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Elevation of Serum Acid Sphingomyelinase Activity in Children with Acute Respiratory Syncytial Virus Bronchiolitis

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Acid sphingomyelinase (ASM) is a lysosomal enzyme that hydrolyzes sphingomyelin into ceramide, a bioactive lipid to regulate cellular physiological functions. Thus, ASM activation has been reported as a key event in pathophysiological reactions including inflammation, cytokine release, oxidative stress, and endothelial damage in human diseases. Since ASM activation is associated with extracellular ASM secretion through unknown mechanisms, it can be detected by recognizing the elevation of secretory ASM (S-ASM) activity. Serum S-ASM activity has been reported to increase in chronic diseases, acute cardiac diseases, and systemic inflammatory diseases. However, the serum S-ASM has not been investigated in common acute illness. This study was designed to evaluate serum S-ASM activity in children with common acute illness. Fifty children with common acute illness and five healthy children were included in this study. The patients were categorized into five groups based on clinical diagnoses: acute respiratory syncytial virus (RSV) bronchiolitis, adenovirus infection, streptococcal infection, asthma, and other infections due to unknown origin. The serum S-ASM activity was significantly elevated at 6.9 ± 1.6 nmol/0.1 mL/6 h in the group of acute RSV bronchiolitis patients compared with healthy children who had a mean level of 1.8 ± 0.8 nmol/0.1 mL/6 h ($p < 0.05$). In the other illness groups, the serum S-ASM activity was not significantly elevated. The results suggest an association of ASM activation with RSV infection, a cause for common acute illness. This is the first report to describe the elevation of serum S-ASM activity in respiratory tract infection.

Keywords: acute bronchiolitis; ceramide; common acute illness; respiratory syncytial virus; secretory acid sphingomyelinase
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Introduction

Acid sphingomyelinase (ASM) is a lysosomal enzyme that catalyzes the hydrolysis of sphingomyelin into ceramide and phosphocholine in acidic conditions in lysosomes. Ceramide is a bioactive lipid that functions as a second intracellular messenger and is implicated in a variety of physiological functions. Thus, ASM activation has been reported as a key event in many biological functions and pathophysiological reactions in human diseases (Schuchman 2010).

In humans, the expression of a single gene, sphingomyelin phosphodiesterase 1 (*SMPD1*), results in two forms of the enzyme, lysosomal ASM (L-ASM) and secretory ASM (S-ASM), that differ in several characteristics including intracellular trafficking processes (Kornhuber et al. 2015). The *SMPD1* gene gives rise to a common mannosylated precursor protein, pre-pro-ASM, which is cleaved to yield pro-ASM. Differential glycosylation and N-terminal as well as C-terminal processing inside the Golgi then lead to the generation of the two ASM forms. While L-ASM is shuttled into the lysosomal trafficking pathway, S-ASM is released into the extracellular space via the constitutive pathway. L-ASM is exposed to cellular pools of Zn^{2+} within the lysosome and saturated with Zn^{2+}. In contrast, S-ASM is located extracellularly and is not saturated with Zn^{2+}. Therefore, S-ASM activities can be measured using an assay buffer containing Zn^{2+} as Zn^{2+}-dependent ASM in various human body fluids, including blood (Takahashi et al. 2000). ASM activation has been found to be associated with extracellular ASM secretion, which can be detected as the increase in S-ASM activity; however, the precise mechanisms of the increase of S-ASM activity remain to be elucidated (Kornhuber et al. 2015).

