Interleukin-13 and Interleukin-5 in Induced Sputum of Eosinophilic Bronchitis*

Comparison With Asthma

Sung-Woo Park, MD, PhD; Hee Kyung Jangm MS; Mi Hyoun An, MS; Ji Won Min, MS; An-Soo Jang, MD, PhD; June-Hyuk Lee, MD; and Choon-Sik Park, MD, PhD

Study objectives: Experimental studies on asthma have indicated that interleukin (IL)-13 induces airway hyperreactivity (AHR). However, it remains unproven that IL-13 is responsible for AHR in asthmatic patients. Eosinophilic bronchitis (EB) shows normal airway responsiveness despite eosinophilic airway inflammation of severity similar to that of asthma. This study evaluated the role of IL-13 in asthma by comparing the sputum IL-5 and IL-13 levels in both groups.

Methods: Comparisons between asthma and EB would clarify the role of IL-13 in AHR. IL-5 and IL-13 were assayed in the sputum and culture supernatants of peripheral blood mononuclear cells (PBMCs) from 22 asthmatic patients, 12 EB patients, and 11 healthy control subjects.

Results: IL-13 levels were higher in the asthmatic patients than in the EB patients or healthy control subjects (p < 0.001). IL-5 levels were similar in the asthmatic patients and EB patients, who had significantly higher levels than those of healthy control subjects. Sputum IL-13, but not IL-5, is inversely correlated with the provocative concentration of a substance causing a 20% fall in FEV1 for methacholine in asthmatic patients (r = -0.502; p < 0.017). IL-13 production by PBMCs was significantly higher in asthmatic patients than in EB patients (p = 0.015), but the levels between EB patients and healthy control subjects was comparable.

Conclusion: The results of the present study indicate that IL-13 is related to AHR in asthmatic patients.

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Key words: airway hyperreactivity; asthma; eosinophilic bronchitis; interleukin-5; interleukin-13

Abbreviations: AHR = airway hyperresponsiveness; EB = eosinophilic bronchitis; IL = interleukin; IQR = interquartile range; PBMC = peripheral blood mononuclear cell; PC20 = provocative concentration of a substance causing a 20% fall in FEV1; PMA = phorbol myristate acetate; Th2 = T helper type 2

Asthma is an airway disorder that is characterized by airway inflammation, variable airflow obstruction, and airway hyperresponsiveness (AHR). Pathologically, asthma shows cellular infiltration of eosinophils, mast cells, and CD4+ lymphocytes, and involves airway remodeling, including subepithelial fibrosis and hyperplasia of goblet cells and smooth muscles. The excessive production of interleukin (IL)-4, IL-5, and IL-13 by T-helper type 2 (Th2) cells is implicated in the development of asthma. IL-5 mobilizes eosinophils from the bone marrow pool, and chemokines, such as eotaxin-1, induce the recruitment of eosinophils into the airway of experimental asthma models. Airway eosinophils, which release mediators that damage the airway epithelium, have been regarded as a major determinant of the AHR observed in asthmatic patients. However, the role of eosinophils in AHR has been challenged by the results of human studies. Neutralization of AHR with the anti-IL-5 antibody decreases peripheral blood and sputum eosinophilia in asthmatic patients but does not affect airway hyperreactivity.

