

Analysis of sputum taken from wheezy and asthmatic infants and children, with special reference to respiratory infections

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Children who are destined to develop asthma are considered to be susceptible to a variety of respiratory pathogens. To elucidate respiratory inflammation among these children, we measured the levels of eosinophil cationic protein (ECP) and tryptase in sputum taken from three different groups of wheezy infants and young children: those with a first wheeze ($n=15$); those with recurrent wheeze ($n=27$); and those with recurrent wheeze with respiratory distress, namely asthma ($n=56$). The numbers of eosinophils or metachromatic cells determined by microscopic analysis of sputum samples were also evaluated in combination with the ECP and tryptase levels. Although neither sputum ECP nor tryptase was a clear discriminative marker that differentiated the three different types of wheezy disease, ECP levels in sputum from the asthma group were significantly higher ($2,269.2 \pm 6,216.8$ ng/g) than those in the recurrent wheezy group ($440.3 \pm 1,199.8$ ng/g) or in the first-wheeze group (209.0 ± 172.9 ng/g). A similar trend was observed with tryptase levels in sputum, but there were no significant differences among the three groups. Sputum taken from asthmatic children showed a marked accumulation of eosinophils. However, an accumulation of eosinophils in sputum (even in the presence of an elevated level of sputum ECP) was not identified in the asthmatic infants <1 year of age. An accumulation of eosinophils in sputum was not evident until children became >1 year old and thereafter the eosinophils rapidly increased in number until the children reached 5 years of age. It was noteworthy that sputa positive for pathogenic bacteria, taken from the 1- and 2-year-old asthmatic infants, had a tendency to show high levels of ECP but a reduced number of eosinophils. Along with the wheezy episodes induced by viral infection, primarily and occasionally in combination with secondary bacterial infection, eosinophil activation and infiltration may develop. These predestined immune reactions to various pathogens might be associated with triggering the onset of asthma.

**Yoko Nagayama, Toshikazu Tsubaki,
Tsuyoshi Toba, Shigeru Nakayama
and Ookusu Kiyofumi**

Chiba Children's Hospital, Department of
Allergy, Immunology and Respiratory Disease,
Midoriku, Chiba, Japan

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Yoko Nagayama, Chiba Children's Hospital,
Department of Allergy, Immunology and
Respiratory Disease, Hetacho 579-1, Midoriku,
Chiba 266-0007, Japan
Tel.: +81-43-292-2111
Fax: +81-43-295-3815
E-mail: yokonagayama@aol.com

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Asthma has been established as a chronic inflammatory state characterized by eosinophilia in the lower respiratory tract. However, there is no consensus as to the definition of asthma and the relevance of eosinophil infiltration during early childhood. Cellular events that occur in the wheezy state remain poorly investigated. It is still a matter of debate as to when and how eosinophil

infiltration is established in the lower respiratory tract of asthmatic children. Several articles have reported investigations of the cellular events in bronchoalveolar lavage (BAL) fluids taken from infants and young children with wheeze (1,2). The reports of BAL analysis showed that asthma was characterized by eosinophilia, whereas infantile wheeze was characterized by neutrophilia in the

BAL fluid. However, one of the reports found that eosinophils in BAL were observed in 27% of the children with infantile wheeze. It has been proposed that inflammatory cells and cytokines limit viral replication. It is probable that these cytokines play a central role in the neutrophil influx and disease protection, but can also induce augmented disease (3,4). Interleukin (IL)-8 and neutrophil recruitment and activation are considered to be the key factors for viral-induced asthma among children (5). In adults also, prominent neutrophilic inflammation in sputum has been reported among asthmatic patients admitted for acute, severe asthma or fatal asthma (6–8). Sputum analysis has been used for the evaluation of asthma patients to elucidate these cellular reactions. Sputum was considered to be less reliable compared to BAL for the assessment of cell counts. By the sequential induction of sputum, the percentages of eosinophils, neutrophils and eosinophil cationic protein (ECP) levels were higher at the start, whereas the percentage of macrophages was high at the end of induction (9). Taking sputum samples is not invasive and facilitates serial examination. Fahy et al. (10,11) showed the value of sputum examination in the diagnosis of asthma by their finding that sputum could provide better quantitative information on asthmatic inflammation than BAL. Sputum analysis data showed that the sputum cells were more diagnostic than BAL (10–13). We have previously shown a pattern of increasing number of eosinophils and metachromatic cells in sputum and nasal smears among asthmatic children, according to age (14). Based on this data, eosinophil accumulation had not yet become established among asthmatic children <1 year of age, but had increased in number in children who were 3 years old. Furthermore, the eosinophil accumulation in sputum occurred at an earlier age than that in nasal smears. In the present study we measured the level of ECP and tryptase in sputum taken from initial or recurrent wheezy children in combination with microscopic examination to elucidate the relationship between factors in sputum and asthma onset.

