

Awarded Article, Annual Meeting of JSA

Creola Bodies in Infancy with Respiratory Syncytial Virus Bronchiolitis Predict the Development of Asthma

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ABSTRACT

Background: Creola bodies (CrBs) in the sputum are an indicator of respiratory epithelial damage and appear specifically in bronchial asthma. We studied the presence and clinical significance of CrBs in infants with respiratory syncytial virus (RSV) bronchiolitis contributing to the development of asthma.

Methods: Aspirated sputum samples were collected from 33 infants admitted with acute RSV bronchiolitis. The samples were then examined for the presence (or absence) of CrBs and classified into the RSV-CrB group and RSV-non-CrB group. Eosinophil cationic protein (ECP) and neutrophil elastase (NE) concentrations in the sputum were compared between the two groups. History of wheeze and asthma was collected at 2 years and 5 years after their discharge from hospital.

Results: CrBs were detected in 23 of the 33 subjects (69.7%). No significant difference in the ECP and the NE concentration were observed between the RSV-CrB group and RSV-non-CrB group. A significant relationship was observed between CrBs detected with RSV bronchiolitis and the development of recurrent wheezing and asthma (after 2 years: relative risk [RR], 3.09; $p = 0.002$; after 5 years: RR 7.00; $p = 0.019$).

Conclusions: These findings suggest that a high rate of CrBs in the sputum is present in infants with RSV bronchiolitis, and notably the CrBs are associated with the progression to recurrent wheezing and asthma.

KEY WORDS

asthma, creola bodies, epithelial damage, RSV

INTRODUCTION

The peak age of onset for childhood asthma is two years, thus providing suitable treatment for young children with asthma is vital to establishing a better prognosis. The 'Japanese Pediatric Guideline for the Treatment and Management of Asthma 2008 (JPGL 2008)' therefore takes a broad approach to asthma in patients aged less than two years including children with recurrent wheezing caused by respiratory viral infections, and recommends early intervention.¹

Severe bronchiolitis in infancy is often caused by Respiratory Syncytial Virus (RSV), numerous studies

have reported associations between severe RSV bronchiolitis in infancy and recurrent wheezing and asthma in later childhood.²⁻⁶ One mechanism considered to underly the development and exacerbation of asthma triggered by respiratory viral infection is airway hyperresponsiveness and airway inflammation due to infection-induced respiratory epithelial damage and desquamation.⁷ A recent study reported that thymic stromal lymphoprotein produced by damaged epithelial cells may strongly elicit a Th2 polarization, and suggests that epithelial cells play a key role in the pathological formation of asthma.⁸

Creola bodies (CrBs) in the sputum are clusters of

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Received 13 December 2009. Accepted for publication 13 May 2010.

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Table 1 Subject characteristics

	RSV-CrB group	RSV-non CrB group	Control	P value
No. of subjects	23	10	5	
Age (months)	4.1 ± 2.7	3.6 ± 3.7	10.3 ± 10.0	N.S.
Gender M : F (%)	13 : 10 (56.5 : 43.5)	4 : 6 (40.0 : 60.0)	2 : 3 (40.0 : 60.0)	N.S.
Family history of atopic diseases No. (%)	11 (47.8)	4 (40.0)	0 (0.0)	N.S.
Illness days (days)	9.1 ± 1.0	9.3 ± 1.2	-	N.S.
Periods of hospitalizations (days)	6.5 ± 1.0	7.0 ± 1.0	-	N.S.

Data are shown as mean ± SEM.; N.S., Not significant.

desquamated epithelial cells which, according to Naylor *et al.*, appear specifically in the sputum of adults with asthma.⁹ Another study has shown that CrBs are associated with major basic protein and airway hyper-responsiveness, and that they are also useful for evaluating simple airway inflammation.¹⁰ We have already reported that CrBs in wheezy infants (excluding RSV infection) is a predictor of recurrent wheezing and progression to asthma for the two years subsequent.¹¹ However, while CrBs are also present in respiratory viral infections such as RSV, there have been no studies to date on the clinical significance of this. We have therefore investigated the effects of desquamated epithelial cells caused by RSV infection on the development of asthma by studying the presence (or absence) of CrBs in infant RSV bronchiolitis and the clinical course of subjects for five years.

