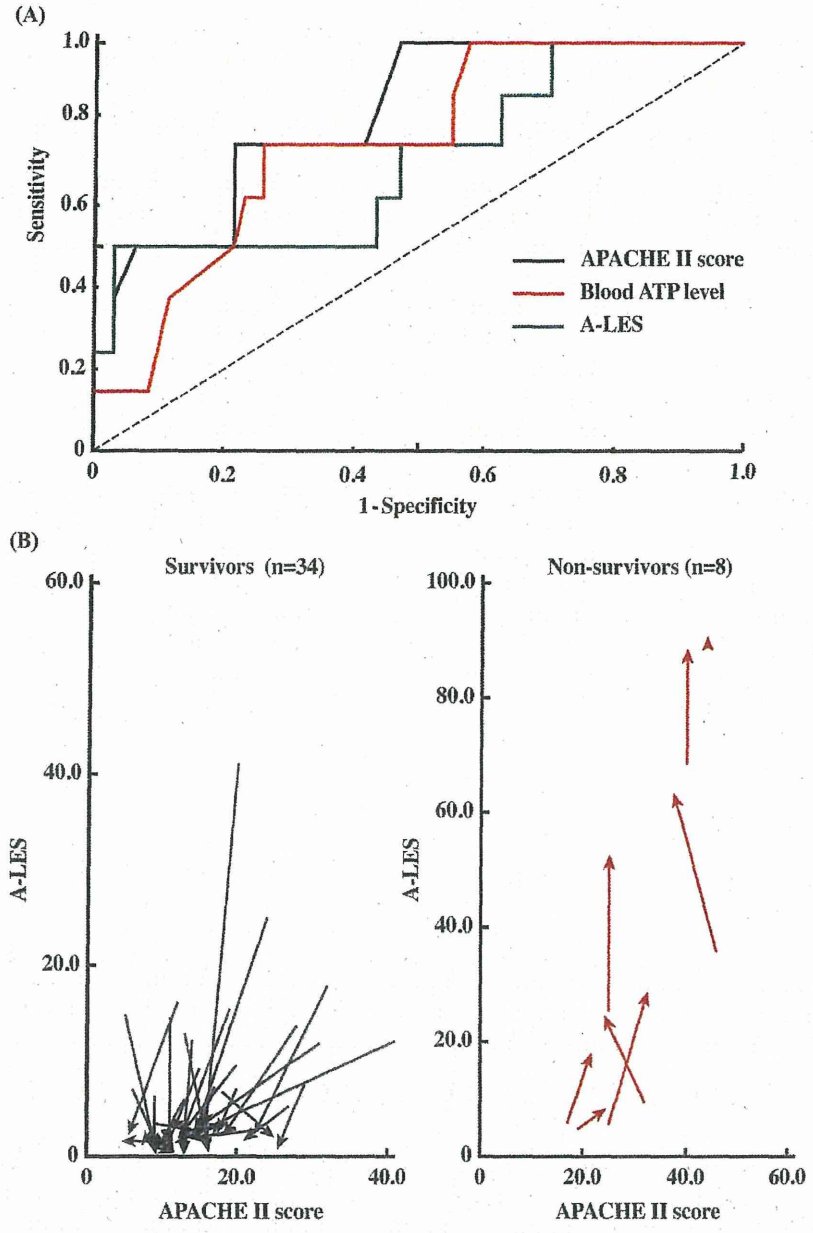


Figure 3. Relationship between APACHE II score and A-LES. (A) ROC analysis in prediction of mortality at the time of ICU admission. Dotted diagonal line = no discrimination. (B) Correlation between changes in APACHE II score and A-LES score during ICU stay in survivors and non-survivors. Data are values measured at two time points: at initial ICU admission and ICU discharge. Arrows indicate from ICU admission to discharge.
doi:10.1371/journal.pone.0060561.g003

A-LES in non-survivors at ICU discharge was 38.9 (21.0 to 67.9), which was significantly higher than the value of survivors ($P<0.01$) (Table 4). These results indicate that A-LES is a highly sensitive prognostic marker of critical illness.

Evaluation of A-LES as a Prognostic Marker and Correlation with APACHE II in ICU Patients

To evaluate A-LES and ATP levels of patients at the time of ICU admission for prediction of mortality, ROC analysis was performed (Figure 3A and Table 5). The values of the area under ROC curve (AUC) for APACHE II, ATP and A-LES were of similar range (>0.5) and measured 0.83, 0.75 and 0.71, respectively, indicating that ATP level and A-LES are as useful as APACHE II score for prediction of mortality.

Figure 3B shows changes in A-LES and APACHE II scores measured during ICU admission. Although APACHE II scores did not sensitively express the change in the critical state of ICU patients, particularly patients with severe illness (APACHE II range, ≥20.0), A-LES reflected well the change in the critical state. Markedly high A-LES values (up to 89.7) in non-survivors and low values in all survivors were observed during ICU admission. These

Table 5. ROC analysis for prediction of mortality in 42 patients at ICU admission.

Variable	AUC	Cut-off value	Sensitivity/specificity (%)
APACHE II score	0.83	>20	71/59
A-LES	0.71	>20	43/94
Blood ATP levels*	0.75	>3	71/74

*The reciprocal of blood ATP levels was used for ROC analysis.
doi:10.1371/journal.pone.0060561.t005

results indicate that A-LES does not only predict mortality at the time of ICU admission in a manner similar to APACHE II, but also provides a sensitive evaluation score of change in illness severity during ICU admission. Figure 4 illustrates the changes in blood ATP, A-LES and APACHE II during ICU admission of representative patients of the three groups (moderately ill patients on admission and discharge, severely ill patients on admission and discharge, and severely ill patients on admission who died during admission). Among the three parameters, A-LES provided the best prognostic information; almost all patients with satisfactory outcome at discharge had A-LES of <5.49, while A-LES during admission was >20.0 in non-survivors.

Discussion

Risk prediction is an important issue in intensive care. The APACHE II score is used as a severity score during the first 24 hours of ICU admission while the SOFA score was developed to estimate morbidity during ICU stay. Although several clinical and laboratory parameters have been evaluated for the prediction of mortality during ICU stay, real-time and easily measurable prognostic biomarker(s) are desirable. During critical illness, the serum contains various PAMP molecules, particularly in severe infection [29], DAMP molecules released by stressed or damaged tissues [17] and host cellular response molecules with regulatory function against these PAMP and DAMP molecules. Among the molecules, alarm reporter(s) might be an important prognostic value. Recently, ATP released from damaged tissues has been classified as a danger signal, alarmin, which induces proinflammatory cytokines, but it is rapidly degraded within few minutes by ecto-ATPase [19]. Although released ATP cannot be analyzed accurately, cellular ATP levels, including blood cell ATP levels, can be measured easily, representing the sum of ATP production and ATP degradation and/or release.

