

DIAGNOSTICS

Bronchodilator Responses after Methacholine and Adenosine 5'-Monophosphate (AMP) Challenges in Children with Asthma: Their Relationships with Eosinophil Markers

YOUNG YOO, M.D.,^{1,2,*} SUNG CHUL SEO, PH.D.,² YOUNG IL KIM, M.D.,² BO HYUN CHUNG, M.D.,¹
DAE JIN SONG, M.D.,^{1,2} AND JI TAE CHOUNG, M.D.^{1,2}

¹Department of Pediatrics, College of Medicine, Korea University, Seoul, Korea.

²Environmental Health Center for Childhood Asthma, Korea University Anam Hospital, Seoul, Korea.

Background. Bronchodilator responsiveness (BDR) and eosinophilic inflammation are characteristic features of asthma. **Objective.** The aim of this study was to compare the relationships of BDR after methacholine challenge or adenosine 5'-monophosphate (AMP) challenge to blood eosinophil markers in children with asthma. **Methods.** Methacholine and AMP challenges were performed on 69 children with mild intermittent to moderate persistent asthma. BDR was calculated as the change in forced expiratory volume in 1 second, expressed as percentage change of the value immediately after the each challenge and the value after inhalation of salbutamol. Serum total IgE levels, blood eosinophil counts, and serum eosinophil cationic protein (ECP) levels were determined for each subject. **Results.** A positive relationship between serum total IgE levels and BDR was found only after the AMP challenge ($R^2 = 0.345$, $p = .001$) rather than after the methacholine challenge ($R^2 = 0.007$, $p = .495$). Peripheral blood eosinophil counts correlated more significantly with BDR after AMP challenge ($R^2 = 0.212$, $p = .001$) than BDR after methacholine challenge ($R^2 = 0.002$, $p = .724$). Both BDR after methacholine challenge ($R^2 = 0.063$, $p = .038$) and BDR after AMP challenge ($R^2 = 0.192$, $p = .001$) were significantly correlated with serum ECP levels. **Conclusion.** BDR after AMP challenge may be more closely related to eosinophilic inflammation, compared with that after methacholine challenge.

Keywords asthma, bronchial provocation, bronchodilator response, child, eosinophils

INTRODUCTION

Asthma is characterized by reversible airflow obstruction, which is thought to be the result of an extensive bronchoconstriction caused by contraction of bronchial smooth muscles and airway eosinophilic inflammation (1). The reversibility of the constricted airway at response in inhaled short-acting β_2 -agonists (SABAs) is highly variable among individuals with asthma (2).

Airway hyperresponsiveness (AHR) is defined as an exaggerated bronchoconstrictive response of the airway to a variety of stimuli that can be divided into direct (3) and indirect (4), and has been largely attributed to structural changes and to inflammatory events in the airway (5). The direct stimuli act directly on specific airway smooth muscle receptors, whereas the indirect stimuli act through intermediate pathways by enhancing the release of mediators from mast cells (6). There is an increasing interest in the role of adenosine 5'-monophosphate (AMP) as an indirect bronchoconstrictor stimulus because it has been indicated that AHR to AMP is a better marker of airway inflammation than direct stimuli such as methacholine.

Bronchodilator responsiveness (BDR), defined as an improvement of forced expiratory volume in 1 second (FEV_1) after inhalation of β_2 -agonists, is usually measured by changes in airflow before and after the administration of

SABAs. BDR has more recently been thought to reflect bronchial lability and to act as a surrogate marker of airway inflammation in asthmatics (7, 8). A previous study has shown the relationship between BDR and airway inflammation markers in children with stable asthma rather than acute exacerbations (7). Thus, it is necessary to estimate the responses to bronchodilators at the acute exacerbation state and to compare their responses to airway inflammatory markers.

Increasing numbers of observations (9–12) indicate that AHR to AMP is more closely related to atopy or eosinophil parameters than AHR to methacholine. However, no such relationship was seen between BDR after AMP-induced bronchoconstriction and BDR after methacholine-induced bronchoconstriction in terms of blood eosinophil parameters.

