

# Influence of allergen avoidance on the eosinophil phase of airway inflammation in children with allergic asthma

Giorgio L. Piacentini, MD, Laura Martinati, MD, Stefania Mingoni, MD, and Attilio L. Boner, MD Verona and Misurina, Italy

**Background:** Exposure to relevant allergens causes an increase in bronchial hyperresponsiveness, as well as an inflammatory reaction at the site of the bronchial mucosa in patients with asthma.

**Objective:** The purpose of this study was to determine whether antigen avoidance can exert an antiinflammatory effect on the eosinophil phase of airway inflammation in children with asthma.

**Methods:** The level of bronchial hyperreactivity and the percentage of eosinophils in sputum samples obtained by inhalation of hypertonic saline solution, were evaluated in a group of asthmatic children allergic to house dust mite before and after a period of antigen avoidance in an Alpine environment (1756 m).

**Results:** At the end of the avoidance period PC<sub>20</sub> increased from a median value (lower and upper quartile: Q1, Q3) of 1.17 (0.74, 4.75) to 3.5 (1.18, 8.87) mg/ml ( $p = 0.02$ ), and eosinophil percentage in the sputum decreased from a median value (Q1, Q3) of 14.02 (3.34, 28.24) to 2.08 (0, 7.4) ( $p < 0.01$ ).

**Conclusion:** A 3-month period of antigen avoidance can significantly reduce the eosinophil phase of airway inflammation, along with bronchial hyperresponsiveness, in patients with asthma. (*J Allergy Clin Immunol* 1996;97:1079-84.)

**Key words:** Asthma, antigen avoidance, bronchial hyperreactivity, sputum, eosinophil, inflammation, altitude

Allergen inhalation in patients with asthma causes mediator release and influx of inflammatory cells into the airway wall, as well as an increase in bronchial responsiveness.<sup>1-6</sup> Eosinophils in particular are considered to be predominant among the inflammatory cells in asthma.<sup>2, 6, 7</sup>

The inflammatory response at the site of airways has been mostly investigated after allergen provocation,<sup>2, 3, 8</sup> whereas no study has investigated the effect of natural avoidance of allergen.

The purpose of this investigation was to evaluate the effect of natural allergen avoidance on the eosinophil infiltration in the airways of asthmatic children allergic to the dust mite. In consideration of the risk, discomfort, and inconvenience of bron-

## Abbreviations used

|      |                                                                |
|------|----------------------------------------------------------------|
| BDP: | Beclomethasone dipropionate                                    |
| BHR: | Bronchial hyperreactivity                                      |
| T1:  | Time of admission to Istituto Pio XII                          |
| T2:  | After a 3-month stay in the allergen-free mountain environment |

choscopy, as well as of the necessity of repeated examinations in the same patients, we examined sputum induced by inhalation of hypertonic saline solution.<sup>9</sup>

## METHODS

### Subjects

Sixteen children (13 boys and 3 girls) with a mean age ( $\pm$ SD) of 10.68 ( $\pm$  1.57) years, who had a history of bronchial asthma and a positive skin prick test response to the house dust mite, were selected from among patients admitted to the residential house "Istituto Pio XII" (Misurina BL, Italy), located at 1756 m in the Italian Alps. The study was performed out of season for

From Clinica Pediatrica, Università di Verona and Istituto Pio XII, Misurina.

Received for publication Dec. 6, 1994; revised June 30, 1995, accepted for publication July 3, 1995.

Reprint requests: Attilio L. Boner, MD, Clinica Pediatrica, Policlinico Borgo Roma, 37134 Verona, Italy.

