

Gender analysis in acute bronchiolitis due to respiratory syncytial virus

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It is reasonable to compare immune reactions between boys and girls because many infections in the early stages are predominant in males. A relationship between immunomodulatory effects of sex hormone surge in boys at early months and infectious diseases is still unclear. We compared clinical features between boys and girls who suffered from wheezing that was initially triggered by acute respiratory syncytial virus (RSV) bronchiolitis. For systemic immune response evaluation, white blood cell (WBC) count, blood eosinophil count, and serum C-reactive protein (CRP) were measured. For local inflammation evaluation, scores for eosinophils and neutrophils in sputum were evaluated microscopically. Patients consisted of 90 boys and 51 girls. Most children were under 6 months of age. WBC counts and serum CRP levels were significantly increased in girls compared with boys. Blood eosinophilia at the acute stage was rarely observed in children after 6 months of age. For local response evaluation, sputum specimens obtained from 42 boys and 29 girls were microscopically examined. Sputum eosinophil score of 2+ and more was observed in boys (6/42) exclusively. In contrast, sputum neutrophilia was commonly observed in boys and girls. From a follow-up study, we confirmed that 28 children with RSV bronchiolitis showed wheezing episodes afterwards. However, their blood and sputum eosinophilia during RSV bronchiolitis did not reflect their subsequent wheezing. We speculated that gender-specific responses to RSV infection might account for male susceptibility. Differences in RSV pathogenicity between boys and girls should be further investigated in terms of asthma progression.

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Respiratory syncytial virus (RSV) is a major cause of respiratory diseases early in life, and affects children <6 months of age (1–3). Although new approaches for preventing RSV disease have been developed (4), bronchiolitis is still increasing worldwide (1). Furthermore, RSV infection may play a pivotal role in the development of recurrent episodes of bronchial obstruction or asthma (5–10). A variety of immune deviations of RSV bronchiolitis has been a matter of ongoing debate (11–16). It was also reported that prognosis of RSV bronchiolitis was not poor, if subjects were followed up for longer years (17, 18). However, it is plausible that altered immune response to RSV plays a key role

in determining asthma progression. Thus, mechanisms leading to accumulation of eosinophils in the lower respiratory tract have been a topic of great interest. Interestingly, younger infants aged 0–2 months old may have a greater chance to develop eosinophilia (16).

The predominance of RSV infection as well as childhood asthma in males is well known but its mechanism has not yet been explored. It was reported that high levels of circulating testosterone for the first few months of life in boys affected somatic growth (19); however, immunomodulatory effects of sex hormones have not been explored. The predominance of RSV infection in males may provide one of the clues to analyze

the pathogenesis of RSV bronchiolitis. A previous study showed that eosinophilic cationic protein (ECP) levels in nasopharyngeal secretions of infants with RSV infection were elevated in girls compared with boys (20). Considering these facts, gender analysis of inflammatory responses should be a good mechanistic target to explore RSV bronchiolitis pathology. We assessed systemic and local immune reactions in relation to eosinophils in children with RSV bronchiolitis.

Methods

Subjects

Infants and young children were referred by doctors in nearby clinics or hospitals to Chiba Children's Hospital. Usually, a nearby doctor would have judged that some patients would need to be hospitalized, and therefore sent them to our hospital. The Department of Allergy mainly received infants and children suffering from wheezing. We decided that all referred children had to be hospitalized, but mild case was exceptionally seen as an outpatient. Hospitalization was purely based on clinical severity. There was no other special selection for hospitalization. Before hospitalization, we evaluated RSV antigens using an Abbott Test Pack RSV test (Abbott Laboratories, Chicago, IL, USA). From 1988 and 2000, RSV antigens were positive in 495 respiratory secretions. Underlying conditions such as neural, cardiac or oto-laryngeal diseases or congenital anomalies which might trigger wheezing, were present in 25 children. These children were consequently excluded from this study. We defined acute RSV bronchiolitis by the presence of audible wheezing without the use of a stethoscope, and respiratory distress using accessory respiratory muscles in addition to chest roentgenogram findings of hyper-inflation. Children having massive pneumonic consolidations extending to two lung fields that might decrease air space were excluded, while children having slight hilar shadow enlargement or atelectasis or consolidation restricted to one lung field were included in this study. By history, wheezing episodes in the past were present in 162 boys and 94 girls. We excluded children who had any wheezing history. Therefore, we studied children with wheezing detected for the first time, exclusively. Ninety boys and 51 girls satisfied our clinical criteria of acute RSV bronchiolitis. Almost all children except five boys and six girls were hospitalized. Two infants required intubation and mechanical ventilation for a short time. There was no fatal case.