This study was designed to compare BDR after AMP challenge and BDR after methacholine challenge with regard to possible links between eosinophil markers in children with asthma. We hypothesized that eosinophil inflammatory parameters more closely reflect BDR after AMP challenge, as a surrogate marker of airway inflammation, than BDR after methacholine challenge in children with asthma.

MATERIALS AND METHODS

Study Population

This study was performed on 69 children with mild intermittent to moderate persistent asthma, aged 6–18 years.

*Corresponding author: Young Yoo, MD, PhD, Department of Pediatrics, Korea University Anam Hospital, 126-1, 5-ga, Anam-dong, Sungbuk-gu, Seoul 136-705, Korea; Tel: +8229205090; Fax: +8229227476; E-mail: yoolina@korea.ac.kr

These patients had physician-diagnosed asthma and a history of episodic wheezing and/or dyspnea during the past year, which was resolved with bronchodilators. They were followed at the Allergy Clinic of Korea University Anam Hospital. The clinical severity of asthma was assessed according to the National Asthma Education and Prevention Program criteria (13). Forty-two were mild asthmatics and 27 were moderate persistent asthmatics. Patients with a history of near-fatal asthma, major exacerbations necessitating the use of systemic corticosteroids, or other respiratory diseases were excluded from the study.

Parents gave written informed consent for their children to participate in the study. The study protocol was approved by Institutional Review Board (No. AN09226-003).

Study Design

This study consisted of assessment of BDR following methacholine-induced and AMP-induced bronchoconstrictions and measurement of blood eosinophil markers. Methacholine inhalation tests were carried out using a modification of the method described by Chai et al. (14), and AMP challenge tests were carried out using a modification of the method of the European Respiratory Society (15). Inhaled SABA was withheld for at least 8 hours, and inhaled corticosteroid, long-acting β_2 -agonists, and leukotriene modifier were withheld for at least 3 days before each challenge. At the time of the test, all patients were free of acute respiratory tract infections and asthma exacerbation for at least 4 weeks.

Methacholine and AMP Challenge Test

Fresh solutions of methacholine and AMP were prepared in a buffered saline solution at concentrations ranging from 0.075 to 50 mg/mL for methacholine and from 3.125 to 400 mg/mL for AMP. Lung function was measured using a computerized spirometer (Microspiro-HI 298; Chest Corporation, Tokyo, Japan), and the largest value of triplicate FEV₁ on each occasion was used for analysis. All patients were required to undergo spirometry in a standard manner and were included in the study if they showed an FEV₁ of at least 70% of the predicted value. A Rosenthal-French dosimeter (Laboratory for Applied Immunology, Baltimore, MD, USA) triggered by a solenoid valve set to remain open for 0.6 seconds, was used to generate an aerosol from a DeVilbiss 646 nebulizer (DeVilbiss Health Care; Somerset, PA, USA) with pressurized air at 20 psi. Each patient inhaled 5 inspiratory capacity breaths of a buffered saline solution and increasing concentrations of methacholine or AMP, respectively, at 5-minute intervals. At each concentration level, FEV₁ was measured 90 seconds after inhalation. The procedure was terminated when the FEV₁ decreased by more than 20% of its post-saline value or when the highest methacholine (50 mg/mL) or AMP (400 mg/mL) concentration was reached.

The percentage decline of FEV₁ from the post-saline value was plotted against the log concentrations of inhaled methacholine or AMP. Methacholine PC₂₀ and AMP PC₂₀ were calculated by interpolating between two adjacent data

points whenever the FEV₁ decreased by more than 20%. The percentage fall of FEV₁ from the mean post-saline value was plotted against log cumulative doses (PD₂₀) of inhaled methacholine or AMP expressed as a breath unit (BU). One BU denotes one inhalation of 1 mg/mL methacholine or AMP. Patients who did not show a 20% decline in FEV₁ after inhalation of the maximal concentration of methacholine or AMP were excluded from the study.

