

Repeatability of allergen-induced airway inflammation

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Background: Allergen inhalation challenge is a useful clinical model to investigate the effects of asthma therapies on allergen-induced airway responses; however, the repeatability of allergen-induced airway inflammation is not known.

Objective: The purpose of this study was to investigate the repeatability of allergen-induced increases in sputum eosinophils. This information will allow the prediction of the number of subjects required in studies evaluating asthma therapies.

Methods: Seventeen subjects completed 2 allergen challenges using the same dose of allergen, at least 3 weeks apart. Allergen-induced airway responses were measured for 7 hours after challenge. Differential cell counts from induced sputum were determined the day before and 7 and 24 hours after challenge; methacholine PC₂₀ was measured the day before and 24 hours after challenge.

Results: The intraclass correlation coefficient for maximum percent late fall in FEV₁ was 0.32 and for the area of the late response was 0.61. The sample size predicted to be necessary to observe 50% attenuation of the maximum percent late fall in FEV₁ and the late area under the curve with a power of 0.95 was 9 subjects. The intraclass correlation coefficient for percent of allergen-induced sputum eosinophils was 0.60 at 7 hours and 0.53 at 24 hours after challenge. With a randomized cross-over study design, the sample size predicted to be necessary to observe 50% attenuation of allergen-induced percent of eosinophils with a power of 0.95 was 5 subjects.

Conclusion: Allergen inhalation challenge with measurements of sputum eosinophils is a noninvasive and reliable method for evaluating the anti-inflammatory effects of asthma therapies. (*J Allergy Clin Immunol* 1999;104:66-71.)

Key words: Allergen challenge, sputum eosinophils, reproducibility, sample size, anti-inflammatory therapy

Allergen inhalation challenge in atopic patients with mild asthma is commonly used as a model to study the mechanisms and effects of treatment on allergic asthma. Inhalation of a relevant allergen by atopic asthmatic patients results in an early fall in the FEV₁. A proportion of these asthmatic patients experience a late fall in FEV₁,

Abbreviations used

AUC: Area under the curve
GSEM: Geometric standard error of the mean
ICC: Intraclass correlation coefficient
MCC: Metachromatic cell

commencing 3 to 7 hours after allergen inhalation¹ and progressively worsening airway inflammation characterized by the presence of activated eosinophils.² Allergen-induced airway eosinophilia has traditionally been measured in bronchoalveolar lavage²⁻⁴ and more recently in sputum.⁵⁻⁷ The technique of sputum induction is advantageous because it is safe and noninvasive and permits repeated samples to be obtained.⁸ The day-to-day reproducibility of cell counts from repeated sputum sampling in stable asthmatic subjects has been reported to be good.⁹

Allergen inhalation challenge has proved to be a repeatable^{10,11} and useful model to assess the bronchoprotective effects of asthma drugs¹²⁻¹⁴ and the relative potency of inhaled corticosteroids.¹⁵ Furthermore, it has been shown to be a useful clinical model to investigate the effects of asthma therapies on allergen-induced sputum eosinophilia in asthmatic subjects.^{5,6,16} The repeatability of allergen-induced airway inflammatory cells measured from sputum has not been investigated, and this is necessary to be able to predict the sample size required to demonstrate a statistically significant attenuation of allergen-induced sputum eosinophilia.

METHODS

Subjects

The data from 17 subjects who had completed 2 allergen inhalation challenges using the same dose of allergen were used to examine the repeatability of allergen-induced airway responses and inflammatory cells measured from induced sputum. Subjects (7 male, 10 female) ranged in age from 19 to 45 years and were non-smokers with mild atopic asthma and allergen-induced early and late bronchoconstrictor responses of at least a 15% fall in FEV₁. All subjects had stable asthma with an FEV₁ greater than 70% of predicted normal value on all study days before allergen inhalation. Subject characteristics are listed in Table I. Subjects were not exposed to sensitizing allergens and did not have asthma exacerbations or respiratory tract infections for at least 4 weeks before allergen challenges. Subjects used no regular medication other than infrequent (less than twice weekly) inhaled β_2 -agonist as required to

