

ATTENUATED METHACHOLINE AIRWAY RESPONSE FOLLOWING REPEAT TESTING IN A MURINE MODEL OF ALLERGIC AIRWAYS DISEASE

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□ *A progressive attenuation of airway reactivity to methacholine is observed in normal individuals with successive bronchial provocation testing. The absence of this attenuation in asthma is thought to be due airway inflammation. The authors investigated this phenomenon in a mouse model of allergic airways disease. Repeated measurements of airway response were carried out in mice sensitized/challenged with ovalbumin or saline, and in untreated mice. Saline-treated and untreated mice showed reduced airway reactivity following repeated testing. This was also observed in ovalbumin-treated mice in the second and third tests compared to the previous test ($P < .05$). This attenuation was not associated with airway inflammation, which remained high in the ovalbumin group. The results suggest that attenuation of airway reactivity with repeated methacholine challenge is due to factors other than airway inflammation.*

Keywords airway reactivity, attenuation, asthma, Balb/c, lung, mice, methacholine, Penh, tachyphylaxis, tolerance

Exposure of the airways to methacholine (MCh) causes airway smooth muscle to contract, resulting in airway narrowing and elevated airway resistance [1]. The extent to which the airways respond to MCh provocation is known as airway reactivity. The level of MCh-induced airway reactivity exhibited is used as a predictor of general airway health and is frequently used to assess and monitor the severity of airway inflammatory diseases, such as asthma.

Interestingly, in healthy individuals, repeated bronchial provocation testing results in a progressive attenuation of MCh-induced airway reactivity with each successive test [2–5]. In contrast, in patients with asthma, the attenuation of airway reactivity is not observed with repeated testing [4–7]. The precise mechanisms contributing to this lack of attenuation of

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airway responses in asthmatic patients remain unclear. Several studies have suggested that this lack of attenuation relates directly to the presence of airway inflammation in patients with asthma [1, 8–10]; however, this is yet to be fully investigated.

A significant number of studies examining allergic airways disease (AADs) utilize murine models and it would be an advantage to make serial measurements of airway reactivity in individual mice. However, the validity of taking this approach remains uncertain due to the potential impact of attenuated airway responses with repeated testing. We hypothesized that airway reactivity would be attenuated in mice following repeated testing and that this is associated with altered airway inflammation. This study applies a murine model to directly examine the role of allergen-induced airway inflammation in preventing attenuation of airway reactivity following repeated measurements in AAD. In addition, this study examines the effect of repeated testing of airway reactivity in the mouse model.

METHODS

Mice

Six-week-old female Balb/c mice were obtained from the Walter and Eliza Hall Institute (Melbourne, Australia). All mice were maintained in a standard animal care facility (Department of Agriculture, University of Melbourne, Australia) and all procedures were approved by the Royal Children's Hospital Animal Ethics Committee.

Ovalbumin-Induced Allergic Airways Disease Model

An ovalbumin (OVA)-induced AAD model of asthma was used [11]. Briefly, 6-week-old mice were sensitized by intraperitoneal (IP) injection on day 0 with 10 μ g Grade V OVA (Sigma Chemical, St. Louis, MO) and 1 mg aluminium potassium sulfate (alum) (Sigma Chemical) in 500 μ L of saline, and challenged with nebulized OVA 1% in phosphate-buffered saline (PBS) on days 14 to 20 ($n = 12$). Saline control mice were injected IP with 500 μ L of saline and challenged with PBS ($n = 12$). Aerosol challenges were carried out on groups of up to 4 mice in a closed-system chamber (24 $\times$ 37.5 $\times$ 15 cm) attached to an ultrasonic nebuliser (NE-UO7; Omron Corporation, Tokyo, Japan) with an output of 1 mL/min and 1- to 5- μ m particle size. Naïve untreated mice were included as an additional control ($n = 12$). In this model of AAD, mice develop increased immunoglobulin E (IgE) and airway inflammation [12–14].