Assessment of Bronchodilator Response

Immediately after the administration of methacholine or AMP was stopped due to a fall in the FEV₁ more than 20% of its post-saline value, BDR test to inhaled salbutamol (Ventolin Evohaler; GlaxoSmithKline, London, UK) was carried out. Four separate doses of salbutamol (400 μ g) were administered from a metered dose inhaler via a spacer (AeroChamber Plus; Trudell Medical International, London, Ontario, Canada); spirometry was performed after 15 minutes (post-BD FEV₁). BDR after methacholine (or AMP) was defined as the change in the FEV₁ %predicted $\times$ 100/post-methacholine (or AMP) FEV₁ %predicted (16).

Skin Prick Test

Skin prick test was performed using 13 common aeroallergens, and atopy was defined as the presence of at least 1 positive reaction to these allergens greater than saline control.

Blood Eosinophil Markers

The number of peripheral blood eosinophils was counted with blood samples containing EDTA using an automated hematology analyzer (Coulter Counter STKS; Beckman Coulter, Fullerton, CA, USA). The serum total IgE levels were measured using a Coat-A-Count Total IgE IRMA (Diagnostic Products Co, Los Angeles, CA, USA) according to the manufacturer's instructions. The serum eosinophil cationic protein (ECP) levels were measured using a commercially available fluoroimmunoassay kit (Pharmacia ECP UniCAP System FEIA; Pharmacia Diagnostics, Uppsala, Sweden), which had a detection limit of less than 2.0 μ g/L. Blood sample collection, serum preparation, and serum ECP measurement were performed according to the manufacturer's instructions.

Statistical Analysis

Data are presented as mean $\pm$ SD. The values for serum total IgE levels, serum ECP levels, and blood eosinophil counts were log transformed before statistical analysis. Methacholine and AMP PC₂₀ values and PD₂₀ values were also log transformed before statistical analysis. Correlations between BDR after each challenge and serum IgE levels, eosinophil counts, or ECP levels were calculated using a linear regression model. Coefficient of determination (R^2) and regression coefficient (β) were obtained from the analysis. Coefficients of determination (R^2) were compared using a Fisher Z transformation and a two-tailed Z test. All statistical analyses were performed using the SPSS statistical package (SPSS Inc, Chicago, IL, USA). A p value of .05 or less was considered to be significant.

RESULTS

The clinical characteristics of the 69 children with mild intermittent to moderate persistent asthma are shown in Table 1. Fifty-two patients (75.4%) had atopy and 53 children (76.8%) had blood eosinophilia (peripheral blood eosinophil counts $\geq 4\%$). Thirteen children used on-demand inhaled SABAs, 19 children used leukotriene receptor antagonists, 16 children inhaled corticosteroids, and 21 children inhaled corticosteroids/long-acting β_2 -agonist in combination treatment for their asthma control.

Pulmonary function test parameters of methacholine and AMP challenges are presented in Table 2. There was no significant difference in the mean ($\pm$ SD) baseline FEV₁ before methacholine and AMP challenges ($89.7 \pm 10.3\%$ predicted vs. $87.8 \pm 10.6\%$ predicted). The mean ($\pm$ SD) FEV₁ ($68.4 \pm 13.3\%$ predicted) immediately after methacholine challenge was not significantly different from the mean FEV₁ ($65.9 \pm 11.7\%$ predicted) immediately after AMP challenge. The mean ($\pm$ SD) FEV₁ after inhalation of salbutamol after methacholine and AMP challenges ($87.6 \pm 11.1\%$ predicted vs. $87.3 \pm 11.7\%$ predicted) were not significantly different, either.

Table 3 shows mean BDR, PC₂₀, and PD₂₀ levels. The mean ($\pm$ SD) Δ FEV₁ was $30.3 \pm 9.4\%$ predicted after methacholine challenge and $32.4 \pm 9.3\%$ predicted after AMP challenge. The geometric mean (range of 1 SD) of methacholine PC₂₀ was 1.53 (0.41–5.71) mg/mL, and that of AMP PC₂₀ was 30.7 (4.95–190.1) mg/mL. The geometric means (range of 1 SD) of methacholine PD₂₀ and AMP PD₂₀ at the end of study were 13.4 (2.70–66.1) BU, and 295.1 (49.5–1757.9) BU, respectively. The geometric mean (range of 1 SD) of inhaled methacholine was 14.2

TABLE 3.—The FEV₁ fall and BDR as well as AHR to methacholine and AMP in asthmatic children.