METHODS

PATIENTS

From December 1999 to April 2001, infants without other concomitant chronic disease (including atopic disease) were hospitalized with RSV bronchiolitis at the Department of Pediatrics of Dokkyo University School of Medicine. Diagnosis was verified using the Abbott Testpack RSV (Abbott Laboratories, Abbott Park, IL, USA).¹² These infants had need oxygen usage, and none had any bacterial infection of the respiratory tract as shown by radiography, increase in C-reactive protein level (<0.4 mg/dl), and/or a positive rhinopharyngeal smear culture for bacteria. Therefore, the 35 infants (18 boys) of whom written informed consent was obtained from their parents or guardians were included in this study. Thus, a final RSV group of 33 infants (18 boys, mean age: 3.9 ± 2.9 months) with appropriate cell components in the sputum were enrolled in this study, excluding 2 subjects with diffuse massive squamous epithelial cells or lack of alveolar macrophages in the sputum. The control group consisted of 5 infants (2 boys, mean age: 10.3 ± 10.0 months) had operation without concomitant acute or chronic airway disease (including atopic disease).

EXAMINATION OF SPUTUM

Sputum was obtained from the subjects using an aspirator at the time of admission, as described previously.¹¹ In detail, the suction device constituted of a two-way tube with a trap. One end of this tube was connected to the aspirator equipment on the wall or an aspirator machine. As coughing drives sputum up to the trachea, coughing was induced by rubbing the anterior neck of the infant over the upper section of the cricoid cartilage. A tube was inserted orally and a specimen was immediately taken during coughing. If the patient cried vigorously, this stimulation would be ineffective. In such a case, it was necessary to immediately take the sputum when coughing was triggered by crying or aspiration. The control group were intubated at surgery, and their sputum specimens were collected by suctioning. Smears of sputum (100 µl) were spread on pairs of glass slides and fixed with ethanol (95%). One slide was stained with Papanicolaou's stain to detect CrBs, which was identified by the presence of a cluster of more than 10 desquamated respiratory epithelial cells.⁹⁻¹¹ The second slide was stained with Hancel's stain and analyzed for cell components. A total of 300 cells per power field line was counted in each preparation.

ASSESSMENT OF PATIENTS

The RSV group was classified into 2 groups, namely, as RSV-CrB group or RSV-non CrB group, by detection of CrBs in the sputum. Characteristics (age, gender, history of atopic disease in family) were compared among the RSV-CrB group, RSV-non CrB group and control group. In addition, illness days and periods of hospitalization as severity of RSV infection were compared among the RSV-CrB group and RSV-non CrB group. We also measured and compared the concentrations (pg/ml) of interleukin (IL)-8, the specific neutrophil-mobilizing cytokine by enzyme-linked immunoassay in the sputum.¹³ Equally, the concentrations of neutrophil elastase (NE, µg/l), which is a neutrophil activity marker, and eosinophilic cationic protein (ECP, µg/l), which is an eosinophil activity marker, were estimated by the Latex concentration method¹⁴ and radioimmunoassay (RIA),¹⁵ respectively. Furthermore, a prospective study was con-

Table 2 Cell differential counts in sputum (% of total cells)

	RSV-CrB group	RSV-non CrB group	Control
Macrophages	3.0 ± 7.1**	1.8 ± 2.3**	51.2 ± 25.5
Lymphocytes	24.4 ± 19.6**	17.0 ± 16.1*	1.5 ± 2.0
Neutrophils	69.2 ± 22.7**	72.2 ± 15.8**	35.0 ± 14.5
Eosinophils	0.2 ± 0.6	0.7 ± 2.2	0.3 ± 0.5

* $P < 0.05$, ** $P < 0.01$ compared to the control.

Table 3 Concentrations of IL-8, NE and ECP in sputum

	RSV-CrB group	RSV-non CrB group	Control
IL-8 (pg/ml)	10428 ± 11008*	6112 ± 3212*	654 ± 1253
NE (μg/l)	1438 ± 1305*	3406 ± 3359*	350 ± 490
ECP (μg/l)	67 ± 130	426 ± 559*	34 ± 39

NE, neutrophil elastase; ECP, eosinophilic cationic protein.