*From the Asthma and Allergy Research Group, Soonchunhyang University, Bucheon Hospital, Bucheon, Korea. Sung-Woo Park and Hee Kyung Jang equally contributed to this work as the first author. This work was supported by grant 01-PJ3-PC6-01GN04-003 from the Korea Health 21 R&D Project, Ministry of Heath & Welfare, Republic of Korea. Manuscript received September 10, 2004; revision accepted February 22, 2005. Reproduction of this article is prohibited without written permission from the American College of Chest Physicians (www.chestjournal.org/misc/reprints.shtml). Correspondence to: Choon-Sik Park, MD, Division of Allergy and Respiratory Medicine, Department of Internal Medicine, Soonchunhyang University Bucheon Hospital, 1174, Jung Dong, Wonmi Ku, Bucheon, Gyeonggi Do, 420–021, Korea; e-mail: mdcspark@unitel.co.kr
tivity. In addition, a lack of association between airway eosinophilia and AHR has been observed in patients with eosinophilic bronchitis (EB). EB is an airway disorder that involves eosinophilic inflammation at a level similar to that seen in asthma, although EB patients have normal airway responsiveness to nonspecific stimulants. This latter responsiveness has remained within the normal range over a long-term follow-up period, even though about half of the EB patients developed recurrent sputum eosinophilia during the follow-up period. Thus, EB may be a good human model to reveal the underlying mechanism behind the AHR of asthma. However, morphometric and cellular analyses revealed no differences in basement membrane thickness and in the numbers of eosinophils, mast cells, and T lymphocytes expressing Th2-type cytokines (ie, IL-4 and IL-5), chemokine receptors (ie, CCR3, CCR5, CCR6, and CXCR3), and activation markers (ie, CD25),

It has been recognized in animal models of experimental asthma that CD4+ T lymphocytes are critical for the events leading to AHR. This process is independent of IL-4 and IL-5, but requires signaling through IL-4R and signal transducer and activator of transcription-6, which are key molecules in the IL-13 pathway. IL-13 overexpression produces all the features of asthma (ie, airway inflammation, remodeling, and hyperresponsiveness), whereas the blockade of IL-13 by the soluble receptor-Fc fusion protein abolishes allergen-induced AHR in experimental asthma settings. IL-13 is also a potent stimulator that induces epithelial cells and smooth muscle cells to produce a variety of chemokines, including monocyte chemotactic proteins and eotaxins. However, a study using eotaxin/IL-5 double-knockout mice confirmed that eosinophils are not required for the IL-13-mediated induction of AHR. Taken together, a series of experimental asthma studies has indicated that IL-13 alone can induce AHR. However, it remains to be proven that IL-13 is the major cytokine involved in the induction of AHR in asthmatic patients.

The levels of proteins and messenger RNA for IL-13 and IL-5 are elevated in the airways of asthmatic patients compared with those of healthy control subjects. However, the exact roles of these cytokines in the airways may be clarified by comparison between asthmatic patients and EB patients, since both of them have a similar degree of airway eosinophilia. Given the unique role of IL-13 in AHR, as revealed in experimental asthma, we speculated that the IL-13 levels should be the same in EB patients as in healthy control subjects, whereas they should be elevated in asthmatic patients. If this is the case, then IL-13 production would be decreased in the EB lung compared with the asthmatic lung, which might be reflected by differences in the production of IL-13 by peripheral blood mononuclear cells (PBMCs). Thus, the aims of this study were as follows: (1) to examine whether the levels of IL-5 and IL-13 in sputum samples differed between EB patients and asthmatic patients; (2) to evaluate whether IL-13 levels were correlated with AHR in asthmatic patients; and (3) to investigate whether IL-13 production by PBMCs was different in patients with the two conditions.

Materials and Methods

Subjects

Twenty-two subjects with asthma, 12 subjects with EB, and 11 healthy control subjects were enrolled into the study. The EB group contained patients who complained of an isolated chronic cough of > 4 weeks duration. EB was diagnosed based on the following criteria: (1) FEV1 and FVC values of > 80% predicted, with a negative response to a short-acting bronchodilator; (2) the absence of bronchial hyperreactivity (ie, a provocative concentration of a substance causing a 20% fall in FEV1 [PC20] for methacholine of > 10 mg/mL); and (3) sputum eosinophilia concentrations of > 3%, as described previously. Asthma was defined on the basis of clinical symptoms according to the criteria of the American Thoracic Society. All of the patients had clinical symptoms that were compatible with asthma. Each patient showed airway reversibility, as documented by a positive bronchodilator response of a 15% increase of FEV1 and/or airway hyperreactivity of < 10 mg/mL methacholine. Only asthmatic patients with disease of mild persistent severity were included based on clinical features. All of the patients receiving therapy with inhaled or systemic steroids within 6 weeks before entering the study were excluded. The healthy control subjects were recruited from hospital personnel who answered in the negative to a screening questionnaire for respiratory symptoms, and who had FEV1 values of > 80% predicted; PC20 values for methacholine of > 10 mg/mL, and normal findings for simple chest radiograms.

