

Effects of single and multiple inhalations of antigen on airway responsiveness in monkeys

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*Airway hyperresponsiveness is an important and characteristic feature of asthma. In monkeys, we have reported that antigen (Ag) inhalation induces a prolonged airway eosinophilia, that chronic airway eosinophilia is associated with marked airway hyperresponsiveness, and that chronic Ag inhalations induce airway eosinophilia and hyperresponsiveness. In this study we have determined the effects of acute Ag inhalation(s) on airway responsiveness to obtain a protocol for the study of the mechanisms involved. Anesthetized and intubated adult male cynomolgus monkeys with a naturally occurring sensitivity to *Ascaris suum* extract were studied. Airway responsiveness (provocative concentration of nebulized and inhaled methacholine that induced a 100% increase in respiratory system resistance [Rrs] [PC_{100}]; twofold decrease regarded as significant) and airway cell composition (bronchoalveolar lavage [BAL]) were determined 1 day before and 20 hours after a single inhalation of *Ascaris* extract, or 3 days before and 3 days after three alternate-day inhalations of *Ascaris* extract. The single inhalation of Ag ($N = 7$) caused an acute increase in Rrs ($307\% \pm 62\%$), an increase in BAL leukocytes, and a decrease in PC_{100} in three animals that was moderate (more than eightfold) in two animals. The mean $\pm$ SE change in log PC_{100} was only -0.25 ± 0.24 . The multiple inhalations of Ag in the same animals caused acute increases in Rrs ($178\% \pm 48\%$, $380\% \pm 83\%$, and $331\% \pm 63\%$, respectively), an increase in BAL granulocytes, and a decrease in PC_{100} in six of seven animals (mean $\pm$ SE change in log PC_{100} was -1.36 ± 0.34) that was moderate in two and severe (more than 80-fold) in three animals. In summary, multiple (but not single) inhalations of Ag induce an increase (usually more than eightfold) in airway responsiveness in most monkeys. (J ALLERGY CLIN IMMUNOL 1991;87:835-41.)*

The extreme sensitivity of the bronchi to inhaled agents (physical, chemical, and physiologic) is an important and characteristic feature of bronchial asthma^{1,2}. The severity of this "airway hyperresponsiveness" correlates with the intensity of asthmatic symptoms,^{2,4} diurnal variations in peak flow rates,⁵ and therapy required.^{2,6}

Although airway hyperresponsiveness in subjects with asthma can remain stable during several years,⁷ responsiveness has been demonstrated to be increased by exposure to allergens,^{3,8-14} air pollutants,¹⁵ viral infections,¹⁶ and certain occupational chemicals.^{4,17-19} Since increased responsiveness converts to increased symptoms, it is important to identify animal

Abbreviations used

Ag:	Antigen
EO:	Eosinophil
Rrs:	Respiratory system resistance
PC_{100} :	Provocative concentration of nebulized and inhaled methacholine required to induce a 100% increase in Rrs
BAL:	Bronchoalveolar lavage
PBS:	Phosphate-buffered saline
log:	Logarithm base 10

models to study the mechanisms involved and to evaluate new therapeutic approaches.

In animals,^{12,13} as well as in man,^{8,9} a single inhalation of an allergen can cause a mild (twofold to sixfold) increase in airway responsiveness. Repeated allergen exposures, such as exposures that occur in man during the pollen season^{3,14} or in an occupational setting,^{4,18,19} have been reported to cause greater (often greater than or equal to tenfold) increases in airway responsiveness. Likewise, we have reported that multiple (four weekly) instillations of Ag-coated beads¹⁰

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Received for publication Sept. 25, 1989.

Revised Nov. 6, 1990.

Accepted for publication Dec. 7, 1990.

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1/1/27352

or ≥ 4 weeks of Ag inhalations¹¹ induce a greater than or equal to a tenfold increase in airway responsiveness in monkeys. Although optimal concerning the magnitude of increase in airway responsiveness induced, the study of the mechanisms involved and new therapeutic approaches during multiple (≥ 4) weeks is limited.

The purpose of this study was to determine the effects of acute (single versus three alternate-day) Ag inhalations on airway responsiveness to inhaled methacholine in monkeys. Since airway inflammation,^{13, 14, 20, 21} especially eosinophil infiltration,^{10, 11, 22, 23} has been hypothesized to play a role in the pathogenesis of airway hyperresponsiveness, airway cellular composition was concomitantly determined.