Materials and methods

Specimen collection and processing

Sputum specimens were obtained from the subjects by using an aspirator or by spontaneous expectoration. Details were as described previously (14,15). Briefly, older children were instructed to cough and produce a sputum specimen, induced by deep forced expiration by

using abdominal muscles. In younger children and in those not familiar with this procedure for production of sputum, the following procedure was used: as coughing drove sputum up the trachea, we rubbed the upper section of their cricoid cartilage to initiate coughing for expectoration. If this method was not successful, a two-way tube with a trap was used. One end of this tube was connected to the aspirator equipment on the wall or an aspirator machine. Coughing was then induced by rubbing the anterior neck of small children, a tube was inserted orally, and a specimen taken quickly during coughing. We repeated this procedure, if necessary, until mucous secretion was identified in the trap tube. Diluent solution was not added.

Sputum cell analysis

The thick or mucoprulent part of the sputum specimen was smeared directly onto a slide glass for microscopic examination. If the specimen was mucoserous, it was centrifuged at 1,600 *g* for 10 min and the sediment collected for microscopic examination. We confirmed the specimen as sputum by the presence of alveolar macrophages in microscopic fields. Specimens containing diffuse massive squamous epithelial cells or no macrophages were excluded from further analyses. However, regardless of the number of neutrophils present, specimens with macrophage accumulation were considered as sputum. During microscopic examination, the number of eosinophils and metachromatic cells were counted in the microscopic fields. Score grades of eosinophils or metachromatic cells were defined as 4+, 3+, 2+, 1+ and $\pm$, and represented >50, 21–50, 11–20, 6–10, and <6 eosinophil or metachromatic cell numbers, respectively, counted from a total of five fields of $\times 1000$ magnification, as described previously (14). The scores of neutrophils/lymphocytes were judged by counting the white blood cells in the same fields. The scores of $\pm$, 1+, 2+, 3+, and 4+ of neutrophils (lymphocytes) represented <10%, 11–20%, 21–59%, 60–89%, and >90%. Macrophage score was determined by direct counting at $\times 100$ magnification. Our scoring methods did not determine the proportion of each cell – neutrophils, macrophages and eosinophils – as a whole but subtle eosinophil infiltration can be detected in selective areas at an early stage (16).

Bacterial culture

The sputum specimen was transferred to the plate and a thick portion was taken selectively and homogenized with the tip of a platinum loop. A

Table 1. Characteristics of the subjects by age in the three wheezy groups

Clinical group	Age								Total
	0–1 month	2–3 months	4–5 months	6–11 months	1 year	2 years	3–4 years	≥5 years	
First wheeze	7	5	1	0	2	0	0	0	15
Mite RAST+	0/4	0/5	0/1	0	1/2	0	0	0	1/12
Only food RAST+	0/4	0/5	0/1	0	0/2	0	0	0	0/12
RSV antigen+	5/7	4/5	1/1	0	0/2	0	0	0	10/15
Pathogenic bacteria+	2/5	0/4	0/1	0	0/2	0	0	0	2/12
Recurrent wheeze	0	0	3	11	10	1	1	1	27
Mite RAST+	0	0	0/3	0/11	3/10	0/1	1/1	0/1	4/27
Only food RAST+	0	0	0/3	2/11	2/10	0/1	0/1	0/1	4/27
RSV antigen+	0	0	3/3	5/10	2/10	0/1	0/1	0	10/25
Pathogenic bacteria+	0	0	1/3	2/8	6/10	0/1	0/0	0/1	9/23
Asthma	0	0	0	7	9	9	11	20	56
Mite RAST+	0	0	0	4/7	3/9	7/9	11/11	17/20	42/56
Only food RAST+	0	0	0	1/7	0/9	0/9	0/11	1/20	2/56
RSV antigen+	0	0	0	2/7	2/7	0/7	1/9	0/11	5/41
Pathogenic bacteria+	0	0	0	2/6	5/8	2/8	1/10	1/13	11/45

RAST, radioallergosorbent test; RSV, respiratory syncytial virus.