We recently established an efficient and improved phenol-based ATP extraction method instead of the chaotropic extraction reagents recommended in the commercially available assay kit, which involves co-precipitation of ATP with insoluble proteins during homogenization and extraction [26]. In the present study, we measured blood ATP levels by a phenol-based extraction reagent and reported the "normal" blood ATP levels in 155 healthy individuals ranging in age from 0 to 92 years. The mean blood ATP level (SD) in healthy subjects was 0.62 (0.19) mM. Age and gender had no significant effect on ATP level, although the values tended to decrease with advancing age, particularly over 60 years of age, and values in males were slightly higher than those in females with some exceptions, probably because of age and gender differences in the number of red blood cells (Table 1).

The present study established the clinical utility of a sensitive and real-time alarm index, A-LES, which consists of ATP and lactate. The median A-LES of patients with satisfactory outcome

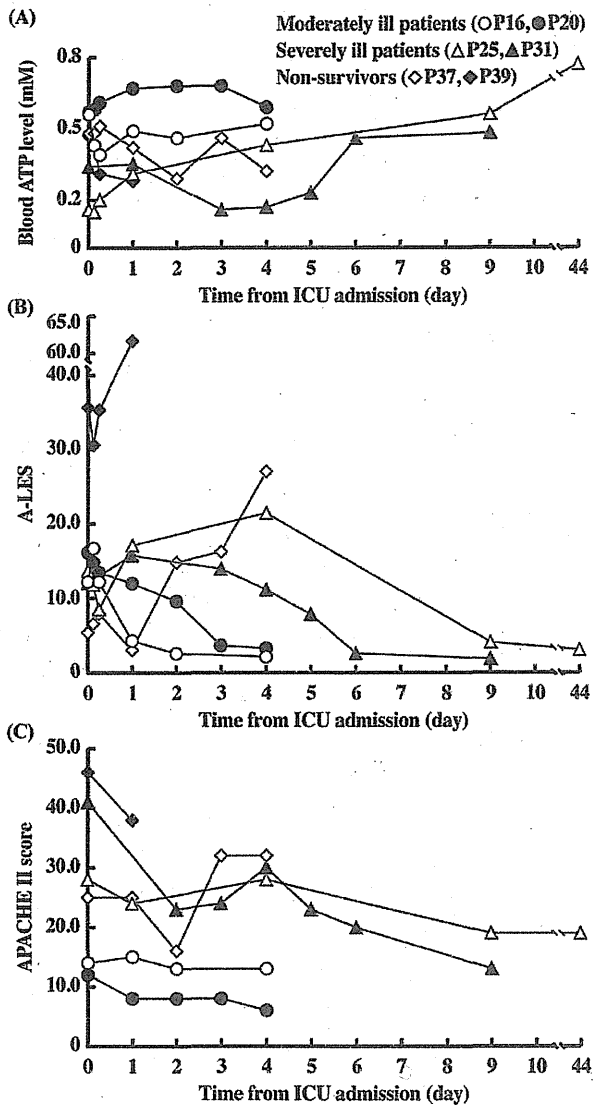


Figure 4. Changes in blood ATP level, A-LES, and APACHE II score of representative examples during the course of ICU stay. Serial changes in the levels of (A) blood ATP level, (B) A-LES, and (C) APACHE II score from admission to discharge from the ICU according to the severity of clinical condition (moderately and severely ill patients and non-survivors).
doi:10.1371/journal.pone.0060561.g004

at ICU discharge was 2.7 (2.1 to 3.3) and that of non-survivors was significantly high at 38.9 (21.0 to 67.9) ($P<0.01$) (Table 4). The A-LES was <20 in almost all moderately ill patients during ICU stay and was ≥20 in a large proportion of the non-survivors (Table 4 and Figure 3B). The results suggest that 20 is a critical cut-off value of A-LES for prediction of survival in the limited number of patients in this study. The change in A-LES ranged from 3.05 to 89.73 in non-survivors whereas the change in APACHE II score was ranged only from 17 to 46 (Table S3 and Figure 3B). These results suggest that A-LES provides better prognostic information compared to the APACHE II score. In addition to the value of A-LES during ICU admission, the AUC values for APACHE II, ATP and A-LES (Figure 3A) indicate that simple measurement of

blood ATP and A-LES at the time of ICU admission predicts mortality in a manner similar to APACHE II, the complex and time-consuming evaluation method.

Although ATP released from damaged cells and tissues [17,19] and from RBC in response to low PO_2, low pH and/or mechanical deformation [30,31] is an emergency signal alarmin, the levels in serum are difficult to monitor because the released ATP is rapidly degraded within few minutes [19]. The major source of ATP in the blood is the RBC and blood ATP levels change hourly with changes in energy and vital status of patients. Therefore, A-LES level is a real-time and sensitive biomarker of vital sign and a marker for prediction of mortality in critically ill ICU patients.

Conclusions

This is the first report on blood ATP levels and A-LES as an alarm biomarker for critical illness during ICU stay and for the prediction of outcome of clinically ill patients at the time of ICU admission, similar to APACHE II score. In addition, A-LES provided further evaluation score of illness severity during ICU stay in addition to APACHE II particularly for those critically ill patients with a score of ≥20.0.

Supporting Information

Table S1 Demographics and clinical details of moderately ill patients (APACHE II score<20). *Blood samples were collected at D0 = ICU day 0 (ICU admission), D1 = ICU day 1 (discharge or death from ICU within 24 hours) and D4 = ICU day 4 (discharge or death from ICU within 4 days). # Blood samples were collected from arterial blood (A) or central venous blood (CV). tHb = total hemoglobin; BS = blood sugar.
(DOC)

Table S2 Demographics and clinical details of severely ill patients (APACHE II score ≥20). *Blood samples were collected at D0 = ICU day 0 (ICU admission), D1 = ICU day 1 (discharge or death from ICU within 24 hours) and D4 = ICU day 4 (discharge or death from ICU within 4 days). # Blood samples were collected from arterial blood (A) or venous blood (V). For abbreviations, see Table S1.
(DOC)

Table S3 Demographics and clinical details of non-survivors. *Blood samples were collected at D0 = ICU day 0 (ICU admission), D1 = ICU day 1 (discharge or death from ICU within 24 hours) and D4 = ICU day 4 (discharge or death from ICU within 4 days). # Blood samples were collected from arterial blood (A). For abbreviations, see Table S1.
(DOC)

Acknowledgments

The authors thank Naoko Masumoto and Miyoko Yamaguchi for the excellent technical assistance.