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TABLE I. Subject characteristics

Subject	Age (y)	Sex	Allergen dose	FEV ₁ % predicted	Time between challenges	Methacholine PC ₂₀ (mg/mL)
1	21	F	HDM 1:2048	79.7	11 mo	0.59
2	23	F	HDM 1:256	85.4	2 mo	0.55
3	21	F	HDM 1:1024	93.4	3 mo	1.14
4	21	F	HDM 1:16	91.9	2 mo	24.3
5	21	F	HDM 1:512	81.9	2 mo	0.87
6	24	M	Grass 1:1024	78.8	10 mo	3.48
7	23	M	Ragweed 1:4	96	4 mo	1.52
8	23	M	Cat 1:16	81.1	5 mo	6.51
9	45	M	Cat 1:32	82.9	1 mo	3.23
10	27	F	Cat 1:1024	80.8	1 mo	0.43
11	24	M	HDM 1:1024	90.7	2 mo	1.20
12	37	M	HDM 1:1024	96	1 mo	2.17
13	24	M	HDM 1:512	83.6	4 mo	2.00
14	21	F	HDM 1:256	95	3 mo	3.15
15	19	F	HDM 1:256	97.3	5 mo	1.5
16	26	F	HDM 1:256	74.7	3 wk	1.25
17	21	F	Ragweed 1:32	89.5	3 wk	0.35
Mean ± SEM	24.8 ± 1.6	7M/10F	3 Cat; 2 ragweed; 11 HDM; 1 grass	87.0 ± 1.7	3.4 ± 0.7 mo	1.63 (GSEM, 1.28)

HDM, House dust mite.

treat their symptoms. All medications were withheld for at least 8 hours before each visit, and subjects were instructed to refrain from rigorous exercise, tea, or coffee in the morning before visits to the laboratory. Signed informed consent was given to participate in the studies, which were approved by the Ethics Committee of McMaster University Health Sciences Centre.

Study design

Seventeen subjects completed 2 allergen inhalation challenges using the same dose of allergen on both occasions. Allergen challenges were separated by at least 3 weeks to a maximum of 11 months (mean, 3.4 months). Each allergen challenge consisted of 3 visits to the laboratory. Baseline measurements of FEV₁, PC₂₀, and differential cell counts from induced sputum were determined the day before the inhalation challenge. Allergen inhalation challenge was carried out the next morning. The FEV₁ was measured for 7 hours after the challenge, and sputum samples were obtained at 7 hours, during the late response. Sputum could not be induced earlier than 7 hours after the challenge because of the requirement of pretreatment with inhaled β_2 -agonists before sputum induction, which would interfere with subsequent measures of FEV₁ values. Methacholine PC₂₀ and sputum samples were obtained again at 24 hours after the challenge.

Laboratory procedures

Methacholine inhalation test. Methacholine inhalation challenge was performed as described by Cockcroft.¹⁷ Subjects inhaled normal saline solution, then doubling concentrations of methacholine phosphate from a Wright nebulizer for 2 minutes. FEV₁ was measured at 30, 90, 180, and 300 seconds after each inhalation. Spirometry was measured with a Collins water-sealed spirometer and kymograph. The test was terminated when a fall in FEV₁ value of 20% of the baseline value occurred, and the methacholine PC₂₀ was calculated.

Allergen inhalation challenge. Allergen challenge was performed as described by O'Byrne et al.¹ The allergen producing the largest skin wheal diameter was diluted in 0.9% saline solution for inhalation. The concentration of allergen extract for inhalation was

determined from a formula described by Cockcroft et al¹⁸ with the results from the skin test and the methacholine PC₂₀. The starting concentration of allergen extract for inhalation was 2 doubling concentrations below that predicted to cause a 20% fall in FEV₁, and the concentration was doubled until an early fall in FEV₁ of at least 20% was measured. The FEV₁ was then measured at 10, 20, 30, 45, 60, 90, and 120 minutes, then each hour until 7 hours after allergen inhalation. The early bronchoconstrictor response was taken to be the largest fall in FEV₁ within 2 hours after allergen inhalation, and the late response was taken to be the largest fall in FEV₁ between 3 and 7 hours after allergen inhalation. The area under the curve (AUC) was determined during the early (0 to 2 hours) and late (3 to 7 hours) response by plotting the response with the use of graphics software (Fig P; Fig P Software Corporation, Durham, NC), which calculated the area of the FEV₁-time response.