METHODS

Animal care and use were performed in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals," Department of Health, Education and Welfare (National Institutes of Health), publication 85-23, 1985. Protocols described below were approved by the Animal Care and Use Committee, Boehringer Ingelheim Pharmaceuticals, Inc.

Animals

Seven adult male cynomolgus monkeys (*Macaca fascicularis*, Charles River Primate Imports, Port Washington, N.Y.), weighing 4.6 to 8.2 kg, were studied. All animals demonstrated a naturally occurring and reproducible respiratory sensitivity to inhaled *Ascaris suum* extract. Animals were studied anesthetized with ketamine hydrochloride (4 mg/kg, intramuscularly; Ketaset; Bristol Laboratories, Syracuse, N.Y.) and xylazine (1 mg/kg, intramuscularly; Rompun; Miles Laboratories, Inc., Elkhart, Ind.), intubated with a cuffed endotracheal tube (5.5 mm inside diameter, Mallinckrodt Critical Care, St. Louis, Mo., catalog No. 86048), and seated in an upright position in a specially designed support chair. Ketamine (4 mg/kg, intramuscularly) was used alone to supplement anesthesia as needed.

Protocol

Airway responsiveness (methacholine PC₁₀₀) followed by airway cell composition (BAL) were determined 1 day before and 20 hours after a single inhalation of *Ascaris* extract or 3 days before (day 0) and 3 days after (day 10) three alternate-day (days 3, 5, and 7) inhalations of *Ascaris* extract. Animals were rested 4 to 6 weeks between studies to allow airway responsiveness and inflammation to return to baseline (pre-Ag) levels. After a moderate (more than eightfold) increase in airway responsiveness was not obtained in most animals with a single Ag inhalation all seven animals were studied in the three alternate-day antigen-inhalation protocol. A letter designation was assigned to each animal for presentation of results.

In subjects with asthma, the peak increase in airway responsiveness occurs 3 to 24 hours after allergen challenge.^{8, 9, 17} Thus, we measured airway responsiveness 20

hours after the single inhalation of Ag. However, for animal health reasons (i.e., to avoid anesthetizing each animal for a fourth time in a 6-day interval), airway responsiveness was not measured 20 hours after the multiple inhalations of Ag. Instead, we chose to measure airway responsiveness 3 days after the multiple inhalations of Ag. We have previously reported,²⁴ and confirm in this study, that airway inflammation (i.e., EO infiltration) still persists 3 days after Ag inhalation.*

Ag and methacholine inhalation challenges

A. suum extract (Greer Laboratories, Lenoir, N.C., catalog No. B-33) was diluted in phosphate-buffered (5 mmol/L, pH 7.4) saline (0.5%), compressed-air nebulized (micronebulizer model 8158, Bird Products Corp., Palm Springs, Calif.), and administered by intermittent positive-pressure breathing (Mark 7A respirator Bird Products Corp.) consisting of 30 inhalations to 20 cm of H₂O in approximately 2 minutes. For each animal, the previously determined concentration of *Ascaris* extract that caused a 100% to 500% acute increase in Rrs was used. Methacholine challenges were performed in an identical manner except that they consisted of only 15 breaths in 1 minute.

Respiratory system resistance

Respiratory system impedance was measured by discrete frequency (4 to 40 Hz in 11 equal logarithmic steps) sinusoidal-forced oscillations superimposed on tidal breathing. Transrespiratory system pressure was measured by side holes in a PE 220 catheter at the tracheal end of the endotracheal tube connected to a differential pressure transducer (Microswitch, model 143PC01D). At the open end of the endotracheal tube, airflow was measured via a pneumotachograph (Fleisch, model O) to a differential pressure transducer (Microswitch, model 163PC01D36), and forced oscillations were applied at an amplitude of 60 ml/sec²⁵ via a loudspeaker (TBL 14-inch woofer, model 2225H). The frequency response of the pressure and flow signals were corrected as described previously.²⁶

The mean of the real or in-phase component of respiratory system impedance over the frequency range was then computed to provide a single value representation of Rrs. Rrs was measured at 3, 7, 10, 15, 20, and 30 minutes after each Ag challenge and at 1 and 3 minutes after each methacholine challenge.