Author Contributions

Conceived and designed the experiments: JC HK MN. Performed the experiments: RO MO EN KS MM KY. Analyzed the data: JC MH. Contributed reagents/materials/analysis tools: JC. Wrote the paper: JC HK.

References

1. Knaus WA, Draper EA, Wagner DP, Zimmerman JE (1985) APACHE II: a severity of disease classification system. Crit Care Med 13: 818–829.
2. Le Gall JR, Loirat P, Alperovitch A, Glaser P, Granthil C, et al. (1984) A simplified acute physiology score for ICU patients. Crit Care Med 12: 975–977.
3. Vincent JL, Moreno R, Takala J, Willatts S, De Mendonca A, et al. (1996) The SOFA (Sepsis-related Organ Failure Assessment) score to describe organ dysfunction/failure. On behalf of the Working Group on Sepsis-Related Problems of the European Society of Intensive Care Medicine. Intensive Care Med 22: 707–710.
4. Teasdale G, Jennett B (1974) Assessment of coma and impaired consciousness. A practical scale. Lancet 2: 81–84.
5. Holtfreter B, Bandt C, Kuhn SO, Grunwald U, Lehmann C, et al. (2006) Serum osmolality and outcome in intensive care unit patients. Acta Anaesthesiol Scand 50: 970–977.
6. Schonhöfer B, Lefering R, Suchi S, Köhler D (2002) Survey of evaluation of scoring systems by intensive care physicians. Intensivmed 39: 240–245.
7. Ishizaka A, Tono-Oka T, Matsumoto S (1984) Evaluation of the proliferative response of lymphocytes by measurement of intracellular ATP. J Immunol Methods 72: 127–132.
8. Garewal HS, Ahmann FR, Schifman RB, Celniker A (1986) ATP assay: ability to distinguish cytostatic from cytocidal anticancer drug effects. J Natl Cancer Inst 77: 1039–1045.
9. Sevin BU, Peng ZL, Perras JP, Ganjei P, Penalver M, et al. (1988) Application of an ATP-bioluminescence assay in human tumor chemosensitivity testing. Gynecol Oncol 31: 191–204.
10. Crouch SP, Kozlowski R, Slater KJ, Fletcher J (1993) The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. J Immunol Methods 160: 81–88.
11. Garland JM, Halestrap A (1997) Energy metabolism during apoptosis. Bcl-2 promotes survival in hematopoietic cells induced to apoptosis by growth factor withdrawal by stabilizing a form of metabolic arrest. J Biol Chem 272: 4680–4688.
12. Nakamura N, Wada Y (2000) Properties of DNA fragmentation activity generated by ATP depletion. Cell Death Differ 7: 477–484.
13. Cook SP, McCleskey EW (2000) ATP, pain and a full bladder. *Nature*; 407: 951–952.
14. Törnroth-Horsefield S, Neutze R (2008) Opening and closing the metabolite gate. Proc Natl Acad Sci U.S.A. 105: 19565–19566.
15. Manson J, Thiemermann C, Brohi K (2012). Trauma alarmins as activators of damage-induced inflammation. Br J Surg 99: 12–20.
16. Ellsworth ML, Ellis CG, Goldman D, Stephenson AH, Dietrich HH, et al. (2009) Erythrocytes: oxygen sensors and modulators of vascular tone. Physiology 24: 107–116.
17. Oppenheim JJ, Yang D (2005) Alarmins: chemotactic activators of immune responses. Curr Opin Immunol 17: 359–365.
18. Lotze MT, Zeh HJ, Rubartelli A, Sparvero LJ, Amoscato AA, et al. (2007) The grateful death: damage-associated molecular pattern molecules and reduction/oxidation regulate immunity. Immunol Rev 220: 60–81.
19. Kobayashi T, Kouzaki H, Kita H (2010) Human eosinophils recognize endogenous danger signal crystalline uric acid and produce proinflammatory cytokines mediated by autocrine ATP. J Immunol 184: 6350–6358.
20. Robinson K, Kongable GL (2010) Lactate in critical illness-implications for monitoring. US Respiratory Disease 6: 52–55.
21. Fredriksson K, Hammarqvist F, Strigård K, Hultenby K, Ljungqvist O, et al. (2006) Derangements in mitochondrial metabolism in intercostal and leg muscle of critically ill patients with sepsis-induced multiple organ failure. Am J Physiol Endocrinol Metab 291: E1044–E1050.
22. Brealey D, Brand M, Hargreaves I, Heales S, Land J, et al. (2002) Association between mitochondrial dysfunction and severity and outcome of septic shock. Lancet 360: 219–223.
23. Carré JE, Orban JC, Re L, Felsmann K, Iffert W, et al. (2010) Survival in critical illness is associated with early activation of mitochondrial biogenesis. Am J Respir Crit Care Med 182: 745–751.
24. Pan HY, Yamada H, Chida J, Wang S, Yano M, et al. (2011) Up-regulation of ectopic trypsins in the myocardium by influenza A virus infection triggers acute myocarditis. Cardiovasc Res 89: 604–613.
25. Kubota M, Chida J, Hoshino H, Ozawa H, Koide A, et al. (2012) Thermolabile CPT II variants and low blood ATP levels are closely related to severity of acute encephalopathy in Japanese children. Brain Dev 34: 20–27.
26. Chida J, Yamane K, Takei T, Kido H (2012) An efficient extraction method for quantitation of adenosine triphosphate in mammalian tissues and cells. Anal Chim Acta 727: 8–12.
27. Knaus WA, Draper EA, Wagner DP, Zimmerman JE (1986) An evaluation of outcome from intensive care in major medical centers. Ann Intern Med 104: 410–418.
28. Weil MH, Michaels S, Rackow EC (1987) Comparison of blood lactate concentrations in central venous, pulmonary artery, and arterial blood. Crit Care Med 15: 489–490.
29. Schenten D, Medzhitov R (2011) The control of adaptive immune responses by the innate immune system. Adv Immunol 109: 87–124.

30. Sprague RS, Ellsworth ML, Stephenson AH, Lonigro AJ (1996) ATP: the red blood cell link to NO and local control of the pulmonary circulation. Am J Physiol 271: H2717–H2722.

31. Ellsworth ML, Ellis CG, Goldman D, Stephenson AH, Dietrich HH, et al. (2009) Erythrocytes: oxygen sensors and modulators of vascular tone. Physiology 24: 107–116.

