

Comparison of the effects of repetitive low-dose and single-dose antigen challenge on airway inflammation

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Background: Airway allergen provocation provides a model to study allergic inflammation in relationship to pulmonary physiology. Allergen provocation is usually administered as a relatively large single-dose challenge that might not reflect a chronic, natural, low-dose airborne allergen exposure.

Objective: We sought to compare the magnitude, characteristic features, and kinetics of airway inflammation induced by means of repetitive low-dose antigen challenges with those factors induced by means of an equivalent single-dose allergen challenge in allergic asthma.

Methods: This was a 2-period crossover study. During separate phases, each subject was administered either a predetermined single-dose antigen challenge or 25% of that dose on each of 4 consecutive days. The airway response to allergen challenge was determined by means of measurement of pulmonary function and sputum features of inflammation, including eosinophil, eosinophil-derived neurotoxin, and fibronectin levels.

Results: Both models of antigen challenge caused significant and equivalent sputum eosinophilia. The immediate decrease in FEV₁ and the FEV₁/forced vital capacity ratio and the increase in sputum eosinophilia, eosinophil-derived neurotoxin, and fibronectin levels occurred gradually over the first 3 low doses and then reached a plateau or tended to decrease with the fourth antigen exposure.

Conclusion: Our data suggest that although the 2 challenge models had similar quantitative effects on lung function and sputum eosinophilia, the qualitative responses and kinetics of these changes were distinct. Repetitive low doses of antigen, as might mimic natural allergy exposure, produced an equivalent inflammatory response to the large single-dose challenge but with a smaller amount of antigen, suggesting that priming and accumulative effects might have occurred. Moreover, our limited data also suggest that immunologic tolerance might be induced by frequent challenges. (*J Allergy Clin Immunol* 2003;111:818-25.)

Key words: Allergen, low-dose provocation, sputum induction, eosinophils

Inhalation of airborne allergen causes an airway eosinophilic inflammatory response in asthma. Consequently, inhaled allergen provocations are used as a model to determine the features of allergen-induced airway inflammation and to identify the mechanisms underlying its development and persistence.^{1,2} Induced sputum provides a noninvasive method to assess lower airway fluid features of inflammation.³⁻⁵ The amount of allergen administered is, in most cases, considerably higher than that encountered under natural conditions to study associated features of inflammatory responses to allergen and possible mechanisms of airflow obstruction in asthma. To compensate for these limitations, investigators have begun to use repeated low-dose antigen provocation,^{6,7} which attempts to simulate events that might occur naturally when patients inhale small amounts of airborne allergens on a daily basis. By using this technique, repetitive low-dose allergen inhalation has been shown to induce a late-phase allergic reaction,⁸ increases in airway hyperresponsiveness (AHR), or both.⁹⁻¹⁵ For example, Sulakvelidze et al⁹ reported that repeated low-dose allergen challenge can cause airway inflammation and AHR without large immediate bronchoconstrictive responses. Direct comparisons of repetitive low-dose allergen with the equivalent single-dose allergen challenge, however, have not been evaluated.

We hypothesized that a chronic low-dose allergen exposure might have a cumulative effect on both lung function and eosinophil recruitment into the airway. The following study was designed to investigate the characteristics, magnitude, and kinetics of airway inflammation induced by means of repetitive low-dose antigen challenges compared with a single-dose antigen provocation. Accordingly, allergic subjects were challenged on 2 separate occasions with either repetitive low doses or an equivalent single dose of allergen, and changes in sputum eosinophilia and pulmonary function were compared.

METHODS

Subjects

Eight nonsmoking allergic subjects were recruited for participation in this study (Table I). Each subject had a positive skin prick test response to one or more aeroallergens with a correlative history, did not require inhaled corticosteroids for asthma treatment, and had no evidence of a respiratory infection or an asthma exacerbation