Parameters	Methacholine	AMP
Δ FEV ₁ , after challenge (%) ^a	30.3 $\pm$ 9.4	32.4 $\pm$ 9.3
BDR (%predicted) ^a	30.7 $\pm$ 18.0	35.2 $\pm$ 23.6
Recovered $\geq 90\%$ FEV ₁ , n (%)	57 (82.6)	59 (85.5)
PC ₂₀ (mg/mL) ^b	1.53 (0.41–5.71)	30.7 (4.95–190.1)
PD ₂₀ (BU) ^b	13.4 (2.70–66.1)	295.1 (49.5–1757.9)

Notes: FEV₁, forced expiratory volume in 1 second; BDR, bronchodilator responsiveness; AHR, airway hyperresponsiveness; AMP, adenosine 5'-monophosphate; PC₂₀, a provocative concentration causing a 20% decline in FEV₁; PD₂₀, a provocative dose causing a 20% decline in FEV₁.

^amean $\pm$ SD; ^bgeometric mean (range of 1 SD).

(4.71–42.8) mg and that of inhaled AMP was 487.5 (108.9–2182.7) mg.

The relationship between actual levels of methacholine PC₂₀ and AMP PC₂₀ and between actual levels of methacholine PD₂₀ and AMP PD₂₀ was depicted in Figure 1. The serum total IgE levels significantly correlated with BDR after AMP challenge (right panel; $R^2 = 0.345$, $p = .001$) but not with the BDR after methacholine challenge (left panel; $R^2 = 0.007$, $p = .495$) (Figure 2). The relationships between peripheral blood eosinophil counts and BDR after methacholine challenge or BDR after AMP challenge are shown in Figure 3. The BDR after AMP challenge (right panel) significantly correlated with peripheral blood eosinophil counts ($R^2 = 0.212$, $p = .001$), but the BDR after methacholine challenge (left panel) did not ($R^2 = 0.002$, $p = .724$).

The serum ECP levels significantly correlated with either BDR after AMP challenge (right panel; $R^2 = 0.192$, $p = .001$) or BDR after methacholine challenge (left panel; $R^2 = 0.063$, $p = .038$) (Figure 4). However, the relationship between BDR after AMP challenge and serum ECP levels showed marginally significant strong correlation compared with BDR after methacholine challenge and serum ECP levels ($p = .060$ for the comparison of coefficients of determination (R^2) of 0.192 and 0.063).

DISCUSSION

In this study, we have investigated the relationships between blood eosinophil markers and the BDR assessed after both methacholine-induced and AMP-induced bronchoconstrictions. We found that serum total IgE levels, blood eosinophil counts, and serum ECP levels

TABLE 1.—Clinical characteristics of asthmatic children.

Parameters	Results (n = 69)
Age (year) ^a	9.5 $\pm$ 2.8
Sex (M/F)	41/28
Atopy, n (%) ^b	52 (75.4)
Total IgE (IU/mL) ^c	295.1 (91.2–955.0)
Asthma medications ^d	13/19/16/21
Blood eosinophil count $\geq 4\%$, n (%)	53 (76.8)
Eosinophil cationic protein (μ g/mL) ^c	21.9 (7.94–60.3)

Note: BMI, body mass index.

^amean $\pm$ SD; ^bdefined as a positive skin prick test; ^cgeometric mean (range of 1 SD); ^dAs required inhaled short-acting β_2 -agonists/leukotriene receptor antagonists/inhaled corticosteroids/inhaled corticosteroids + long-acting β_2 -agonist.

TABLE 2.—Pulmonary function parameters in asthmatic children.