Blood specimen

White blood cells (WBCs) were counted mechanically. The Coulter STKR system (Beckman Coulter, Fullerton, CA, USA) was used for counting WBCs using the method of electric impedance. WBC differentiation was doubly checked. First, differentiation was made automatically using a three-color-pattern recognition method using Hitachi 8200 (Hitachi, Tokyo, Japan). Then, confirmation was made microscopically by a technician. Blood eosinophil count was calculated by using total blood WBC count and eosinophil fraction percentage. Note that WBC differentiation was not evaluated during emergency times such as nights and holidays. The first blood test was performed when RSV antigens were positive, and the second test was carried out within 2 wk after the first blood test. Amount of serum C-reactive protein (CRP) was determined by assessing turbidity photometrically with the commercial kit Lpiaace CRP-L (Mitsubishi-Chemical, Tokyo, Japan).

Sputum specimen

Sputum specimen from subjects was obtained using an aspirator (21, 22). In brief, an aspirator with a two-way tube having a trap in the middle was used. Coughing was induced by rubbing the anterior neck of infants, and then the tube was inserted orally to quickly take a specimen during coughing. This procedure was repeated, if necessary, until mucous secretion was caught in the trap. Diluents were not added. A thick or mucopurulent portion of specimen was selected, and smeared on a glass slide for microscopic examination. If a specimen looked mucoserous, it was centrifuged at 1600 *g* for 10 min, and sediments were used for microscopic examination. Reliable sputum samples for microscopic analysis were judged, based on cellular components. Specimens were confirmed as sputum by the presence of alveolar macrophages. Specimens containing diffuse massive squamous epithelial cells or a lack of WBC accumulation were excluded from further consideration, because accumulation of macrophages or WBC mainly neutrophils, reflected sputum quality. Above all, neutrophils were the main cellular components in sputum. During microscopic examination, eosinophils in specific microscopic fields were counted, and assigned a score. A score of 4+, 3+, 2+, 1+, and $\pm$ represented > 50, 21–50, 11–20, 6–10, and < 6 eosinophils in a total of five fields, with an objective lens of 1000 $\times$ magnifications, respectively as described in our previous paper.

Neutrophil score in sputum was assessed by the ratio of neutrophils to mononuclear cells microscopically. A neutrophil score of 4+, 3+, 2+, 1+ represented >90%, 89–60%, 59–21%, <20% of neutrophils, respectively. Ratios of neutrophils to mononuclear cells differed for every microscopic field; therefore, neutrophil scores represented a wide range of ratios of neutrophils to mononuclear cells. As a result, one score covered a wide range of neutrophil ratio. For example, a neutrophil score of 3+ represented 89–60% of neutrophils together with 11–40% of mononuclear cells. On the contrary, a neutrophil score 2+ represented 59–21% of neutrophils together with 41–79% of mononuclear cells. This ratio between eosinophils and neutrophils was determined by differentiation of 300 WBC in sputum. Sputum specimens were obtained successfully from 42 boys and 29 girls, and were used for further analysis. Fifteen specimens from boys and seven specimens from girls were discarded because of poor quality after application on slides for microscopic examination.

Measurements of specific immunoglobulin E (IgE) for mites, egg whites, milk were done using commercial kits of CAP radioimmunoallergosorbent assay (CAP-RAST). Kits were from Pharmacia Pharmaceutical, Uppseda, Sweden (FEIA CAP system with immunocap). Serum-specific IgE levels were classified according to six scores. Score 0 represented <0.35 U/ml; score 1 was for 0.35–0.7 U/ml; score 2 was for 0.7–3.5 U/ml. We considered scores of ≥ 2 for mites, and scores of ≥ 1 for foods (egg white or milk) as positive scores.