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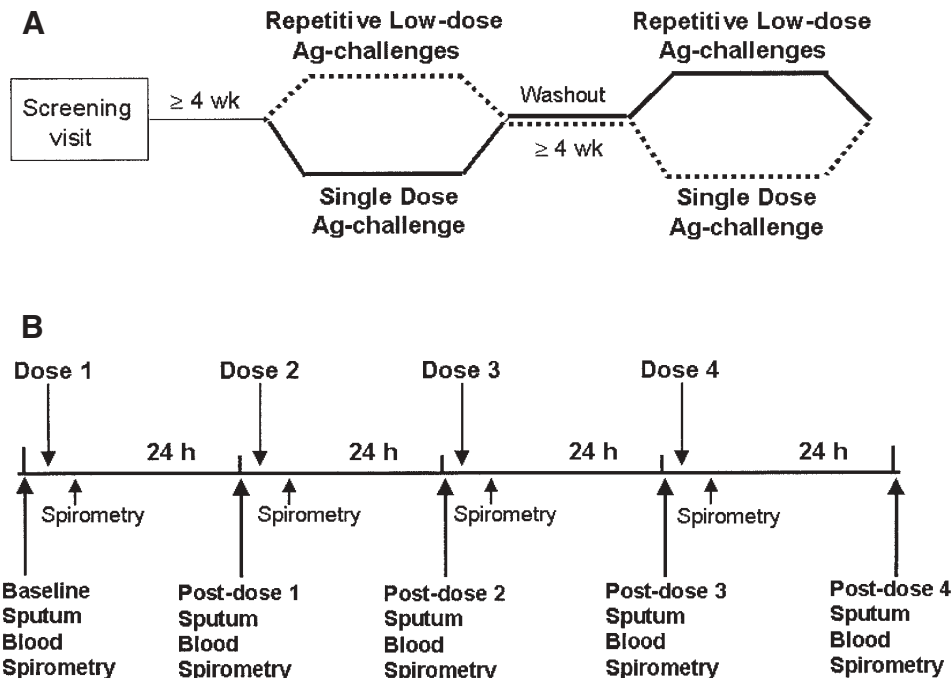


FIG 1. Study design. **A**, Crossover design. After the screening visit, each subject was evaluated on 2 separate occasions: repetitive low-dose antigen challenge and single-dose antigen challenge. The washout time was at least 4 weeks between these 2 arms. **B**, Repetitive low-dose arm assessment design. Each of the low-dose antigen challenges was equal to 25% of the single-dose antigen PD₂₀. Pulmonary spirometry was performed before and after each challenge. Sputum and blood samples were collected at baseline and 24 hours after each low-dose antigen challenge.

Abbreviations used

AHR: Airway hyperresponsiveness
EDN: Eosinophil-derived neurotoxin
FVC: Forced vital capacity

within 4 weeks. The study was approved by the University of Wisconsin-Madison Health Sciences Committee, and informed consent was obtained from each subject.

Study design and allergen challenge

This was a 2-period antigen challenge crossover study (Fig 1). Each subject underwent a screening evaluation to establish baseline spirometry, methacholine responsiveness, and response to skin prick antigen testing. Subjects were screened with a graded nebulization of antigen delivered through a nebulizer attached to a French-Rosenthal dosimeter to determine the dose of antigen to be administered during the study phases. Baseline spirometry was measured and then redetermined 10 minutes after 5 breaths of the diluent solution (normal saline, Bayer Allergy Products). If there was no significant response to diluent challenge (FEV₁ remained within 10% of baseline), 5 breaths of antigen were inhaled; spirometry was repeated 10 minutes later. Consecutively higher concentrations of antigen were administered until the FEV₁ decreased by 20% or greater from baseline values. Antigen PD₂₀ was calculated by means of linear interpolation of the last 2 doses on the dose-response curve by using a computer program (PD20, Madison Scientific Software) and expressed in cumulative breath units. The calculated antigen PD₂₀ was then either administered as a single-dose challenge or divided into 4 equivalent amounts (each dose was

equal to 25% of the single-dose antigen PD₂₀) for the low-dose provocation that was administered over 4 consecutive days. The antigen challenges were done between 8 and 10 AM to minimize the effect of circadian rhythms on response to antigen. The crossover periods between single-dose and low-dose challenges were separated by a 4-week minimal washout period (Fig 1). Some individuals had their single-dose challenge completed first, whereas others had their repetitive low-dose challenges first to control for order effects. Spirometry was performed before each challenge and then every 10 minutes for 30 minutes to determine the immediate FEV₁ decrease. Sputum was induced at baseline and 24 hours after each challenge. Peripheral blood samples were collected at each sputum induction for white blood cell counts and differentials.

Sputum induction and processing

Sputum inductions were performed as previously described.¹⁶ Sputum samples were collected and treated with an equal volume of 0.1% dithiothreitol (Sputolysin, Calbiochem Corp). Sputum cells were collected for cell counts and viability. Cytospin preparations were made for differential cell counts. Sputum supernatants were stored at -80°C until analysis.