Sputum analysis. Sputum was induced and processed by the method described by Pizzichini et al.⁹ Subjects inhaled 3%, 4%, and then 5% saline solution for 7 minutes each. The induction was stopped when an adequate sample was obtained or if the FEV₁ dropped 20% from baseline. Cell plugs with little or no squamous epithelial cells were selected from the sample, separated from saliva, and weighed. Samples were aspirated in 4 times their volume of 0.1% dithiothreitol (Sputolysin, Calbiochem Corp, San Diego, Calif) and 4 times their volume of Dulbecco's PBS (Life Technologies Inc, Grand Island, NY). The cell suspension was filtered through a 52 μ m nylon gauze (BNSH Thompson, Scarborough, Ontario, Canada) to remove debris and centrifuged at 425g for 10 minutes; then cells were resuspended in Dulbecco's PBS. The total cell count was determined with the use of a Neubauer hemocytometer chamber (Hausser Scientific, Blue Bell, Pa) and expressed as the number of cells per milliliter of sputum. The cell suspension was adjusted to a concentration of 0.75 to 1.0 $\times$ 10⁶/mL, and cytopins were prepared on glass slides with the use of 50 μ L of cell suspension and a Shandon III cytocentrifuge (Shandon Southern Instruments, Sewickly, Pa) at 300 rpm for 5 minutes. Differential cell counts were obtained from the mean of 2 slides with 400 cells counted per slide stained with Diff Quik (American Scientific Products, McGaw Park, Ill). The same observer counted all study slides, with a high reproducibility of the cell counts using these methods.⁹

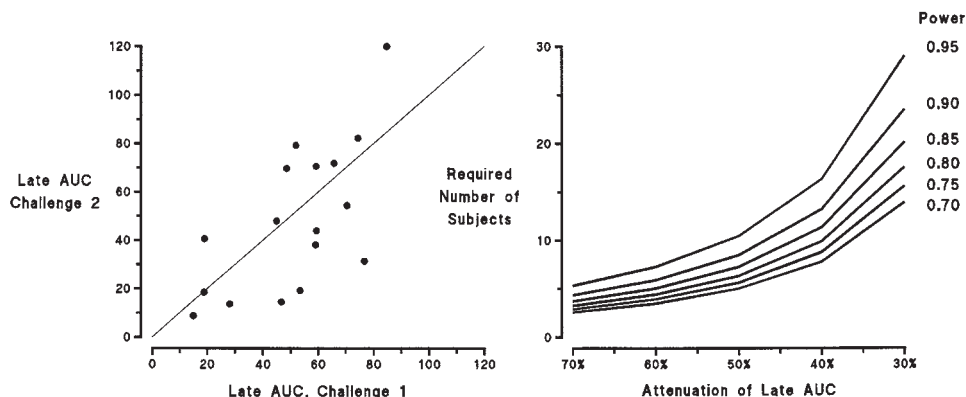


FIG 1. Late AUC after allergen challenges 1 and 2 (*left panel*) and power curves allow estimation of sample size for expected attenuation the late AUC (*right panel*).