0.01-ml sample was then streak-inoculated directly onto trypticase-soy agar, supplemented with 5% sheep blood and chocolate agar (BBL; Becton-Dickinson, Tokyo, Japan), and incubated overnight at 35°C in an atmosphere of 7% CO₂. The bacterial species were identified according to standard methods (17). The bacterial count was semi-quantitatively estimated from the distribution of colonies in the streak culture. The criteria for positive bacterial culture was dependent on the number of pathogenic bacteria. A count of pathogenic bacteria of $\geq 10^5$ /ml, or at least 10-fold greater than counts for the non-pathogenic varieties such as *Neisseria* and *Streptococcus viridans*, was judged to be a positive bacterial finding.

Mediator assays

Sputum specimens were put on a plate and 50–200 µl from the mucous portion was transferred to a tube. The amount of specimen used depended on its characteristics (i.e. viscous or serous). After weighing a selected portion of the specimen, 1–2 ml of saline and beads were added to the tube, mixed by vortex, and centrifuged for 30 min at 40,000 g. The supernatant was stored until required for analyses. ECP and tryptase levels were measured by using the Pharmacia ECP RIA[®] (Pharmacia, Upsalla, Sweden) and Pharmacia Tryptase RIACT[®]. Specific immunoglobulin E (IgE) to mite, egg white, and milk, in serum, were measured by enzyme-linked immunosorbent assay (ELISA), using the Pharmacia Immuno CAP-RAST[®] system (Pharmacia). Respiratory syncytial virus (RSV) antigen was analysed in nasal secretion or sputum using a

commercial kit (Abbott Test Pack RSV test, Abbott Laboratories, Abbott Park, IL, USA). A specimen was not taken from healthy controls owing to the difficulty of obtaining specimens. Therefore, as a control, we measured ECP levels in saliva taken from asthmatic children, 4–10 years of age, who were able to expectorate saliva.

Subjects

Subjects who received treatment as an outpatient, or were admitted to the Chiba Children's Hospital because of an acute wheezy condition, were enrolled in this study after exclusion of the following: congenital abnormalities; foreign bodies; tumors; cardiac or neural diseases; and tracheo-laryngomalacia and other otopharyngeal problems such as crycopharyngeal coordinate insufficiency. Patients with generalized immunodeficiency or cystic fibrosis were also excluded. The wheezy children were divided into three clinical groups according to the number and severity of previous wheezy episodes. The three groups were as follows: Group 1, first wheeze (n=15); Group 2, recurrent episodes of mild wheeze but occasionally moderate or severe wheeze not more than twice previously (n=27); and Group 3, recurrent episodes of apparent wheezing diagnosed clinically as asthma (n=56). The distribution of the patients by age in each group is shown in Table 1 in combination with their atopic diathesis and presence of RSV infection or pathogenic bacteria. The grading of mild, moderate, and severe wheezing was made by the clinical impressions of airway narrowing characterized by sound or respiratory distress.

Table 2. Distribution of sputum cell scores in the three wheezy groups

Clinical group	Macrophage				Eosinophil					Neutrophil				
	±	+	2+	≥3+	±	+	2+	3+	4+	±	+	2+	3+	4+
First wheeze (n=15)	5	7	0	3	12	0	2	1	0	1	3	3	8	0
Recurrent wheeze (n=27)	6	7	2	12	18	0	3	2	4	1	2	1	21	2
Asthma (n=56)														
0–2 years (n=25)	6	10	5	4	12	1	2	1	9	0	2	3	17	3
3–12 years (n=31)	5	5	6	15	1	1	0	4	25	3	1	0	27	0

Ultimately, the diagnosis of asthma among infants and small children depended on their history of hospital admission. Admissions among young asthmatic children in Group 3 were frequent (more than twice), whereas admission among wheezy children in Group 2 did not occur more than once. The majority of asthmatic children had been followed-up regularly at our hospital. At sputum sampling, the admission rate was 15/15 (100%) in the first wheezy group, 19/27 (70.4%) in the recurrent wheeze group and 38/56 (67.9%) in the asthmatic group. The number of children who already had specific IgE to mite, which is the major allergen in Japan, was 42/56 (75.0%) in the asthmatic group but low in the other two groups. The number of children with pathogenic bacteria in sputum was two of 12 (16.7%) in the first wheezy group, nine of 23 (39.1%) in the recurrent wheezy group, and 11/45 (24.4%) in the asthmatic group. The bacterial species were restricted to the three major pathogens: *Hemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis*. The distribution of bacterial species in each of the three groups was as follows: two of *M. catarrhalis* in Group 1; three of *H. influenzae*, one of *S. pneumoniae*, one of *M. catarrhalis*, and four of mixed infection (with two of: *H. influenzae*, *S. pneumoniae*, *M. catarrhalis*), in Group 2; and four of *H. influenzae*, three of *S. pneumoniae*, one of *M. catarrhalis*, and two of mixed infections (as described for Group 2), in Group 3. There was no mixed infection with three pathogens.