Follow-up study

In general, we obtained information about outcomes of RSV bronchiolitis if a patient returned to our clinic because of another wheezing episode until 2002. In 2002, we sent letters to 52 patients' families. We successfully received answers from 19 families (19 infants). Twenty letters were returned due to a move. Interval from bronchio-

litis to inquiry was 4–7 yr in four infants, 8 yr and longer in 15 infants.

Statistical analysis

Parameters were analyzed using a repeated measures analysis of variance (one-way balanced ANOVA). Continuous variables were compared using a Student's *t*-test or by analysis of variance with the Turkey test or multiple comparisons. Categorical variables were compared by using Chai analysis. Statistical significance was defined as $p < 0.05$. All data are reported as means $\pm$ s.d. unless otherwise stated. Calculations were performed with a statistical package (Stat Mate 3 for Windows, Tokyo, Japan).

Ethics

The Institutional review boards approved the study protocols.

Results

Clinical features of children with RSV bronchiolitis

Age distribution in combination with febrile episodes at the acute stage of RSV bronchiolitis is shown in Fig. 1. For infants under 1 month of age, there was a large difference in numbers between boys ($n = 10$) and girls ($n = 2$). Incidence of febrile episodes in each month of age differed between boys and girls. Febrile incidence was higher in younger boys than girls under 6 months old, but was more frequent in girls after half a year of life. After 6 months of age, all girls (13/13) were febrile, while 18/25 (72%) of boys were febrile (Chi-square test, $p < 0.05$). Table 1 shows comparisons of RSV bronchiolitis by gender and months of age with clinical symptoms and laboratory results, abnormal shadows in chest roentgenograms, serum specific IgE antibodies and family histories. Hyper-inflation in chest roentgenograms was seen among all infants but often associated with atelectasis or a variety of abnormal shadows. Hyper-inflation

Fig. 1. Age distribution of children with respiratory syncytial virus (RSV) bronchiolitis in combination with febrile episodes in the acute stage, ■: Febrile, □: Not febrile.

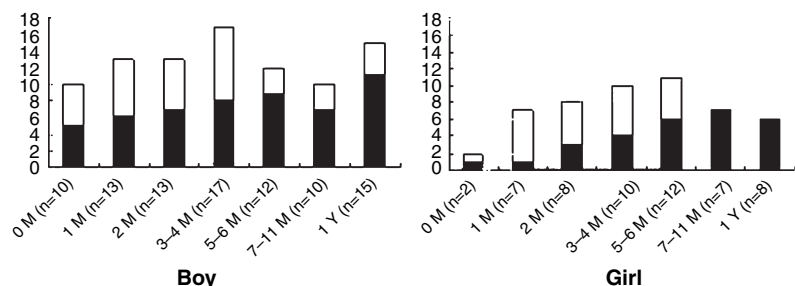


Table 1. Clinical background of children with respiratory syncytial virus bronchiolitis

	Boys (n = 90)	Girls (n = 51)
Febrile		
0–1 m	11/23	2/9
2–6 m	21/42	13/29
7–11 m	7/10	7/7
12 < m	11/15	6/6
Total	49/90	28/51 (Not significant)
Pneumonia		
0–1 m	4/23	4/9
2–6 m	21/42	10/27
7–11 m	6/9	3/5
12 < m	11/15	6/6
Total	51/89	23/47 (Not significant)
Specific IgE to mite		
0–1 m	0/8	0/3
2–6 m	2/22	0/17
7–11 m	1/6	0/4
12 < m	2/6	0/4
Total	5/42	0/28 (Not significant)
Specific IgE to egg or milk		
0–1 m	0/8	1/3
2–6 m	6/21	4/15
7–11 m	2/4	1/4
12 < m	1/6	1/4
Total	9/39	7/26 (Not significant)
Eczema (patient)	12/91	5/51 (Not significant)
Family history of asthma	32/91	16/51 (Not significant)
Family history of allergy	15/91	8/51 (Not significant)

alone without any abnormal shadows was observed in 38 of 89 (42.7%) boys, and, in 24 of 47 (51.1%) girls (no significant difference). Positive results for specific IgE antibodies against mites were obtained in boys (5/42, 11.9%) exclusively. For specific IgE antibodies for egg white or milk, rates were similar in boys (9/39,

23.1%) and in girls (7/26, 26.9%). There was no significant difference in allergic family histories between boys and girls.