Challenge test	Parameters	Pre-challenge	Post-challenge	Post-bronchodilator
Methacholine	FEV ₁ (%predicted)	89.7 $\pm$ 10.3	68.4 $\pm$ 13.3	87.6 $\pm$ 11.1
	FVC (%predicted)	93.3 $\pm$ 10.2	75.9 $\pm$ 12.7	91.5 $\pm$ 11.2
	FEV ₁ /FVC (%)	82.2 $\pm$ 6.9	70.5 $\pm$ 7.9	81.8 $\pm$ 6.8
AMP	FEV ₁ (%predicted)	87.8 $\pm$ 10.6	65.9 $\pm$ 11.7	87.3 $\pm$ 11.7
	FVC (%predicted)	90.3 $\pm$ 10.6	72.7 $\pm$ 12.8	89.2 $\pm$ 12.5
	FEV ₁ /FVC (%)	81.0 $\pm$ 7.6	68.1 $\pm$ 7.9	81.6 $\pm$ 7.3

Notes: AMP, adenosine 5'-monophosphate; FEV₁, forced expiratory volume in 1 second; FVC, forced vital capacity.

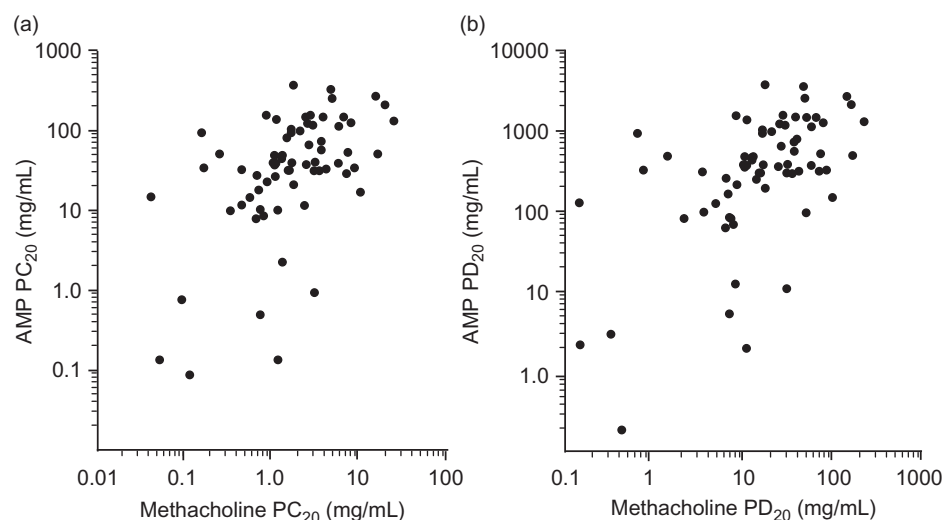


FIGURE 1.—Actual PC₂₀ to the methacholine and AMP (a) and actual PD₂₀ to the methacholine and AMP (b).

