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The Release of Leukotrienes in the Respiratory Tract during Infection with Respiratory Syncytial Virus: Role in Obstructive Airway Disease

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ABSTRACT. Samples of nasopharyngeal secretions from a group of 73 infants with bronchiolitis or upper respiratory illness alone during infection with respiratory syncytial virus were analyzed for leukotriene C_4 (LTC_4) content using a reverse-phase high-pressure liquid chromatography assay with confirmation by radioimmunoassay. Titers of respiratory syncytial virus (RSV)-specific IgE in nasopharyngeal secretion (NPS) specimens were determined using an enzyme-linked immunosorbent assay. The highest concentrations of LTC_4 were found in the first 3 to 8 days after the onset of illness, and LTC_4 was detectable in progressively lower concentrations in samples obtained up to 28 days after the onset of illness. LTC_4 was detected in samples of NPS obtained in the acute phase of illness from 67% of infants with bronchiolitis due to RSV and in 33% of samples of NPS obtained during the same interval from infants with upper respiratory illness alone ($p < 0.025$). Concentrations of LTC_4 in children with bronchiolitis were 5-fold higher (1271 pg/ml) than the mean concentration of LTC_4 in children with upper respiratory illness (224 pg/ml, $p < 0.02$). LTC_4 was detected in 83% of the children developing an RSV-IgE response and in 24% of subjects not developing an RSV-IgE response ($p < 0.001$). Quantities of LTC_4 measured in NPS were directly correlated with the magnitude of the RSV-IgE response in secretions ($r = 0.33$, $p < 0.02$). These studies lend support to previous investigations suggesting that severe bronchiolitis due to RSV results from IgE-mediated hypersensitivity reactions to viral antigens, with release of chemical mediators of airway obstruction. Their implications should be considered in new approaches to therapy of RSV bronchiolitis. (*Pediatr Res* 24: 504–507, 1988)

Abbreviations

NPS, nasopharyngeal secretions
URI, upper respiratory illness
RSV, respiratory syncytial virus
LTC_4, leukotriene C_4
ELISA, enzyme-linked immunosorbent asay
PBS, phosphate-buffered saline
HPLC, high-pressure liquid chromatography
RIA, radioimmunoassay

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Infection with RSV is the most common cause of bronchiolitis in infancy (1). Previous studies have indicated that infants with RSV bronchiolitis exhibit increased levels of virus-specific IgE and histamine in respiratory tract secretions in comparison to individuals with URI alone due to RSV (2, 3). In addition to histamine, other mast cell products, especially LTC_4 and LTD_4, have been shown to be potent mediators of bronchoconstriction and increased airway mucus secretion (4-6). However, their role in wheezing associated with respiratory infection in infancy has not been determined. Bronchoactive and vasoactive mediators may also be released by other effector cells that are present in the respiratory tract during acute viral infection (7, 8). Our study was designed to determine the presence of LT in the nasopharyngeal mucosa during RSV infection and to evaluate their potential role in RSV-induced wheezing in infancy and childhood.

MATERIALS AND METHODS

Subjects. A total of 73 children with RSV infection hospitalized during the winters of 1986 and 1987 was enrolled in this study. A diagnosis of bronchiolitis with or without pneumonia was made in 48 children on the basis of physical examination by a single member of the study team and a careful review of radiographs with a radiologist. The other 25 subjects had URI alone without wheezing. The subjects included 56 infants less than 1 yr of age (38 with bronchiolitis, 18 with URI) and 17 children from 1 to 6 yr of age (10 with bronchiolitis, seven with URI). Assays for LTC_4 were carried out by an individual who had no knowledge of the clinical diagnosis.

Sample collection. After explanation of the goals and risks of the study and obtaining of signed statements of informed consent, samples of NPS were collected for identification of RSV infection for determination of the RSV-specific IgE antibody titer, as well as for determination of LTC_4 content. Samples were obtained by direct aspiration into polyethylene catheters passed into the nasopharynx (9). Infection with RSV was confirmed in one aliquot of secretions by indirect immunofluorescence assays as previously described (9). The sensitivity and specificity of this assay are in excess of 92%. The supernatant of this specimen was used to determine the magnitude of the RSV-IgE response. Measured titers of RSV-IgE were standardized to a protein content of 1 mg/ml of fluid (2). An additional aliquot of secretions (subsequently measured to be between 0.3 and 0.5 ml in all cases) was obtained for LTC_4 determination also by direct aspiration into a polyethylene catheter and rinsing with 0.5 ml of PBS prepared with HPLC-grade water (Aldrich, Milwaukee, WI). Samples for determination of LTC_4 content were trans-

ported on ice to the laboratory and stored at −70° C. Measured concentrations of LTC_4 were standardized to 0.1-ml volumes of secretion.