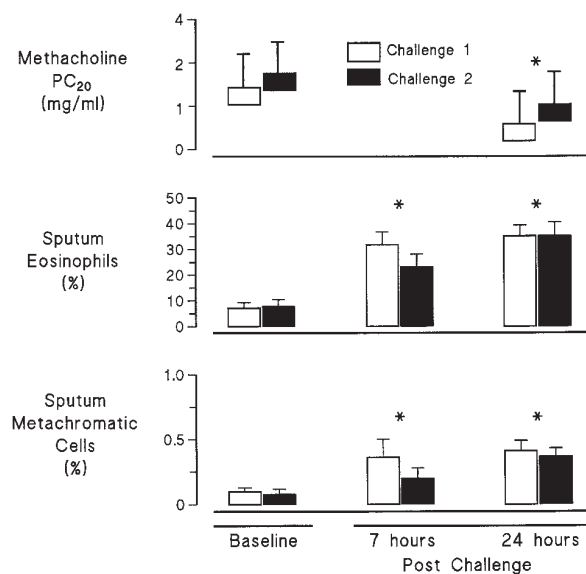


FIG 2. Methacholine PC₂₀ (*top panel*), percent of sputum eosinophils (*middle panel*), and percent of sputum MCCs (*bottom panel*) measured at baseline and 7 and 24 hours after allergen challenge 1 (*open bars*) and allergen challenge 2 (*solid bars*). Sputum values are reported as means and SEM; methacholine PC₂₀ values are reported as geometric means and GSEM. **P* < .05 change from baseline.

Metachromatic cells (MCCs) were enumerated on slides stained with toluidine blue for basophil and mast cell counts, which were obtained from the mean of 2 slides, with 1500 cells observed on each slide.

Statistical analysis

All summary statistics are expressed as means $\pm$ SEM except for methacholine PC₂₀. Methacholine PC₂₀ measurements were made by linear interpolation of log dose-response curves resulting in logarithmic values for PC₂₀, which were then subjected to statistical analysis and expressed as geometric mean and geometric standard error of the mean (GSEM). The repeatability of allergen-induced changes was assessed by the reliability coefficient (intraclass correlation coefficient [ICC]) as the proportion in the variance between

the 2 measurements compared with the variance between subjects.¹⁹ The relationship between the allergen-induced late airway response and sputum eosinophils were examined with the correlation coefficient. Sample sizes for nonrepeated measurements (early and late responses) were predicted with the variance of the difference between challenge 1 and challenge 2.²⁰ Sample sizes for repeated measurements (sputum inflammatory cells) were predicted with the time/challenge interaction error term from ANOVA tables as an estimate of variance.

RESULTS

Airway responses

The maximum early percent fall in FEV₁ was 27.2% $\pm$ 2.3% and 28.9% $\pm$ 2.4% after challenges 1 and 2, respectively (ICC, 0.40). The maximum late percent fall in FEV₁ was 24.8% $\pm$ 1.6% and 23.9% $\pm$ 2.5% for challenges 1 and 2, respectively (ICC, 0.32). The AUC of the late response was 51.6 $\pm$ 5.0 L·min after challenge 1 and 48.7 $\pm$ 7.3 L·min after challenge 2 (ICC, 0.61). The sample size predicted to be necessary to observe 50% attenuation of both the maximum late percent fall in FEV₁ and the late AUC, with a power of 0.95, is 9 subjects (Fig 1).

The methacholine PC₂₀ decreased from baselines of 1.6 mg/mL (GSEM, 1.3 mg/mL) and 2.1 mg/mL (GSEM, 1.3 mg/mL) to 0.5 mg/mL (GSEM, 1.3 mg/mL; *P* < .001) and to 1.1 mg/mL (GSEM, 1.3 mg/mL; *P* < .002) after challenges 1 and 2, respectively (Fig 2).