予防接種概論

13. アジュバント

木戸 博*

はじめに

2013年，新型の高病原性鳥インフルエンザH7N9が発生した。今のところ感染は限局的であるが，パンデミックインフルエンザウイルスH1N1 2009の例にもあるように，十分な対策を講ずる必要がある。さまざまな感染症のなかでも重症化による死亡例の多いインフルエンザの場合，現状のワクチンは以下の問題を抱えており早急な対策が望まれている。①死亡例の多い2歳以下の乳幼児に「安全で有効なワクチン」がいまだに開発されていない。②現状の皮下注射型ワクチンは，血液中の抗ウイルス抗体誘導効果は優れているが，ウイルス侵入門戸の気道粘膜IgA（粘膜免疫）誘導効果と細胞性免疫誘導効果が低く，そのため感染防御効果が不十分である[1~4]。これらの問題を克服する試みの一つが，2歳以下の乳幼児にも使用可能な安全性と防御効果の高い粘膜ワクチン開発で，そのためのアジュバント開発を必要としている。一方で，アジュバントによる免疫力の活性化は，アジュバント病として自己免疫疾患などの副反応リスクを伴うことがある。

1. これまでにヒトに使用されてきたアジュバントと開発途上アジュバント

ワクチンの免疫力を高め，抗体産生持続を良くするためにワクチンに加える物質をアジュバントという。これまでにわが国で使用されてきたアジュバントは，ジフテリア，破傷風，百日せきなどの沈降ワクチンに加えられているアルミニウム塩がある。国際的にはアルミニウム塩以外に，ISCOM（immune stimulating complex），MF59，MDP，Squalene，サポニン成分のQS21，これらの混合剤のAS01（liposome/MPL/QS21），AS03（vitamin E/squalene in oil/water emulsion），AS04（MPL/aluminium salt），AS15（liposome/MPL/QS21/CpG 7909）などが知られている。これらのアジュバントは主に皮下注射，筋肉内注射ワクチン用として添加されてきたが，今後はより感染防御効果の強い粘膜免疫ワクチンのためのアジュバント開発が必要とされている。

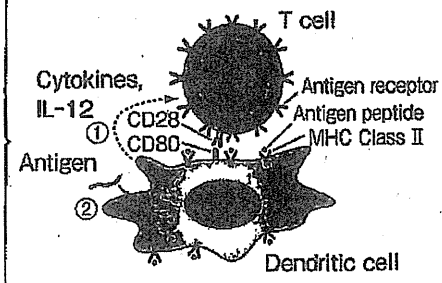
これまでに開発されてきた主要な粘膜免疫ワクチンアジュバントを表[5]に示す。絶えず異物と病原体の侵入リスクにさらされている気道粘膜，腸管粘膜では，粘膜上皮細胞の繊毛運動と細胞表面を循環している粘液によって，異物の侵入を防御するシステムが整っている。そのためワクチン抗原を直接体内に物理的に投入する皮下注射や筋肉内注射に比べ，粘膜免疫ワクチンではより多くの抗原量と強力なアジュバントを必要とする[6]。これまでに開発されてきたアジュバントは，2つのグループに分けることができる。第一のアジュバントグループは粘膜の樹状細胞刺激物質である。代表的なアジュバントにコレラ毒素（CT），大腸菌易熱化毒素（HLT），Toll-like receptor（TLR）刺激物質のPoly(I：C)，CpG DNA，各種サイトカイン，TLRシグナル関連物質群があげられる。これらのアジュバントは一般に抗体誘導効果に優れているが，アジュバント単独でも樹状細胞を強く刺激することから，まれに自己抗体を誘導する副反応を伴うと考えられる。HLTを添加したVirosome Vaccineの場合，一過性の顔面神経麻痺（Bell's palsy）が高率（25.2%）に発症して市場から撤退した例が記憶に新しい[7]。第二のアジュバ

KIDO Hiroshi
*徳島大学疾患酵素学研究センター
〔〒770-8503 徳島市蔵本町3丁目18-15〕
TEL 088-633-7423 FAX 088-633-7425
E-mail：kido@ier.tokushima-u.ac.jp

表　これまでに開発された主要な2種類の粘膜アジュバント

Mucosal adjuvant and Delivery system	Route responses	Systemic responses	Mucosal
①粘膜の樹状細胞刺激物質			
Toxin-based adjuvants			
CT	ON	+	+
mutant CT	N	+	+
HLT	ON	+	+
mutantH LT	N	+	+
CTA/HLTB	ON	+	+
mutant CTA/HLTB	N	+	+
mutant PTX	N	+	+
mutant STX	N	+	+
STXB	N	+	+
Cytokine-based adjuvants			
IL-1	N	+	+
IL-6	N	+	−
IL-12	N	+	+
Type I IFN	N	+	+
Innate immunity associated adjuvants			
Lptn	N	+	+
RANTES	N	+	+
Defensin	N	+	−
CpG DNA	ON	+	+
Poly (I : C)	ON	+	+
Saponin (QS-21)	O	+	+
②粘膜の樹状細胞への抗原運搬体			
ISCOM	ON	+	+
Liposome	N	+	+
Live attermated vectors			
rSalmonella	O	+	+
rBCG	N	+	−
Chitosen	N	+	+
Mucosal DNA vaccine	ON	+	+
Edible vaccine	ON	+	+
Pulmonary Surfactant (SF-10)	N	+	+

(Yuki ら[5], 2003 を改変)



ントグループは粘膜樹状細胞への抗原運搬体関連物質である。このアジュバントは抗原を効率良く樹状細胞に運搬して抗体を誘導する。そのため，このアジュバントの多くはアジュバント単独で樹状細胞の刺激性は低く安全性は高いが，抗体誘導効果の低いものが多い。最近筆者らは，肺の生体成分，肺サーファクタントに「抗原運搬体アジュバント」活性を見出し[3]，物質としての安全性を基盤に有効性をCT並みに増加させた人工合成粘膜アジュバント，SF-10[8]を開発した。本稿ではSF-10を中心に最近の開発状況を述べてみたい。

2. 肺サーファクタントに類似した安全性と有効性の高い人工合成粘膜アジュバントSF-10

肺サーファクタントは，肺胞Ⅱ型細胞から絶えず分泌され，肺胞表面を覆うことで肺の表面張力を低下させて肺呼吸を可能にしている生体物質である。肺内の半減期は6〜7時間と短く，絶えず合成と分解を繰り返している。肺サーファクタントの50%は肺胞Ⅱ型細胞に取り込まれて再利用