A total of two to four wk after the onset of acute illness, the subjects were reevaluated and a second sample of secretions was obtained. A small number of infants with documented RSV infection could not be studied for LTC_4 content at the time of acute illness but were tested during convalescence. Thus, 64 infants were studied during the acute phase of illness, and 17 of these returned to have a convalescent sample obtained. An additional nine subjects were studied only during the convalescent period.

RSV-IgE assay. Titers of RSV-specific IgE were measured by an ELISA previously described (2). In brief, RSV grown in the laboratory was purified and concentrated by sucrose-gradient centrifugation. Uninfected tissue culture cells were similarly processed for use as a control. The virus or control preparations (0.1 ml) were fixed overnight to the bottom of wells of polyvinyl micro-ELISA plates (Dynatech Laboratories, Alexandria, VA) after dilution to 1:100 in carbonate buffer (pH 9.6). On the next morning the plates were washed three times with a washing solution consisting of PBS and 0.05% Tween 20 (Fisher Scientific, Fairlawn, NJ). Test wells were then incubated with 0.1 ml of serial 2-fold dilutions of secretions in PBS. After 2 h of incubation at 37° C, the plates were washed and incubated for 2 more h with 0.1 ml of horseradish peroxidase-conjugated goat anti-human IgE (Miles Laboratories, Elkhart, IN) diluted 1:00 in PBS and Tween 20, with 1.5% fetal-calf serum added. After another washing, 0.1 ml of O-phenylenediamine (Sigma Chemical, St. Louis, MO) in an 0.08% solution in PBS, with 10 μl of hydrogen peroxidase added, was incubated in wells for 30 min. End-points were determined by spectrophotometer at 488 nm. Positive readings (indicating the presence of RSV-IgE) were those in which the optical density in wells coated with RSV was more than twice that in wells coated with similarly processed uninfected cell preparations. Known positive assays could be blocked by incubation of the goat anti-IgE with purified human IgE but not human IgA, IgG, or light chains. Positive assays could also be blocked by incubation of secretions with RSV-infected tissue culture cells followed by centrifugation but not by incubation with uninfected tissue culture cells (2).

Measurement of leukotrienes. The NPS samples were initially centrifuged at 1500 × *g* in 4°C for 20 min. A 0.1-ml volume of the supernatant, containing secretion in PBS, was mixed with 4 vol of ethanol, incubated for 2 h on ice, and centrifuged as before. The supernatant was vacuum extracted to dryness in a rotary evaporator and the residue obtained was subsequently dissolved in 20% methanol and applied to a Sep-Pak C_{18} cartridge (Millipore Waters Associates, Millford, MA) activated with 10 ml of methanol. The sample thus prepared was subjected to vacuum extraction to dryness and the residue redissolved in 500 μl of 30% methanol. A total of 5 ng of purified prostaglandin B_2 (Sigma Chemical Co., St. Louis, MO) was introduced into the sample as an internal standard. The sample was then injected into a C_{18} reverse-phase column (Beckman, San Ramon, CA) and eluted isocratically in an HPLC system (Pharmacia, Piscataway, NJ) with a mixture of 80% methanol, 20% water, 0.05% trifluoroacetic acid, and 0.5% triethylamine (Pierce, Rockford, IL). Each sample was tested in duplicate. Purified preparations of LTC_4 (generously given by J. Rokach, Merck Frost, Montreal, Quebec, Canada) were used as standards and tested in parallel with the sample. Recovery of LTC_4 was 86 ± 7%.

RIA for LTC_4. The presence of LTC_4 in materials determined to be positive by HPLC was verified by a specific RIA (New England Nuclear, Boston, MA). Samples collected after separation with HPLC were dried and then dissolved in an assay buffer. A tracer, containing [^{3}H]LTC_4, was added to the samples along with specific anti-LTC_4 antibody. After incubation, absorption with charcoal suspension and centrifugation at 1500 × *g* for 15 min, the supernatant was added to Atomlight, a scintillation solution (DuPont, Boston, MA). The radioactivity was measured in a liquid scintillation counter (Beckman LS9000, Fullerton, CA). Recovery of LTC_4 from the HPLC in the RIA was 76 ± 9%.

