

Removal of bronchoalveolar cells augments the late eosinophilic response to segmental allergen challenge

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Summary

Background In patients with quiescent asthma, macrophages are the most prevalent cells recovered by bronchoalveolar lavage (BAL). Through activation via their FcεRII receptors or by acting as antigen-presenting cells, macrophages could, in theory, promote the late airway response to allergen.

Objective In order to investigate the importance of macrophages and other airway luminal cells in inducing the late airway response, a novel washout experiment was designed.

Methods Five patients with ragweed-allergic asthma underwent bronchoscopy and segmental bronchial challenge with either normal saline or short ragweed extract in two segments of one lung. In a third segment of the opposite lung, 12 successive BALs (25 mL each) were performed, followed by challenge with an identical dose of short ragweed (washed-challenged segment). After 24 h, all three challenged segments underwent BAL.

Results Initially, in the washed-challenged segment, over 80% (mean 80.4%, range 68–88%) of the recoverable airway dwelling cells were removed. Unexpectedly, 24 h later these same washed-challenged segments contained more eosinophils in the BAL than the challenged segments from the opposite lung ($P = 0.033$).

Conclusions Removing the majority of airway luminal cells followed by allergen bronchoprovocation increased the number of eosinophils recovered 24 h after challenge. Our results suggest that in quiescent allergic asthma, the airway luminal cells are protective and attenuate the late eosinophilic response to allergen challenge.

Keywords segmental bronchoprovocation, eosinophils, macrophages, lymphocytes, interleukin-5, bronchoalveolar lavage, successive washout lavage

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Introduction

Analysis of airway cells and fluids recovered by bronchoalveolar lavage (BAL) from patients with allergic asthma has been instrumental in advancing our knowledge of the pathophysiological mechanisms of the inflammatory response in asthma. Symptomatic patients have increased numbers of eosinophils and increased quantities of eosinophil granule proteins and eosinophil-active cytokines, such as IL-5 and granulocyte-macrophage colony-stimulating factor (GM-CSF), in their BAL fluids [1–3]. After allergen challenge in patients with allergic asthma, the numbers of eosinophils from the late (after 16, 24 or 72 h) BAL fluids increase dramatically [4–6] and are associated with increased numbers of CD4 lymphocytes [7] and activated CD4 lymphocytes [8]. Analysis of the post-allergen BAL fluids has also shown increased levels of eosinophil granule proteins and IL-5 and increased mRNA for IL-5 in the BAL cells [4,5]. IL-5 concentrations in these fluids correlate

with both eosinophil and lymphocyte numbers [9]. The differential percentages of airway luminal cells recovered from asymptomatic patients with allergic asthma are quite similar to normal non-smokers and consist of mostly macrophages (75–95%), lymphocytes (5–20%) and a few granulocytes (0–5% eosinophils, 0–5% neutrophils, < 1% metachromatic cells) [5,7,9,10].

Pulmonary macrophages, recovered by BAL performed on rats [11] and on patients with allergic asthma [12] possess FcεRII receptors. Recent evidence from bronchial biopsies demonstrates that some macrophages from patients with asthma possess FcεRI receptors as well [13]. Pulmonary macrophages from allergic patients can be activated by cross-linking IgE to produce cytotoxic molecules and cytokines [13,14]. Further, macrophages recovered from patients with asthma, but not macrophages recovered from normal subjects, produce GM-CSF [15].

Therefore, we tested the hypothesis that airway macrophages are important in the induction of the late eosinophil response to allergen challenge. If valid, an attenuation of the late eosinophilic response should occur when these airway luminal cells are removed from a bronchial subsegment by sequential lavages before allergen challenge. A novel successive lavage (washout)

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method compared the 24 h results between an allergen-challenged pulmonary segment and a washed and challenged pulmonary segment.