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0091-6749/96 \$5.00 + 0 1/1/67525

TABLE I. Subject characteristics

| Patient No. | Sex | Age (yr) | Weight (kg) | Height (cm) | Asthma duration (yr) | Severity score | Treatment |
|-------------|-----|----------|-------------|-------------|----------------------|----------------|-----------|
| 1           | M   | 11       | 29          | 136         | 10                   | 2              | None      |
| 2           | M   | 8        | 24          | 126         | 7                    | 3              | LBDP      |
| 3           | M   | 13       | 42          | 149         | 3                    | 3              | LBDP      |
| 4           | F   | 12       | 51          | 144         | 11                   | 1              | None      |
| 5           | M   | 11       | 43          | 145         | 10                   | 4              | LBDP      |
| 6           | M   | 12       | 36          | 145         | 11                   | 2              | None      |
| 7           | M   | 11       | 36          | 137         | 8                    | 3              | None      |
| 8           | M   | 10       | 38          | 139         | 8                    | 3              | Th + HBDP |
| 9           | M   | 12       | 51          | 147         | 9                    | 3              | Th + HBDP |
| 10          | F   | 11       | 35          | 148         | 10                   | 4              | Th + HBDP |
| 11          | M   | 8        | 38          | 140         | 7                    | 4              | Th + HBDP |
| 12          | M   | 8        | 25          | 128         | 5                    | 3              | None      |
| 13          | M   | 10       | 38          | 133         | 7                    | 3              | LBDP      |
| 14          | F   | 10       | 30          | 139         | 7                    | 4              | Th + HBDP |
| 15          | M   | 12       | 31          | 138         | 7                    | 3              | None      |
| 16          | M   | 12       | 30          | 137         | 7                    | 3              | NS + HBDP |

LBDP, BDP (200 µg/day); Th, theophylline; HBDP, BDP (750 µg/day); NS, nedocromil sodium.

pollens to avoid interference with the experimental design for those patients who were also sensitized to pollens. None of the patients had had respiratory tract infections for at least 1 month before the beginning of the study.

The parents were asked to answer a questionnaire to assess the severity of asthma according to the clinical scoring method designed by Aas<sup>10</sup> at the time of recruitment. Subjects' characteristics and the duration and severity of asthma are listed in Table I. The study was approved by the Istituto Pio XII Ethics Committee, and both the children and their parents gave informed consent.

### Study design

All of the children who took part in the study were from families residing in the Northeastern area of Italy, below 300 m above sea level. The mean level of house dust mite in this area, as measured by the guanine test, is class 2 positive.<sup>11</sup> In contrast, the routine around-the-year determinations of house dust mite content in the dust from the residential house Istituto Pio XII has always shown a class 0 guanine test result.<sup>11</sup> Furthermore, because of the Alpine weather conditions, the ground in Misurina is usually covered with snow from November to May, reducing to a short summer period the presence of pollens in the atmosphere (unpublished data).

All of the children were selected from among those who applied for admittance to the residential house and who had spent at least 3 months at sea level, being exposed to seasonal and perennial allergens before arrival in the Alps.

Airway responsiveness to methacholine and percentage of eosinophils in sputum of our subjects were

evaluated in late September, at the time of admission to Istituto Pio XII (T1) and in December, after a 3-month stay in the allergen-free mountain environment (T2). The evaluation of airway responsiveness to methacholine was performed at the time of admission to the residential house, and the sputum was induced 24 hours after the methacholine challenge for all the subjects. The evaluations at T2 were performed in the same order and at the same time of day as evaluations at T1 for each subject.

None of the children had received oral corticosteroids for at least 1 month before or during the study. At the time of admission to the study (patient selection), 10 subjects were receiving inhaled  $\beta_2$ -agonists when needed, and 10 were also treated with inhaled corticosteroids. Six of them (Table I) were receiving beclomethasone dipropionate (BDP), 250 µg three times daily in association either with theophylline (four patients) or nedocromil sodium (one patient): five patients discontinued BDP the day before their arrival at the residential house, and one patient discontinued BDP 2 days before arrival, but all six of these patients continued to receive theophylline or nedocromil sodium. The remaining four patients, who were treated with low-dose BDP (100 µg twice a day), were instructed to discontinue the treatment 7 to 10 days before their arrival in the alpine environment. Inhaled  $\beta_2$ -agonists were allowed when needed throughout the study period, but they were withheld at least 24 hours before measurement of bronchial responsiveness.