The sputum specimens were collected before antibiotic therapy was started.

In Group 3, all asthmatic children, except for four <5 years of age, were taking oral theophyllin on a daily basis. Combination therapy with theophyllin and inhalation of cromoglycate solution was given to 22 of 36 asthmatic children <5 years of age. Inhaled steroids were not used among asthmatic children in this age-group. Among children ≥ 5 years of age, all except four were given theophyllin on a daily basis. Inhaled steroid was administered to three asthmatic children at the sputum examination and had been given to four children at some time-

point previously. No subject was given oral or parenteral corticosteroid. In Group 2, daily oral theophyllin was used as prophylaxis in seven children. Neither steroid nor cromoglycate solution was used in this group.

Statistical analysis

Statistical analysis was performed as follows. The difference among the sputum samples of the three groups in regard to ECP levels, tryptase levels, alveolar macrophage (dust cell) scores, and eosinophil scores was analyzed by using the Kruskal–Wallis analysis of variance (anova) by ranks test. Values statistically significant by this method were then analysed by using the multiple comparison procedure (Scheffes test). Correlation between ECP levels and eosinophil scores was analyzed by using the Spearman rank correlation coefficient. Differences of eosinophil scores in sputum between sputum samples that were positive and negative for bacteria was analyzed by the chi-square test. Difference of ECP levels between specimens positive and negative for bacteria was analyzed by the Mann–Whitney *U*-test.

Ethics

The study was approved by the Regional Ethics Committee for Human Research at the Chiba Children's Hospital.

Result

Sputum cell analysis

Macrophage, eosinophil, and neutrophil counts in each clinical group are shown in Table 2. Sputum cells taken from the infants in Group 1 were characterized by low numbers of macrophages and eosinophils. Higher numbers of macrophages and eosinophils were observed in the other groups. Among asthmatic children, a relationship between macrophage score and age was observed. The macrophage scores in Group 1 were significantly lower than those in asthmatic children >2 years of age ($p < 0.05$, Kruskal–