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Several clinical studies have been conducted to measure the blood S-ASM activity in various human diseases, and increased blood S-ASM activity has been observed in many different disease states (Kornhuber et al. 2015). Thus far, the increase in blood S-ASM activity has been thought to reflect inflammation, cytokine release, oxidative stress, and endothelial damage in pathophysiological reactions (Kornhuber et al. 2015). In addition, as the endothelium is known to be a rich source of S-ASM, diseases with endothelial involvement actually appear to present a marked elevation in peripheral S-ASM activity. Mild elevations of blood S-ASM activity were observed in Alzheimer's disease (Lee et al. 2014), type 2 diabetes mellitus (Górska et al. 2003), stable angina pectoris (Pan et al. 2014), acute myocardial infarction (Pan et al. 2014), chronic heart failure (Doehner et al. 2007), post-traumatic stress disorder (Hammad et al. 2012), and in response to ionizing radiation treatment in cancer patients (Sathishkumar et al. 2005). Marked elevations of blood S-ASM activity were observed in unstable angina pectoris (Pan et al. 2014), Wilson disease (Lang et al. 2007), sepsis (Claus et al. 2005), systemic inflammatory response syndrome (Kott et al. 2014), lymphohistiocytosis (Takahashi et al. 2002; Jenkins et al. 2013), non-alcoholic fatty liver (Grammatikos et al. 2014), hepatitis C (Grammatikos et al. 2014), systemic vasculitis inflammatory renal disease (Kiprianos et al. 2012), and in patients with alcohol dependency undergoing withdrawal (Mühle et al. 2014). Thus, ASM has a potential pathophysiological role in these diseases.

Interestingly, there has been several reports describing the therapeutic effects of ASM inhibition in some disease models, in which ASM inhibition, using a functional inhibitor of acid sphingomyelinase, alleviated the severity of disease state, suggesting that ASM could be a possible therapeutic target (Kornhuber et al. 2010, 2011).

In this study, serum S-ASM activities were measured in children with common acute illness to clarify how ASM contributes to the pathophysiology of common acute illness.

Material and Methods

Patient sampling

Fifty children who visited the Ogachi Central Hospital and Omagari Kousei Medical Center between June 2014 and March 2017 as outpatients and five healthy children were enrolled in this study. Thirty-three were boys and 17 were girls between 0 month and 169 months of age. The five healthy children were aged from 6 to 8 years. The patients' demographic characteristics are summarized in Table 1. Patients complained of various symptoms including fever, cough, and sore throat, and some of them were admitted to the hospitals.

Blood samples were collected on their first visit or admission, and were additionally collected before discharge from some of them. Laboratory testing was performed to detect infectious pathogens, including respiratory syncytial virus (RSV), adenovirus and group A beta-hemolytic streptococcus, depending on clinical symptoms and physical signs. Acute RSV bronchiolitis was diagnosed based on criteria that included cough, increase in respiratory rate, chest retraction, prolongation of expiratory time, sibilant rhonchi, and hyperinflated lungs on chest X-ray. RSV infection was confirmed by antigen detection test of nasopharyngeal discharges (Capilia™ RSV Neo, Shizuoka, Japan). Diagnoses of adenovirus and streptococcal infections were based on positive results by the antigen detection tests of pharyngeal discharges (Capilia™ Adeno Neo, Shizuoka, Japan) and throat swabs (Capilia™ Strep A, Shizuoka, Japan), respectively.

Serum S-ASM activities were determined in the 50 patients and the 5 healthy children who were controls in this study.

Medical records were reviewed for age, sex, clinical symptoms, complications, and laboratory data, including white blood cell count, platelet count, and levels of hemoglobin, alanine aminotransferase, aspartate aminotransferase, lipids, electrolytes, and C-reactive protein. Ethical approval was obtained from the Ethics Committee of Akita University, Graduate School of Medicine, in Akita, Japan. Written informed consent was obtained from the parents of the patients and controls enrolled.

Measurements of serum S-ASM and Interleukin-6.