### Methacholine provocation test and sputum induction

Bronchial hyperreactivity (BHR) was evaluated by a methacholine bronchial provocation test<sup>11, 12</sup> performed

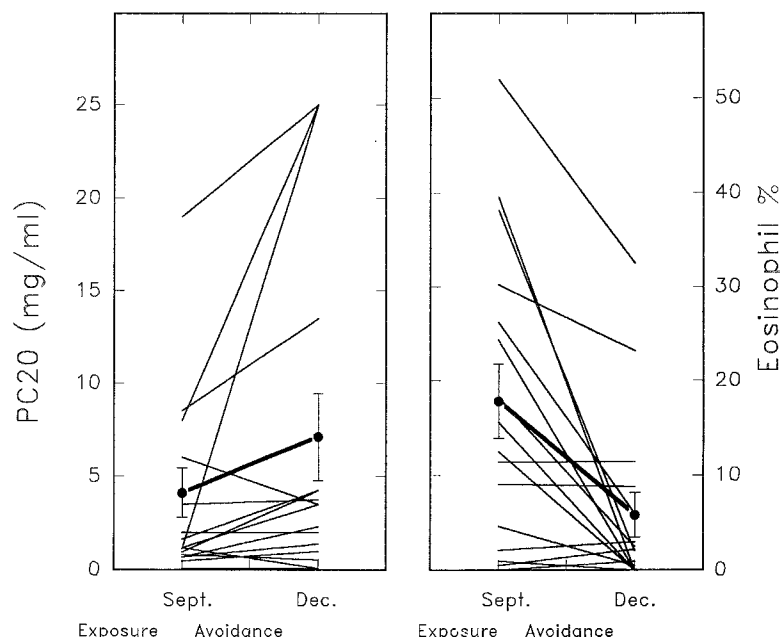


**FIG. 1.** Plots represent individual changes for  $PC_{20}$  and eosinophil percentage of total cells in the sputum (*Eosinophil %*) before and after allergen avoidance. *Thick lines* show changes in the study population, which, for graphic purposes, have been expressed as means  $\pm$  SEM.

with a MeFar dosimeter (MeFar, Brescia, Italy).<sup>11</sup> The concentration of methacholine required to produce a fall of 20% in  $FEV_1$  defined as a provocative concentration causing a 20% fall in  $FEV_1$  ( $PC_{20}$ ), was calculated as described by Rosenthal.<sup>13</sup>

Sputum production was induced by inhalation of hypertonic saline solution with a method similar to the one described by Pin et al.<sup>8</sup> Briefly, hypertonic saline solution was nebulized by using an ultrasonic nebulizer (UN202; Medical Service, Brescia, Italy), for four periods of 5 minutes each, for a total time of 20 minutes. Starting concentration was 4% NaCl delivered for a total of 10 minutes, which was followed by a 5% NaCl solution delivered for an additional 10 minutes. Before inhalation of hypertonic saline solution, all the patients were premedicated with inhaled salbutamol (2 puffs, 200  $\mu$ g) to inhibit possible bronchoconstriction. The  $FEV_1$  was recorded before and 10 minutes after salbutamol administration and then every 5 minutes during the saline inhalation. If  $FEV_1$  fell by more than 20% of the postbronchodilator value or troublesome symptoms occurred, nebulization was to be discontinued.

After each period of nebulization, subjects were instructed to rinse their mouths and then to cough sputum into a Petri dish. In the laboratory, the Petri dish was placed against a dark background, making the identification of sputum portion in the watery saliva easier. Plugs arising from the lower respiratory tract were selected by visual inspection, suspended in dithiothreitol for 30 minutes at 37°C, and then centrifuged at 1500 rpm (400 *g*) for 10 minutes at 12°C. Supernatant

was removed, and cells were washed twice in 1 ml 0.9% saline solution. The pellet was resuspended in 0.9% saline solution, and the samples were transferred to slides by cytocentrifugation (600 rpm for 5 minutes) with Cytospin 3 centrifuge (Shandon, Inc., Pittsburgh, Pa.), fixed in alcohol 95% and then stained with May-Grunwald-Giemsa stain. Slides were coded to be counted in blinded fashion. A differential cell count was obtained by counting at least 400 cells. Results were expressed as mean percentage of total cells counted by two independent observers.