Fibronectin and eosinophil-derived neurotoxin detection

Total soluble fibronectin in sputum supernatants was determined by means of a sandwich ELISA.¹⁷ Briefly, ELISA plates were coated with a predetermined optimal concentration of purified monoclonal anti-human fibronectin antibody (clone 1601, Biodesign International). Nonspecific binding sites were blocked with 1% BSA/0.05% Tween/PBS, and test samples were added and incubated overnight at 4°C. Fibronectin was detected with a biotinylated, horseradish peroxidase-conjugated rabbit anti-human fibronectin

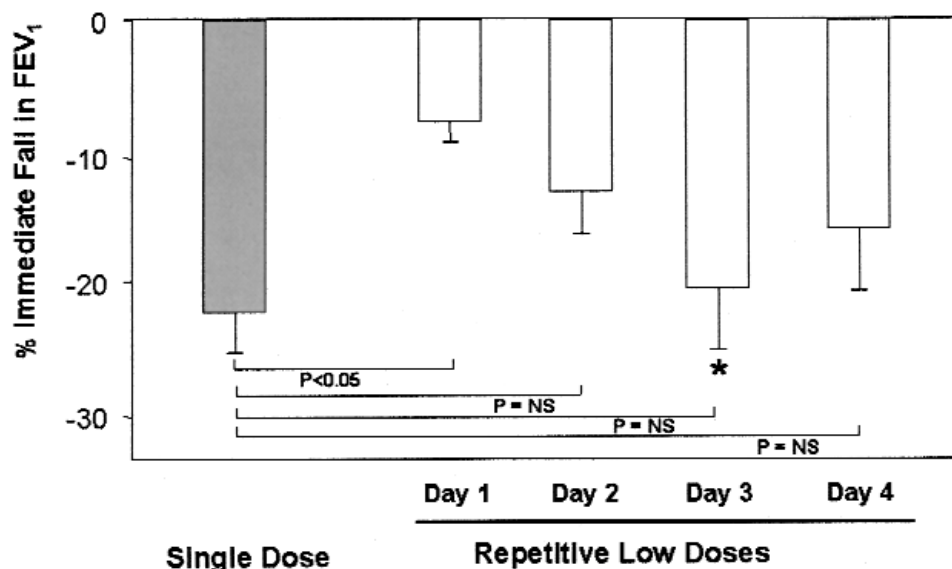


FIG 2. The percentage of immediate decrease in FEV₁ after antigen challenge. The percentage of immediate decrease in FEV₁ was measured after both single-dose (shaded bar) and repetitive low-dose (open bars) antigen challenges. Bars represent means $\pm$ SEM. **P* < .05 versus day 1.

TABLE I. Subject characteristics

Subject no.	Age (y)	Sex	Antigen	Antigen PD ₂₀ (CBU)	Meth PC ₂₀ (mg/mL)	FEV ₁ (% predicted)
1	27	F	Ragweed	46.8	20.0	98
2	22	M	HDM	2.3	0.3	81
3	27	M	Ragweed	37.5	2.3	94
4	31	M	Cat dander	100.0	2.8	84
5	25	F	Cat dander	5.5	1.7	91
6	29	F	HDM	10.7	2.9	83
7	22	M	HDM	2.6	0.5	78
8	19	M	Ragweed	66.0	18.8	116

CBU, Cumulative breath units; Antigen PD₂₀, dose of antigen that caused a 20% decrease in FEV₁; Meth PC₂₀, concentration of methacholine that caused a 20% decrease in FEV₁; HDM, house dust mite.

antibody (DAKO Corp) with a sensitivity of less than 12 ng/mL. Eosinophil-derived neurotoxin (EDN) in sputum supernatants was determined by means of competitive radioimmunoassay (sensitivity of 2 ng/mL).¹⁸

Statistical analysis

Data are presented as medians with interquartiles of 25% and 75% or means $\pm$ SEM (for normally distributed data). The Wilcoxon signed-rank test (or a paired *t* test for normally distributed data) was used to compare the responses to single-dose or each repetitive low-dose antigen challenge with baseline values. Correlations were determined by using the Spearman rank order correlation test. A *P* value of less than .05 was considered significant. Statistical analyses were performed with the SigmaStat software package (Jandel Scientific Software).

