

Nonspecific refractoriness to adenylyl cyclase stimulation in alveolar macrophages from infants with recurrent bronchiolitis

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Epidemiologic studies suggest an association between recurrent bronchiolitis in children younger than 3 years of age and diagnosis of asthma later in life. Bronchoalveolar lavages from 20 infants with recurrent wheezing and 18 nonwheezy control subjects were analyzed to determine whether alveolar macrophages of wheezy infants present abnormalities similar to those described in adults with asthma. Alveolar macrophages from both groups responded in vitro, in a concentration-dependent manner, to prostaglandin E₂, salbutamol, and forskolin, drugs that increase cyclic adenosine monophosphate levels. However, alveolar macrophages from infants with recurrent wheezing accumulated less cyclic adenosine monophosphate than those from control subjects in response to all three stimulations. These results are in agreement with the reduced cyclic adenosine monophosphate response to different agonists demonstrated in leukocytes from patients with asthma, and suggest that this refractoriness could be one of the precipitating events in the development of asthma observed in a large proportion of infants who have had bronchiolitis. (J ALLERGY CLIN IMMUNOL 1994;93:885-90.)

Key words: Wheezing, recurrent bronchiolitis, asthma, infants, bronchoalveolar lavage, adenylyl cyclase, macrophage, β -adrenergic agonists

The diagnosis of asthma in infants is frequently missed or delayed.^{1, 2} Difficulties in distinguishing infants who wheeze only in response to viral infections from those who wheeze in response to additional precipitating factors may partly explain this.³ Further difficulties arise from poor efficacy of the usual antiasthmatic treatments, such as β -adrenergic agonists and corticosteroids, in infants.⁴⁻⁶ In any case, recurrent bronchiolitis is clearly associated with bronchial asthma, especially when a family background of allergic disease is present.⁷ In addition, respiratory syncytial virus, bronchiolitis, and asthma have common humoral and cellular immune mechanisms.⁸⁻¹⁰ For instance, eosinophil degranulation has been im-

Abbreviations used

BAL: Bronchoalveolar lavage
cAMP: Cyclic adenosine monophosphate
FB: Flexible bronchoscopy
PGE₂: Prostaglandin E₂

plicated in the pathogenesis of bronchiolitis.¹¹ Furthermore, increased nonspecific bronchial responsiveness, a major feature of asthma, is also observed in infants with bronchiolitis.¹²

In infants with frequent or persistent wheezing who respond poorly to bronchodilators, flexible bronchoscopy (FB) is indicated for alternative diagnosis,¹³⁻¹⁵ because diseases leading to recurrent wheezing are numerous.

We have previously shown that alveolar macrophages from adults with asthma display a reduced adenylyl cyclase increase in response to salbutamol and prostaglandin E₂ (PGE₂).¹⁶ The aim of this study was to verify whether similar abnormalities occur in cells from infants with recurrent wheezing who underwent FB for clinical reasons. The demonstration that adults and infants with asthma have common cellular mechanisms might

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Received for publication Mar. 25, 1993; accepted Sept. 23, 1993.

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0091-6749/94 \$3.00 + 0 1/152639

TABLE I. General markers of the bronchoalveolar lavage from infants

	Volume (ml)	Cell number (million)	Viability (%)	Protein content (mg/ml)
Control group (<i>n</i> = 18)	33.3 ± 2.7	6.7 ± 0.9	68.3 ± 2.8	0.22 ± 0.05
Bronchiolitis group (<i>n</i> = 20)	30.7 ± 1.6	9.2 ± 1.4	63.5 ± 3.9	0.18 ± 0.03

Values represent means ± SEM.

allow for an earlier diagnosis and lead to development of preventive and therapeutic measures.¹⁷