Statistical methods. Differences in titers of RSV-IgE or of LTC_4 content in nasopharyngeal secretions among patient groups were calculated by Student's *t* test. Chi-square analysis was used to compare the rates of detection of RSV-IgE and LTC_4 among groups. Correlation coefficients were calculated using standard methods.

RESULTS

Temporal kinetics of LTC_4 in NPS. Concentrations of LTC_4 measured in secretions on different days after the onset of illness are shown in Figure 1. Maximum concentrations of LTC_4 were observed from 3 to 8 days after the onset of illness. Measureable concentrations appeared to decline at 9 to 10 days after the onset of illness and persisted in low levels in some individuals up to 28 days after the onset of illness but not beyond.

Relationship of LTC_4 content in NPS to form of illness. Concentrations (mean ± SD) of leukotriene measured in samples of NPS from patients with different forms of illness due to RSV are shown in Table 1. During the acute phase of illness, LTC_4 was detectable in 29 of 43 (67%) infants with bronchiolitis but in only seven of 21 (33%) infants with upper respiratory illness alone ($p < 0.025$). Mean concentrations of LTC_4 were more than 5-fold higher in infants with bronchiolitis due to RSV than in infants with URI alone ($p < 0.02$). LTC_4 was detectable in low concentrations in approximately 25% of subjects studied in the convalescent period, with no difference in frequency of detection in the two illness groups.

Relationship of RSV-IgE response to presence of LTC_4. The relationship of the development of an IgE antibody response to RSV with the release of LTC_4 in NPS is illustrated in Table 2. Samples for determination of RSV-IgE titers were available from 64 subjects. LTC_4 was detectable in secretions of 24 of 29 (83%) individuals developing an RSV-IgE response but in only 10 of 35 (29%) individuals not developing a detectable RSV-IgE response ($p < 0.001$).

The mean concentration of LTC_4 detected in samples of NPS from children with positive RSV-IgE responses was 736 ± 680

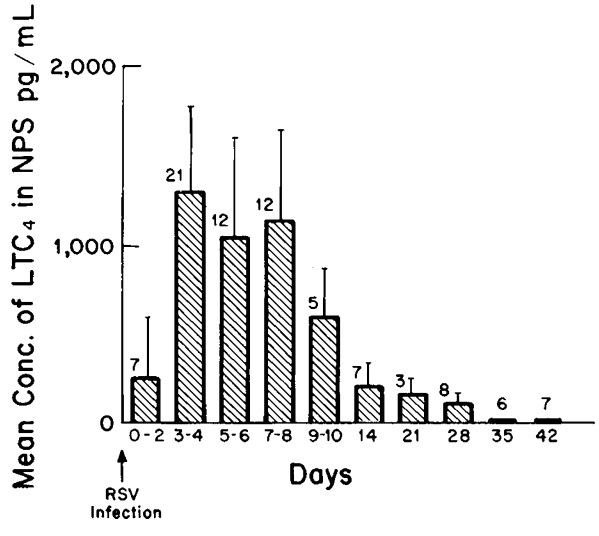

Fig. 1. Concentration of LTC_4 in samples of nasopharyngeal secretions obtained from patients with RSV infection. *Hatched bars* indicate mean; intervals indicate 1SD; and figures over *bars* indicate number of subjects studied at each interval. *Horizontal axis* refers to days after the onset of symptoms of respiratory illness.

Table 1. *Presence of LTC_4 in nasopharyngeal secretions of infants and children with RSV infection*

Diagnostic group	Days after onset of illness	No. studied	No. (%) with LTC_4	Group mean LTC_4 concentration (pg/0.1 ml)
Bronchiolitis	0–7	43	29 (67)*	1271 ± 239†
	14–42	18	5 (28)	139 ± 52
URI alone	0–7	21	7 (33)*	224 ± 114†
	14–42	8	2 (25)	125 ± 77

* $p < 0.025$.
† $p < 0.02$.

Table 2. *Relationship of development of RSV-IgE response to presence of LTC_4 in respiratory secretions*

RSV-IgE response	LTC_4 in NPS: Present *n*	Present %	Absent *n*	Absent %
Positive *n* = 29	24	83*	5	17
Negative *n* = 35	10	29*	25	71
Total 64	34		30	

* $p < 0.001$.

pg/0.1 ml, which was significantly higher than the mean concentration of LTC_4 in subjects without a detectable RSV-IgE response (202 ± 331 pg/0.1 ml, $p < 0.02$). A weaker overall correlation of the magnitide of the RSV-IgE response of individual patients with the LTC_4 content of NPS was noted ($r = 0.33$, $P < 0.02$).