ASM activities were determined following the methods previously described (Konno et al. 2015). Briefly, S-ASM levels were assayed using a buffer containing Zn^{2+} as Zn^{2+}-dependent S-ASM. Zn^{2+}-independent ASM was concurrently assayed using a buffer with ethylenediaminetetraacetic acid (EDTA). The assay used ^{14}C-labeled sphingomyelin (PerkinElmer, MA, USA) as a substrate. A standard 200 μL assay mixture consisting of 100 μL serum and 50 μL assay buffer with 4% Triton X-100 (1.0 M sodium acetate, pH 5.0) was used (final concentration of Triton X-100, 1%). Final concentrations of EDTA and Zn^{2+} in the assay buffer were 0.02 mM and 0.1 mM, respectively. To determine the optimum pH of sphingomyelin hydrolysis by serum, assays were performed using 0.1 M glycine HCl buffer (pH 3.0), 0.1 M acetate buffer (pH 4.0 and pH 5.0), and 0.1 M phosphate buffer (pH 6.0 and pH 7.0) with either EDTA or Zn^{2+}. The reaction was initiated by addition of 50 μL of substrate (20 nmol, ^{14}C-labeled sphingomyelin, and 0.08 μCi/20 nmol) in 0.2% taurodeoxycholic acid. Assay mixtures were incubated at 37°C for 6 h. The assay was terminated with 200 μL ice-cold 30% trichloroacetate and 400 μL 2.5% bovine serum albumin. Tubes were briefly vortexed and allowed to settle for 5 min at room temperature before centrifugation (5 min, 3,000 rpm). The supernatant (500 μL) was carefully aspirated and transferred into glass scintillation vials. Radioactivity was measured directly after mixing with 4.5 mL Clearsol II (Nakalai Tesque, Kyoto, Japan) in a liquid scintillation counter LSC 950 (Aloka, Tokyo, Japan).

Serum interleukin-6 (IL-6) was assayed using Cymax™ Human IL-6 ELISA (Abofrontier, Seoul, Korea).

Statistical analysis

Data were analyzed using the IBM SPSS statistics 22.0 software package and the results are presented as the mean ± standard deviation (SD). A Students' unpaired t-test was used to compare the mean differences between two groups. A p-value < 0.05 was considered statistically significant.

Results

Diagnosis and categorization of the patients

The patients were categorized into five groups based on clinical diagnosis: acute RSV bronchiolitis, adenovirus infection, streptococcal infection, asthma, and other infec-

Table 1. Demographic characteristics of the 50 patients with common acute illness.

Age (months)	31.3 ± 35.6 (0-169)
Sex (number) (%)	
Male	33 (66.0%)
Female	17 (34.0%)
Diagnosis	
Acute RSV bronchiolitis	6 (12.0%)
Adenovirus infection	5 (10.0%)
Streptococcal infection	9 (18.0%)
Asthma	6 (12.0%)
Infections due to unknown origin	24 (48.0%)
White blood cells (/μL)	11,542 ± 5,156.1
C-reactive protein (mg/dL)	1.6 ± 2.2

RSV, respiratory syncytial virus.

Table 2. Distribution of patients based on the serum levels of S-ASM.

S-ASM (nmol/0.1 mL/6 h)	Number of patients
5-	7*
4-4.99	5
3-3.99	11
2-2.99	14
1-1.99	11
0-0.99	2

*Data included six patients with acute RSV bronchiolitis.

tions due to unknown origin. Six children were diagnosed with acute RSV bronchiolitis and all of them were admitted to the hospital. Five patients were diagnosed with adenovirus infection and nine patients were diagnosed with streptococcal infection. The other groups had 6 patients with asthma and 24 patients with acute infections due to unknown origin (Table 1).

S-ASM activities in children with common acute illness

The mean serum S-ASM activity was 3.2 ± 1.9 (0.73-9.92) nmol/0.1 mL/6 h in the 50 patients, whereas healthy children had a mean level of 1.8 ± 0.8 nmol/0.1 mL/6 h. The serum S-ASM activities were greater than 4.0 nmol/0.1 mL/6 h in 12 patients, including all of the six patients with acute RSV bronchiolitis in whom the activities were greater than 5.0 nmol/0.1 mL/6 h (Table 2). The serum S-ASM activities were significantly elevated at 6.9 ± 1.6 nmol/0.1 mL/6 h only in the group of acute RSV bronchiolitis patients compared to healthy children (Fig. 1). In the other groups, serum S-ASM activities were mildly elevated; however, they were not significantly higher compared to healthy children. Since the patients with acute RSV bronchiolitis were all within 1 year of age, serum S-ASM activities could be compared between the two groups: acute RSV bronchiolitis and other common acute diseases in patients less than 1 year of age. Significantly, higher serum S-ASM activities were found in the patients with acute RSV bronchiolitis (Fig. 2). The effects of the pH of the buffer on the hydrolysis of sphingomyelin by serum were investigated in a case with acute RSV bronchiolitis. Under assay conditions, ASM activity was optimal at approximately pH 5.0 in both EDTA and Zn^{2+} assay buffers (Fig. 3). In the four patients with acute RSV bronchiolitis, the high serum S-ASM activities decreased into the normal range before their discharge from the hospital (Fig. 4).