Materials and methods

Patient selection

Six non-smoking patients with asthma, as defined by the American Thoracic Society criteria [16], with a history of exacerbations of allergic rhinitis and asthma symptoms during the ragweed pollen season were studied. Because of coughing, one patient could not complete the washout so the procedure was discontinued and the patient was dropped from the study. All patients were skin tested with serial dilutions of ragweed pollen extract and all produced at least a 10×10 mm weal with surrounding flare in response to a skin prick test with ragweed extract. Because endotoxin is a potent activator of macrophages, an endotoxin-free ragweed extract, previously tested using a modified Limulus assay, was used for skin testing and for segmental allergen challenge (Greer Laboratories Inc., Lenoir, NC, USA; L56-106) [17]. All patients had an FEV₁ greater than 90% of predicted, had no upper respiratory tract

infection for at least 4 weeks prior to segmental bronchoprovocation, and had not required systemic or topical corticosteroids or sodium cromolyn within 2 months before the study. The patients were asymptomatic and gave written informed consent; the study was conducted outside of the ragweed pollination season. Table 1 summarizes the patient demographic and bronchoprovocation information. The study was approved by the Mayo Clinic Institutional Review Board.

Bronchoscopy, segmental washout, and bronchoprovocation

As shown schematically in Fig. 1, an Olympus P20 D bronchoscope was advanced to a subsegmental level until it could not be advanced further and from this 'wedged' position, a BAL was performed with two 60 mL instillations of normal saline (baseline). In a nearby segment, 5 mL of saline was instilled as a control (24-h sham). Likewise, in another nearby segment (24-h antigen, Ag), ragweed allergen was administered based on the skin test reactivity (230 PNU in two patients, 500 PNU in three patients) in 5 mL normal saline. The bronchoscope was moved to the opposite lung and in a fourth segment (Ag wash), 12 sequential BALs using 25 mL saline per instillation were performed; each of the 12 successive 25 mL BAL wash

Table 1. Patient data

Patient	Age	M/F	FEV ₁ * (F%)	Nature of asthma	Medication†	O ₂ saturation during procedure	SRW segmental challenge done (PNU)
1	48	M	90	Seasonal Exercise-induced Post-viral	Albuterol	86–88	500
2	20	F	91	Seasonal	Albuterol	92–95	230
3	30	M	99	Seasonal	Albuterol	92–94	500
4	42	M	94	Seasonal Exercise-induced	Albuterol	88–92	230
5	41	F	105	Seasonal	Albuterol	84–90	500

*% of predicted value. †Albuterol inhalation was used as needed for dyspnoea.

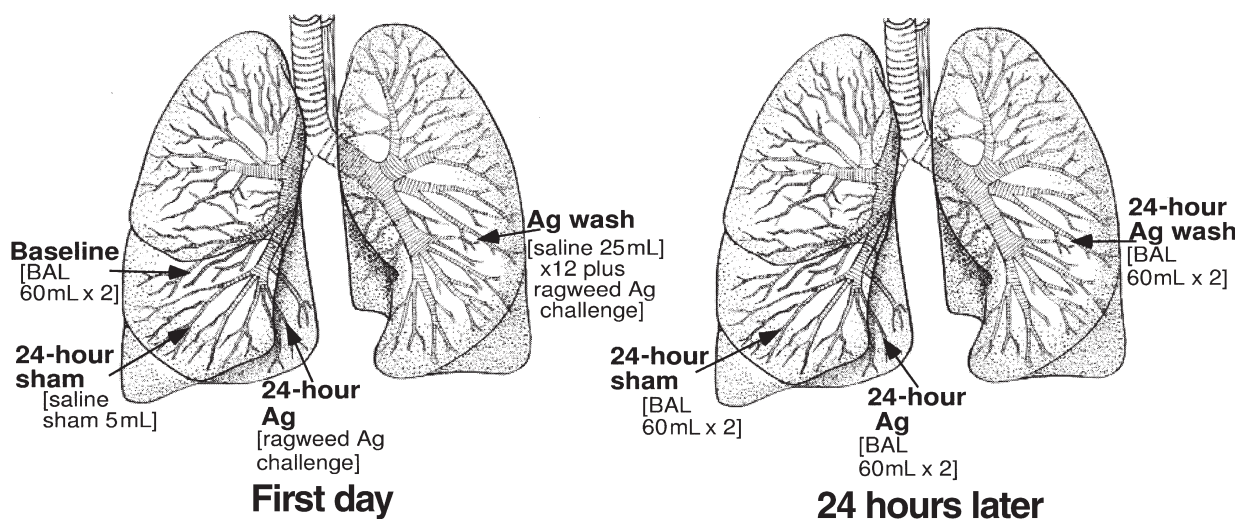


Fig. 1. Schematic representation of the sequential lavage and allergen challenge. On the first day four segments were treated as follows: the baseline segment received two 60-mL saline lavages; the 24-h sham segment received a 5-mL saline sham treatment; the 24-h Ag segment received a 5-mL ragweed antigen challenge; the Ag wash segment in the opposite lung received 12 sequential lavage treatments of 25 mL saline per lavage followed by a 5-mL ragweed antigen challenge. Twenty-four hours later the 24-h sham, the 24-h Ag and the 24-h Ag wash segments were each lavaged twice with 60 mL of saline per lavage.