Relationship of patient age to presence of LTC_4. The relationship of the presence of an RSV-IgE response and the presence of LTC_4 in patients older than or younger than 12 months of age at the time of infection is shown in Table 3. LTC_4 was detected in secretions of 18 of 23 (78%) RSV-IgE positive patients in the first year of life and in six of six (100%, p = NS) individuals developing an IgE response who were older than 12 months of age at the time of infection. In a similar fashion, 27% of individuals younger than 12 months of age who did not develop an RSV-IgE response had detectable LTC_4 in their secretions, whereas 12% of RSV-IgE-negative individuals older than 12 months of age at the time of infection had LTC_4 present in their secretions (P = NS). Overall, 51% of subjects younger than 12 months of age and 50% of patients more than 12 months of age at the onset of illness had detectable LTC_4 in NPS, indicating that patient age was not as important factor as was the development of an RSV-IgE response in determining the quantity of Ltc_4 released into secretions.

Table 3. *LTC_4 release in respiratory secretions analyzed by patient age at time of infection*

Patient age (mo)	Number with detectable LTC_4/no. tested: RSV-IgE positive	RSV-IgE negative	Total
<12	18/23 (78%)	7/26 (27%)	25/49 (51%)
>12	6/6 (100%)	1/8 (12%)	7/14 (50%)

DISCUSSION

The sulfidopeptide LT (LTC_4, LTD_4, and LTE_4) are among the major mast cell mediators responsible for the late-phase of bronchospasm in asthma (10). LTC_4 and LTD_4 are the most potent constrictors of smooth muscle and stimulators of airway mucus production (4–6). In addition, they increase vascular permeability and induce pulmonary edema (4, 11, 12). Results of this study, demonstrating that concentrations of LTC_4 are greater in infants with wheezing at the time of RSV infection than in infants with URI alone, suggest that the LT may play an important role in the pathogenesis of episodes of RSV-induced wheezing. Maximum release of LTC_4 was observed from 3 to 8 days after the onset of symptoms of respiratory infection, the time at which airway obstruction after RSV infection is most severe. LTC_4 release was also correlated with production of RSV-specific IgE. In other studies from this laboratory (13), LTC_4 has been quantitated in respiratory secretions obtained from individuals with nonrespiratory illnesses. The mean LTC content in these samples (158 pg/0.1 ml) was similar to the LTC content of secretions obtained from patients with URI alone in the present study (224 pg/0.1 ml). Patients with bronchiolitis due to viral agents other than RSV have not been studied extensively.

LTC_4 and LTD_4 are released after stimulation of human mast cells (14–16) and other human inflammatory cells (7, 8). A variety of inflammatory cells is present in the lung in individuals with bronchiolitis (17). An alternative hypothesis might be that release of IgE, histamine, LTC_4 and other compounds into the respiratory tract might be greater in cases of bronchiolitis than in cases of URI because of the greater inflammatory response in the lower airway in bronchiolitis. Previous studies comparing infants with RSV pneumonia without wheezing to infants with bronchiolitis have demonstrated that, despite a presumably equivalent amount of inflammation, IgE responses and histamine release are greater in the group with bronchiolitis (2). Antibody responses in other isotypes were similar in each group (18). No patients with RSV pneumonia without wheezing were available in the current investigation. Nevertheless, the results of the previous studies indicate that RSV-IgE production is unrelated to inflammation. In our study, LTC_4 was detectable more often in individuals developing an RSV-IgE response than in those not developing such a response ($p < 0.001$) and was of greater magnitude in RSV-IgE responders ($p < 0.02$). A more modest correlation of LTC_4 release with the absolute magnitude of the RSV-IgE responses was observed (r = u 0.33, $p < 0.02$). Therefore, some LTC_4 may have been released nonspecifically by inflammatory cells in the lung, but LTC_4 release was apparently enhanced by RSV-IgE synthesis, which would not be related to inflammation. Overall the findings support a primary role for production of RSV-specific IgE and IgE-directed mediator release in the pathogenesis of bronchiolitis. It may be that individuals who release lower concentrations of LTC_4 as a result of non-IgE-mediated mechanisms develop milder forms of bronchiolitis, whereas individuals who develop an RSV-IgE response release greater quantities of LTC_4 and histamine and experience more severe forms of illness.

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