Sputum

The number of eosinophils per milliliter of sputum increased significantly from 11.4 $\pm$ 2.3 $\times 10^4$ /mL and 12.5 $\pm$ 4.3 $\times 10^4$ /mL (ICC, 0.36) at baseline to 114.1 $\pm$ 36.2 $\times 10^4$ /mL and 78.9 $\pm$ 18.4 $\times 10^4$ /mL (ICC, -0.02) at 7 hours after challenges 1 and 2, respectively, and to 192.1 $\pm$ 45.8 $\times 10^4$ /mL and 91.4 $\pm$ 16.8 $\times 10^4$ /mL (ICC, 0.34) at 24 hours after challenges 1 and 2, respectively (*P* < .001). The percent of sputum eosinophils increased significantly after allergen challenges from 7.2% $\pm$ 2.1% and 7.9% $\pm$ 2.6% (ICC, 0.56) at baseline to 31.8% $\pm$ 5.0% and 23.5% $\pm$ 4.8% (ICC, 0.60) at 7 hours after chal-

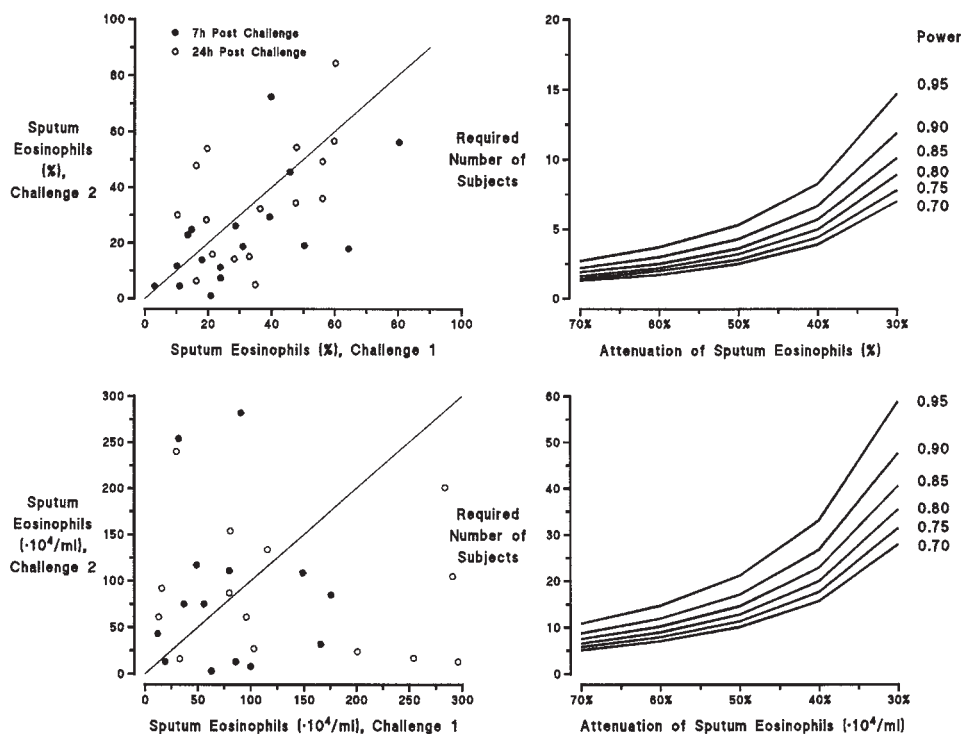


FIG 3. Percent of sputum eosinophils (*top left*) and number of eosinophils per milliliter of sputum (*bottom left*) at 7 hours (*filled circles*) and 24 hours (*open circles*) after allergen challenges 1 and 2 and power curves for percent of sputum eosinophils (*top right*) and number of eosinophils per milliliter of sputum (*bottom right*) allow estimation of sample size for expected attenuation of allergen-induced sputum eosinophils.

lenges 1 and 2, respectively, and to $35.2\% \pm 4.2\%$ and $35.2\% \pm 5.2\%$ (ICC, 0.53) at 24 hours after challenges 1 and 2, respectively ($P < .001$; Fig 2). These eosinophil counts were more reproducible when expressed as percentage than when expressed as the total number per milliliter of sputum. The sample size necessary to observe a 50% attenuation of allergen-induced percentage of sputum eosinophils, with a power of 0.95, is 5 subjects and for the number of eosinophils per milliliter of sputum is 21 subjects (Fig 3).