instillations was aspirated back into the injecting syringe. Preliminary observation of four healthy volunteers showed that after 12 washes, the cumulative recoveries of total cells were flattening, suggesting that a plateau was being approached. An identical dose of allergen to that used for the 24-h Ag challenge in the opposite lung was immediately instilled.

Promptly after each challenge (24-h sham, 24-h Ag and Ag wash), an additional 5 mL of saline was injected followed by two injections of 5 mL of air to flush the bronchoscope and to distribute the challenge material within the bronchopulmonary segment. Twenty-four hours later, bronchoscopy with BAL was repeated in the 24-h sham, 24-h Ag, and 24-h Ag wash segments using two 60 mL instillations of normal saline per segment.

During these procedures, oxygen saturation was measured by pulse oximetry (Table 1). Pulmonary function was measured before and after bronchoscopy and did not drop appreciably (< 15%). One patient became febrile 12 h after bronchoscopy and was treated by administration of antipyretics and antibiotics.

Processing of the BAL fluid and cells

The 12 wash syringe samples, the baseline BAL samples and all 24-h BAL samples from the challenged segments were passed through a nylon mesh and centrifuged at 400 g for 10 minutes. Resuspended cells were counted to yield total cells. Cytospin preparations were stained with Diff Quick (VWR Scientific Co., Chicago, IL, USA) and differential cell counts (at least 400 cells) were performed to identify macrophages, lymphocytes, neutrophils, eosinophils, metachromatic cells and epithelial cells. The cell-free BAL supernatant fluid specimens were frozen in aliquots at -20°C . Cells present in the 12 wash syringe samples were counted after each successive wash, and the cumulative cell counts for each cell type were calculated. Assuming a negative exponential growth model, non-linear regression was used to extrapolate the curve for each patient [18]. The predicted values from the fitted curves were used to estimate the number of recoverable cells that would be removed if successive 25 mL BAL instillations were continued through 35 washes (estimated total recoverable cells). This allowed a calculation of the percentage of recoverable cells removed (total cells actually removed/estimated total recoverable cells) $\times 100\%$.

IL-5 radioimmunoassay

A double antibody radioimmunoassay measured interleukin (IL)-5 [9]. Briefly, Immulon-4 wells (Dynatech Laboratories, Chantilly, VA, USA) were coated with monoclonal rat anti-human IL-5 (a gift of DNAX Laboratories, Palo Alto, CA, USA). Test samples of unconcentrated BAL fluids and serial dilutions of IL-5 were added to the wells, incubated and washed. I^{125} -labelled polyclonal rabbit anti-IL-5 IgG was used as the second antibody. The lower limit of detection of the assay was 13 pg/mL. There was no cross-reactivity with GM-CSF and IL-4.

Statistical analysis

The sample size of $n=6$ was chosen after weighing statistical concerns and the practical limitations of patient recruitment and resource requirements for this rigorous investigation. To maximize statistical power, a one-sample repeated-measures design was employed whereby measurements from each of four treatment conditions were obtained for each patient. One patient was dropped from the study because they could not

complete the washout procedure. Thus, the analysis was performed using a sample size of $n=5$. In general, for a one-sample comparison a sample size of $n=5$ provides statistical power of 82% to detect an effect size of 1.65 standard deviations ($\alpha=0.05$, two-sided paired t -test).