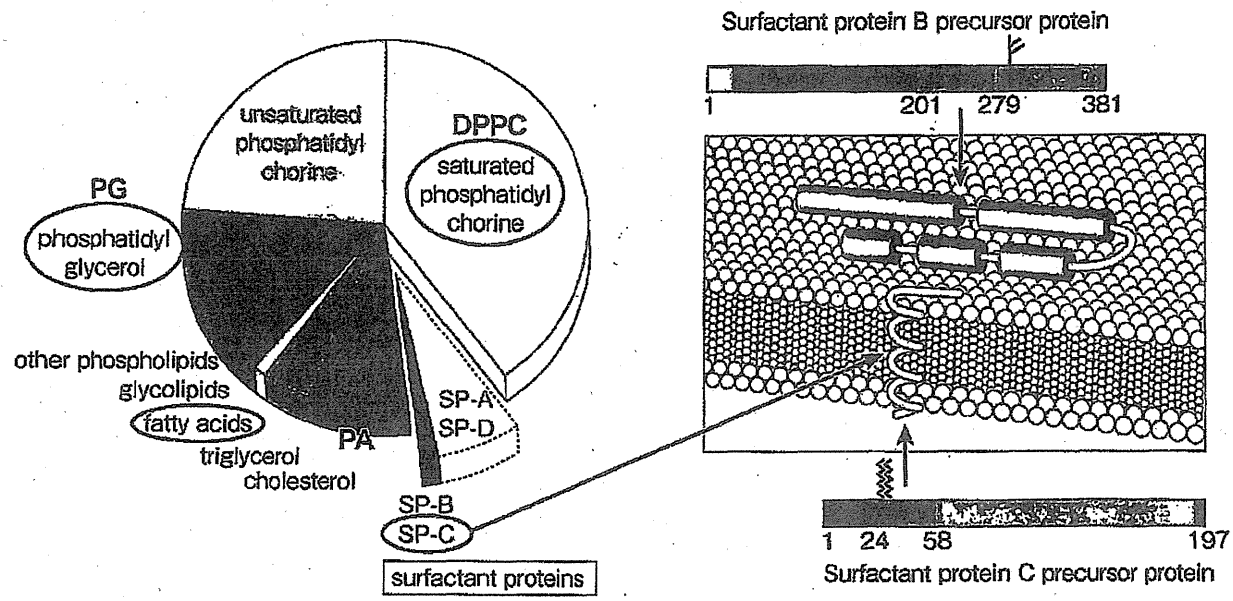


図1　粘膜アジュバント活性に必要な肺サーファクタントの構成成分
青丸で囲んだ成分が，最小必須成分と同定された。
(Whitsett JA, et al：Hydrophobic surfactant proteins in lung function and disease. N Engl J Med 347：2141-2148, 2002)

されているが，残りの50%はマクロファージと樹状細胞に選択的に取り込まれて分解される。筆者らは，マクロファージと樹状細胞へのサーファクタントの流れに，さまざまなワクチン抗原を乗せて抗体誘導を引き起こすことを試みた。すなわち，樹状細胞への肺サーファクタントの取り込みを鼻腔粘膜で再現させて，経鼻粘膜ワクチンとして利用する構想である。臨床の現場では，肺サーファクタントは未熟児呼吸窮迫症候群の特効薬として過去20年安全に使用されてきた実績がある。しかしこの薬剤は，牛の肺から精製される生物製剤で，高価であるだけでなく牛海綿状脳症のリスクを完全に除くことが困難と推定された。そこで粘膜アジュバント活性のある人工合成肺サーファクタントの作成が試みられた。肺サーファクタントは多数の脂質成分の複合体であるが，この中で粘膜アジュバント活性に必要な成分は，図1に示すsaturated phosphatidyl chorine (DPPC)，phosphatidyl glycerol (PG)，脂肪酸 (PA) の phosphatidic acid，surfactant protein C (SP-C) であることを同定した[9]。SP-Cは脂質膜貫通領域をもつペプチドで樹状細胞への取り込みに必須な成分であるが，難溶性で工業生産には向かない。そこで溶解性が良く粘膜アジュバント活性をもった類似のペプチドがスクリーニングされ，K6L16ペプチドを得て人工合成品が完成した。さらに加えたワクチン抗原の運搬効率を高めるため，増粘剤として作用するcarboxy vinyl polymerを添加した人工合成粘膜アジュバントSF-10[8]が完成した。

SF-10アジュバントによるマウスを用いた抗インフルエンザ抗体の誘導効果を図2[8]に示す。比較対象として，TLR3を介する強力な樹状細胞活性化剤のPoly(I：C) の結果を示した。鼻汁の抗インフルエンザIgA抗体は，生物製剤の天然型肺サーファクタント (NSF) に比べてSF-10は約15倍，Poly(I：C) に比べても約10倍と，これまで報告されている粘膜アジュバントのなかでは最も効果的な抗体誘導効果を示した。血液の抗インフルエンザIgG抗体においても，NSFの150倍，Poly(I：C) の約2倍と効果的な抗体誘導効果を示した。SF-10の作用は，Th2タイプのIgG1誘導のほかに，Th1タイプのIgG2aの誘導を効果的に引き起こした。しかしIgEの誘導はみられず，経鼻粘膜アジュバントとしては副反応の少ない理想