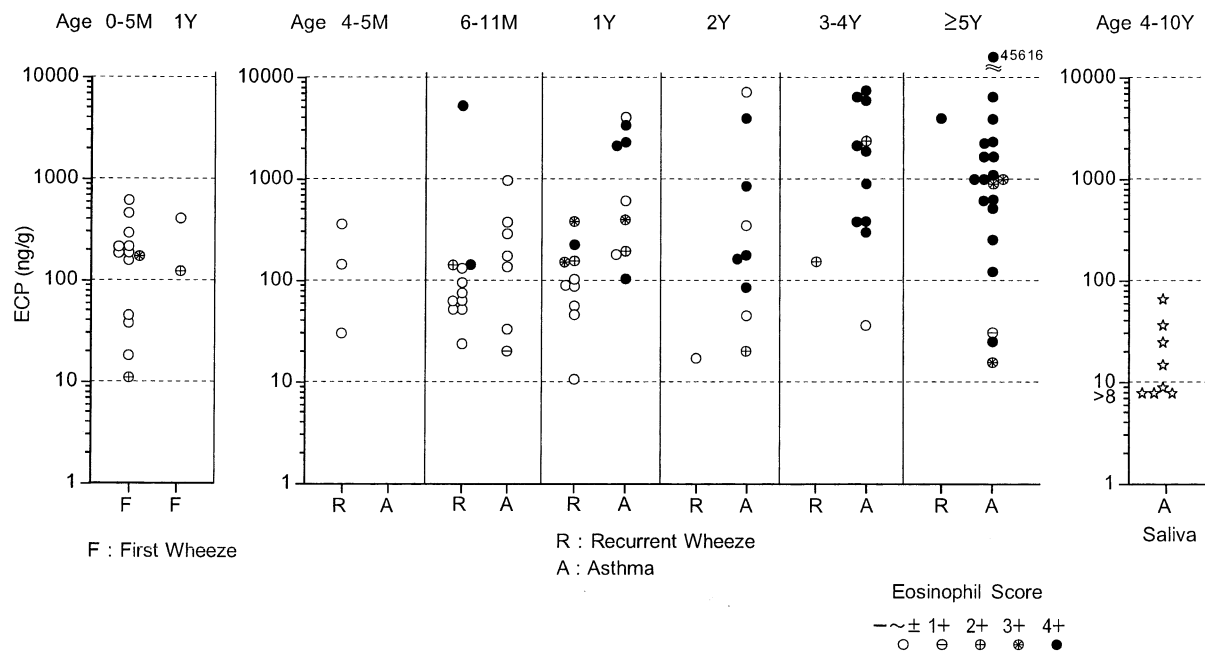


Fig. 1. Eosinophil cationic protein (ECP) in sputum.

Wallis and Scheffe tests). In the asthmatic group, macrophage scores among children 0–2 years of age were significantly lower compared with those ≥ 3 years of age ($p < 0.05$, Kruskal–Wallis and Scheffe tests). Eosinophil accumulation in sputum was established in asthmatic children

≥ 3 years of age. Eosinophil scores were significantly different between the three clinical groups ($p < 0.01$, Kruskal–Wallis and Scheffe tests). Neutrophil scores were not significantly different among the three groups by Scheffe test, but were by Kruskal–Wallis test ($p < 0.05$).

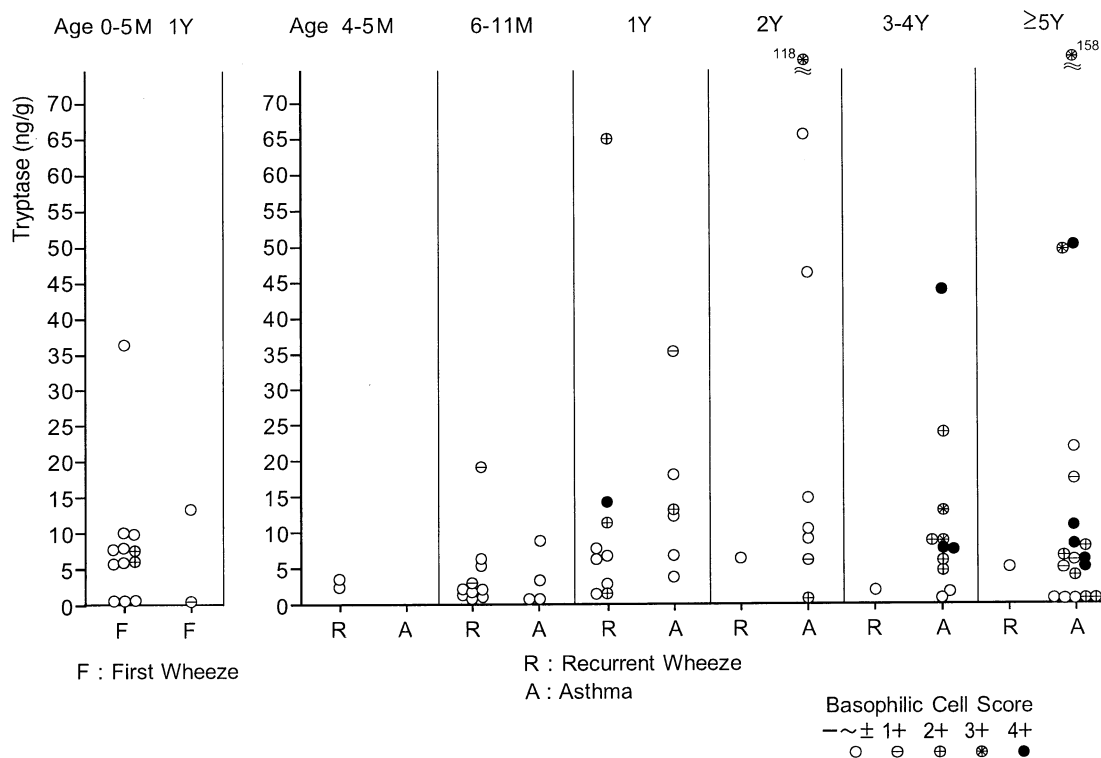


Fig. 2. Tryptase in sputum.