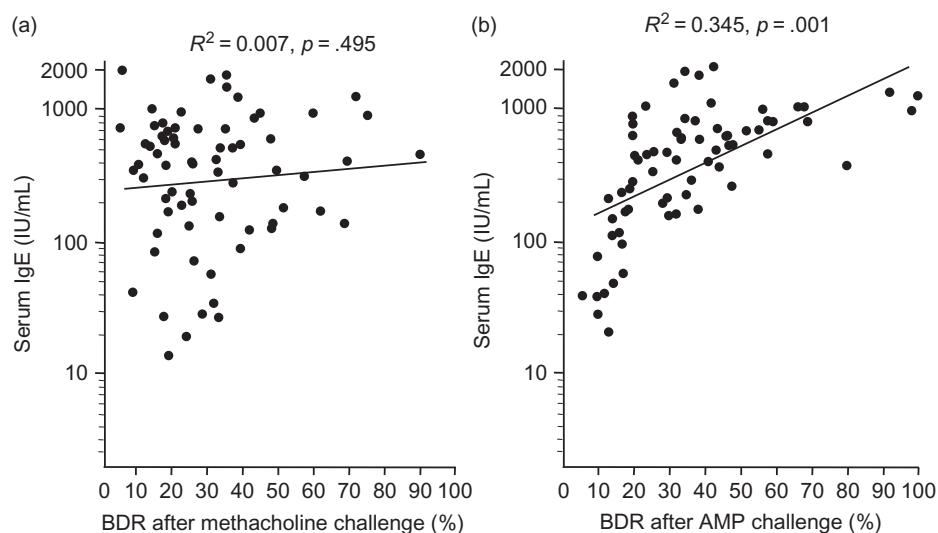


FIGURE 2.—Relationships between bronchodilator responsiveness after methacholine-induced bronchoconstriction (a) and after AMP-induced bronchoconstriction (b) against serum IgE concentrations in asthmatic children.

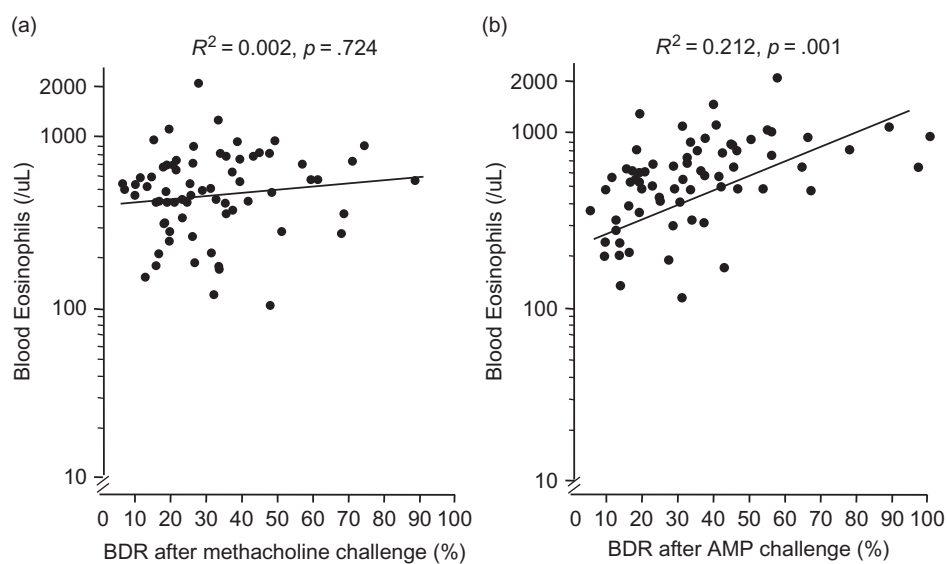


FIGURE 3.—Relationships between bronchodilator responsiveness after methacholine-induced bronchoconstriction (a) and after AMP-induced bronchoconstriction (b) against blood eosinophil counts in asthmatic children.