Total cell numbers and percentages of differential cell counts from each patient's BAL and from each lavage from the Ag wash segments were tabulated. Because the distribution of values for the cellular response to allergen challenge was markedly skewed, a log transformation [$\log(\text{count} + 1)$] was employed for statistical comparisons of cell counts among the four lavaged segments. The [$\log(\text{count} + 1)$] was employed because in a few instances no cells of a given type were present in the BAL fluids and the log of zero is undefined. Cell counts from the baseline segments were compared with those from the 24-h sham segment using the paired t -test. Cell counts were compared among the 24-h sham, 24-h Ag and 24-h Ag wash segments using repeated measures analysis of variance and the paired t -test. All tests were 2-tailed.

Results

By using 12 successive 25 mL BALs, we were able to remove between 68% and 88% (mean 80.4%) of the estimated recoverable cells from the Ag wash segments in five patients with allergic asthma. As shown in Fig. 2, the cumulative cell counts and the cumulative macrophage counts for each successive 25-mL lavage were used to construct a series of washout curves. Assuming a negative exponential growth model, non-linear regression was used to extrapolate these curves to estimate the numbers of cells that would have been removed after 35 successive 25-mL BALs. Because these negative exponential growth curves plateau, they provide theoretical limits where no additional cells should be obtained with further lavages. The total cells removed from the washout procedure in the five patients (Table 2) ranged from 4.5 to 24.2×10^6 with the total macrophages and lymphocytes removed ranging from 3.3 to 17.8×10^6 and 1.0 to 5.1×10^6 , respectively.

When BAL fluids from the 24-h sham segments were compared with those from the baseline segments, no significant differences were noted for any cell types ($P>0.127$ in all cases). From repeated measures analysis of variance, comparing BAL fluids from 24-h sham, 24-h Ag and 24-h Ag wash, no significant differences were found for total cells ($P=0.196$), neutrophils ($P=0.278$), lymphocytes ($P=0.404$) or metachromatic cells ($P=0.445$). However, significant differences were detected for macrophages ($P=0.002$) and eosinophils ($P=0.001$). As shown in Fig. 3, the macrophage counts from

Table 2. Cumulative total cells, macrophages and lymphocytes removed by the 12 sequential lavages for each patient

Patient No.	Total cells removed $\times 10^6$	Total macrophages removed $\times 10^6$	Total lymphocytes removed $\times 10^6$
1	24.2	17.8	5.1
2	4.5	3.3	1.0
3	19.0	16.0	2.1
4	10.4	8.0	1.8
5	7.5	4.7	2.0