Study Design and Procedures

Subjects visited twice consecutively. At the first visit, CBC and differential counts were obtained, a chest radiogram was taken, and an allergen skin-prick test, a short-acting bronchodilator test, and a sputum induction test were conducted. At the second visit, the subjects underwent methacholine inhalation test. The PC20 test for methacholine was conducted using the method of Juniper et al, and the results are expressed as the PC20. Atyposis was determined by skin prick tests using 48 common inhalant allergens, including dust mites (Dermatophagoides farinae and Dermatophagoides pteronyssinus), cat fur, dog fur, fungus, cockroach, grass, tree, and ragweed pollen (Bencard; Brentford, UK). The test result was regarded as positive when the wheal size was equal to or larger than that of the histamine control. The numbers of total cells and differential cells were counted in peripheral venous blood samples (Coulter Counter; Beckman Coulter Co; Miami, FL). The Ethics Committee of Soonchunhyang University Hospital approved the study, and informed written consent was obtained from each study subject.
**Isolation of PBMCs and IL-13 Assays**

PBMCs were isolated from asthmatic patients, patients with EB, and healthy control subjects (Histopaque 1077; Sigma Chemical Co; St. Louis, MO). Briefly, 30 mL of heparinized peripheral blood was layered onto 15 mL of the density gradient separation reagent (ie, Histopaque 1077). After centrifugation at 400g for 30 min, the mononuclear cell interface was collected and washed twice with phosphate-buffered saline solution. A differential cell count was performed. The collected PBMCs were cultured at 5 x 10^6 cells/mL in RPMI 1640 medium that contained 10% fetal bovine serum. The PBMCs were stimulated with phorbol myristate acetate (PMA) [100 ng/mL; Sigma Chemical Co] and calcium ionophore (1 µM; Sigma Chemical Co) together for 16 h. Then, the supernatants were collected for IL-13 measurements.

**Assays for IL-5 and IL-13**

The levels of IL-13 and IL-5 were measured using the quantitative sandwich enzyme immunoassays with enzyme-linked immunosorbent assay kits according to the recommendations of the manufacturer (for IL-5: Endogen; Woburn, MA; for IL-13: Pharmingen, San Diego, CA). The detection limits for IL-5 and IL-13 were 2.0 and 3.5 pg/mL, respectively. Values below these thresholds were assigned a value of 0 pg/mL. The interassay coefficient of variability of the IL-5 and IL-13 assays was < 10%, and the intraassay coefficient of variability was < 10% across the range of concentrations. For validation of the assay, we performed spiking experiment using 10 samples in dithiothreitol-treated sputum, and the mean (± SEM) recovery rates of IL-13 and IL-5 were 86.2 ± 0.1% and 83.2 ± 0.6%, respectively.

**Statistical Analysis**

The results are expressed as the median (interquartile range [IQR]), unless otherwise specified. The concentrations of IL-13 and IL-5 were corrected by the dilution factor. A statistical software package (SPSS/PC+; SPSS; Chicago, IL) was used for the analysis. Differences between groups were compared using the nonparametric Mann-Whitney U test. Correlations between the data were assessed using the Spearman rank test. Differences were considered to be statistically significant when the p value was < 0.05.

**Results**

**Comparisons of Clinical Characteristics and Cellular Profiles in Sputum Samples**

Subject characteristics of age, sex, smoking, atopy, PC_{20} for methacholine, and respiratory function measurements are shown in Table 1. There were no differences in FEV1 values between the groups of EB patients, healthy control subject, and asthma patients. Sputum cell counts were similar between the groups of EB and asthma patients, although both group had significantly higher total cell numbers than the healthy control subject group (p < 0.05). The percentages of eosinophils in sputum were significantly higher in the EB and asthma groups than in the healthy control group (p < 0.01), but were similar in the former two groups. In peripheral blood differential counts, the counts of eosinophils were significantly higher in the asthma group (p < 0.01) and EB group (p < 0.05) than in the healthy control subject group, but were similar in the former two groups. The counts of basophils did not differ among the three groups.