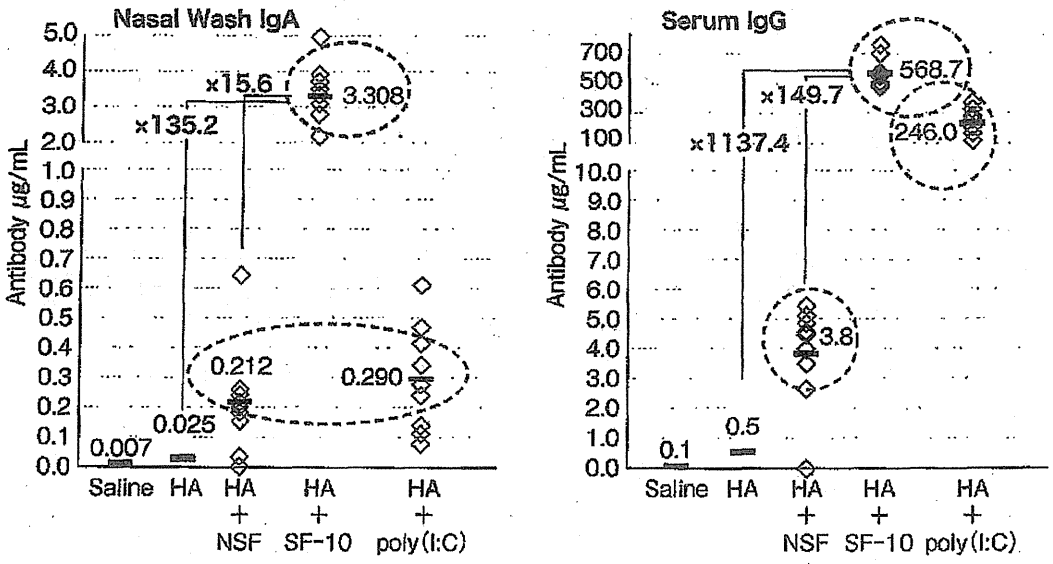


図2　SF-10アジュバント添加経鼻インフルエンザワクチンによるマウス鼻汁中の抗ウイルスIgA抗体と血中のIgG抗体の誘導効果

マウスにインフルエンザスプリットワクチン（haemagglutinin HA量0.1 μg/head）と，アジュバントとして牛由来の天然型肺サーファクタント（NSF），人工合成肺サーファクタント（SF-10），TLR3アゴニストのPoly（I：C）を添加して，それぞれ2週間隔で3回経鼻接種し，その後2週目の鼻汁中の抗インフルエンザIgA抗体と血中IgG抗体を測定した。青色の点線で囲んだ領域は，これまで文献で報告されている抗体誘導領域を示し，黒色の点線で囲んだ領域は，SF-10の添加で抗体誘導が増強された領域を示す。

（Kimotoら[8]，2013を改変）

的な抗体誘導パターンといえる。さらに**図3**に示すように，血中の液性免疫IgG，IgA誘導を主体とする現行の皮下注射ワクチンに比べて，血中のIgG，IgAと気道粘膜の分泌型IgA，さらにTh1タイプの抗体誘導を介する細胞性免疫誘導を起こすSF-10経鼻インフルエンザワクチンは，インフルエンザウイルスを濃厚感染させたマウス生存率において，皮下注射に比べて有意差をもって効果的な感染防御効果を示した。これらの効果は，離乳期直後のミニブタ[6]，カニクイザルでも確かめられている。またこれまでの動物実験であげられた副反応には，局所炎症，サイトカンの増加，白血球の減少などがあげられるが，これらの副反応はみられなかったことからSF-10の安全性は高いと推定された。

おわりに

高病原性鳥インフルエンザを始めとする，新型インフルエンザの脅威にさらされている今日，従来の皮下注射型ワクチンに代わって，強い感染予防効果と感染拡大阻止をもつ粘膜免疫ワクチンの実用化が望まれる。とくに死亡率の高い2歳以下の乳児にも適用化能なワクチンとして粘膜ワクチンに期待が集まっている。そのために，安全で有効なアジュバントの開発が必須であり，その有力な候補として生体成分の肺サーファクタントに類似した人工合成粘膜アジュバントSF-10があげられ，実用化に向けた取り組みが進んでいる。

Key words：粘膜アジュバント，肺サーファクタント，経鼻ワクチン

文献

1) Clements ML, Betts RF, Tierney EL, et al：Serum and nasal wash antibodies associated with resistance to experimental challenge with influenza A wild type virus. J Clin Microbiol 24：157-160, 1986
2) Tamura SI, Asanuma H, Ito Y, et al：Super cross-protective effect of nasal vaccination to subcutaneous inoculation with influenza henagglutinin

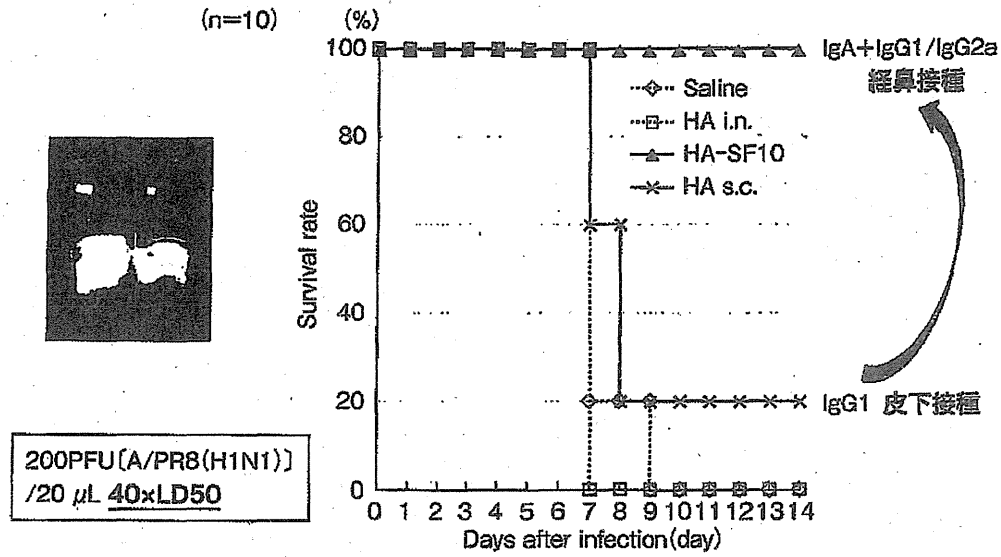


図3　SF-10アジュバント添加経鼻インフルエンザスプリットワクチンと皮下注射ワクチンの感染防御効果の違い

マウスにインフルエンザスプリットワクチン（haemagglutinin HA量0.1 μg/head）（HA i. n.）とコントロールとしての生理食塩水（Saline）の経鼻投与，アジュバントとして人工合成肺サーファクタント（SF-10）をスプリットワクチンに添加した経鼻投与（HA-SF-10）を2週間隔で3回行った。皮下注射は，常法に準じて同量のプリットワクチンを2週間隔で2回免疫した（HA s. c.）。最終免疫後の2週目に，病原性の強いIAV/PR8/34（H1N1）ウイルスを半数致死量の40倍経鼻濃厚感染させて，生存率の推移を14日間観察した。1群10匹のマウスで構成している。図には示していないが，同一条件の実験条件で，最終免疫2週目の抗インフルエンザ抗体価を測定して，Th2タイプのIgG1抗体とTh1タイプのIgG2a抗体，鼻汁中のIgA抗体価の有意な増加を確認している。
（Kimotoら[8]，2013を改変）

vaccine. Eur J Immunol 22：477-481, 1992
3) Mizuno D, Ide-Kurihara M, Ichinomiya T, et al：Modified pulmonary surfactant is a potent adjuvant that stimulates the mucosal IgA production in response to the influenza virus antigen. J Immunol 176：1122-1130, 2006
4) Davis SS：Nasal vaccines. Adv Drug Deliv Rev 51：21-42, 2001
5) Yuki Y, Kiyono H：New generation of mucosaladjuvants for the induction of protective immunity. Rev Med Virol 13：293-310, 2003
6) Nishino M, Mizuno D, Kimoto T, et al：Influenza vaccine with Surfacten, a modified pulmonary surfactant, induces systemic and mucosal immune responses without side effects in minipigs. Vaccine 27：5620-5627, 2009
7) Mutsch M, Zhou W, Rhodes P, et al：Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. N Engl J Med 350：896-903, 2004
8) Kimoto T, Mizuno D, Takei T, et al：Intranasal influenza vaccination using a new synthetic mucosal adjuvant SF-10-Induction of potent local and systemic immunity with balanced Th1 and Th2 responses. Influenza Other Respi Viruses. 2013 May 26　doi：10.1111/irv.12124［Epub ahead of print］
9) Mizuno D, Kimoto T, Takei T, et al：Surfactant C is an essential constituent for mucosal adjuvanticity of Surfacten, acting as an antigen delivery vehicle and inducing both local and systemic immunity. Vaccine 29：5368-5378, 2011