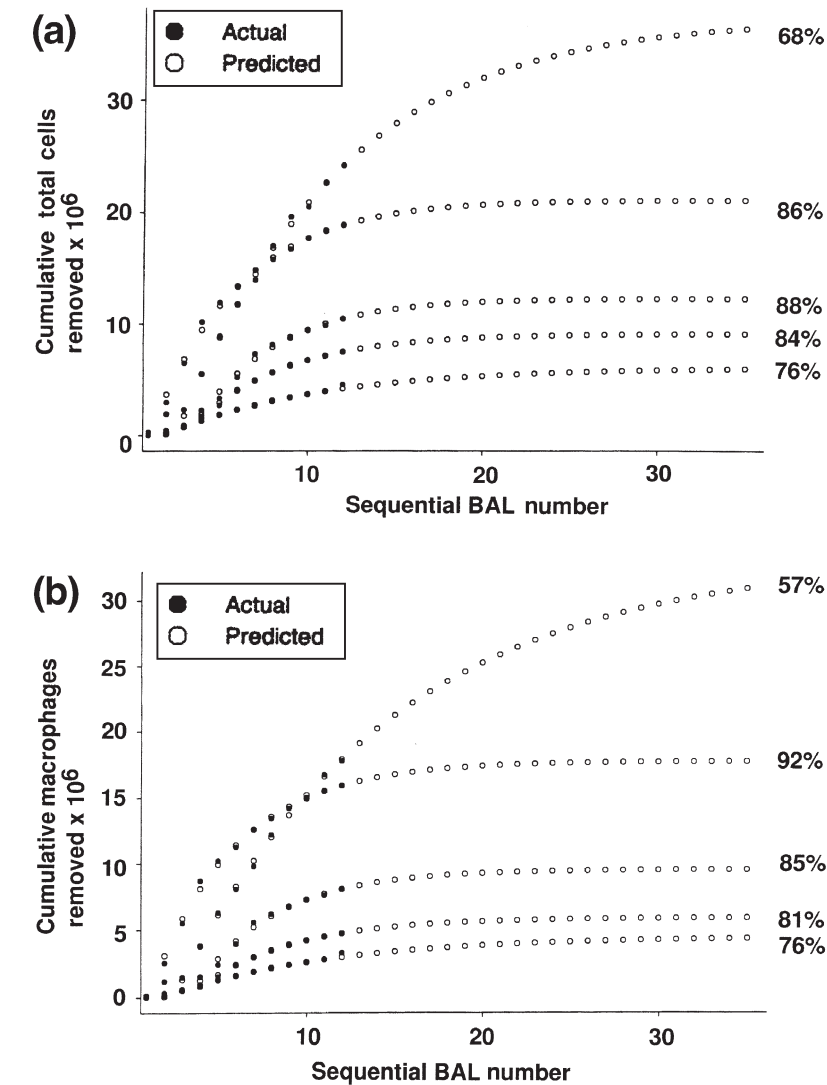


Fig. 2. Cumulative numbers of cells vs. sequential BAL number. The cumulative total cells (a) and total macrophages (b) removed with each consecutive syringe are plotted, showing the curves of declination (filled symbols). These curves are then extrapolated assuming a negative exponential growth model (open symbols). The point at which the curve flattens is the theoretical point at which no further cells would be obtained with additional washouts (estimated total recoverable cells). Actual cells recovered/estimated total recoverable cells $\times 100\%$ equals the theoretical percentage of cells removed by the washout. As shown on the right, between 68% and 88% of theoretically recoverable total cells and between 57% and 92% of theoretically recoverable macrophages were removed.

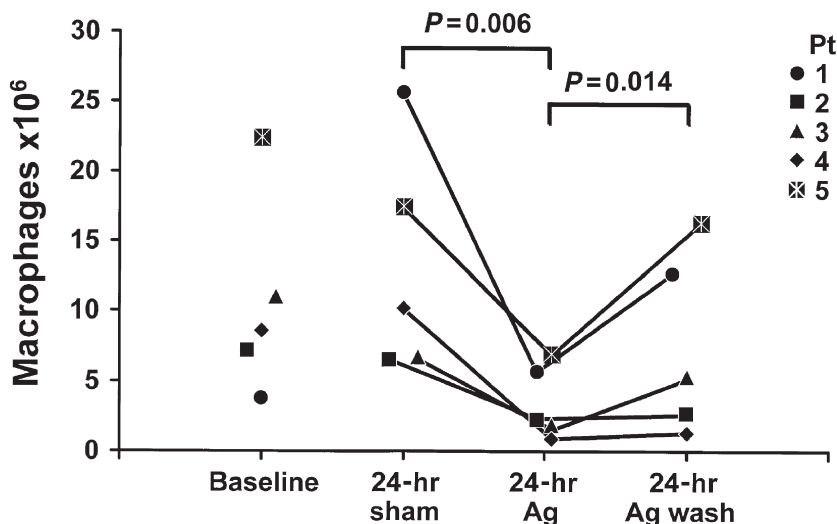


Fig. 3. Macrophage counts recovered from each patient. There was a significant decrease in macrophages between the saline-challenged segment (24-h sham) and the allergen-challenged segment (24-h Ag). The difference between the allergen-challenged segment (24-h Ag) and the washed out and allergen-challenged segment (24-h Ag wash) was also significant.

Fig. 4. Eosinophil counts recovered from each patient. The saline-challenged segment (24-h sham) showed no differences in eosinophils from baseline. The allergen-challenged segment (24-h Ag) produced significantly more eosinophils than the 24-h sham. The allergen-challenged segment that had been washed out (24-h Ag wash) had significantly more eosinophils when compared with the 24-h Ag and the 24-h sham segments.