**Comparison of Cytokine Levels in Induced Sputum Samples**

IL-13 was detected in the sputum of 100% of the asthmatic patients, 100% of the EB patients, and 65% of the healthy control subjects (Fig 1, top, A). IL-5 was detected in the sputum of 90% of the asthmatic patients, 100% of the EB patients, and 74% of the healthy control subjects. The median IL-13 level in the asthma group was significantly higher than that in the EB group (81.2 pg/mL [IQR, 55.8 to 170.2 pg/mL] vs 34.2 pg/mL [IQR, 25.3 to 63.6 pg/mL], respectively; p = 0.001). There was no difference in the mean IL-13 levels between the EB group and healthy control group (34.2 pg/mL [IQR, 25.3 to 63.6 pg/mL] vs 26.8 pg/mL [IQR, 0 to 42.4 pg/mL], respectively; p = 0.132). The median IL-5 levels were significantly higher (p = 0.001) in the EB and asthma groups than in the healthy control group (107.4 pg/mL [IQR, 85.3 to 143.8 pg/mL] and 91.4 pg/mL [IQR, 73.5 to 124.6 pg/mL] vs 31.4 pg/mL [IQR, 0 to 42.4 pg/mL], respectively) [Fig 1, bottom, B]. There was no significant difference of sputum IL-5 levels between the EB group and the asthma group (p = 0.67). There was no difference in IL-13 and IL-5 levels by the presence of atopy in the three groups (Fig 1).
In the asthma group, PC20 methacholine levels inversely correlated with sputum IL-13 levels ($r = -0.502; p = 0.017$) [Fig 2], but not with sputum IL-5 levels ($r = 0.092; p = 0.15$) and sputum eosinophil percentages ($r = -0.068; p = 0.17$).

Comparisons of IL-13 Production by PBMCs Among the Asthma, EB, and Control Groups

PBMCs were composed of lymphocytes and monocytes, and the proportion of eosinophils and basophils was < 1%. IL-13 was not generated spontaneously by cultured PBMCs from any of the study subjects. However, IL-13 was detected in all of the culture supernatants 16 h after stimulation with PMA and calcium ionophore. The median IL-13 generation was significantly higher in the asthma group (700.9 pg/mL; IQR, 420.4 to 1215.7 pg/mL) than in either the EB group (384.7 pg/mL; IQR, 289.3 to 665.1 pg/mL; $p = 0.015$) or healthy control group (238.0 pg/mL; IQR, 63.2 to 555.2 pg/mL; $p = 0.001$). IL-13 production was comparable in the latter two groups ($p = 0.098$) (Fig 3). IL-13 production did not correlate with the basophil counts in the peripheral blood from either the EB or asthma groups ($p > 0.05$).

**Discussion**

The main object of this study was to reveal a difference in the levels of IL-13 in the sputum between EB and asthma patients. In this study, we demonstrated that the sputum IL-13 levels were significantly higher in asthmatic patients than in patients with EB, who showed levels of IL-13 that were similar to those of healthy control subjects. IL-5 levels were similar between asthmatic patients and patients with EB. Since the absence of AHR is a basic characteristic of EB, comparisons of the clinical and biochemical findings of EB and asthma patients provide clues as to the mechanism of AHR.