Measurement of Airway Response

Methacholine-induced airway reactivity was measured using an unrestrained whole-body plethysmograph (Buxco Electronics, Troy, NY) [15, 16]. Airway response is expressed as an enhanced pause (Penh). The algorithm for Penh compares the mean amplitude of the early part of the expiratory phase to the mean amplitude of the later part of the expiratory phase as a function of the proportion of maximal expiratory and inspiratory box pressure. In brief, the Bio-system was first calibrated with a rapid injection of 1 mL of air into the mouse chambers. Mice were then placed in individual chambers and allowed to settle for approximately 10 to 15 minutes, after which a baseline Penh reading was taken. Following the baseline reading, the mice were exposed to increasing concentrations of aerosolized MCh (3.125, 6.25, 12.5, 25, 50, and 100 mg/mL in PBS) for a period of 2 minutes each. After each MCh exposure Penh values were recorded at 20-second intervals and the mean calculated over 5 minutes to give a single Penh value for each MCh dose.

Methacholine-induced airway reactivity was measured every 24 hours over 4 days (21 to 24) to investigate attenuation of responses in OVA-sensitized/challenged, saline-sensitized/challenged, and untreated mice. A group of mice sensitized/challenged to OVA that were tested for MCh-induced airway reactivity on 1 day only (day 21) served as an additional control.

Bronchoalveolar Lavage (BAL)

BAL was performed 1 day after the final MCh challenge (day 24 in mice receiving 4 tests, day 21 in mice receiving only a single test). The BAL consisted of 3 1-mL lavages with cold PBS containing 10% fetal calf serum. The BAL cells were washed in PBS and resuspended in 1 mL PBS containing 5% fetal calf serum. Total BAL cell counts were determined manually by trypan blue exclusion. Differential counts of eosinophils, neutrophils, lymphocytes, and monocytes were determined on cytospin smears of BAL samples (4×10^5 cells) stained with DiffQuik (Life Technologies, Auckland, New Zealand).

Statistical Analysis

Data are expressed as mean $\pm$ SEM. One-way analysis of variance was used to test for significance in comparisons between groups in BAL cell count. Two-way analysis of variance was used to test for the influence of repeated airway response testing on MCh dose-response curves. In addition, significant differences in airway response at individual MCh doses were assessed using a Bonferroni post-test. A 95% confidence interval was

used. Prism GraphPad software (San Diego, CA) was used for all statistical analyses.

RESULTS

Attenuation of Airway Reactivity With Repeated Measurements

Repeated measurements of MCh-induced airway reactivity were performed over 4 consecutive days in OVA-sensitized/challenged, saline-sensitized/challenged, and untreated mice to examine for attenuation of airway response. At the first measurement, the OVA-sensitized/challenged mice demonstrated significantly increased airway reactivity as compared to both saline-sensitized/challenge mice ($P < .001$) and the untreated mice ($P < .001$). Comparisons at individual doses of MCh revealed significantly increased airway reactivity in OVA-sensitized/challenged mice as compared to the saline-sensitized/challenge mice ($P < .05$) and the untreated mice ($P < .05$) at the higher MCh doses of ≥ 25 mg/mL (Figure 1).

With repeated testing of MCh-induced airway reactivity, saline-sensitized/challenged mice and untreated control mice both exhibited decreased airway reactivity with each successive measurement (Figure 2A and B). In saline-sensitized/challenged mice, MCh-induced airway reactivity was significantly reduced on each subsequent day of testing ($P < .05$ for day 1 versus 2, 2 versus 3, 3 versus 4). In untreated mice, MCh-induced airway reactivity was significantly lower after the second test as compared to

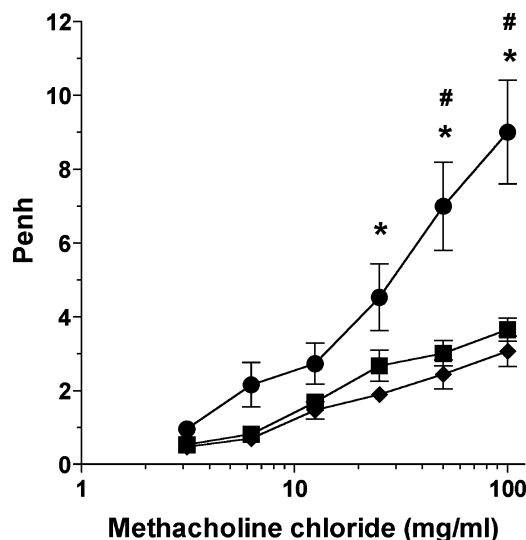


FIGURE 1 MCh-induced airway reactivity (Penh) in Balb/c mice. OVA-sensitized/challenged (●), saline-sensitized/challenged (■), and untreated (◆) mice. * $P < .05$ versus saline-sensitized/challenged mice. # $P < .05$ versus untreated control mice. $n = 12$ mice/group.