* $P < 0.05$ compared to the control.

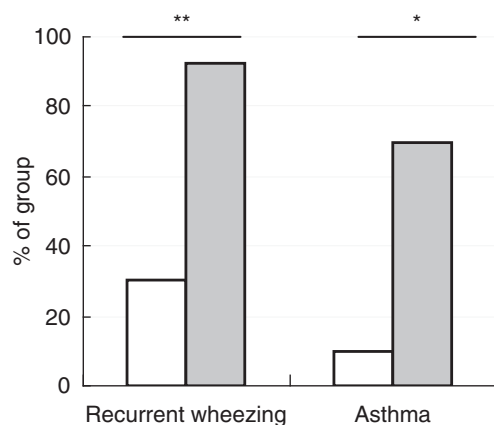


Fig. 1 Prevalence of recurrent wheezing and asthma with/without CrB in RSV bronchiolitis group. Open column indicates RSV-non CrB group; and closed column represents RSV-CrB group. RSV bronchiolitis with CrB had significantly higher rate than those without CrB. * $p < 0.05$, ** $p < 0.01$.

ducted to investigate the 3 or more wheezing episodes for less than 2 years and the development of asthma for less than 5 years after their discharge from hospital by the physicians.

STATISTICAL ANALYSIS

Data are expressed as mean ± SD. Differences were assessed by the Mann-Whitney's *U*-test. The percentage between two groups were compared using the Fisher's exact test. For factor analysis, univariate and multivariate logistic regression analyses were used. A *p*-value less than 0.05 was considered significant.

ETHICS

The study was approved by the Regional Ethics Committee for Human Research at the Hospital of Dok-

kyo University School of Medicine. The parents of all patients participating in this study gave their informed oral and written consent.

RESULTS

DETECTION OF CrB IN RSV BRONCHIOLITIS

CrBs in the sputum were observed in 23 of the 33 subjects in the RSV group (69.7%), but not in any members of the control group.

DEMOGRAPHIC CHARACTERISTICS

The backgrounds of patients in the RSV-CrB, RSV-non CrB and control groups are shown in Table 1. No significant difference was observed in any of the groups in terms of age, gender, or family history of atopic diseases. Illness days and periods of hospitalization compared among the RSV-CrB group and RSV-non CrB group resulted in no significance.

CELL COMPONENTS IN SPUTUM

Cell components in the sputum counts for each group are shown in Table 2. Almost no eosinophils were seen in any of the groups, and no significant difference was observed. However, lymphocytes and neutrophils were significantly high in both the RSV-CrB and RSV-non CrB groups compared with the control group (lymphocytes: RSV-CrB group vs control group: $p = 0.001$, RSV-non CrB group vs control group: $p = 0.02$, neutrophils: $p = 0.006$ and $p = 0.004$, respectively).

CONCENTRATIONS OF IL-8, NE AND ECP IN SPUTUM

The concentrations of IL-8, NE and ECP in the sputum for each group are shown in Table 3. The IL-8 concentration was significantly high in both the RSV-CrB and RSV-non CrB groups compared with the control group (RSV-CrB group vs control group: $p =$

Table 4 Contributing factors for progression to recurrent wheezing and asthma

Factor	Level	Recurrent Wheezing				Asthma	
		<i>p</i> value	RR	95%CI	<i>p</i> value	RR	95%CI
Gender	M/F	1.00	1.08	0.61-1.93	1.00	0.81	0.28-2.38
Age (<3 months)	Y/N	0.06	0.46	0.18-1.16	0.64	0.62	0.16-2.29
Family history of atopic diseases	Y/N	0.08	1.86	0.93-3.70	0.07	4.66	0.70-31.0
CrBs	Y/N	0.002	3.09	1.18-8.06	0.02	7.00	1.04-46.9

RR, relative risk; CI, confidence intervals.