RESULTS

Subject characteristics

Data on subject characteristics are shown in Table I. Eight subjects were enrolled in this study. Five of them (subjects 1-5) completed both the repetitive low-dose and single-dose challenge arms of the study. Of those 5 sub-

jects, 3 had their single-dose antigen challenge first, and 2 had the repetitive low-dose challenge first. Subjects 6 through 8 could not complete the 2 study arms and were not included in subsequent analysis. Two of these subjects moved out of town, and one individual was unable to complete the low-dose challenge phase of the study.

Pulmonary function

Of the 5 subjects who completed both arms of the protocol, the decrease in lung function in response to inhaled antigen challenge was similar during the single-dose and repetitive low-dose antigen challenges (Fig 2). There was an immediate decrease in FEV₁ (22.0% $\pm$ 2.8% [mean $\pm$ SEM]) during single-dose antigen challenge. When subjects received 25% of the single-dose antigen PD₂₀ on day 1, there was an immediate decrease in FEV₁, but the decrease was not as great as that seen with the single-dose challenge. By day 3 of the repetitive low-dose challenges, however, the immediate decrease in FEV₁ was equivalent (25.0% $\pm$ 5.8%) to that seen with the single-dose antigen challenge, even though only 75% (25% $\times$ 3

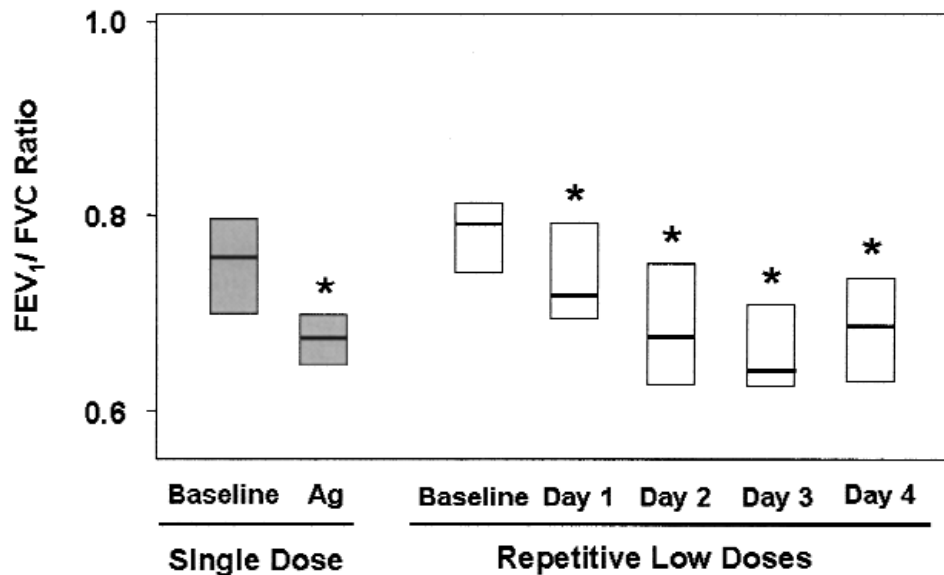


FIG 3. The decrease in FEV₁/FVC ratios after antigen challenge. The FEV₁/FVC ratio was calculated at baseline and 24 hours after both single-dose (shaded bars) and repetitive low-dose (open bars) antigen challenges. Boxes represent medians with 25% and 75% quartiles. **P* < .01 versus patients' own baseline values.

times) of the antigen PD₂₀ had been administered. Interestingly, the decrease in FEV₁ reached a plateau by the fourth challenge. Twenty-four hours after antigen challenge, the percent predicted FEV₁ values were slightly reduced after both single-dose antigen challenge (90.0% ± 5.3% before challenge vs 86.0% ± 4.7% 24 hours after challenge) and the third repetitive low-dose antigen challenge (93.0% ± 3.5% before challenge vs 89.0% ± 5.1% 24 hours after challenge).

The FEV₁/forced vital capacity (FVC) ratio is a sensitive index to express and evaluate the changes in expiratory airway resistance.¹⁹ This index was used to further measure airflow obstruction after antigen challenge. There was a striking decrease in the FEV₁/FVC ratio after both the single-dose and the repetitive low-dose antigen challenges (Fig 3). After the second low-dose challenge (ie, 50% of the antigen PD₂₀), the decrease in the FEV₁/FVC ratio was significantly reduced from baseline and equivalent to the decrease seen after the single-dose challenge (100% of the antigen PD₂₀). Similar to the immediate decrease in FEV₁, the decrease in the FEV₁/FVC ratio was also attenuated by the fourth low-dose challenge.