In an attempt to determine the reason for the elevation in serum S-ASM activity, the serum S-ASM activities were compared with the levels of C-reactive protein in the patients; however, it was not significantly correlated (data not shown). Thereafter, the levels of serum IL-6 were determined in the patients, and it was found that the levels of serum IL-6 were not significantly correlated with the serum S-ASM activities (Fig. 5).

Discussion

In the present study, we measured the serum S-ASM activities in children with common acute illness including viral infections, bacterial infections, and asthma. Among the participants in this study, serum S-ASM was specifically elevated in the patients with acute RSV bronchiolitis. Several forms of sphingomyelinase, encoded by independent genes, have been identified in humans, and with different optimal activities at acidic, neutral, or alkaline pH levels. The serum from a patient with acute RSV bronchiolitis showed the optimum activity at approximately pH 5.0 and was stimulated with the addition of Zn^{2+} to the assay buffer, confirming the presence of S-ASM. The patients with acute RSV bronchiolitis were all aged less than 1 year; however, serum S-ASM activities were not significantly elevated in the other patients aged less than 1 year. The high levels of

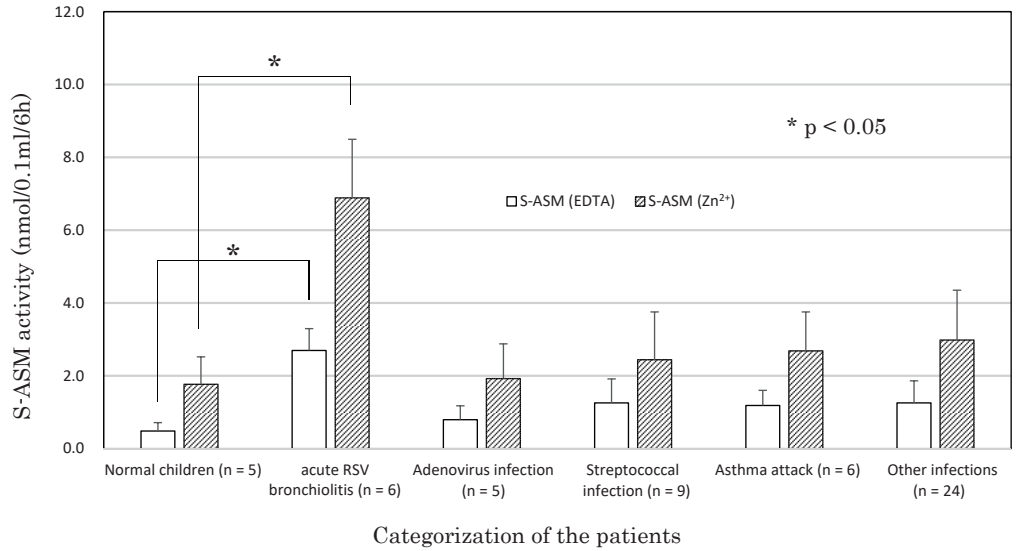

Fig. 1. Serum S-ASM activities in children with common acute illness.
Serum Zn^{2+}-dependent and -independent S-ASM activities were determined in patients who were categorized into five groups of patients based on clinical diagnosis: acute RSV bronchiolitis, adenovirus infection, streptococcal infection, asthma, and other infections due to unknown origin. The serum Zn^{2+}-dependent S-ASM activity was significantly elevated only in the group of acute RSV bronchiolitis patients ($p < 0.05$).