0.011, RSV-non CrB group vs control group: $p = 0.026$). Similarly, the NE concentration was significantly high in the RSV groups (RSV-CrB group vs control group: $p = 0.045$, RSV-non CrB group vs control group: $p = 0.026$). Although no significant difference in the ECP concentration was observed between the RSV-CrB group and the control group, it was significantly high in the RSV-non CrB group compared with the control group ($p = 0.026$). No significant difference in the IL-8, NE and the ECP concentration were observed between the RSV-CrB group and RSV-non CrB group.

CUMULATIVE PREVALENCE OF RECURRENT WHEEZE AND ASTHMA

Figure 1 shows the results of our investigation on the association between sputum CrBs and development of recurrent wheezing and asthma in post-RSV bronchiolitis patients. The incidence of recurrent wheezing in subjects aged up to two years was 92.8% for the RSV-CrB group and 30.0% for the RSV-non CrB group ($p = 0.002$). Furthermore, the development of asthma in subjects aged up to five years was 70.0% for the RSV-CrB group and 10.0% for the RSV-non CrB group ($p = 0.019$).

RISK FACTORS OF CUMULATIVE RECURRENT WHEEZE AND ASTHMA

We investigated whether background factors considered to be related to the development of asthma (in the present study, these factors were male infants, aged less than three months, and family history of atopic diseases) and CrBs are linked to the progression to recurrent wheezing and asthma (Table 4). No link to gender, lower age, or family history of atopic diseases was found, with CrBs providing the only significant correlation to recurrent wheezing ($p = 0.002$, relative risk [RR] 3.09, 95% CI 1.18 to 8.06) and asthma onset ($p = 0.019$, RR 7.00, 95% CI 1.04 to 46.9).

DISCUSSION

The present study revealed that CrBs indicating respiratory epithelial damage were present in high rates in infants with RSV bronchiolitis. The study also suggests that the presence of CrBs in infants with RSV bronchiolitis is a factor which has a considerable influence on the development of recurrent wheezing

and asthma within at least five years.

CrBs are clusters of desquamated epithelial cells which, when present in the sputum of both childhood and adult asthma, serve as an indicator of epithelial damage. We previously reported that cell differential counts in the sputum of wheezy infants (excluding those with RSV infection) showed that neutrophils were predominant and that CrBs was also present among 15 of the 23 cases (65%), making it a predictor of recurrent wheezing and progression to asthma within two years.¹¹ We have also reported that young children with asthma aged less than three years, in whom we observed CrBs, exhibited high IL-8 and NE concentrations compared with IL-5 and ECP concentrations in the sputum, as well as the presence of neutrophilic inflammation.¹⁶ Additionally, Wang *et al.* reported that RSV infection enhances neutrophils adhesion to the epithelium and that activated neutrophils augment the epithelial cell damage.¹⁷ Neutrophil activation is therefore presumed to contribute to airway epithelial damage in wheezy infants and young children with asthma. The present study has also compared the neutrophil counts and IL-8 and NE concentrations of the RSV-CrB, RSV-non CrB and control groups. However, although our study demonstrated a significantly high level of sputum neutrophils, IL-8 and NE in both the RSV-CrB and RSV-non CrB groups compared with the control group, no significant difference was observed between the RSV-CrB group and the RSV-non CrB group. No significant difference in the eosinophil counts and the ECP concentration were also observed among two groups. These results suggest that not only neutrophil and eosinophil activation but also the other mechanism might be related to the desquamation of epithelial cells. Furthermore, there were no association between severity of RSV infection and the presence of CrBs in the present study. The mechanism of epithelial cell desquamation in asthma and respiratory viral infections remains insufficiently understood. Interestingly, a recent study has reported that the production of excess active oxygen from epithelial cells infected by respiratory viruses may be responsible for triggering oxidative stress, and may also induce epithelial damage as well as the production of various cytokines, chemokines and growth factors.^{18,19} Accordingly, we consider that examination of the role of oxidative stress in con-

tributing to RSV infection-induced epithelial cell desquamation will lead to an understanding of the expression mechanism of CrBs.