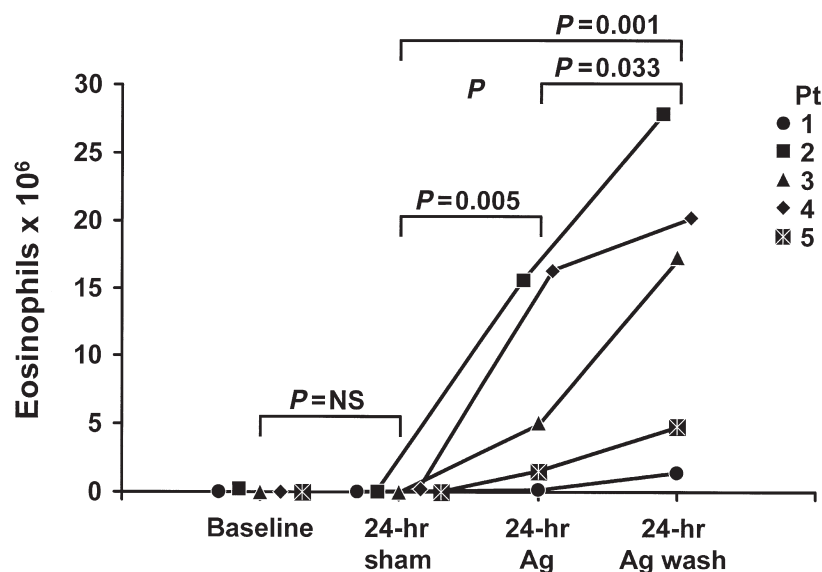


Table 3. IL-5 levels in lavage fluids

Patient No.	IL-5 Levels (pg/mL)			
	Baseline	24-h Sham	24-h Ag	24-h Ag Wash
1	< 13*	< 13	< 13	< 13
2	< 13	< 13	3068	4871
3	< 13	< 13	96	829
4	< 13	< 13	2597	5579
5	< 13	< 13	< 13	< 13

*The lower limit of detection for IL-5 was 13 pg/mL.

the 24-h Ag segment were significantly lower than those from the 24-h sham ($P = 0.006$) and also lower than those from the 24-h Ag wash ($P = 0.014$).

As shown in Fig. 4, the eosinophil counts from the 24-h Ag and 24-h Ag wash segments were significantly higher than those from the 24-h sham ($P = 0.005$ and $P = 0.001$, respectively) and the eosinophil counts from the 24-h Ag wash were significantly higher ($P = 0.033$) than those from the 24-h Ag. Measurement of IL-5 (Table 3) showed detectable levels in the post-challenge 24-h Ag segments in three patients; in these patients, IL-5 levels were greater in the 24-h Ag wash segments compared with the 24-h Ag segments. For both the 24-h Ag and 24-h Ag wash segments, there was a trend towards a positive correlation between eosinophil numbers and IL-5 levels ($r = 0.87$, $P = 0.054$ for each segment; Spearman rank correlation).

Discussion

We compared bronchopulmonary segments 24 h after segmental allergen challenge. Segments depleted of cells by sequential BAL showed increased numbers of eosinophils when compared with control segments. We believe that these observations are important for two reasons. First, our model demonstrates that the bronchoalveolar compartment can be altered by depleting

the majority of recoverable airway cells, mostly macrophages and lymphocytes. This novel challenge method will be a useful model in both animals and humans to probe these cells' contributions during the late cellular response to allergen or other challenges.

Secondly, this model tested the hypothesis that recoverable airway cells augment the late eosinophilic response to allergen challenge. If so, the eosinophils in the late phase response should decrease. Surprisingly, the numbers of eosinophils increased, suggesting that when present, these recoverable airway cells inhibit or suppress the late eosinophilic response to allergen. Thus, resident airway cells may be protective at the baseline, pre-challenge state in patients with asymptomatic allergic asthma. Alternatively, the numbers of allergen-specific cells might be relatively increased in the Ag wash segments and thereby augment the late phase inflammation. This report is the first successful attempt to alter the segmental allergen challenge model in humans; the results do not support a role for luminal airway macrophages or lymphocytes in inducing the late cellular response to allergen.

In our model, the macrophage count decreased in the 24-h Ag segment compared with the sham (saline) segment, but the 24-h Ag wash segment macrophage count was higher than the 24-h Ag segment (Fig. 3). Other segmental challenge studies have shown variable results. For example, two have shown no significant rise in macrophages 6–48 h after pulmonary allergen challenge [4, 19], whereas others have shown a slight rise [5]. The number of macrophages reflected in the total number recovered from post-challenge BAL could be spuriously altered by the increased activation state of these macrophages [20], which, in turn, increases their adhesiveness. This could cause increased adherence to either the bronchoalveolar wall or sides of collecting vials, resulting in an underestimation of the number of these cells. We found increased numbers of macrophages in the 24-h Ag wash segments compared with the 24-h Ag segment; these results are likely to reflect the increased late response to allergen as manifested by increased eosinophils. The macrophages could be allergen-specific and might augment the eosinophil-associated inflammation by production of cytokines, such as

GM-CSF. In the model used by Dupuis, increased numbers of macrophages were observed only in the segment challenged with the highest dose of allergen; this segment also had the highest eosinophil count [5].