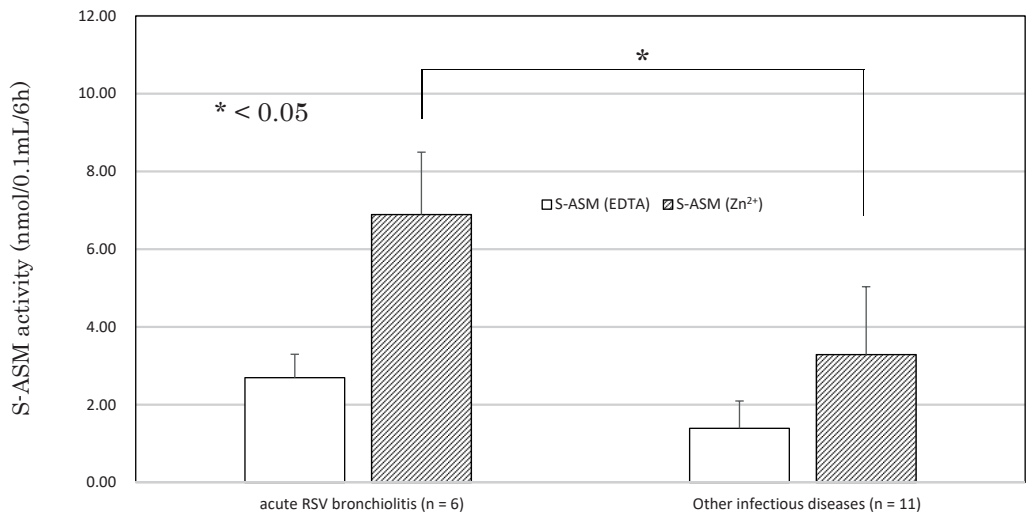

Fig. 2. Serum S-ASM activities in acute RSV bronchiolitis vs. other infectious diseases in patients aged less than 1 year.
Serum Zn^{2+}-dependent S-ASM activities were compared between the two groups: acute RSV bronchiolitis vs, other common acute diseases in patients less than 1 year of age. The higher serum Zn^{2+}-dependent S-ASM activities were observed in the patients with acute RSV bronchiolitis. ASM activity was measured in both EDTA and Zn^{2+} assay buffers.

serum S-ASM were also shown to decrease into the normal range before their discharge from the hospital. Additionally, previous studies showed that the levels of blood S-ASM remain nearly constant from birth to adulthood (Takahashi et al. 2002; Knapp et al. 2005). Collectively, high serum S-ASM activities were reactive outcomes to RSV infection, but not innate in the patients.

Recently, we showed elevation of serum S-ASM activity in patients with acute phase of Kawasaki disease using the same assay method described above (Konno et al. 2015). In that study, the patients showed high levels of serum S-ASM activity of 3.85 ± 1.46 nmol/0.1 mL/6 h, whereas the healthy children showed levels of serum S-ASM activity of 1.15 ± 0.10 nmol/0.1 mL/6 h. The serum S-ASM activities in the patients with acute RSV bronchiolitis were much higher than those of serum S-ASM activities in the patients with acute phase of Kawasaki disease, suggesting a more specific association of ASM activation with RSV infection than with Kawasaki disease.

ASM has been found to play a critical role during infections by microorganisms (Kornhuber et al. 2015). For instance, ASM was reported to be rapidly activated during