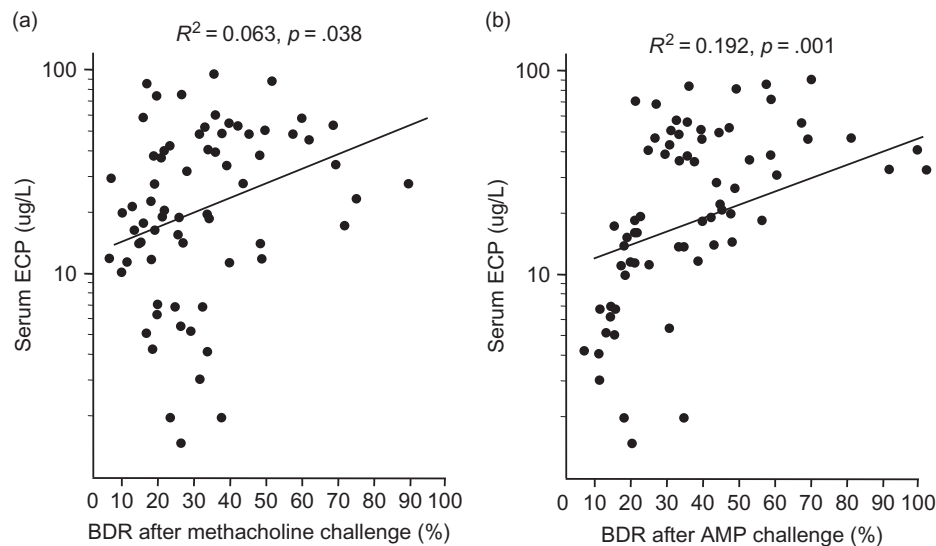


FIGURE 4.—Relationships between bronchodilator responsiveness after methacholine-induced bronchoconstriction (a) and after AMP-induced bronchoconstriction (b) against serum eosinophil cationic protein (ECP) concentrations in asthmatic children.

were more strongly related to the BDR after AMP challenge than to the BDR after methacholine challenge. To the best of our knowledge, this is the first study that has compared the BDR after both the challenges regarding their relationships to blood eosinophil markers in children with asthma.

Usually, BDR is assessed as the change in airflow before and after the administration of a β_2 -agonist. However, the baseline airway tone may influence the subsequent BDR because it is a dynamic measure of bronchodilation from baseline (16). In this study, we excluded subjects who showed the difference of baseline FEV₁s greater than 3% between each challenge for participating further study. A previous study with pediatric asthmatics (17) has demonstrated that although they did not find any significant baseline spirometric differences on the basis of asthma severity, after multivariate analysis, asthma severity was the only significant factor for the BDR. In this study, we did not intend to compare BDR according to the underlying severity of the disease directly, but physicians should be aware that severe asthma itself may be associated with an increased BDR.

It is expected that airflow may not increase if it is already fully dilated at baseline by asthmatic treatment. This study addressed this issue by measuring BDR after induction of bronchoconstriction by provocation tests. This challenge-rescue method of measuring bronchodilation was suggested as a method for overcoming the variability of FEV₁ prior to the measurement of bronchodilation (18). In addition, chemical challenges, such as methacholine or AMP tests, have the advantages of being easily controlled, compared with bronchoconstriction in acute asthma attack, using standard doses and producing immediate bronchoconstriction (19). Several studies (20–22) have also demonstrated that induced bronchoconstriction and its reversal by inhaled bronchodilators is a useful and standardized model employed to estimate the

effectiveness of BDR. However, despite the usefulness of this approach, BDR is also confounded by spontaneous recovery from the induced bronchoconstriction. Although spontaneous return for 15 minutes after bronchoconstriction is rare (23), investigators should be aware of this possibility.

In this study, we defined BDR as the change in % predicted FEV₁ over the pre-bronchodilator value. Among the several indices of bronchodilator responses, many investigators have suggested that Δ FEV₁ %predicted is the best index of bronchodilator response, because it takes into account the confounding effects of height, gender, and pre-bronchodilator FEV₁ values (18, 24). The average BDR was 30.7% after methacholine challenge and 35.2% after AMP challenge in this study, which were comparable to the moderate asthma attack.

We performed methacholine and AMP bronchial challenges, which induced the direct effects on airway smooth muscles or the indirect effects of inflammatory mediators on smooth muscles, respectively. In this study, the correlations between blood eosinophil counts and the BDR after AMP challenge were stronger than those between eosinophil counts and the BDR after methacholine challenge. The relationships between serum ECP levels and the BDR after AMP challenge tend to correlate stronger than between serum ECP levels and the BDR after methacholine challenge ($p = .060$) for the comparison of coefficients of determination (R^2). These results suggest that blood eosinophil markers are better reflected by BDR assessed after AMP-induced bronchoconstriction than after methacholine-induced bronchoconstriction.

Regarding AHR, previous studies have supported the fact that AHR to AMP more closely reflects inflammatory events than AHR to methacholine (9, 10). Indeed, AMP is thought to act through mediators released from activated mast cells, thus AHR to AMP reflects underlying airway inflammation more precisely

(25). Our finding that reversibility to a SABA after AMP-induced bronchoconstriction was more closely related to blood eosinophil markers than reversibility after methacholine-induced bronchoconstriction is novel result. Bronchodilator response after AMP challenge are significantly related to eosinophil markers, so subjects with eosinophilia may show a high level of reversibility to salbutamol when acute asthma attack develops.