感染症内科，1(6)：544-551，2013

特集 インフルエンザ―最近の動向

インフルエンザと生体防御*

木戸　博**
高橋悦久**
山根一彦**

Key Words : influenza, host defence, immunoglobulin A(IgA), influenza-associated encephalopathy, mucosal immunity

はじめに

「インフルエンザと生体防御」を論ずるにあたって，①インフルエンザウイルスの感染感受性を決定している粘膜免疫とワクチン接種の問題，②インフルエンザ感染で問題となる感染重症化の機序とその治療について最新知見を加えて概説したい．医療現場でいまだによく認識されていない①に関する事項として，現行の皮下注射ワクチンは血液中にIgG抗体を誘導して肺炎防止効果を示すが，感染予防に関与する気道粘膜局所の分泌型IgAの誘導効果はなく，予防効果の不十分なワクチンであることがあげられる．さらに，治療の現場で頻繁に使用されている抗インフルエンザ薬は，体内ウイルス抗原の産生を効果的に抑制するため，獲得免疫が不十分になる副反応を伴う．特にこの現象は，免疫メモリの低い小児で明確で高い再感染として現れる．一方，②の感染重症化の機序とその治療については，多くの感染者は治癒するが一部のハイリスク者で重症化して入院，死亡することへの対策が急務となっている．特に新型インフルエンザ，高病原性鳥インフルエンザの流行時への対応として，感染重症化の機序の解明とその治療法の開発が急がれる．本稿では，これらの問題について最新情報を概説する．

ウイルスの体内侵入を防御している粘膜免疫とワクチン接種

インフルエンザウイルスは気道粘膜と腸管粘膜で最初に感染して増殖する．ウイルス侵入部位の生体防御物質は，体内で最も多量に作られ粘膜表面を覆っている分泌型IgA(SIgA)抗体である．この抗体は，交叉免疫性が高くウイルスの亜型が違っても反応するため，侵入門戸で防御するには最適の防御物質である．図1は，2006/2007年のインフルエンザシーズン前に鼻汁を採取して抗インフルエンザSIgA抗体価を測定し，翌年5月にインフルエンザ感染の有無を調査した結果である[1]．感染者群は，迅速診断キットで感染陽性が確認された群で，非感染者群は自己申告によるもので感染の自覚症状のなかった人たちである．非感染者群のなかには，感染していたが自覚症状が軽く医師の診断を受けなかった人，感染していたが迅速診断キットで感染陽性と出なかった人が含まれるため，図に示すように抗体価の幅は大きい．縦軸は，抗インフルエンザSIgA抗体価/全IgA値×100で表しており，抗インフルエンザ抗体の占める割合を示している．感染者は例外なく鼻汁中の抗体価の低いこ

* Influenza and host defence system.

** Hiroshi KIDO, M.D., Ph.D., Etsuhisa TAKAHASHI, M.S. & Kazuhiko YAMANE, M.S.: 共同利用・共同研究「酵素学研究拠点」徳島大学疾患酵素学研究センター・KOSOKEN〔〒770-8503 徳島県徳島市蔵本町3-18-15〕; Institute for Enzyme Research, The University of Tokushima, Tokushima 770-8503, JAPAN

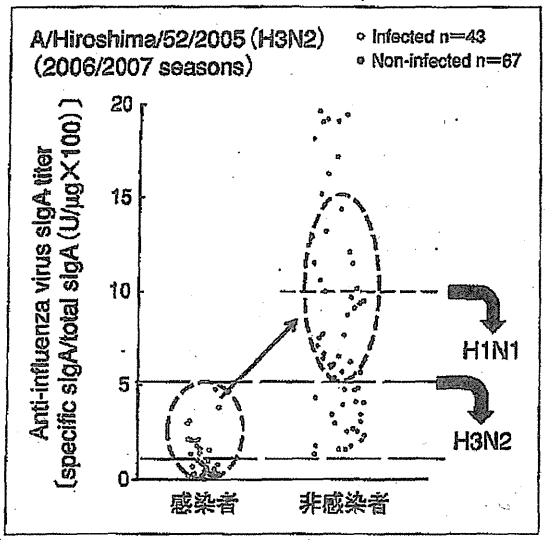


図1　鼻汁中の抗インフルエンザ抗体価とインフルエンザ感染感受性

2006/2007インフルエンザシーズン前の10月に鼻汁を採取し，翌年5月に感染履歴の調査を行った．この年の流行株はA/Hiroshima(H3N2)と同型であることから，抗インフルエンザSIgA抗体価は，A/Hiroshima/52/2005(H3N2)に対する抗体価で評価した．

とがわかる．感染者の示す抗体価の上限値は，ウイルス亜型によって異なる．図に示すようにH3N2型に比較してH1N1型ではその値が高い傾向にある．インフルエンザワクチンの皮下注射では血液中の抗インフルエンザIgG抗体価は接種者の多くで増加して，感染防御の目標値としているヘマグルチニンインヒビション(HI)価≧40に到達すると思われる．公衆衛生学的調査の場合，どのくらいのインフルエンザウイルスに曝露されたかを個々の事例で判定することができないため，曝露の有無を無視して調査する場合が多い．一方でインフルエンザウイルス感染者を対象にして調査を実施した場合，ウイルス曝露条件化下での調査となり，その結果は重要な情報を提示する[2]．論文に報告された感染者集団では約70%にワクチン接種履歴があり，HI価≧40が確認されてワクチン接種の効果は達成できているがインフルエンザに罹患した患者である．これらの患者の特徴は，鼻汁の抗インフルエンザSIgA抗体価が例外なく図1に示す基準以下であった．すなわち，インフルエンザの感染感受性を決定している重要な因子として，血液中のIgG抗体価よりはむしろ気道粘膜のSIgA抗体価があげられることを示している．