The number of MCCs per milliliter of sputum increased significantly after inhaled allergen, from $1.9 \pm 0.8 \times 10^3/\text{mL}$ and $2.0 \pm 0.8 \times 10^3/\text{mL}$ at baseline to $15.2 \pm 6.8 \times 10^3/\text{mL}$ and $12.5 \pm 4.5 \times 10^3/\text{mL}$ at 7 hours after challenges 1 and 2, respectively, and to $26.4 \pm 5.6 \times 10^3/\text{mL}$ and $20.7 \pm 8.4 \times 10^3/\text{mL}$ at 24 hours after challenges 1 and 2, respectively ($P = .004$). The percentage of MCCs also increased significantly after inhaled allergen ($P < .001$; Fig 2), with higher postallergen reproducibility at 7 hours (ICC, 0.42) and 24 hours (ICC, 0.55) after challenge than the number of MCCs per milliliter of sputum at 7 hours (ICC, 0.17) and 24 hours (ICC, 0.12) after challenge.

The number of neutrophils per milliliter of sputum did not change after inhaled allergen ($P = .30$); however, the percentage of neutrophils decreased significantly ($P = .03$) from $47.0\% \pm 1.3\%$ and $46.0\% \pm 1.5\%$ at baseline to

$37.7\% \pm 1.3\%$ and $35.1\% \pm 1.4\%$ at 24 hours after the allergen challenges 1 and 2, respectively.

There was a significant correlation between the maximum percentage of late fall in FEV_1 and the allergen-induced increase in the percentage of sputum eosinophils at 7 hours ($r, 0.38$; $P = .03$) and 24 hours ($r, 0.49$; $P = .004$) after allergen.

DISCUSSION

This study has demonstrated that allergen-induced sputum eosinophilia is a reproducible measurement that contributes to the magnitude of the allergen-induced late airway response. Furthermore, this study demonstrates that the area of the late response after allergen challenge is a reproducible measurement and confirms previous observations on the reproducibility of the allergen-induced maximal late fall in FEV_1 .¹⁰

Reproducible allergen-induced late responses require careful administration of a suitable dose of relevant allergen and stability of the subjects studied. The subjects enrolled in these studies were characterized as having mild, stable asthma with FEV_1 values more than 70% of predicted value and with stable airway responsiveness before the allergen challenge. Also, the studies were performed when the subjects were not exposed to seasonal allergies. The baseline sputum eosinophil counts of 7%

are higher than expected for individuals with mild, stable asthma because of 4 subjects with baseline sputum eosinophil counts ranging between 10% and 40%. Despite this, these subjects were asymptomatic, with FEV₁ values of more than 85% of predicted value. Lack of individual consistency in airway responses and cell recruitment after allergen challenge with the same dose of allergen can result from variability in the allergen challenge itself, including day-to-day variation in diluting and nebulizing the allergen. Slight changes in airway responsiveness between challenges alter the magnitude of the allergen-induced airway responses and inflammation. Furthermore, measurements of sputum inflammatory cells are affected by poor cell viability and morphologic condition. Inflammatory cells expressed as absolute numbers are critically dependent on the total cell count, which has been shown to be nonreproducible,⁹ largely because of variable airway secretions in the sputum sample. These factors, however, are present in all studies designed to deliver repeated allergen challenges and must be included as realistic sources of variability between repeated measurements in the same individual.

In this study, allergen-induced late airway responses were measured either by recording the maximum percent fall in FEV₁ occurring between 3 and 7 hours after allergen or by constructing a time-FEV₁ curve and measuring the AUC from 3 to 7 hours after allergen. We examined the reproducibility of the late response measured by maximal percent fall in FEV₁ and AUC, because AUC may be a more sensitive indicator of the magnitude of the late response than a single time-point maximal fall in FEV₁ value. We observed a higher ICC for the AUC, demonstrating that this is indeed a more reproducible measurement than the maximal fall in FEV₁ value. However, the sample sizes necessary to achieve a significant 50% attenuation of the late response by either of these measurements are identical, requiring 9 subjects. A low ICC, however, does not necessarily indicate poor reproducibility, because a low range of measurements prevents a high ICC. For safety, allergen challenges are designed to produce airway responses within a narrow range.