Silvestri et al. (26) demonstrated highly significant positive correlations between blood eosinophilia and responsiveness to salbutamol in atopic asthmatic children but not in non-atopic asthmatic children. Although statistically insignificant, another previous study reported that changes in FEV₁ after inhalation of β_2 -adrenergic agent were higher in allergic rhinitis patients with higher serum total IgE levels than those with lower total IgE levels. In this study, serum total IgE levels were also significantly related to BDR after AMP challenge but not to BDR after methacholine challenge. Based on these results, it is conceivable that serum total IgE may be a determinant of airway tone. In this study, the contribution of atopy or eosinophilic inflammation to bronchial reversibility after indirect challenge was unclear. This can be partially explained by the previous studies (7, 8) that reported that BDR may be a marker for inflammation. This is consistent with the result that inhaled steroid-induced reduction of airway eosinophils is associated with an attenuation of BDR in asthmatics (27) and treatment with omalizumab reduces the response to AMP without decreasing the response to methacholine (28). Therefore, changes in airway inflammation can cause changes in airway physiology.

On the other hand, several reports failed to show any relationship between eosinophil counts and reversibility in FEV₁ after bronchoconstriction. Zietkowski et al. (29) have shown no significant relationship between blood eosinophil counts and changes in FEV₁ after inhalation of a bronchodilator in adult asthmatic patients. Zielen et al. (30) also demonstrated no significant differences in eosinophil counts between responders (improvement of FEV₁ > 15% after salbutamol) and non-responders. The reasons why they could not demonstrate a positive relationship between reversibility and eosinophil counts may be that there is little room for improvement of FEV₁ from the baseline and subsequent small bronchodilator responses.

To the best of our knowledge, this is the first report comparing the relationships of the BDR after direct and indirect challenges with eosinophil markers in asthmatic children, although one previous study reported similar relationships in adult asthmatics (31). However, they did not compare BDR after direct challenge and BDR after indirect challenge regarding their relationships with eosinophil markers. In this study, it was found that BDR after AMP challenge is more closely related to eosinophilic inflammation than BDR after methacholine challenge, which is consistent with the assumption that greater reversibility in FEV₁ indicates a greater degree of airway inflammation (26).

In this study, although participating patients have mild intermittent to moderate persistent asthma, their FEV₁ was decreased by 30.3% immediately after methacholine challenge and by 32.4% immediately after AMP challenge, corresponding to a moderate to severe asthma attack. Much greater reductions in lung function can occur with acute severe asthma attacks and the loss of the responsiveness to bronchodilators appears to be consequently greater.

The most important finding in this study is the strong association between eosinophilic inflammation and the degree of BDR after AMP challenge. Although we did not measure airway inflammation directly, it is speculated that the degree of airway reversibility is a good indicator of changes in eosinophilic inflammation. Based on these results, it is evident that eosinophils may be considerably involved in bronchial smooth muscle contractility and reversibility during indirect challenge tests in asthmatic children.

Both eosinophilic inflammation and increases in FEV₁ in response to bronchodilators are important characteristic features of asthma. Therefore, the correlation between these two factors is thought to be highly significant. Several studies have used the response to bronchodilators as an indicator of inflammation (7, 8, 26). The results of this study suggest that BDR is more closely related to eosinophilic inflammation after indirect challenge than after direct challenge and can be used an effective clinical tool to predict airway inflammation at acute asthmatic attacks.

CONCLUSIONS

BDR following AMP challenge may be more closely related to eosinophilic inflammation, compared with that following methacholine challenge. It is stressed that clinicians should be aware of this fact and be careful to interpret bronchodilator responses.

ACKNOWLEDGMENT

This study was supported in part by Korea University Grant (K0933151) and Grant for the Environmental Health Center for Childhood Asthma (2010–2011), Ministry of Environment, Republic of Korea.

DECLARATION OF INTEREST

The authors declare no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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