Sputum and blood cellular characteristics after single-dose or repetitive low-dose antigen challenges

The percentage of squamous and epithelial cell contamination in the whole sputum was 21.0% ± 2.1% (mean ± SEM) for all 35 samples, and the sputum leukocyte viability averaged 82.0% ± 1.2%. The percentage of sputum eosinophils significantly increased 24 hours after

either the single-dose or repetitive low-dose antigen challenges (Fig 4, A). When subjects received 25% of the antigen PD₂₀ on day 1, there was a small but significant increase in sputum eosinophils (4.0% [2.5%-7.3%] vs 0.1% [0.1%-1.5%] at baseline, *P* < .05); this, however, was not as great as that achieved with the single-dose challenge (11.0% [10.0%-13.0%]). By the second day, when subjects had received a total of 50% of the predetermined antigen PD₂₀, the sputum eosinophil response to antigen (17.0% [4.0%-19.3%]) was equivalent to that observed with the single-dose PD₂₀ and reached a plateau with each subsequent antigen exposure (20.0% [10.3%-23.3%] on day 3 and 16.0% [5.5%-22.0%] on day 4). Moreover, when data from each repetitive low-dose antigen challenge were analyzed, the increases in sputum eosinophil counts had a significant and inverse correlation with the reduced FEV₁/FVC ratios (Fig 4, B; *r*_s = -0.517; *P* = .019).

The percentage of sputum lymphocytes also significantly increased after both the single-dose (7.1% ± 1.1% before challenge vs 13.8% ± 2.4% 24 hours after challenge, *P* < .05) and the third low-dose challenges (5.6% ± 1.1% before challenge vs 8.6% ± 2.0% 24 hours after challenge, *P* < .05). The increase in eosinophils and lymphocytes resulted in a corresponding reduction in the percentage of sputum macrophages at 24 hours after the single-dose and the third repetitive low-dose antigen challenges. Neither the percentage nor the absolute numbers of sputum neutrophils changed after antigen challenges. There was no change in peripheral white blood cell counts or differentials after single-dose antigen challenge. A small but significant increase in peripheral white blood cell counts occurred after the second day of repet-