Airway responsiveness

Airway responsiveness was assessed by determining the concentration of nebulized and inhaled methacholine that

*Note that the multiple Ag inhalation protocol differs from the interim "week 2" reported in reference 11 in two ways: (1) airway responsiveness was measured 3 days after the last Ag challenge in this study versus 4 to 5 days after challenge in reference 11 (see reference 34 for consequence of this difference) and (2) the animals in this study were better characterized and controlled; all animals had been found to have a reproducible acute bronchoconstriction to Ag, and "threshold" Ag concentrations were selected for each animal individually.

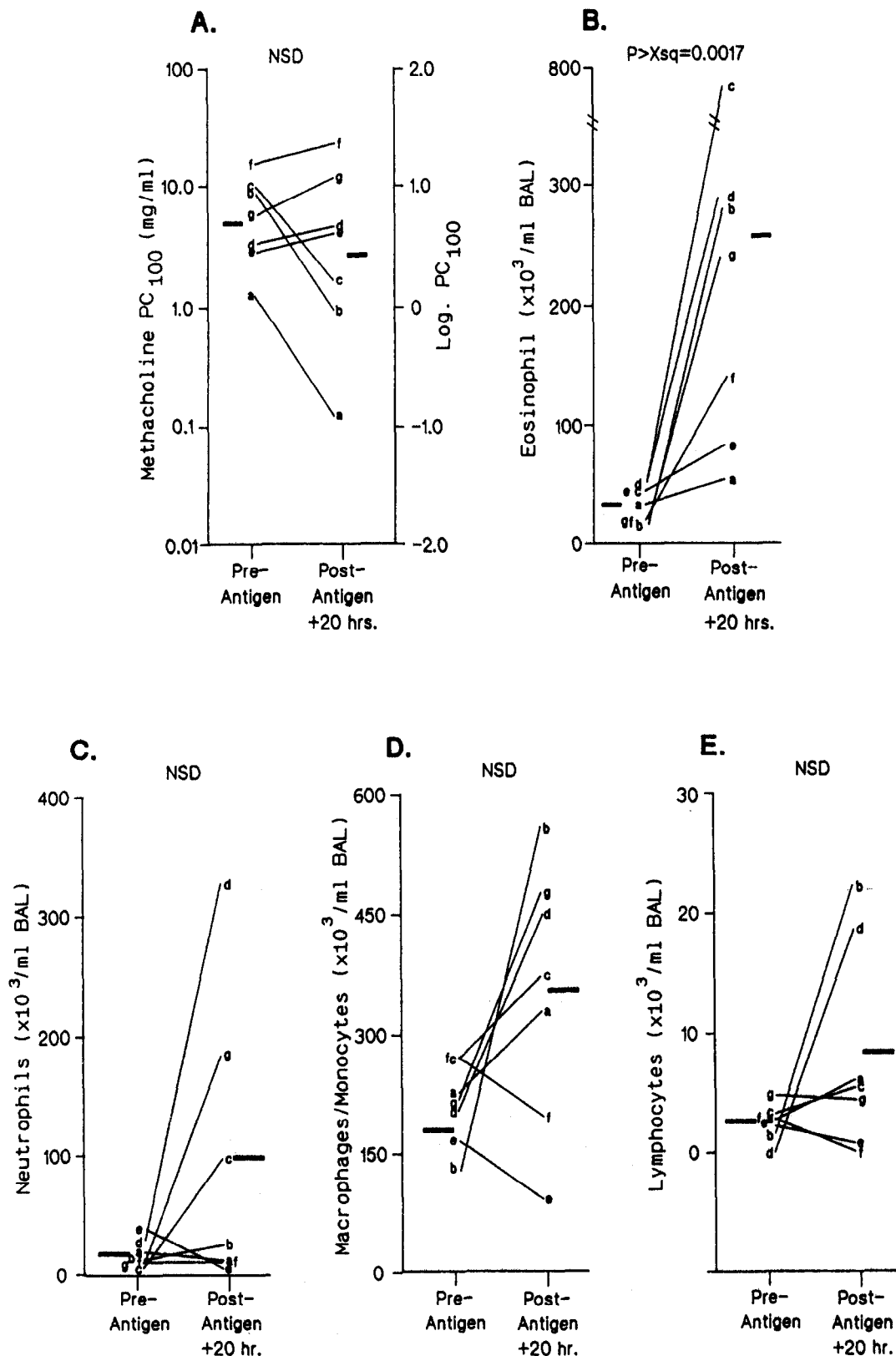


FIG. 1. Single inhalation of Ag-induced changes. **A.** In inhaled methacholine PC₁₀₀. **B.** In BAL EOs. **C.** In BAL neutrophils. **D.** In BAL macrophages/monocytes. **E.** In BAL lymphocytes. *Xsq*, Kruskal-Wallis test (chi-square approximation); *NSD*, no significant difference. Each letter represents an individual monkey.

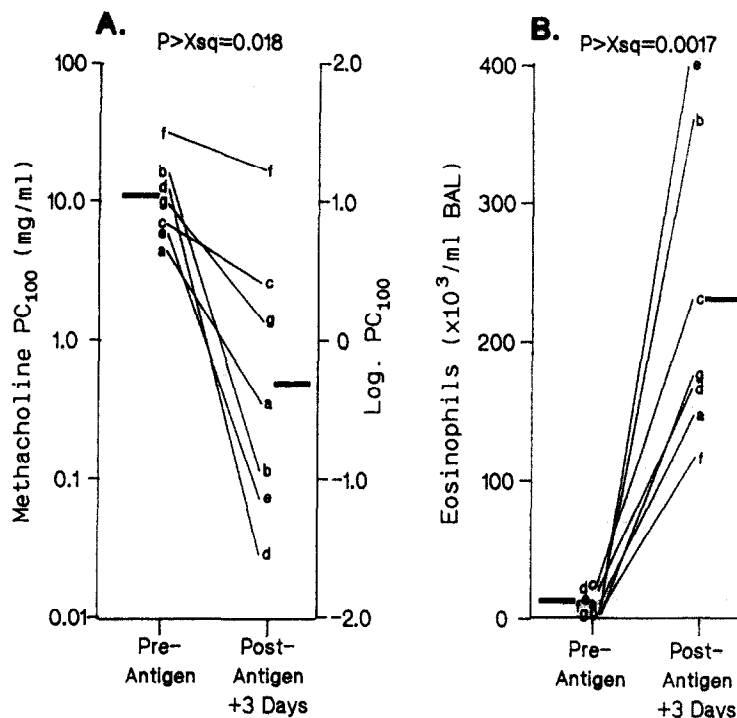


FIG. 2. Multiple inhalations of Ag-induced changes. **A.** In inhaled methacholine PC_{100} . **B.** In BAL EOs. Xsq , Kruskal-Wallis test (chi-square approximation). Each letter represents an individual monkey.