### Statistical analysis

Results are expressed as median and lower and upper quartile (Q1, Q3). Wilcoxon's rank sum test on paired data was used for comparing  $PC_{20}$  and eosinophil percentage between the times of the study. Correlation between changes in the investigated parameters during the period of avoidance, expressed as difference between results at the different times ( $PC_{20}$  and change in eosinophils), was evaluated by Spearman's rank correlation test. Reproducibility of cell counts was assessed by calculation of the coefficient of variation between repeated measures.<sup>14</sup>

### RESULTS

We examined the changes in methacholine airway responsiveness and sputum eosinophil counts during the 3 months of residence in the Alps. Individual  $PC_{20}$  values (in milligrams per milliliter) are plotted

in Fig. 1. Between T1 and T2, PC<sub>20</sub> increased from 1.17 (0.74; 4.75) to 3.5 (1.18; 8.87) ( $p = 0.02$ ).

None of the subjects experienced bothersome symptoms during inhalation of hypertonic saline solution. For none of the subjects, did the fall in FEV<sub>1</sub> exceed 10%, and therefore nebulization never had to be stopped. A significant decrease in the percentage of eosinophils in the sputum was observed at T2 in comparison with T1, respectively being 14.02 (3.34; 28.34) versus 2.08 (0; 7.4) ( $p < 0.01$ ). The individual values are plotted in Fig. 1.

A significant negative correlation between change in PC<sub>20</sub> and change in eosinophils was observed ( $r = 0.55$ ,  $p = 0.032$ ). The coefficient of variation between repeated measures was always below 10%.

## DISCUSSION

The eosinophil granulocyte is regarded as a potent proinflammatory cell with considerable tissue-injuring capacity, which may be directly responsible for the epithelial mucosal damage in patients with asthma<sup>7</sup> through oxygen-dependent and oxygen-independent mechanisms.<sup>15-23</sup> Moreover, a direct correlation between eosinophil cationic protein and eosinophil protein X secretion and the exposure to relevant allergens has also been shown,<sup>24</sup> further confirming the major role of eosinophils in the allergic inflammation underlying bronchial asthma.

In recent years, information obtained from bronchial biopsies, brushing, or lavage showed that many of the inflammatory features, which are observed in airways of subjects who have died of asthma, may also be found even in patients with mild or moderate disease.<sup>6, 7, 25, 26</sup> Because of the possible occurrence of wheezing immediately after a bronchoscopy, all the previously named methods of sample collection are not recommended in subjects with moderate to severe BHR, or for repeated examinations,<sup>3, 7, 26</sup> and they are seldom used in children with asthma. Therefore for the purposes of this study, we chose the method of hypertonic saline-induced sputum,<sup>9</sup> which is considered a safe and noninvasive alternative.<sup>8, 9, 27, 28</sup> A significant increase in the number of eosinophils has been previously shown in the sputum of asthmatic patients after allergen-induced asthma, which was correlated with the degree in increase of BHR and with the magnitude of late asthmatic reaction.<sup>8</sup> Nevertheless, no study evaluating the effect of natural exposure-avoidance to relevant allergens on the bronchial inflammatory infiltration in children with asthma has been published.