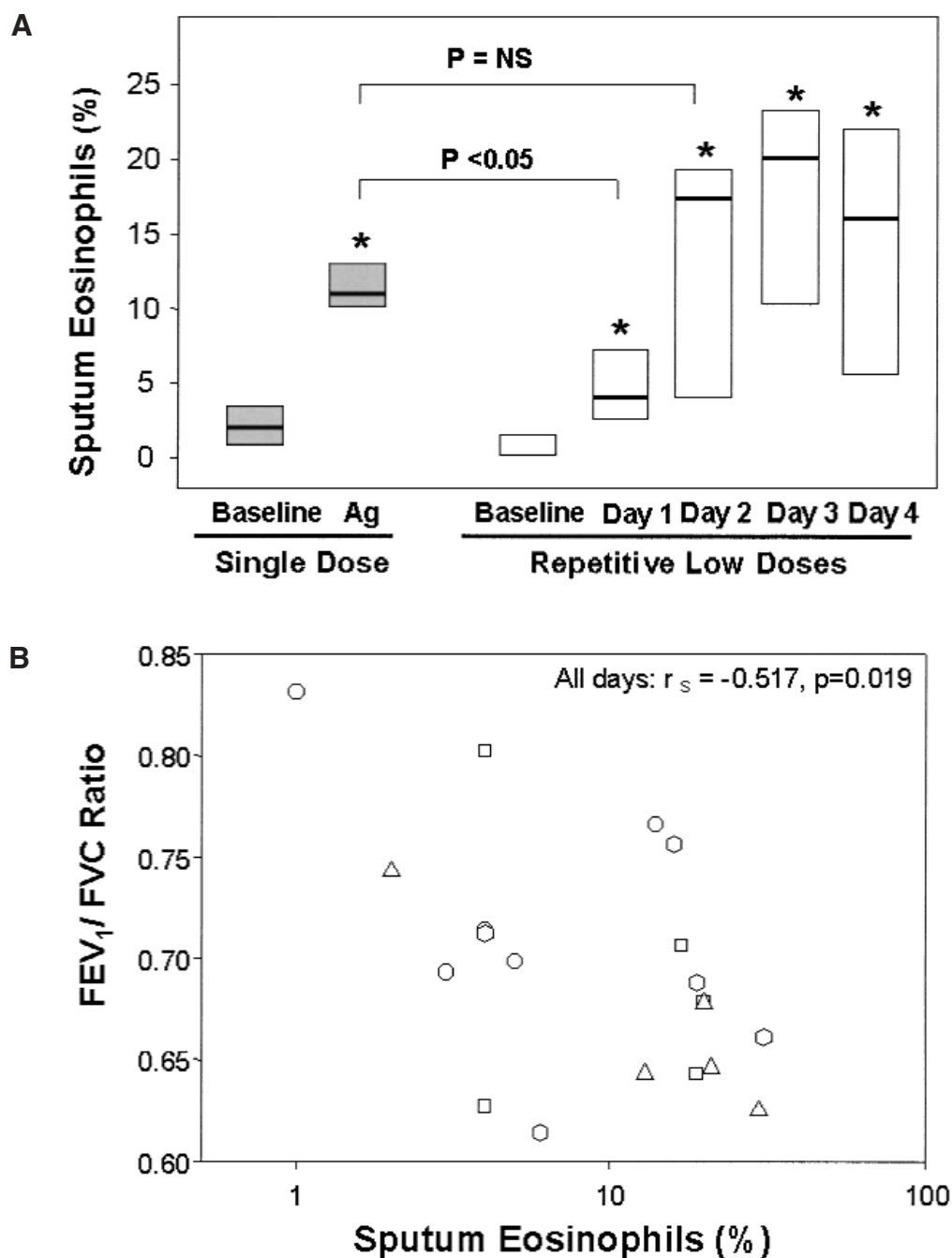


FIG 4. The increase in sputum eosinophils and their relationship with FEV₁/FVC ratios after antigen challenge. **A**, The percentages of sputum eosinophils were determined at baseline and 24 hours after both single-dose (shaded bars) and repetitive low-dose (open bars) antigen challenges. Boxes represent medians with 25% and 75% quartiles. $*P < .01$ versus patients' own baseline values. **B**, The relationship of sputum eosinophils and FEV₁/FVC ratios after low-dose antigen challenges. Data represent values obtained from each repetitive low-dose antigen challenge in 5 subjects. Values for all data points were an $r_{s\text{ all}}$ value of -0.517 and a P value of $.019$. Circles represent the first-day low-dose challenge ($r_s = -0.100$), squares represent the second-day low-dose challenge ($r_s = -0.103$), triangles represent the third-day low-dose challenge ($r_s = -0.700$), and hexagons represent the fourth-day low-dose challenge ($r_s = -0.200$).

itive low-dose antigen challenge ($5508 \pm 246/\text{mm}^3$ at baseline vs $6176 \pm 340/\text{mm}^3$ after challenge, $P < .05$), and a significant increase in blood eosinophils was also

noted after the fourth repetitive low-dose antigen challenge ($1.4\% \pm 0.5\%$ at baseline vs $3.6\% \pm 0.7\%$ after challenge, $P < .05$).