induced a 100% increase in Rrs (PC_{100}). This was accomplished by administering increasing concentrations of methacholine (diluted with phosphate-buffered saline) in half-logarithmic steps (at 7-minute intervals) until a >100% increase in Rrs from baseline was obtained. The PC_{100} was then calculated by linear regression analysis of the last 2 or 3 points on the logarithm methacholine concentration versus percent increase in Rrs plot. A change of more than twofold in the PC_{100} was regarded as significant, although a reproducibility of $\pm 50\%$ between multiple measurements has been found for this measurement in our laboratory.¹¹

BAL

Airway cell composition was assayed by BAL. A pediatric fiberoptic bronchoscope (model BF-3C4, Olympus Corp., Lake Success, N. Y.) was guided past the carina until it wedged into, typically, a fifth to seventh generation bronchus. A single 15 ml aliquot of bicarbonate-buffered (0.5 mmol/L) normal saline (pH 7.4) was then infused and gently aspirated (return volume ranged from 7 to 10 ml) through a channel in the bronchoscope. Total leukocytes per milliliter of BAL was determined with a Coulter counter (model Z₈₁, Coulter Electronics, Hialeah, Fla.) Differential cell counts (a total of 200 cells counted) were performed on Wright-Giemsa-stained cytocentrifuge (Cytospin, model 2, Shandon, Oakland, Calif.) preparations. To prevent the effects of a prior BAL on subsequent BAL determinations,

the BALs performed before and after Ag challenge were done on opposite sides of the lungs.