Laboratory test results of children with RSV bronchiolitis

Blood tests. Blood WBC counts are shown in Fig. 2. Mean $\pm$ s.d. of WBC was 10274.7 ± 3689.7 in boys ($n = 86$), and 11902 ± 4475.6 in girls ($n = 49$). These figures were significantly different (t -test, $p < 0.05$). Similarly, serum CRP levels were higher in girls than in boys. For serum CRP levels from 0 to 7 mg/dl, mean $\pm$ s.d. value of CRP was 0.66 ± 1.06 mg/dl in boys, and 1.22 ± 1.60 mg/dl in girls (t -test, $p < 0.05$). The number of infants with CRP levels > 1 mg/dl was 15 of 79 (19%) for boys, and 17 of 45 (37.8%) for girls. These figures were significantly different (Chi-square test, $p < 0.05$). During RSV bronchiolitis, blood eosinophil count increased along days of illness. Comparison between blood eosinophil count at the first test (RSV positive) and the second test by age is shown in Fig. 3 (boys) and Fig. 4 (girls), respectively. There was a significant difference in blood eosinophil counts between the first test and the second test. In boys, mean $\pm$ s.d. of blood eosinophil count was 87.47 ± 127.49 ($n = 63$) at the first test, and 269.24 ± 246.67 ($n = 50$) at the second test (t -statistics, $p < 0.001$). On the contrary, mean $\pm$ s.d. of blood eosinophil count in girls was 104.26 ± 159.61 at the first test ($n = 42$), and 276.81 ± 314.36 at the second test ($n = 28$) (t -statistics, $p < 0.05$). Blood eosinophil count was inversely related to months of age. Mean $\pm$ s.d. of eosinophil count at the first test

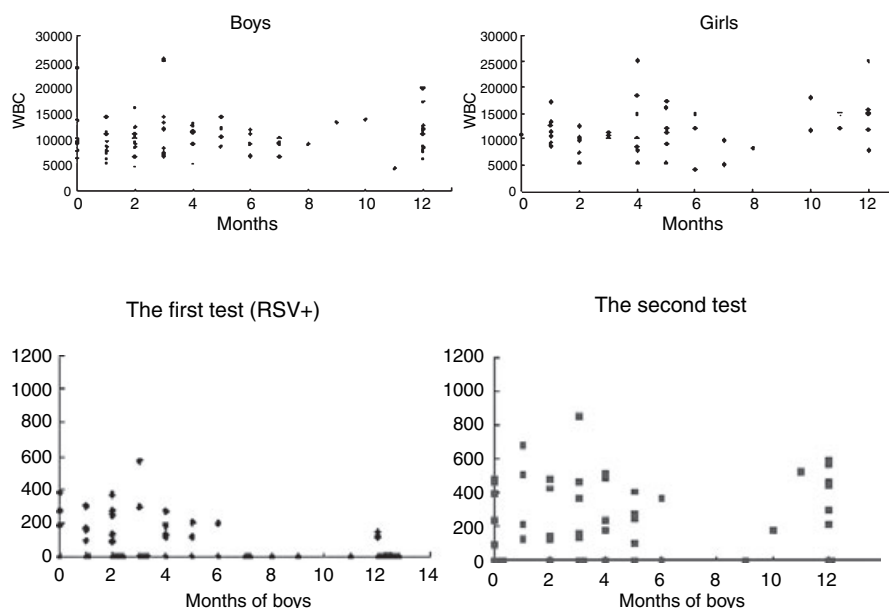


Fig. 2. White blood cells in the acute stage of RSV bronchiolitis. Circles represent individuals. Left is boys and right is girls.