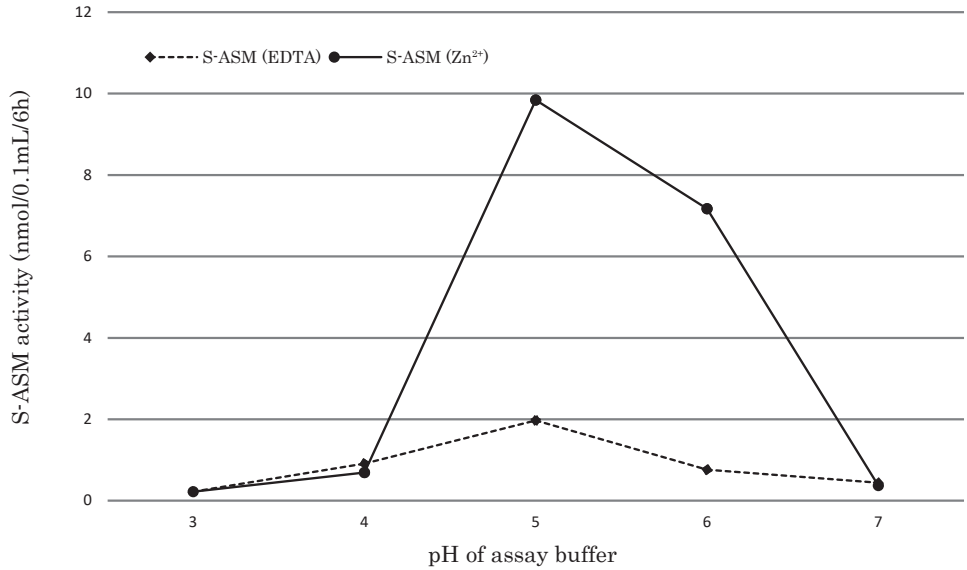

Fig. 3. Optimum pH of serum S-ASM activity in a case with acute RSV bronchiolitis.
The effects of the pH of the buffer on the hydrolysis of sphingomyelin by serum were investigated in a case with acute RSV bronchiolitis. Under assay conditions, ASM activity was optimal at approximately pH 5.0 in both EDTA and Zn^{2+} assay buffers.

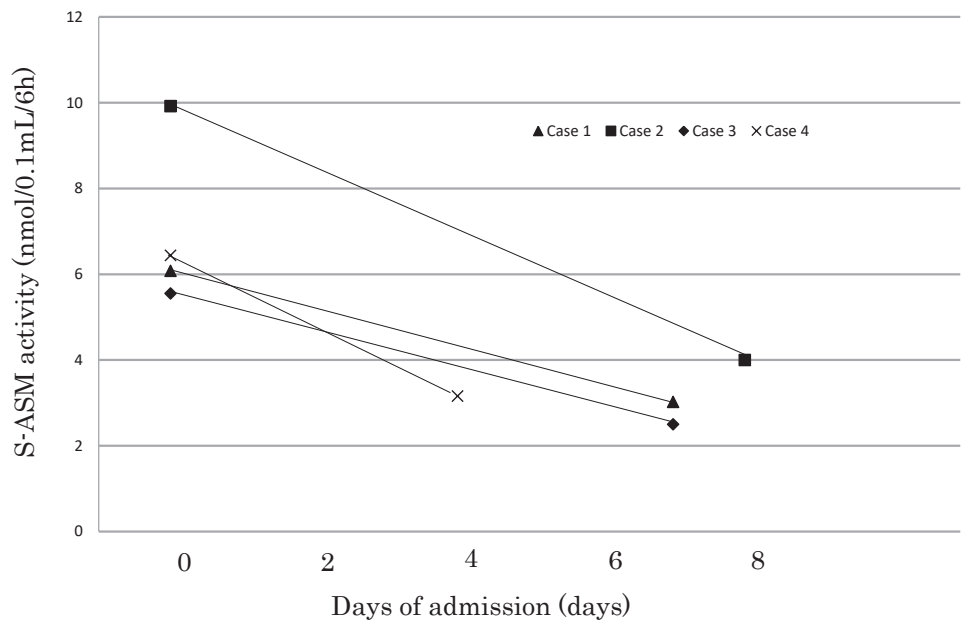

Fig. 4. Profiles of serum S-ASM activity in four patients with acute RSV bronchiolitis.
In the four patients (case 1, case 2, case 3, and case 4) with acute RSV bronchiolitis, high serum Zn^{2+}dependent S-ASM activities decreased into the normal range before their discharge from the hospital. In each of the patients, serum S-ASM was measured on two occasions throughout the clinical course, shown by days from admission.

the entry of sindbis virus into neuroblastoma cells (Jan et al. 2000). In another experiment, ASM and ceramide were determined to be key molecules for the infection of human cells with rhinoviruses (Grassmé et al. 2005). Infection of human epithelial cells by several rhinovirus strains was found to trigger a rapid activation of the ASM and the resulting ceramide-enriched membrane platforms were shown to be significant for rhinovirus entry (Grassmé et al. 2005). Another study reported that ASM activity and sphingomyelin presence are necessary for efficient infection of cells by ebolavirus (Miller et al. 2012). Thus far, ASM has not been reported to be associated with RSV infection; however, our results suggest that ASM could play a critical role in RSV infection.