Statistics

Analysis of variance was performed with the nonparametric Kruskal-Wallis test (chi-square approximation). Correlations were performed with Pearson's product-moment and Spearman's ranks of the variables. A p value of > 0.05 was considered not significant.

RESULTS*

The single inhalation of Ag caused an acute increase in Rrs ($307\% \pm 62\%$) that peaked 10 to 15 minutes after challenge, an increase in airway leukocytes (267 ± 19 to $694 \pm 142 \times 10^3$ /ml of BAL; $p > \chi^2 = 0.018$), and a decrease in methacholine PC_{100} in three animals that was moderate (more than eightfold) in two animals (Fig. 1, A). The increase in airway leukocytes consisted of an infiltration of EOs in all animals (Fig. 1, B), neutrophils in three animals (Fig. 1, C), macrophages/monocytes in five animals (Fig. 1, D), and lymphocytes in four animals (Fig. 1, E). The magnitude and direction of the change in airway responsiveness (methacholine PC_{100}) did not

*All values are mean $\pm$ SE.

TABLE I. Comparison of change in methacholine PC₁₀₀ to the bronchoconstriction and leukocyte infiltration induced by a single inhalation of Ag*

Animal	Change in log PC ₁₀₀	Increase in Rrs (%)	Leukocyte Infiltration (× 10 ³ /ml BAL)			
			EO	Neut	Mac/Mono	Lympho
a	-1.02	494	23	297	105	3.3
b	-0.95	278	266	-33	421	20.5
c	-0.75	181	668	87	104	2.7
d	0.16	244	242	6	253	18.6
e	0.21	525	38	-6	-73	-1.6
f	0.24	66	124	-2	-78	-3.0
g	0.36	360	221	178	264	-0.4
Mean	-0.25	307	226	76	142	5.8
SE	± 0.24	± 63	± 82	± 46	± 69	± 3.7

Neut, Neutrophils; Mac/Mono, macrophages/monocytes; lympho, lymphocytes.

*No significant correlations were found.

TABLE II. Comparison of change in methacholine PC₁₀₀ to EO infiltration induced by multiple inhalations of Ag to the change in methacholine PC₁₀₀ induced by single inhalation of Ag*

Animal	Multiple inhalations of Ag		Single inhalation of Ag
	Change in log PC ₁₀₀	EO infiltration (× 10 ³ /ml BAL)	Change in log PC ₁₀₀
d	-2.66	144	0.16
b	-2.13	352	-0.95
e	-1.95	409	0.21
a	-1.07	135	-1.02
g	-0.97	174	0.36
c	-0.43	206	-0.75
f	-0.27	109	0.24
Mean	-1.36	218	-0.25
SE	± 0.34	± 44	± 0.24

*No significant correlations were found.

correlate with the intensity of the acute increase in Rrs or with the magnitude/existence of the infiltration of any leukocyte subtype (Table I).

The multiple inhalations of antigen caused acute increases in Rrs (178% ± 48%, 380% ± 83%, and 331% ± 63%, respectively), an increase in airway leukocytes (209 ± 42 to 553 ± 129 × 10³/ml of BAL; $p > \chi^2 = 0.0088$), and a decrease in methacholine PC₁₀₀ in all seven animals that was moderate (more than eightfold) in two and severe (>80-fold) in three animals (Fig. 2, A). The increase in airway leukocytes consisted of a marked infiltration of EOs (Fig. 2, B) and slight infiltration of neutrophils (8.5 ± 2.6 to 31.0 ± 7.5 × 10³/ml of BAL; $p > \chi^2 = 0.010$). There was no significant infiltration of macrophages/monocytes (179 ± 36 to 209 ± 40 ×

10³/ml of BAL) or lymphocytes (3.0 ± 1.2 to 3.0 ± 1.3 × 10³/ml of BAL). The magnitude of the increase in airway reactivity (decrease in PC₁₀₀) did not correlate with the intensity of the EO infiltration or with the magnitude/direction of the change in airway reactivity (PC₁₀₀) induced by the single inhalation of Ag (Table II).

In both protocols, the value of the baseline Rrs at the time of determination of airway responsiveness was not altered by the prior Ag inhalation(s).