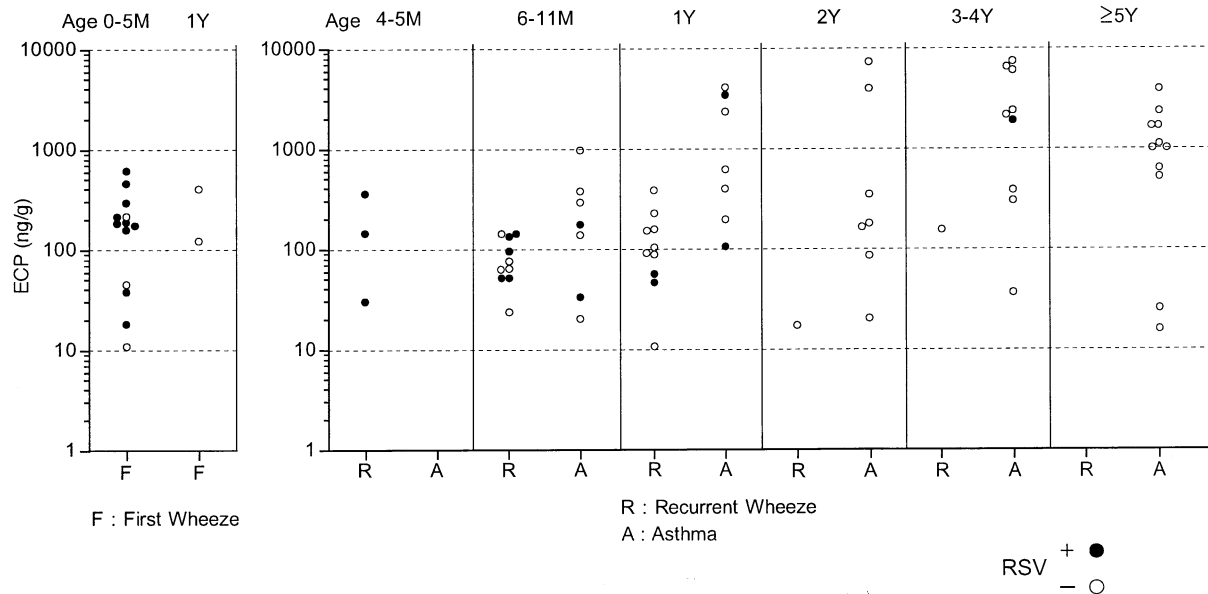


Fig. 3. Respiratory syncytial virus (RSV) antigen and eosinophil cationic protein (ECP) in sputum.

ECP and tryptase levels in sputum in combination with eosinophil and metachromatic cell scores

The levels of ECP in sputum (s-ECP) or tryptase in sputum (s-tryptase) with the relevant cell scores are plotted in Figs 1 and 2. The widely distributed s-ECP or s-tryptase levels were not discriminatory indices of the three groups and did not reflect the number or severity of wheezy episodes. Although s-ECP levels varied widely, they tended to show markedly high levels among asthmatic children >1 year of age. As a result, s-ECP levels in the asthma group were significantly higher ($2,269.2 \pm 6,216.8$ ng/g) than those in the recurrent wheezy group (440.3 ± 1199.8 ng/g) and those

in the first wheezy group (209.0 ± 172.9 ng/g). The mean and SD values of s-ECP were significantly different between the three clinical groups, as determined by using Kruskal–Wallis and Scheffe statistics ($p < 0.01$). The patterns of tryptase levels in the three groups were similar to those of s-ECP. The mean and SD values of tryptase were: 8.0 ± 9.0 ng/g in Group 1, 7.3 ± 13.0 ng/g in Group 2, and 17.4 ± 29.5 ng/g in Group 3. There was no significant difference in tryptase levels among the three groups. Concerning the relationship between s-ECP levels and score of eosinophils (as judged by microscopic examination), a discrepancy between two makers – high s-ECP level and