Infants with severe acute RSV bronchiolitis are well known to have increased plasma cytokine concentrations

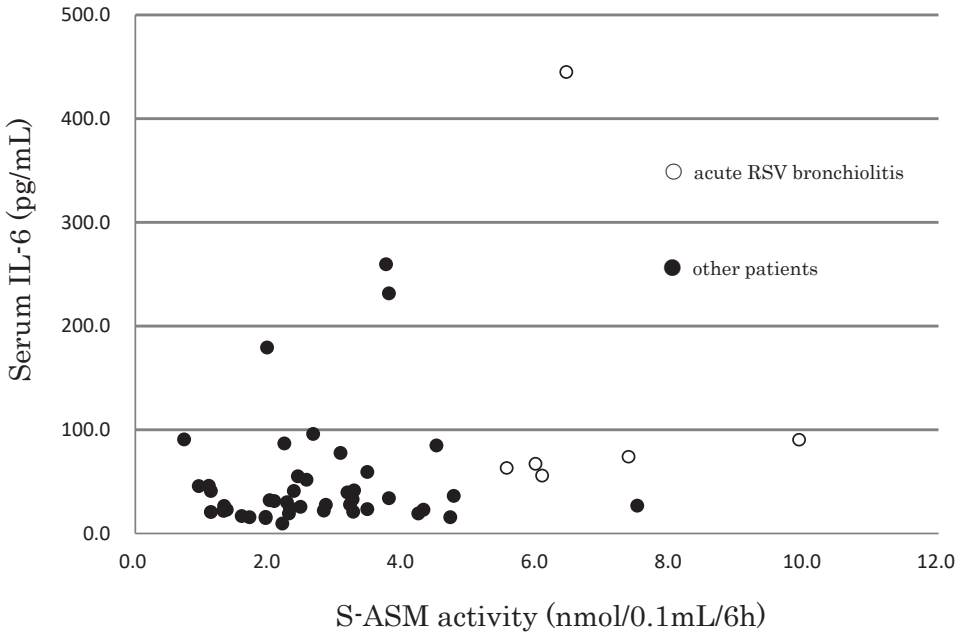

Fig. 5. Serum S-ASM activity and IL-6 in 50 patients.
Serum IL-6 was measured in the 50 study patients, showing that the levels of serum IL-6 were not correlated with the serum S-ASM activities.

(Mella et al. 2013). S-ASM is also known to be a cytokine-responsive enzyme that increases upon stimulation with interleukin (IL)-1β or tumor necrosis factor-α (TNF-α) (Marathe et al. 1998). These suggest that the elevation of serum S-ASM might originate from the response to some specific cytokines induced by RSV infection in infants. In the present study, levels of serum IL-6 were determined in the patients, but they were not correlated with the levels of serum S-ASM activity (Fig. 5). Further studies, such as analyses of multiple cytokines or chemokines, may reveal the possible mechanism of ASM activation in acute RSV bronchiolitis.

This report is also the first to describe the elevation of serum S-ASM activity in respiratory tract infection. In this study, a group categorized as other infections due to unknown origin actually included a few patients with respiratory infections, but they did not show high levels of serum S-ASM activity. Further studies of S-ASM might be needed to clarify the original site and the mechanism of ASM secretion in respiratory tract infections.

In summary, children with common acute illness can show an elevation of serum S-ASM activity. This suggests that ASM activation might play a role even in the pathophysiology of common acute illness. Serum S-ASM activities were specifically elevated in children with acute RSV bronchiolitis, suggesting an association of ASM activation with RSV infection.

Conflict of Interest

The authors declare no conflict of interest.

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