DISCUSSION

Nonspecific airway hyperresponsiveness, as assessed clinically by responsiveness of the airways to inhaled histamine, methacholine, exercise, or cold air, is a characteristic feature of asthma.^{1, 2} Although

the mechanisms underlying the pathogenesis of airway hyperresponsiveness are not known, results from many studies suggest that leukocyte infiltration^{10, 11, 13, 20-23, 26} and/or mediators released by resident or infiltrating cells^{12, 20, 21, 23, 27-29} are involved. We have previously reported that in monkeys (1) Ag inhalation induces an acute neutrophil and more chronic EO airway infiltration,²⁴ (2) a chronic idiopathic airway eosinophilia is associated with severe (>80-fold) airway hyperresponsiveness,³⁰ and (3) multiple (four weekly) intratracheal instillations of Ag-coated beads¹⁰ or ≥ 4 weeks of Ag inhalations¹¹ induce marked increases in airway EOs and responsiveness (more than or equal to tenfold). To obtain an acute protocol for studying the mechanisms/mediators involved in Ag-induced airway hyperresponsiveness, we compared the effects of a single versus multiple (three alternate-day) inhalations of *A. suum* extract on airway responsiveness and leukocyte composition in "allergic" monkeys.

The single inhalation of Ag resulted in an acute bronchoconstriction and, as measured 20 hours later, an infiltration of leukocytes (primarily and most consistently EOs), as well as an increase in airway responsiveness (decrease in inhaled methacholine PC₁₀₀) in three of seven animals that was moderate (more than eightfold) in two animals. These effects, as well as the frequency with which they occurred, are similar to effects reported for man; that is, in allergic subjects with asthma, a single inhalation of Ag induces a predominately eosinophilic leukocytic infiltration²⁶ and an increase (usually mild, less than eightfold) in airway responsiveness in some individuals.^{8, 9}

In contrast, the multiple inhalations of Ag induced, as assayed 3 days after the last challenge, an increase in airway responsiveness in six of the seven monkeys that was moderate (more than eightfold) in two and severe (>80-fold) in three animals. These effects are also consistent with effects reported for man. During the pollen season, the airway responsiveness of allergic subjects with asthma has been reported to increase in all individuals, although the increase is in varying degrees.^{3, 4} Similarly, repeated exposure to occupational allergens has been demonstrated to increase airway responsiveness in sensitive individuals.^{4, 18, 19}

In both the single and multiple inhalation of Ag protocols, the magnitude/direction of change in airway responsiveness did not correlate with the intensity of the leukocyte infiltration. This finding conflicts with our previous studies^{10, 11, 29} and emphasizes that leukocyte infiltration alone is not enough to infer their direct involvement in the pathogenesis of airway hyperreactivity. Rather, the duration of the infiltrate, the

amount (degree of activation) and type (stimulator or inhibitor) of mediators released by the infiltrating and resident cells, as well as the interactions occurring between the infiltrating and resident cells (e.g., EOs and airway epithelium), must be evaluated.

Of interest in this study was the lack of a correlation between the magnitude of the increase in airway responsiveness induced by the multiple Ag inhalations and the magnitude/direction of change in airway responsiveness induced by the single Ag inhalation. This observation suggests that different mechanisms may be involved. Possibly the occasional mild increases in airway responsiveness after a single Ag exposure are caused by acute nerve-ending sensitization²⁸ and/or edematous airway-wall thickening,³⁰ whereas the consistent and usually moderate to severe increases in airway responsiveness after multiple Ag exposures are caused by these factors plus damage/denudation of airway epithelium^{23, 32} and/or nonedematous (tissue hypertrophy elicited) airway-wall thickening.³³ In any case, the similarities between effects reported in this study and effects reported by other investigators in subjects with asthma suggest that this animal model provides an excellent system to evaluate such questions.

In summary, as has been previously reported for subjects with asthma, we found that multiple (but not single) inhalations of Ag induce an increase (usually more than eightfold) in airway responsiveness in most monkeys. These findings indicate that this animal model may be useful and have added advantages for investigations into the mechanisms involved in the pathogenesis of airway hyperreactivity and asthma. In addition, these results emphasize, once again, that multiple allergen exposures can induce or enhance airway hyperresponsiveness. Thus, avoidance of chronic exposure to allergens is important in reducing asthma symptoms.

We thank Mr. John P. Sabo for his assistance with the statistical analysis of the results.

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