Brightling and coworkers analyzed Th2 cytokine expression by T cells, and revealed similar levels of IL-4 and IL-5 in asthma and EB patients; they did not evaluate IL-13 expression. Recently, they compared IL-13 levels in sputum samples between asthmatic patients and patients with EB. Their data also have shown that IL-13 levels were higher in asthmatic patients than in EB patients. But they did not reveal the correlation between sputum IL-13 levels and AHR in the asthmatic patients. Although we measured the concentrations of IL-4 in the sputum samples, they were below the detection limit of our enzyme-linked immunosorbent assay system (data not shown). In the present study, there were no differences in the levels of IL-5 between patients with the two conditions. This indicates that levels of IL-5 and the airway eosinophilia mediated by these cytokines are not related to AHR in asthmatic patients. We also demonstrated that the PC20 for methacholine was significantly correlated with sputum IL-13 levels, even though it was not strong.
but not with IL-5 levels and eosinophil percentages in asthmatic patients. These results indicate that IL-13 is closely associated with AHR in asthmatic patients and is not mediated by the overproduction of eosinophil-active cytokines, including IL-5. This provides clinical evidence for the role of IL-13 in AHR development, which has been demonstrated in animal models.\textsuperscript{18,19,31}

AHR in asthmatic patients may be attributed to the direct effects of IL-13 on epithelial cells\textsuperscript{32} and the reduction of the β-adrenergic responsiveness of human airway smooth muscle cells.\textsuperscript{33} IL-13 also induces bronchial smooth muscle proliferation via the augmented expression of the cysteinyl leukotriene 1 receptor.\textsuperscript{34} Brightling and coworkers\textsuperscript{11} demonstrated differences in the localization of mast cells in the airways of EB and asthma patients. They performed morphometric and cellular analyses, and revealed that mast cell infiltration in the smooth muscle is significantly higher in asthma patients than in either EB patients or healthy control subjects. Thus, the lack of mast cells in smooth muscle may be a major contributory factor to the maintenance of normal airway reactivity in EB patients.\textsuperscript{11} This concept seems reasonable when one considers the biological effects of IL-13 on smooth muscle and

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure1.png}
\caption{IL-13 (top, A) and IL-5 (bottom, B) levels in the induced-sputum from asthmatic patients (BA), patients with EB (EB) and healthy control subjects (NC). Open and closed symbols represent atopy and nonatopy subjects, respectively. Horizontal bars represent median values of the three groups.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure2.png}
\caption{Correlation between sputum IL-13 levels and the PC\textsubscript{20} for methacholine in the asthmatic patients. There was a significant inverse correlation between IL-13 levels and PC\textsubscript{20} for methacholine levels ($r = -0.484; p = 0.02$).}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure3.png}
\caption{Comparison of IL-13 levels in the supernatants of cultured PBMCs 16 h after stimulation with PMA and calcium ionophore between asthmatic patients, patients with EB, and healthy control subjects. See the legend of Figure 1 for abbreviations not used in the text. Horizontal bars represent the median values of the three groups.}
\end{figure}
whether IL-13 production by airway mast cells would be different between the two conditions.

The difference in IL-13 levels in sputum between two conditions may be attributed to the altered synthesis of IL-13 by the inflammatory and immune cells in the airway. We performed the in vitro study to compare the production of IL-13 by PBMCs to investigate differences in IL-13 production by PBMCs from EB and asthma patients. We confirmed that the production of IL-13 was significantly higher in asthmatic patients than in EB patients (Fig 3). Both basophils and T cells in the peripheral blood may secrete IL-13 after activation with either non-specific stimuli or specific antigens. However, in the present study, the proportion of basophils or eosinophils was <1% in PBMCs isolated from asthmatic patients and EB patients. These data suggest that differences in IL-13 production may be due to the decreased potential of IL-13 production by peripheral blood cells other than basophils and eosinophils.

As an additional finding of the present study, there was no difference in the levels of IL-5, which plays a key role in the development of airway eosinophilia in asthma patients. Bearing in mind the biological key role in the development of airway eosinophilia in asthma patients and EB patients, we investigated differences in IL-5 production by peripheral blood cells other than basophils and eosinophils.

In conclusion, the results of the present study indicate that IL-13 is related to AHR in asthmatic patients. The normal levels of IL-13 in the airways of EB patients may be due to the normalized production of IL-13, which leads to the normal responsiveness of the EB airway. This study provides clinical evidence for the concept that the altered production of IL-13 can be the main mechanism of AHR in experimental asthma.

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