This study and others²¹ have demonstrated that there is a direct relationship between the magnitude of the late response to allergen and the magnitude of airway eosinophilia. Furthermore, eosinophils and eosinophil cationic protein levels have been inversely correlated with the reduction in FEV₁.^{9,22,23} Eosinophils may release cytotoxic mediators, such as eosinophil cationic protein and major basic protein, which are shown to be elevated in the airways of asthmatic individuals²⁴ and may lead to airway damage.^{22,25} We have shown that the presence of eosinophils contributes to the late airway response; however, the presence of mediators from other inflammatory cells present after allergen challenge may also contribute to the late airway response.

Eosinophils and MCCs have been implicated as major participants in IgE-driven allergic responses and are elevated in the airway after allergen inhalation.^{2,3} We have demonstrated a reproducible allergen-induced increase in

the number of sputum eosinophils and a less reproducible, but statistically significant, increase in the number of sputum MCCs. These measurements from induced sputum confirm observations made from bronchial biopsy specimens and bronchoalveolar lavage fluid^{2,3,26} and demonstrate that increases in eosinophils and MCCs can be reliably measured in sputum samples after an allergic asthmatic response. In contrast, the percentage of neutrophils, but not total numbers of neutrophils, decreased 24 hours after allergen inhalation as a result of the increased percentage of eosinophils, which peaked at 24 hours. Allergen-induced changes in sputum neutrophil counts are not consistent.^{5,7,27,28} If the neutrophil is involved in the airway response to inhaled allergen, it may reflect an early nonspecific response that occurs with acute tissue injury unlike the prolonged eosinophilia, which occurs in response to specific allergic stimuli.

Allergen-induced eosinophilia is a useful measurement in the assessment of the anti-inflammatory properties of asthma drugs. Based on estimates of variance with the use of repeated measures ANOVA, the sample size necessary to achieve a 50% attenuation in the number of sputum eosinophils after allergen with a power of 0.95 is 21 subjects and with a power of 0.70 is 10 subjects (Fig 3). The poor reproducibility of the total cell count in sputum,⁹ which is influenced by the viscosity and volume of airway secretions and salivary contamination during the sputum induction procedure, lowers the ICC and adds considerably to the amount of variability in a repeated measures analysis. The sample size required to demonstrate suppression of the number of eosinophils per milliliter of sputum is larger than that required to demonstrate suppression of the percentage of eosinophils, where a sample size of 5 subjects is necessary to achieve a 50% attenuation in the percent of sputum eosinophils after allergen with a power of 0.95 (Fig 3). Despite the variability in the number of eosinophils per milliliter of sputum, we have previously demonstrated effects of asthma therapies that correspond to their effects on the allergen-induced late response with a sample size of not more than 10 subjects.^{5,6}

Repeated measures ANOVA is a powerful statistical tool for randomized cross-over studies. When applied to measurements of sputum eosinophils, repeated measures ANOVA allows for practical sample sizes to demonstrate attenuation in the number of sputum eosinophils. If, for example, the number of allergen-induced eosinophils were measured at only 1 time point (ie, 7 or 24 hours) after challenge, the predicted sample size required to statistically demonstrate a 50% reduction in the number of eosinophils per milliliter of sputum by using a paired *t* test would be approximately 30 to 70 subjects. Thus sputum induction allows for repeated sampling of airway inflammatory cells, yielding a more sensitive measure of variance.

In conclusion, this study has demonstrated that enumeration of sputum eosinophils for the assessment of allergen-induced airway inflammation is a reproducible measurement. With the introduction of novel therapies

for the treatment of asthma²⁹⁻³¹ and comparisons with therapies known to possess anti-inflammatory properties,^{5,15,16} allergen-induced sputum eosinophilia may provide a valuable clinical tool for assessing their anti-inflammatory effects in the airways.

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