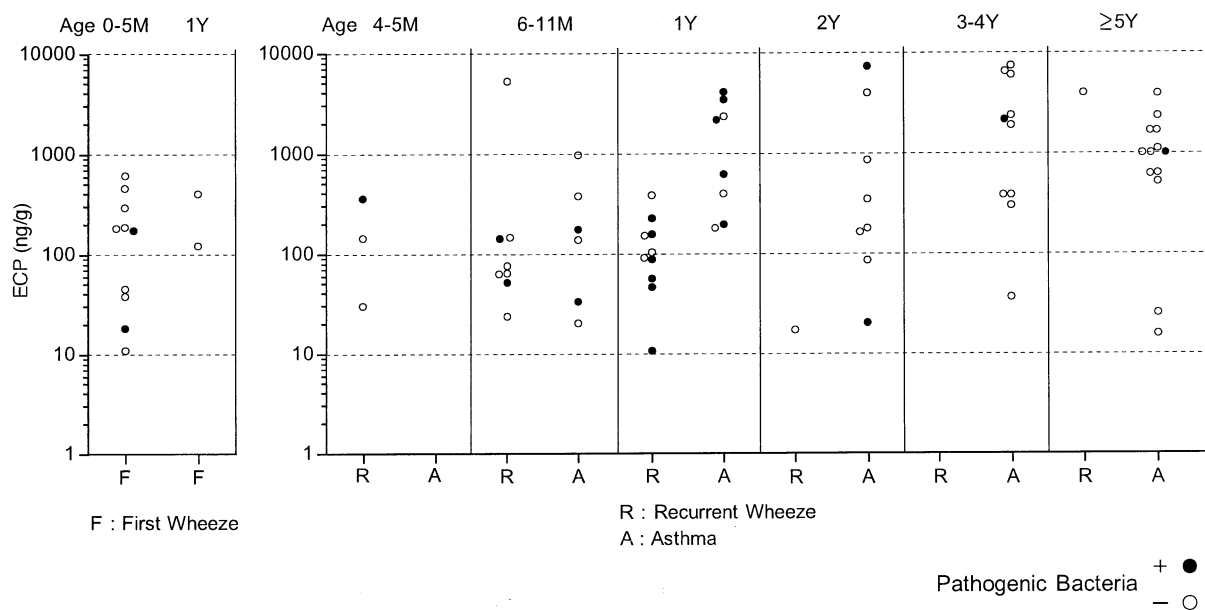


Fig. 4. Pathogenic bacteria and eosinophil cationic protein (ECP) in sputum.

Table 3. Comparison of eosinophil and neutrophil scores in sputum between bacteria positive and negative sputum

	Pathogenic bacteria positive (n=7)					Pathogenic bacteria negative (n=9)				
	±	+	2+	3+	4+	±	+	2+	3+	4+
Eosinophil	3	0	2	0	2	2	0	0	1	6
Neutrophil	0	0	1	5	1	0	0	3	5	1

low eosinophil score, and vice versa – was observed. In Group 2, a high s-ECP level was accompanied with a high score of eosinophils. In Group 3, a high eosinophil score in sputum was established regardless of s-ECP levels in children ≥ 5 years of age. The correlation between s-ECP level and eosinophil score was confirmed by Spearman's rank correlation coefficient in Group 2 ($r=0.79$, $p<0.01$) and Group 3 ($r=0.43$, $p<0.01$). The relationship between s-ECP and RSV antigen was illustrated in Fig. 3. As for RSV infection, there was no relationship between s-ECP level and the presence of RSV antigen.

Relationship between s-ECP levels and bacterial pathogens

A relationship between s-ECP levels and the presence of bacteria is shown in Fig. 4. A positive bacterial culture was associated with an increase of s-ECP levels among young asthmatic children (1 and 2 years of age). However, this association was not present among asthmatic infants and the non-asthmatic children in Groups 1 and 2. We analyzed the sputum cells and mediators (eosinophil, neutrophil, and ECP) from asthmatic children of these ages. The change in number of sputum cells in association with the presence of bacterial infection is shown in Tables 3 and 4. Sputum containing bacteria tended to have high levels of s-ECP but a reduced number of eosinophils. The mean s-ECP level in the presence of bacteria was higher ($2,548 \pm 2,725$ ng/g) than in sputum without bacteria ($983 \pm 1,387$ ng/g), but the differences were not significant (Mann–Whitney *U*-test). The proportion of specimens with an eosinophil score of $\geq 3+$ was two out of seven in sputum with bacterial pathogens and seven out of nine in sputum without bacterial pathogens. These figures were significantly different (chi-square test, $p<0.05$).