The concept that certain airway dwelling cells might be protective is not new. In general, macrophages and certain lymphocyte populations are reported to suppress inflammation [21,22]. For example, dichloromethylene diphosphonate (Cl_2MDP) encapsulated liposomes eliminated alveolar macrophages in allergen-sensitized mice; this treatment resulted in an increased IgE response to the allergen challenge, suggesting a protective role for the resident macrophages [21]. Similarly, when murine alveolar macrophages were depleted with intratracheal Cl_2MDP liposomes, mice challenged with TNP-KLH (trinitrophenyl-keyhole limpet haemocyanin) showed a markedly enhanced immune response [22]. Taken together, these studies support a suppressive role of resident alveolar macrophages in immune inflammation. Airway lymphocytes might also be protective. Analyses of airway lymphocytes before allergen challenge have shown that increased numbers of BAL CD8 lymphocytes reduce the likelihood of a late phase bronchoconstrictive reaction [23]. Future studies of this phenomenon should focus on macrophage and lymphocyte products that might alter the late inflammatory reaction to allergen.

Our study did not address whether proteins removed during the 24-h Ag wash procedure might account for the increased eosinophil response. Quantitatively, the major antibody in alveolar and bronchial lining fluid is secretory IgA. Antigen-specific sIgA could, in theory, through initial contact and binding of allergen, reduce the antigen-specific IgE binding, thus attenuating both the immediate and late allergic reactions. Experimental evidence suggests the contrary. For example, *in vitro* studies suggest that sIgA may actually augment allergic reactions by inducing eosinophil degranulation [24] through specific sIgA [25]. Ragweed-specific sIgA in nasal lavage fluids increases during seasonal pollenosis when patients are most symptomatic [26]. However, there are no reports of attempts to modify an allergic reaction through sIgA reduction.

Alveolar lining fluid also contains surfactant proteins. These proteins stabilize the alveolar wall through reduction of surface tension [27] and are immunomodulatory. For example, surfactant proteins inhibit both the proliferative response of lymphocytes cultured with mitogens [28] and cytokine (TNF- α and IL-6) production by macrophages activated with endotoxin [29]. In ovalbumin-sensitized guinea-pigs, pretreatment of the airway with natural lung surfactant before allergen challenge significantly reduced the pulmonary response [30]. The lower levels of surfactant proteins after the sequential BAL might permit an allergic reaction of greater magnitude. We did not quantify the amount of surfactant material removed during the washout procedure. Studies in humans using surfactant pretreatment of a pulmonary segment before segmental allergen challenge would be of interest.

An additional control segment where sequential washout without Ag challenge would have been desirable, but was omitted because of the prolonged intubation time. Could the washout procedure have altered the mucosal permeability, thus enhancing the cellular influx in response to cytokine signals? If so, the IL-5 levels should have remained constant while the numbers of eosinophils increased when the two challenged

segments were compared. In the three patients where IL-5 was detectable, however, all of the IL-5 concentrations were higher in the 24-h Ag wash segments (Table 3).

Could the sequential washout procedure have activated epithelial cells? In our patients, the successive washout lavages removed a mean of only 9.8×10^2 epithelial cells, a number not likely to be important. Teran et al. observed that bronchial intubation and saline control injection alone resulted in neutrophil recruitment that correlated with IL-8 concentrations [31]. The source of IL-8 was shown to be the epithelium. Eosinophils are recruited *in vitro* by IL-8 [32]. Thus, epithelial perturbation by bronchoscopy and repeated BAL could explain our results.

In conclusion, sequential washout of airway dwelling cells before bronchoprovocation with allergen increased the number of eosinophils recovered at 24 h. Thus, our results suggest that in patients with quiescent allergic asthma, the resident airway cellular population is most likely to be protective against the late eosinophilic response to allergen challenge.

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