METHODS

Reagents

Culture medium (RPMI 1640) was purchased from Jacques Boy SA (Reims, France), and penicillin, streptomycin, and amphotericin B were purchased from Gibco (Grand Island, N.Y.). Heat-inactivated fetal calf serum was purchased from Boehringer Mannheim (Mannheim, Germany). Salbutamol, PGE₂, forskolin, and 3-isobutyl-1-methylxanthine were purchased from Sigma Chemical Co. (St. Louis, Mo.). Stock solutions of PGE₂ and forskolin were made in ethanol (10 mmol/L), and further dilutions were made in saline solution (final concentration of ethanol in the culture medium was 1%). Tritiated cyclic adenosine monophosphate (cAMP) was purchased from Amersham International Ltd. (Little Chalfont, Bucks, England), and the cAMP binding protein used in the assay of cAMP was prepared from rabbit muscle.¹⁸ Reagents for the protein assay were purchased from Pierce Chemical Co. (Rockford, Ill.).

Patients

The study population consisted of 38 infants younger than 3 years of age who were divided into two groups, wheezers and nonwheezers. Twenty infants (14 boys and 6 girls; mean age, 15.8 ± 1.8 months) had experienced recurrent or persistent wheezing. In all cases severity and/or frequency of symptoms (more than once a month) and poor responsiveness to bronchodilators justified the use of FB in the search for an alternative diagnosis. None of these infants had been treated with β-adrenergic agonists (for 2 weeks) or corticosteroids (for 3 weeks) before the study began. At this time, 16 infants were free of symptoms, and four were considered to have moderate persistent wheezing.¹ FB was performed remote from any acute episode of bronchiolitis or acute upper respiratory illness.

In the remaining 18 infants (13 boys and 5 girls; mean age, 20.0 ± 2.3 months), FB was indicated for a nonwheezy-related disease: pulmonary tuberculosis (6), recurrent nonwheezy bronchitis (7), suspicion of tracheobronchial foreign body (1), persistent atelectasis (3), and mediastinal adenopathy (1). In infants with recurrent nonwheezy bronchitis, FB was performed to diagnose tracheobronchomalacia, after underlying dis-

eases such as cystic fibrosis, immune deficiency, or recurrent aspiration were excluded.¹⁹ In all infants oxygen transcutaneous saturation before examination was greater than 95%.

Preparation of alveolar macrophages

Bronchoscopy was performed by the same operator and at the same hour (8:30 to 10:30 AM) with a flexible bronchoscope (BF3C20; Olympus Winter & IBE GMBH, Hamburg, Germany), which was passed through the nose after local anesthesia was induced.²⁰ After examination, the extremity of the bronchoscope was wedged in the right middle or lower lobe. A first aliquot of 5 to 10 ml of 0.9% NaCl at 37° C was injected and retrieved by gentle aspiration. This sample was used for viral detection by immunofluorescence. Then 4 to 6 aliquots were injected and reaspirated to a total volume of 4 to 5 ml/kg body weight. Bronchoalveolar lavages (BALs) were collected into sterile polyethylene vials, filtered through saline-moistened gauze, and sedimented by centrifugation (470g for 10 minutes) at room temperature. The pellets were resuspended in a culture medium (RPMI 1640) supplemented with N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid buffer (10 mmol/L), heat-inactivated fetal calf serum (3%), penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (0.25 µg/ml). An aliquot was removed for determination of cell numbers and viability. The remaining cell suspension was plated at a density of 0.5 × 10⁶ cells/ml in 22 mm tissue culture wells (Costar Corp., Cambridge, Mass.) and kept at 37° C in a humidified atmosphere containing 5% CO₂. After 1 hour, nonadherent cells were discarded, and the remaining cells (>95% alveolar macrophages) were incubated for 1 additional hour in serum-free RPMI medium.