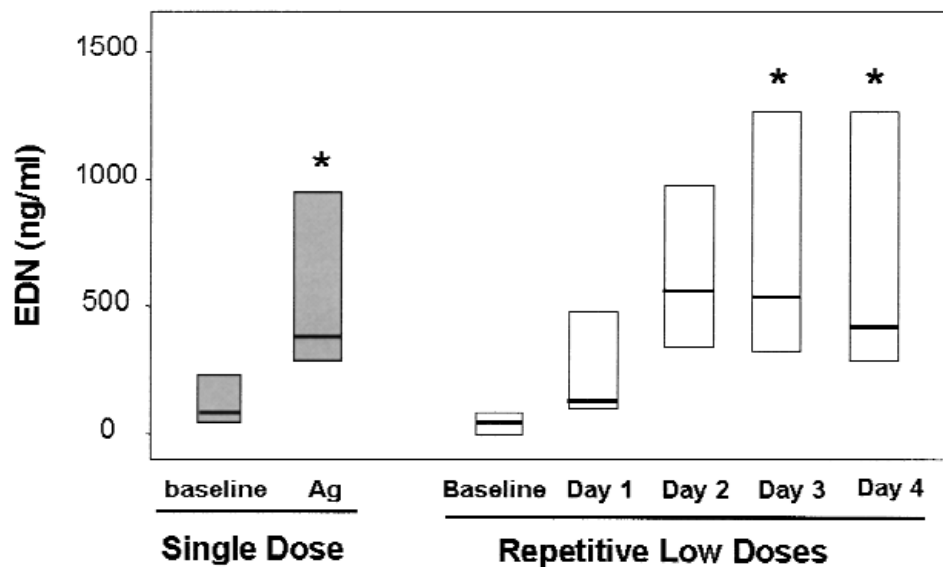


FIG 5. Concentrations of sputum EDN after antigen challenge. Concentrations of EDN in sputum supernatant fluids were measured at baseline and 24 hours after both single-dose (shaded bars) and repetitive low-dose (open bars) antigen challenges. Boxes represent medians with 25% and 75% quartiles. * $P < .05$ versus patients' own baseline values.

Sputum EDN concentrations after single-dose or repetitive low-dose antigen challenges

EDN concentrations in sputum supernatants followed the changes in sputum eosinophils (Fig 5). The increase in sputum EDN concentrations was statistically significant after the single-dose (362 ng/mL [295-962 ng/mL] vs 116 ng/mL [24-24 ng/mL] at baseline, $P < .01$) and third (410 ng/mL [252-1105 ng/mL] vs 78 ng/mL [36-97 ng/mL] at baseline, $P < .01$) and fourth (313 ng/mL [251-1115 ng/mL], $P < .01$) low-dose antigen challenges. Moreover, in the repetitive low-dose challenge arm, when data from each challenge day were analyzed together, the increased sputum EDN level had a significant positive correlation with the increased sputum eosinophil count ($r_s = 0.672$, $P < .001$) and had a significant inverse correlation with the FEV₁/FVC ratio ($r_s = -0.684$, $P < .001$).

Sputum fibronectin concentrations after single-dose or repetitive low-dose antigen challenges

Sputum fibronectin concentrations increased significantly after the single-dose (16.9 μ g/mL [6.7-19.8 μ g/mL] vs 11.0 μ g/mL [4.4-11.8 μ g/mL] at baseline, $P < .05$) and third low-dose (20.0 μ g/mL [15.4-23.0 μ g/mL] vs 6.3 μ g/mL [4.6-10.4 μ g/mL] at baseline, $P < .05$) antigen challenges. Interestingly, when data from each low-dose challenge day were analyzed together, the concentrations of fibronectin in sputum supernatants had a significant positive correlation with the increased sputum eosinophil counts ($r_s = 0.717$, $P < .001$).

DISCUSSION

We found a significant and equivalent eosinophilic airway inflammatory response to either a single-dose or a repetitive low-dose antigen challenge. With repetitive low-dose challenges, sputum eosinophilia and EDN release gradually increased, and the immediate decrease in FEV₁ and FEV₁/FVC ratios decreased, both in a day-by-day fashion. Changes in airway function and inflammation peaked by the third day of low-dose challenge and then reached a plateau or had a tendency to decrease with the fourth low-dose exposure. Although the 2 challenge models had similar quantitative effects on the eosinophilic airway inflammatory response, the qualitative aspects of this reaction to inhaled antigen were distinct. The repetitive low-dose antigen challenge model might simulate events that occur with natural and chronic environmental allergen exposure and might explain gradual worsening of airflow obstruction and airway inflammation when allergen is inhaled in low doses but on a more continuous basis.

Two consecutive days of low-dose antigen challenges induced an equivalent degree of sputum eosinophilia as that produced by a single large-dose challenge, even though only 50% of the antigen dose had been given. Moreover, after antigen challenge, sputum eosinophils were probably activated as indicated by increasing concentrations of the granule protein EDN, which followed the same kinetic pattern as sputum eosinophils ($r_s = 0.672$, $P < .001$). In addition, an equivalent decrease in FEV₁ and the FEV₁/FVC ratio was achieved with only 75% of the amount of single-dose antigen when the smaller but repetitive doses of antigen were administered, suggesting a cumulative effect on airflow obstruction by the repetitive low-dose antigen challenges.