さらに，抗インフルエンザ薬と粘膜免疫の問題が明らかになっている．具体的には，抗インフルエンザ薬を服用した場合，翌年の再感染率が高くなることを示すretrospective studyが報告された[3]．この論文では，抗インフルエンザ薬として経口投与のoseltamivirと吸入薬のzanamivirが選ばれた．詳細は論文にゆだねるが，oseltamivirとzanamivirの投薬を受けた患者では，鼻汁中の抗インフルエンザSIgA抗体価が有意に低く抑制されており，血液の抗インフルエンザIgG抗体価についても軽度であるが同様な抑制傾向がみられた．その結果図2に示すように，翌年の再感染率では，抗インフルエンザ薬を投与していない群が8.6%であるのに対して，oseltamivir投与群で37.3%，zanamivir投与群で45%と有意に高い値を示した．この獲得免疫の抑制効果[3)~5)]は特に免疫メモリーの低い小児で明確であるが，これを補正して獲得免疫を増強するイムノモジュレーターとしてclarithromycin(CAM)の新たな薬効が見出された[3)5)]．気道や鼻腔粘膜の抗原提示細胞は，侵入してきたインフルエンザウイルス抗原の抗原情報を把握して，その情報を粘膜局所の抗体産生B細胞に情報を伝え，IgMからの分化を促すクラススイッチが起きてSIgA産生が高まる．CAMはこの過程のなかで，抗原提示細胞が分泌するシグナル伝達物質のB cell activating factor of the TNF family(BAFF)の発現を増加させ，B細胞のクラススイッチを促進するactivation-induced cytidine deaminase(AID)を著明に増加させることから，CAMのアジュバント効果が証明された[6)]．抗インフルエンザ薬の使用は，このようにウイルス抗原量を低下させて獲得免疫能を弱めるが，CAMを併用することでこの不利な抗インフルエンザ薬の作用が軽減されることが判明した．

インフルエンザ感染の重症化機序とその治療法

インフルエンザに感染者のほとんどが治癒するが，感染者の一部の人で重症化して死に至ることがある．この場合，重症化の原因はウイルス侵入によって誘発された生体防御機能と深く

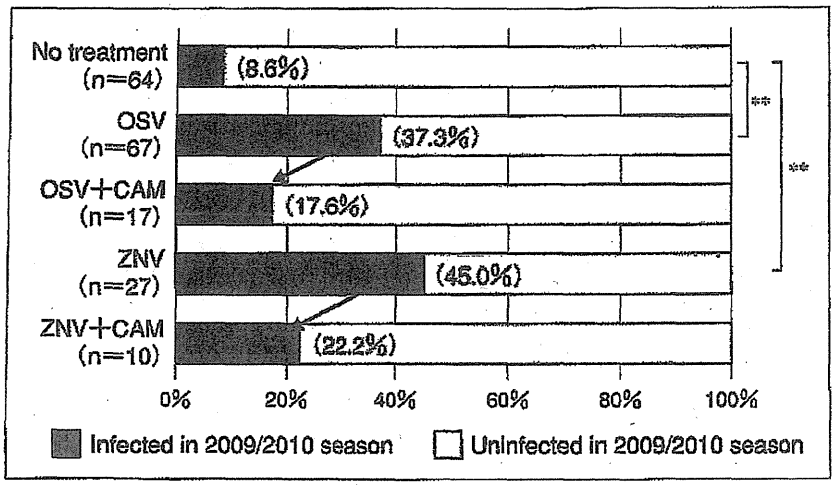


図2　抗インフルエンザ薬の単独投与とclarithromycinとの併用投与を実施した患者の翌年のインフルエンザ再感染率

2008/2009インフルエンザシーズンのインフルエンザ感染小児に，無投薬群(64名)，oseltamivir投与群(67名)，oseltamivir＋clarithromycin投与群(17名)，zanamivir投与群(27名)，zanamivir＋clarithromycin投与群(10名)に対して，翌シーズン(2009/2010)のインフルエンザ再感染率を調査した．** $P<0.01$ (Fisher's exact test with Bonferroni correction)．

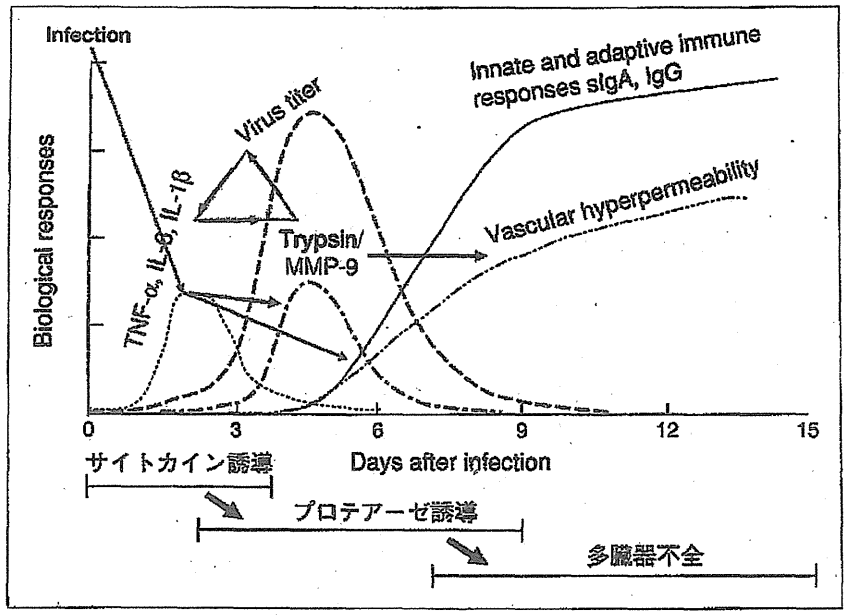


図3　インフルエンザ感染後に惹起される気道における生体反応の経時変化

関係している．重症化の際には抹消循環不全に由来する多臓器不全がみられるが，その時期は免疫系が発動する時期と重なっている[7)~9)]．図3に，インフルエンザウイルスが最初に気道に感染して増殖する際にみられるさまざまな生体応答を示す[9)]．感染後の最も早い生体応答が，炎症性サイトカインのTNF-α, IL-6, IL-1β等の誘導で，感染後1～2日をピークに増加して続いて起こるさまざまな生体反応の引き金を引く．生体にとってこれらのサイトカインが有利に働く反応が，生体防御系(自然免疫系，獲得免疫系)の発動で，感染初期の細胞性免疫系と感染4日以後の液性免疫(抗体産生)に代表され，これらの応答によってウイルスは通常体外に排除される．一方これらのサイトカインは同時に，重症化に結びつく体内因子のtrypsinとmatrix metallo-