Determination of intracellular levels of cAMP in alveolar macrophages

Adherent cells were incubated in 0.3 ml/well tris-HCL buffer (50 mmol/L, pH 7.4) containing 0.2 mmol/L 3-isobutyl-1-methylxanthine (a phosphodiesterase inhibitor) and ethylenediaminetetraacetic acid (2 mmol/L). Incubations were carried out for 3 minutes at 37° C in the presence of increasing concentrations of agonists (as described in the figure legends), and the reaction was stopped by heating the plates in boiling water for 3 minutes to empty the intracellular content

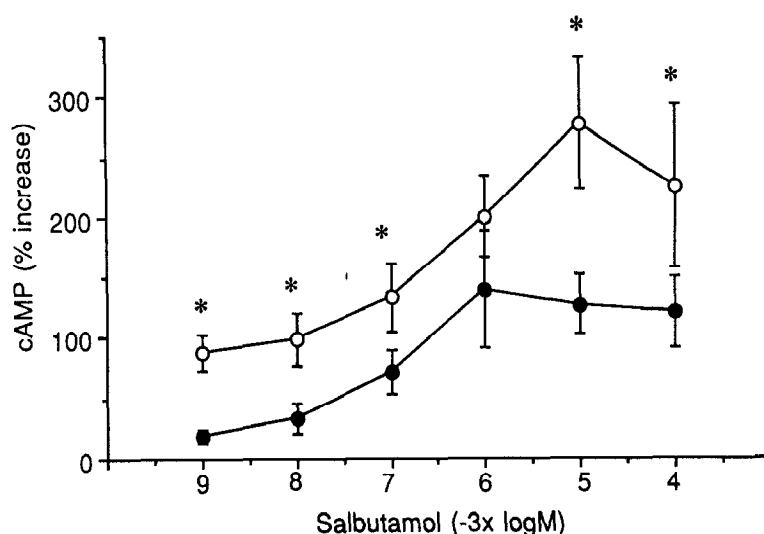


FIG. 1. Effect of salbutamol on the intracellular cAMP levels of alveolar macrophages from infants. Cells from 20 infants with recurrent bronchiolitis (●) and 18 nonwheezing control subjects (○) were incubated for 3 minutes at 37° C in the presence of increasing concentrations of salbutamol (3×10^{-9} to 3×10^{-4} mol/L). * $p < 0.05$.

of cAMP into the extracellular medium. The medium was transferred to Eppendorf tubes (Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) and centrifuged (12,000g for 2 minutes) to produce supernatants that were analyzed for cAMP content with a competitive protein-binding assay.²¹ The limit of detection was 0.1 to 0.2 pmol cAMP per 50 μ l sample. The monolayers were scraped for protein determination. Results were expressed as picomoles of cAMP contained in 10^6 cells.

Additional methods

Total cell counts, viability, and number of epithelial ciliated cells were evaluated with a Malassez hemocytometer (Preciss, Paris, France) in 0.5% trypan blue. The protein content was determined in supernatants and cell scrapings with the use of bicinchoninic acid,²² and the yield was approximately 5×10^6 cells per milligram of protein. Protein content was used to correct the number of adherent cells.

Analysis of results

Comparisons between the two groups were performed with the Mann-Whitney U test for unpaired data.

RESULTS

General markers of the BAL fluid recovered from infants

The mean values for volume of BAL recovered, total cell counts, viability, and protein content of the BAL supernatants are shown in Table I. The values of the two groups were not significantly different, but when compared with the values from lavages performed in adults, lower cell vi-

ability and higher protein content were noted. The percentage of ciliated cells (most of them nonviable) was slightly higher in BAL from infants with bronchiolitis ($11.3\% \pm 1.8\%$), compared with control subjects ($7.2\% \pm 1.8\%$), and both values were higher than those reported in adults (1% to 5%).²³