The contribution of eosinophils to asthma pathophysiology has yet to be fully established. Whereas some reports failed to detect significant correlations between sputum eosinophilia and airflow obstruction, FEV₁, or the FEV₁/FVC ratio in asthma,²⁰⁻²² other studies demonstrate significant correlations between baseline FEV₁ and the percentage of sputum eosinophils^{23,24} and eosinophil cationic protein.^{4,25} Our study shows that although there was not a significant gradual decrease in baseline FEV₁ (prechallenge lung function) over time after daily repetitive low-dose antigen challenges, the immediate airflow obstruction (FEV₁/FVC ratio) after antigen challenge correlates closely with sputum eosinophilia ($r_s = -0.517$, $P = .019$), as well as sputum EDN concentrations ($r_s = -0.684$, $P < .001$), suggesting a linkage between the increasing presence of eosinophils and airflow obstruction.

A number of possible mechanisms might explain our observations. First, a daily low-dose antigen challenge might cause an accumulation of inflammatory cells and their products in the airway, such as eosinophils and eosinophil granule protein (ie, EDN), to intensify allergen-driven inflammation with repetitive provocation. We have shown that eosinophils and EDN persist for up to 48 hours after antigen challenge both in sputum¹⁶ and in bronchoalveolar lavage fluid.²⁶ The persistence of eosinophils and their granule proteins could then cause ongoing injury to the airway to enhance the bronchial reaction with repetitive antigen challenges. Second, cumulative and nonresolving effects in the airway (including circulating or airway cells, as well as the airway tissues themselves) prime the airway response (greater immediate decrease in FEV₁) and contribute to further activation of the eosinophils. We propose that when the first low-dose antigen was inhaled into the lung, a small number of leukocytes, including eosinophils and lymphocytes, were recruited into the airway, where they persisted and were primed. Twenty-four hours later, before resolution of the initial inflammation could occur, a second low dose of antigen further activated existing airway leukocytes and enhanced leukocyte influx into the lung. Third, fibronectin might contribute to the airway inflammatory process by increasing eosinophil recruitment and survival.^{27,28} A highly significant correlation ($r_s = 0.717$, $P < .001$) was found between sputum fibronectin concentrations and sputum eosinophilia. These findings parallel observations of increased fibronectin concentrations in bronchoalveolar lavage fluid,^{29,30} suggesting that local fibronectin production in the airspace might promote the persistence of allergic airway inflammation.

There are inherent limitations to our findings. First, we did not measure AHR after daily low-dose challenges and thus cannot compare the effects of low-dose versus single-dose challenge on this outcome. Nonetheless, AHR to methacholine is known to be enhanced by repeated low-dose antigen challenges.^{9,10,12,13,15,31} Thus we propose that the increased AHR might be a consequence of or contributor to the priming processes of the cumulative effects on airway responses and eosinophilic

inflammation that follow repetitive challenges. Second, although 8 subjects were enrolled in this study, only 5 of them completed both the repetitive low-dose and single-dose challenge arms. Although our observations are made on the basis of a small number of subjects, it should be pointed out that this was a 2-period crossover study that, despite a limited number of subjects, showed significant differences between the 2 challenge methods. Finally, it should be noted that although the cumulative effects on pulmonary function and eosinophilia in response to antigen were observed with the first 3 low-dose challenges, there was a plateau or a tendency toward decrease by the fourth low-dose challenge. We do not have sufficient information to conclude whether this represents the beginning of downregulation or tolerance of the inflammatory response because additional challenges were not performed. However, reduction in the late asthmatic response has been reported after repeated low-dose allergen exposure, suggesting that immunologic tolerance can be induced with repetitive inhalation challenge.³² Whether intermittent low-dose challenges (separated by more than 24 hours) would prevent tolerance induction is not currently known.

In summary, we directly compared the effects of repetitive low-dose challenge with an equivalent single dose of inhaled antigen on airway physiology and eosinophilia. Although both methods of antigen exposure caused an immediate decrease in FEV₁ and the FEV₁/FVC ratio, as well as sputum eosinophilia, a smaller amount of antigen was required to achieve an equivalent degree of the airway response when lower doses of antigen were administered repetitively. Our findings suggest that repetitive low-dose antigen challenge, as likely occurs with chronic environmental exposure, might have a priming or accumulative effect on airway inflammation and might provide insight into events leading to chronic persistence of airway inflammation and eventual airway remodeling in asthma.

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