Discussion

As there is a strong association between respiratory viral infection and asthma attacks (18), the link between infection and asthma onset has been debated. Both diseases have a common pathology in the lower respiratory tract, in terms of inflammation. The present study evaluated cel-

lular components and mediators in sputum taken from three different clinical groups. From our experience, a sputum specimen with many cellular components was easy to obtain owing to the presence of infection. s-ECP and s-tryptase levels varied markedly and there was considerable overlap between the three clinical groups. However, the numbers of s-eosinophils or s-metachromatic cells reflected the clinical diagnosis. Prominent eosinophil accumulation was reached by 5 years of age among asthmatic children, regardless of s-ECP levels. In contrast, the asthmatic infants <1 year of age, who already possessed specific IgE to inhalant allergen, did not show an increase in the number of s-eosinophils. Specimens were obtained from infants and young children who were mostly at the acute stage of wheezing, requiring hospital admission. The majority of specimens in this study contained many white blood cells, which were mainly neutrophils. Only a small number of patients had lymphocytes in the sputum, but this phenomenon may be transient. The discrepancy of high s-ECP and low s-eosinophil number may be the result of eosinophil degranulation occurring without a new influx of eosinophils. It was noteworthy that this discrepancy was observed among asthmatic children of 1–2 years of age. This age-range was the transitional zone in terms of eosinophil accumulation. Recently, Gibson et al. (19) has shown the heterogeneity of the inflammatory response by using sputum taken from asthmatic children. He compared the sputum ECP levels between children with eosinophilic influx and those with combined neutrophilic and eosinophilic influx. The median value of ECP was 666 ng/ml in the eosinophil-only group and 2,146 ng/ml in the mixed eosinophil/neutrophil group. They speculated that there was greater eosinophil

Table 4. Comparison of ECP levels in sputum between pathogenic bacteria positive and negative sputum

ECP (ng/g)	Pathogenic bacteria positive (n=7)	Pathogenic bacteria negative (n=9)
2000–4000	4	2
600–2000	1	1
100–600	1	5
<100	1	1

degranulation in the children with mixed cell infiltration. They also showed an increased number of mast cells and higher IL-8 levels in the mixed group. IL-5 levels were selectively higher in the eosinophilic group. They suggested that mast cells, as well as neutrophils, might be candidate stimulators of eosinophils (6). Considering these facts, s-ECP levels in the present study might also be influenced by the presence of neutrophils in the sputum. s-ECP levels were high in sputum samples that were positive for bacteria and obtained from asthmatic or recurrent wheezy children. Furthermore, the specimen from an asthmatic girl, 2 years of age, had a high s-ECP level of 7,640 ng/g without an increase in eosinophil number but with a marked accumulation of neutrophils (4+) and bacterial pathogens. A marked increase in the ECP level, but with no pronounced increase of eosinophils, has been previously documented in the bronchial lavage of a patient who experienced a fatal asthma attack (7). In that study, the levels of ECP, neutrophil elastase, histamine, and IL-8 were similar between bacterial-positive and -negative BAL specimens, in patients receiving parenteral corticosteroid therapy during mechanical ventilation. The patients of the present study were not treated with systemic steroids at the examination. Some researchers have speculated that ECPs originate from other cell types, such as neutrophils and basophils (20–22). In cystic fibrosis, the concentrations of s-ECP were not different from those found in patients with asthma. Furthermore, acute pulmonary exacerbation raised the levels of s-ECP and IL-8 (21). In neonates, tracheal-aspirate ECP concentrations were elevated during the first week after birth in infants who subsequently developed bronchopulmonary dysplasia (BPD) (22). Sputum taken from patients with cystic fibrosis had the ability to induce ECP release from eosinophils derived from asthmatics (23). From BAL, an increased number of neutrophils in the upper (93%) or lower (76%) airways have been reported in patients with acute bronchiolitis (24). Furthermore, increases in the numbers of neutrophils and ECP were shown during acute RSV infections, with a decrease occurring after 3 days of illness (25).

Fahy et al. (26) compared the ECP and tryptase levels in sputum and saliva taken from asthmatic and control subjects. The mean level of ECP in saliva was 26.1±4.7 ng/ml, whereas the level of tryptase in saliva was very low. In the present data, the maximum value of ECP in saliva was 65 ng/g, obtained from asthmatic children who were 4 years of age. Otherwise, ECP levels were lower than 30 ng/g. The influence of saliva

contamination was considered not to be great because the mucopurulent portion of sputum was selected for the study.

Although sputum analysis still has much to be desired as a clinical diagnostic tool, we have shown that airway eosinophilia occurred during wheezy episodes. The younger the child, the progressively less likely it was to find eosinophils, even when the infants had wheeze. Although it is unclear as to whether the ECP comes from degranulated eosinophils or whether they originate from other cells, such as neutrophils, infections may initiate the production of toxic mediators which could contribute to the development of asthma. Either way, the finding of ECP could be relevant in relation to the subsequent development of full asthma pathology. Further investigation of the eosinophil role during respiratory infection may indicate a causative association with asthma. However, only longitudinal studies will be able to demonstrate this association.

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