Response to adenylyl cyclase stimulation in alveolar macrophages

Mean baseline levels of cAMP were slightly higher, although not significantly different, in macrophages collected from patients with recurrent wheezing as compared with control subjects (8.8 ± 1.7 pmol/ 10^6 cells vs 7.4 ± 1.6 pmol/ 10^6 cells). Alveolar macrophages responded to salbutamol at concentrations as low as 3 nmol/L with increased production of cAMP. The response observed in macrophages from wheezy infants was significantly reduced as compared with that of the control group (Fig. 1). Similarly, alveolar macrophages from infants with recurrent wheezing were less sensitive to the stimulating effect of PGE₂ (Fig. 2). When alveolar macrophages from both groups of infants were incubated with increasing concentrations of forskolin (2×10^{-7} to 2×10^{-4} mol/L), a significant increase in cellular cAMP was observed only for high concentrations (2×10^{-5} to 2×10^{-4} mol/L), and this increase was significantly higher in alveolar macrophages from infants in the control group (Fig. 3).

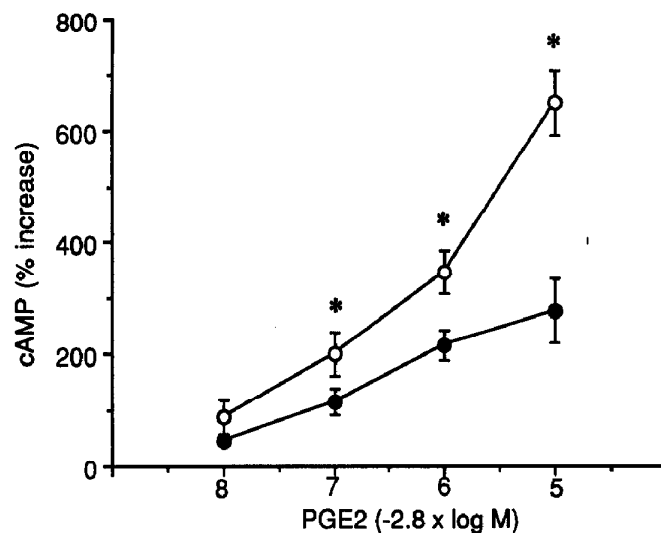


FIG. 2. Effect of PGE₂ on the intracellular cAMP levels of alveolar macrophages from infants. Cells from eight infants with recurrent bronchiolitis (●) and eight nonwheezing control subjects (○) were incubated for 3 minutes at 37° C with increasing concentrations of PGE₂ (2.8×10^{-8} to 2.8×10^{-5} mol/L). * $p < 0.05$.

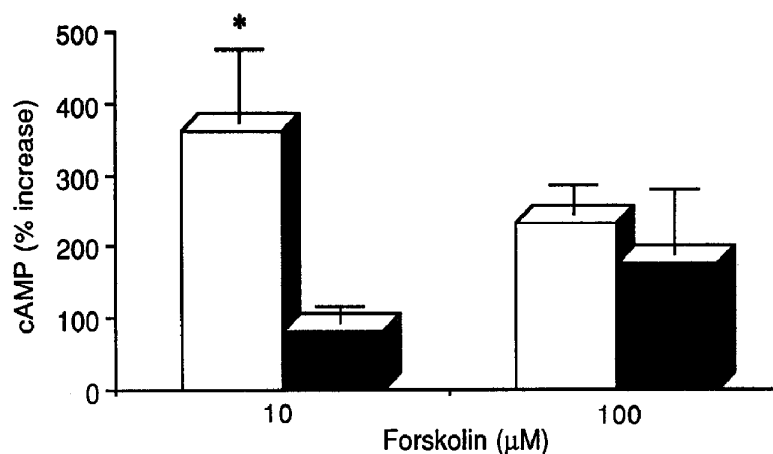


FIG. 3. Effect of forskolin on the intracellular cAMP levels of alveolar macrophages from infants. Cells from six infants with recurrent bronchiolitis (■) and from six nonwheezing control subjects (□) were incubated for 3 minutes at 37° C in the presence of forskolin (10 or 100 μmol/L). * $p < 0.05$.