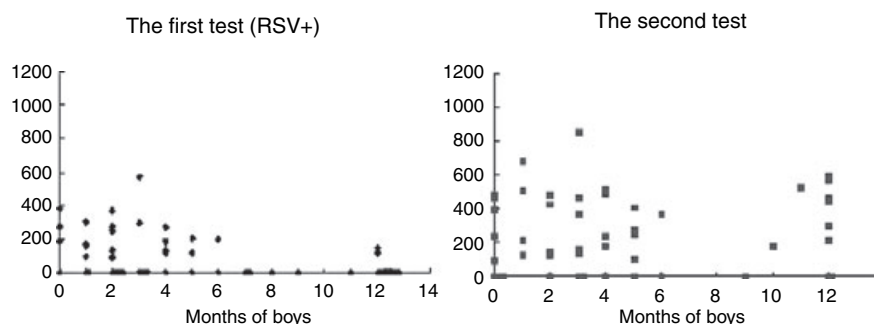
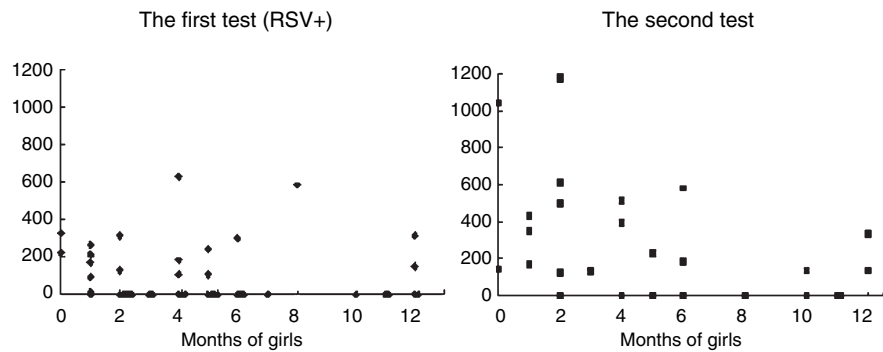


Fig. 3. Fluctuations of blood eosinophils at the first test (left) and the second test (right) during RSV bronchiolitis in boys. Circles or squares represent individuals.

Fig. 4. Fluctuations of blood eosinophils at the first test (left) and the second test (right) during RSV bronchiolitis in girls.



was 183.1 ± 150.9 in 0–1-month-old infants, 68.2 ± 101.2 in 5–6-month-old infants, 40.9 ± 131.8 in 7 months and older infants, respectively. Blood eosinophil counts in the first test among boys and girls aged 0–1 months were significantly higher than those for infants aged 5–6 months (t -statistics, $p < 0.01$) and those aged ≥ 7 months (t -statistics, $p < 0.001$). Blood eosinophil count decreased in number among children after 6 months of age, especially in boys. In boys, blood eosinophil count at the acute stage before 6 months of age (129.46 ± 138.34) was significantly higher than that after 6 months of age (24.58 ± 59.93) (t -statistics, $p < 0.05$). However, blood eosinophil counts between boys and girls, even restricted to infants over 4 months of age were not significantly different.

Eosinophil and neutrophil scores of sputum specimens

Fig. 5 shows the relationships between infant's age and their sputum eosinophil scores. One sputum specimen obtained from an infant under 1 month of age was scored 4, and two sputum specimens from infants under 1 month were scored 0. In total, sputum eosinophil with a score of 2+ and more affected six specimens, but five of them were obtained from infants aged 0–3 months. Sputum eosinophilia was not observed in infants over 4 months of age except for one. All infants having sputum eosinophilia were boys, while, girls did not show any local eosinophilia throughout all months. On the contrary, sputum neutrophilia was observed commonly in boys and girls. Most sputum specimens showed accumulating neutrophils. Neutrophil score in

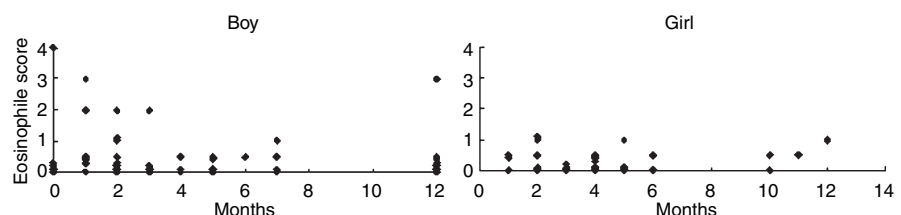
sputum specimens was as follows: score 3 in 62 specimens, score 2 in five specimens, and score 4 in four specimens.