In the present study, the RSV bronchiolitis group with CrBs were observed had a significantly high rate of recurrent wheezing and asthma onset compared with the group without CrBs. RSV bronchiolitis has been shown in numerous cohort studies to be an important risk factor in the progression to recurrent wheezing and asthma onset. Stein *et al.* reported that a history of infant RSV lower respiratory infection is associated with the frequency of childhood wheezing, suggesting that prolonged airway hypersensitivity induced by viral infection may be present in children.⁵ Moreover, several studies have demonstrated that respiratory viral infection-induced epithelial cell desquamation is associated with in the progression to recurrent wheezing and asthma onset. First, Piedimonte *et al.* have attributed one of the mechanisms for the development of asthma induced by respiratory viral infections to increased neurogenic inflammation due to stimulation of exposed C-fibers resulting from epithelial cell desquamation.²⁰ Increased neurogenic inflammation is thought to cause airway hypersensitivity, constriction and edema, as well as reduced ciliary function which in turn leads to a reduction in foreign body removal function and susceptibility to invasion by antigenic substances such as allergens, viral infection, smoking and air pollution. In addition, a damaged epithelium may trigger all the events leading to airway remodeling by releasing mitotic and fibrogenic growth factors, which may promote smooth muscle proliferation, angiogenesis, and increased collagen deposition, resulting in reticular basement membrane thickening.^{21,22} Although the mechanism whereby associated between the presence of CrBs and development of recurrent wheezing and asthma is not known, we speculate that our results are because RSV-induced epithelial cell desquamation in infants causes the development of airway hypersensitivity for a period of several years.

Targeting neonatal infants with a family history of atopic disease, Kusel *et al.* has studied whether the onset of asthma up to the age of five is related to the presence of respiratory viral infection and atopic sensitization.²³ The study revealed that wheezy lower respiratory tract illnesses caused by RSV or rhinovirus in the first year of life are important contributors to persistent wheeze and asthma in 5-year-old children, particularly in those who are atopic sensitized during infancy. This findings suggests that both infantile lower respiratory viral infections and atopy-associated inflammation may interact synergistically to drive asthma pathogenesis. The present study did not reveal any significant difference among any of the groups in terms of family history of atopic diseases. Neither gender, lower age, nor family history of atopic diseases was associated with the progression

to recurrent wheezing and asthma, with CrBs providing the only significant correlation. CrBs were therefore considered to be an independent risk factor in the progression to recurrent wheezing and asthma even among RSV bronchiolitis without family history of atopic diseases. The present study did not investigate inhaled allergen sensitization and thus we were unable to verify the interaction of allergen sensitization indicated by Kusel *et al.* However, the fact that RR values for CrBs were high in the cases of recurrent wheezing and asthma onset leads us to believe that alleviating epithelial damage caused by respiratory viral infection as well as preventing exposure to inhaled allergens is important for asthma prevention.

In conclusion, this study is the first to show that CrBs in the sputum are expressed at high rate and that respiratory epithelial damage is present in infants with RSV bronchiolitis. Furthermore, by conducting a prospective study for five years we demonstrate that notably the CrBs are associated with the progression to recurrent wheezing and asthma. Screening for CrBs in infancy with RSV bronchiolitis may be important to identify high-risk group of development of asthma.

ACKNOWLEDGEMENTS

This work was supported in part by the Health Science Research Grants (Research on Immunology and Allergy) from the Ministry of Health, Labour and Welfare of Japan.

REFERENCES

1. Japanese Society of Pediatric Allergy and Clinical Immunology. [*Japanese Pediatric Guideline for the Treatment and Management of Asthma 2008*]. Tokyo: Kyowa Kikkaku, 2008 (in Japanese).
2. Sigurs N, Gustafsson PM, Kjellman B *et al.* Severe respiratory syncytial virus bronchiolitis in infancy and asthma and allergy at age 13. *Am J Respir Crit Care Med* 2005; **171**:137-41.
3. Korppi M, Piippo-Savolainen E, Korhonen K, Remes S. Respiratory morbidity 20 years after RSV infection in infancy. *Pediatr Pulmonol* 2004; **38**:155-60.
4. Weber MW, Milligan P, Giadom B *et al.* Respiratory illness after severe respiratory syncytial virus disease in infancy in the Gambia. *J Pediatr* 1999; **135**:683-8.
5. Stein RT, Sherrill D, Morgan WJ *et al.* Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. *Lancet* 1999; **354**:541-5.
6. Stensballe LG, Simonsen JB, Thomsen SF *et al.* The causal direction in the association between respiratory syncytial virus hospitalization and asthma. *J Allergy Clin Immunol* 2009; **123**:131-7.
7. Folkerts G, Nijkamp FP. Virus-induced airway hyperresponsiveness. Role of inflammatory cells and mediators. *Am J Respir Crit Care Med* 1995; **151**:1666-73.
8. Soumelis V, Reche PA, Kanzler H *et al.* Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. *Nat Immunol* 2002; **3**:673-80.
9. Naylor B. The shedding of the mucosa of the bronchial tree in asthma. *Thorax* 1962; **17**:69-72.