A body of evidence from our previous studies showed that moving atopic asthmatic children who were mainly sensitized to mites from low to high altitude is associated with a reduction not only in nonspecific parameters such as BHR,<sup>29</sup> eosinophil protein X-eosinophil cationic protein levels,<sup>30</sup> and IL-2R,<sup>31</sup> but also in specific markers of antigen exposure, such as the levels of specific IgE to mites and antigen-induced release of histamine from circulating basophils,<sup>11</sup> as well as allergen sensitivity, late allergen-induced bronchial reactions, and enhancement of BHR by allergen challenge.<sup>32</sup> These observations suggest that the improvement observed in patients with asthma after a stay at high altitude is due to a reduction in allergen exposure. This idea is also supported by the demonstration of undetectable levels of mites in the residential House Istituto Pio XII, where routine around-the-year determinations performed by guanine test always showed class 0 results,<sup>11</sup> which indicates that samples contain less than 2  $\mu\text{g}$  of group I antigen (Der p 1, Der f 1) per gram of dust.<sup>33</sup> In contrast, in the area of the family houses, relevant concentrations of mites have been shown throughout the year.<sup>11</sup> Indeed, guanine tests performed throughout the year in the family houses of the patients who were guests of the Istituto Pio XII showed a mean class of 2,<sup>17</sup> which indicates a level of group I antigen greater than 2  $\mu\text{g}/\text{gm}$  of dust for all the samples and suggests a level greater than 10  $\mu\text{g}/\text{gm}$  in most cases (80%).<sup>33</sup>

The results of this report show that the decrease in bronchial responsiveness observed in our previous investigations, after long periods of mite avoidance in a mountain environment, is associated with a reduction in airway inflammation as well. Indeed, in this study significant reductions in both bronchial reactivity and eosinophil percentage in the sputum have been observed. Furthermore, the correlation observed between the changes in these parameters confirms a close relationship between airway inflammation, bronchial responsiveness, and exposure to relevant allergens. In this study, the magnitude of the changes may be in part driven by the treatment with inhaled steroids in 10 of 16 patients before their arrival at the mite-free mountain environment. In fact, greater changes could have been hampered because of the antiinflammatory treatment at the time of admission to the study. On the other hand, it is also possible that withdrawal of inhaled steroid could cause a rebound increase in eosinophils at the time of the first evaluation, followed by a natural leveling off to lower levels in the following weeks. At present, we

do not have data that clearly show that this does not occur. However, in order to rule out this possibility we have subanalyzed the data from this study, according to the steroid treatment discontinuation. Assuming that withdrawal of low-dose steroids would not have had an effect on the number of eosinophils in sputum after 7 to 10 days of withdrawal of low-dose BDP we have excluded from the analysis the six patients treated with higher doses of BDP. The statistics still show a significant difference in the number of eosinophils in the sputum between the time of arrival in the Alpine environment and 3 months later ( $p = 0.024$ ). Even if we exclude all the children treated with inhaled steroids, a significant reduction ( $p < 0.05$ ) in the number of eosinophils can be observed in the sputum of the six remaining children who were untreated before admission to the study, thus confirming the role of antigen avoidance in the decrease of bronchial eosinophilia in our study group.

Although knowledge on the pathogenesis of asthma is increasing, the dynamics of the inflammatory process are still unclear, and the mechanism of local tissue eosinophilia is largely unexplained. The sequence of cell migration, the way in which this inflammatory process is resolved and the sequence of the structural changes are not completely explained. The regulation and activation of eosinophilic inflammation in patients with asthma are currently being investigated. In particular, a potential role of adhesive molecules in different stages of the inflammatory response, including adhesion of blood eosinophils to vascular endothelium at the beginning of migration into the respiratory tissue, as well as the steps that follow, such as the interaction of the respiratory tissue extracellular matrix proteins with other cells, has also been proposed.<sup>34</sup>

The possibility of obtaining cellular samples from the bronchi with a safe, inexpensive method and without discomfort, as with the method of induced sputum production, applied to a model of natural antigen exposure-avoidance opens new perspectives for a more comprehensive analysis of some of the cellular inflammatory events at the site of the bronchial mucosal cells in children with allergic asthma.

At present, this is the first demonstration of the effect of allergen avoidance at high altitude on the eosinophil phase of airway inflammation in children with asthma, supporting the value of environmental measures to control asthma in house dust mite-sensitive subjects.<sup>35, 36</sup>

We thank Prof. Frederick Hargreave, for his suggestions and for the final review of the manuscript, and Dr. Andrew Luyang for the revision of the English form of the final manuscript.

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