Follow-up study. After RSV bronchiolitis diagnosis, 25 infants came back to our hospital because of recurring wheezing episodes. Moreover, we obtained follow-up information from 19 of 52 patients by letter inquiry as mentioned in Methods. Twelve of 19 children were reported to have recurrent wheezing afterwards, while seven children did not. We assessed these 12 children with recurrent wheezing. In addition, we assessed another 16 patients who actually revisited our hospital because of subsequent wheezing. Blood eosinophil counts during RSV bronchiolitis in these 28 children are shown in Fig. 6, whereas, those in seven children, whose parents answered 'no subsequent wheezing', are shown in Fig. 7. Mean $\pm$ s.d. of eosinophil counts at the first test in children with subsequent wheezing was 143 ± 64.56 , while, those in children without subsequent wheezing was 85.71 ± 55.34 (not significant). Eosinophil count during RSV bronchiolitis was not a predictable marker for wheezing. Similarly, 14 sputum specimens collected from children with subsequent recurrent wheezing during RSV bronchiolitis did not reveal eosinophil scores of 2 and more.

Discussion

Although much attention has been paid to the link between RSV infection and asthma onset, the role of eosinophilic inflammation because of RSV has remained unclear. The present

Fig. 5. Sputum eosinophils in the acute stage of RSV bronchiolitis left is for boys and right is for girls.



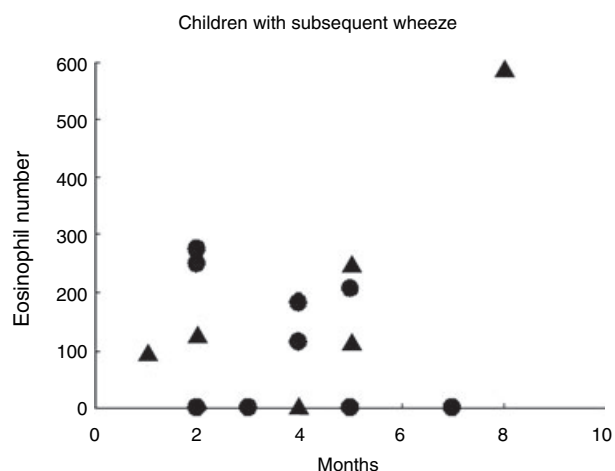


Fig. 6. Blood eosinophils during RSV bronchiolitis in infants with subsequent wheezing. Circles represent boys and triangles represent girls.



Fig. 7. Blood eosinophils during RSV bronchiolitis in infants without subsequent wheezing. Circles represent boys and triangles represent girls.

gender analysis showed immunologic differences between boys and girls through clinical and laboratory results in children with RSV bronchiolitis. Here we demonstrate new information about gender difference. A major advance is the demonstration that laboratory responses characterized by WBC counts and serum CRP levels at acute stage were predominant in girls. On the contrary, eosinophil counts fluctuated depending on infant's age and gender. Blood eosinophilia was more prominent in boys than girls in the early months. However, boys rarely showed blood or sputum eosinophilia after 6 months of age. In contrast, girls showed no sputum eosinophilia. Febrile episodes were frequent in boys of early months, but in girls of late months. Collectively, clinical features of RSV bronchiolitis differed when infants were less than or more

than 6 months old. We must be cautious about evaluating eosinophil counts in young infants under half a year of life. Initial T-cell priming against antigens appears to occur prenatally via transplacental transfer. This is consistent with previous studies showing that fetal immunologic propensity might be left in young infants. In our study, infant's age was inversely related to blood eosinophil count at the acute stage of RSV bronchiolitis. Moreover, long-term persistence of RSV in a guinea-pig model was shown to result in eosinophilic response accompanied with airway hyper-responsiveness. These changes might be pertinent to the pathogenesis of post-bronchiolitis wheezing and asthma in children (23). These facts also suggested that occurrence of eosinophilia might be multi-factorial. Prediction of asthma by eosinophil count may be difficult because different factors may provoke eosinophilic accumulation. Eosinophil activation relevant to RSV infection reported by previous clinical studies showed a variety of results. That might be explained by the diversity of infants' backgrounds and ages.