10. Motojima S, Ogata H, Makino S *et al*. Creola bodies asthmatic attacks and the major basic protein in sputa [abstract]. *J Allergy Clin Immunol* 1989;**83**:281.
11. Yamada Y, Yoshihara S. Creola bodies in wheezing infants predict the development of asthma. *Pediatr Allergy Immunol* 2004;**15**:159-62.
12. Swierkosz EM, Flanders R, Melvin L, Miller JD, Kline MW. Evaluation of the Abbott TESTPACK RSV enzyme immunoassay for detection of respiratory syncytial virus in nasopharyngeal swab specimens. *J Clin Microbiol* 1989;**27**:1151-4.
13. Norzila MZ, Fakes K, Henry RL, Simpson J, Gibson PG. Interleukin-8 secretion and neutrophil recruitment accompanies induced sputum eosinophil activation in children with acute asthma. *Am J Respir Crit Care Med* 2000;**161**:769-74.
14. Hafner G, Erbes H, Drosdat H, Lotz J, Ebrental W, Würzburg U. Evaluation of a new assay for the determination of PMN elastase- α 1-proteinase inhibitor complexes in EDTA and citrated plasma. *Clin Lab* 1997;**43**:3-9.
15. Ogata H, Motojima S, Fukuda T, Makino S. [Creola bodies and the eosinophil cationic protein in sputum in acute asthmatic attacks with respect to their clinical significance]. *Arerugi* 1990;**39**:1567-75 (in Japanese).
16. Yoshihara S, Yamada Y, Abe T, Lindén A, Arisaka O. Association of epithelial damage and signs of neutrophil mobilization in the airways during acute exacerbations of paediatric asthma. *Clin Exp Immunol* 2006;**144**:212-6.
17. Wang SZ, Xu H, Wraith A, Bowden JJ, Alpers JH, Forsyth KD. Neutrophils induce damage to respiratory epithelial cells infected with respiratory syncytial virus. *Eur Respir J* 1998;**12**:612-8.
18. Hosakote YM, Liu T, Castro SM, Garofalo RP, Casola A. Respiratory syncytial virus induces oxidative stress by modulating antioxidant enzymes. *Am J Respir Cell Mol Biol* 2009;**41**:348-57.
19. Suliman HB, Ryan LK, Bishop L, Folz RJ. Prevention of influenza-induced lung injury in mice overexpressing extracellular superoxide dismutase. *Am J Physiol Lung Cell Mol Physiol* 2001;**280**:69-78.
20. Piedimonte G, Rodriguez MM, King KA, McLean S, Jiang X. Respiratory syncytial virus upregulates expression of the substance P receptor in rat lungs. *Am J Physiol* 1999;**277**:L831-40.
21. Holgate ST, Roberts G, Arshad HS, Howarth PH, Davies DE. The role of the airway epithelium and its interaction with environmental factors in asthma pathogenesis. *Proc Am Thorac Soc* 2009;**6**:655-9.
22. Barbato A, Turato G, Baraldo S *et al*. Epithelial damage and angiogenesis in the airways of children with asthma. *Am J Respir Crit Care Med* 2006;**174**:975-81.
23. Kusel MM, de Klerk NH, Kebabdzic T *et al*. Early-life respiratory viral infections, atopic sensitization, and risk of subsequent development of persistent asthma. *J Allergy Clin Immunol* 2007;**119**:1105-10.