DISCUSSION

Our results show that alveolar macrophages from infants with recurrent bronchiolitis have a decreased sensitivity to the stimulating effect of salbutamol, PGE₂, and forskolin, as compared with infants in the control group. These results are consistent with results of previous studies of adults, which demonstrate reduced adenylyl cyclase stimulation in alveolar macrophages¹⁶ and circulating leukocytes in patients with asthma.²⁴⁻²⁸

No significant differences were observed in basal or stimulated cAMP levels between infants

with tuberculosis ($n = 6$) and those with recurrent nonwheezing bronchitis ($n = 7$) in the control group (data not shown). Therefore it is highly unlikely that our control group would exhibit hyperresponsiveness and that the wheezy infants would exhibit normal responsiveness.

The mechanisms leading to the described modifications of the responsiveness of alveolar macrophages from adults or infants to drugs that increase cAMP are still unclear. Cell exposure to catecholamines induce homologous desensitization, which has been attributed to receptor modi-

fications,^{29, 30} whereas exposure to other factors such as prostaglandin E results in reduced adenylyl cyclase response not only to prostaglandin E but also to isoproterenol, GppNHp (the nonhydrolyzable guanosine triphosphate analog) or sodium fluoride through alterations at the guanine regulatory protein level.³¹

In agreement with observations in patients with untreated asthma in whom decreased β -adrenergic responsiveness was demonstrated,³² our study, performed in infants receiving no adrenergic therapy, also suggests that the refractoriness cannot be entirely accounted for by drug treatment.

In addition, forskolin, which directly affects the catalytic subunit,³³ also displayed reduced maximal response in intact cells from infants with recurrent wheezing, suggesting a decrease in the activity of the catalytic subunit, though it has been proposed that forskolin may also affect the guanine nucleotide protein.³⁴ It is clear that alterations distal to the receptor are involved in the refractoriness observed in cells from infants with recurrent wheezing.

We could not correlate in vitro hyporesponsiveness of alveolar macrophages with clinical hyporesponsiveness to β -adrenergic agonists because infants with a satisfactory response to bronchodilators were considered to have asthma and thus did not require bronchoscopy. Therefore it is possible that our selected group of infants with recurrent bronchiolitis does not represent all infants with asthma. However, though the response to β -adrenergic agonists should provide a strong argument for asthma, in this age group, even when the diagnosis of asthma is made confidently, the response to β -adrenergic treatment is often unsatisfactory.³⁵ Moreover, in adults with asthma successfully treated with β -adrenergic agonists, hyporesponsiveness to β -agonists is known to persist.¹⁶

Because bronchiolitis most often results from viral infection, respiratory viruses could be involved in the decreased sensitivity observed. Indeed, respiratory viral infections induce airway hyperreactivity, possibly through an impaired β -adrenergic response,³⁶ and may thus be included among the factors involved in β -adrenergic refractoriness. However, a viral hypothesis alone cannot account for the hyporesponsiveness, because in our study, examination was performed remote from an acute episode of bronchiolitis or an acute upper airway infection; and immunofluorescence testing did not reveal the presence of respiratory syncytial virus.

Our results establish a link, at the cellular level, between wheezing in infants and asthma in adults, which is in agreement with the natural history of asthma, because 50% of cases of asthma in children and adults begin before the age of 2 years.³⁷ The previous identification of eosinophilic cationic protein¹¹ and of leukotriene C₄ in the nasopharyngeal secretions,³⁸ in correlation with the severity of bronchiolitis, suggested that infants and adults may have common cellular mechanisms for the described diseases.

Finally, our findings confirm that alveolar macrophages of infants respond to β -adrenergic stimulation and may help us to understand the mechanisms of asthma in infancy. Some of the infants will probably stop wheezing in early childhood and will not become asthmatics.³⁹ A prospective study to correlate in vitro data and clinical follow-up is in progress. This might help to distinguish, among infants with recurrent bronchiolitis, those who have asthma and allow the institution of preventive and therapeutic measures.

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