Previously, local reactions in bronchoalveolar fluid (BALF) from young children have been reported (24–28). Neutrophilic dominance in BALF in young infants with RSV bronchiolitis was reported in most of these studies. The report by Everard et al. (27) did not support that excessive lymphocyte activity was responsible for the pathology of RSV bronchiolitis. Inflammatory indices did not correlate with RSV concentration (26). The bronchoalveolar lavage (BAL) cellular profile in an asthma group was characterized by eosinophils, whereas neutrophil ratio was higher in the bronchiolitis group. However, Marguet et al. (25) reported that several cytokines, ECPs and cellular components were in common between children with asthma and bronchiolitis. In our sputum analysis, eosinophilia was observed exclusively in boys under 4 months of age. In addition, follow-up study revealed that eosinophilia in the early months did not predict subsequent wheezing. We previously showed the advantage of sputum analysis for detecting subtle local trafficking of eosinophils (29). For ECP, we showed a positive relationship between respiratory eosinophilia and ECP levels in sputum from aged children with wheezing, but this association was not clear in younger children. Local eosinophilia did not always parallel ECP levels in the BAL study (25). Sigurs et al. (30) showed that nasal ECP/albumin ratios after 6 months of age were significantly higher than during acute RSV infection. Otherwise they did not find any predictable value in local or blood

ECP. Regardless of asthma history in the family, bronchiolitis was the highest independent risk factor for asthma at 7 yr of age (5). Heymann et al. (31) assessed blood eosinophil counts during wheezing episodes, and showed that eosinophil counts remained high among children suffering wheezing compared with control children after the age of 4 yr, but were indistinguishable between the two groups before the age of 4 yr. Koller et al. (10) found that infants who went on to have persistent episodes of wheezing later in life had higher serum levels of ECPs at the time of their first acute illness, when compared with infants who would not subsequently have more than three such episodes. Similarly, Martinez et al. (7) showed that persistent wheezers had eosinophilia during their lower respiratory infection, whereas blood eosinophil counts in transient early wheezers who stopped wheezing by 6 yr were significantly reduced during the acute stage. The mean age of their subjects for lower respiratory infection was around 8.4 months old for transient early wheezers, while it was 11.3 months old for persistent wheezers. Their subjects were older than those in our report. Usually, acute bronchiolitis because of RSV occurs at younger ages within 3 months of age.

Many factors mentioned above may contribute to the pathogenesis of RSV bronchiolitis. Subjects, in this study, might reflect Japanese RSV bronchiolitis in general, because infants were referred because of severe enough, acute infectious signs that required hospitalization. They did not have a special background. We cannot tell as to whether Japanese infants suffering from wheezing requiring hospitalization may have a unique clinical outcome that may be different from other counterparts in the USA or Europe. Martinez et al. (6) reported that early wheezing was associated with persistent wheezing in Hispanic groups but not in other major ethnic groups. Above all, gender was a critical factor in RSV bronchiolitis. Zylaya et al. (20) reported that ECP levels in nasopharyngeal aspirates were exclusively higher in girls than in boys. Consistently, our study showed that blood eosinophil count was suppressed in number in boys after 6 months of age. It is noteworthy that five boys showed specific IgE antibodies to mites exclusively, in this study. Gender differences for sensitization to inhaled antigens could potentiate the predominance of asthma onset in male. In view for neonatal endocrinology, circulating testosterone levels in boys are high during the early months. However, roles of sex hormones in this critical period have not been explored. Most

probably, immunologic influence could not be ignored because male hormones were generally immunosuppressive (32).

An inevitable respiratory infection and genetic susceptibility of asthma diathesis are not mutually exclusive. Risk factors and respiratory infections in the development of childhood asthma are now partly clarified with information from longitudinal cohort studies, but still controversial. Gender analysis of inflammatory response might contribute further knowledge for linking factors to asthma progression.

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