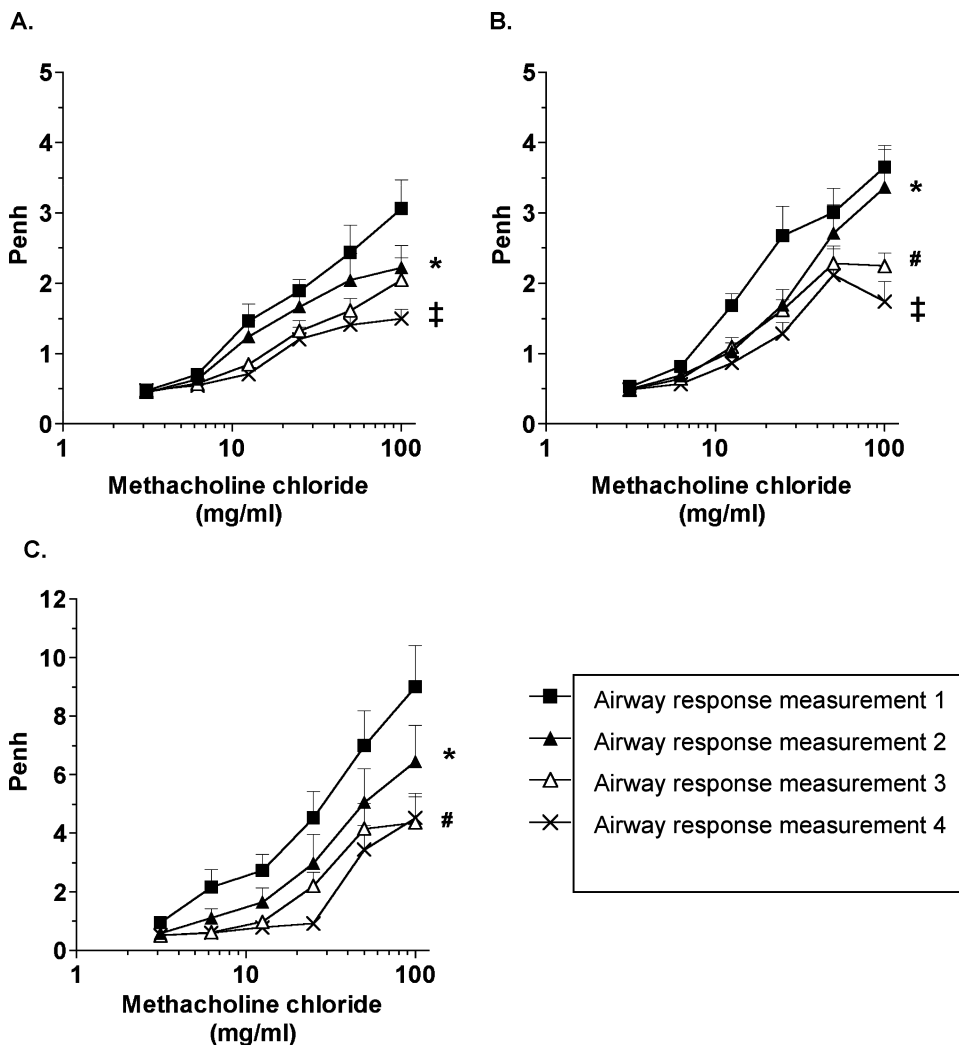


FIGURE 2 Effect of repeated testing on MCh-induced airway reactivity in Balb/c mice. (A) Untreated control mice; (B) mice sensitized/challenged with saline; (C) mice sensitized/challenged with OVA. * $P < .05$ for comparison of MCh dose-response curve after 2 tests versus 1 test. # $P < .05$ for comparison of MCh dose-response curve after 3 tests versus 2 tests. † $P < .05$ for comparison of MCh dose-response curve after 4 tests versus 3 tests. $n = 12$ mice/group.

the first ($P < .05$) and after the fourth test as compared to the third ($P < .05$). Surprisingly, despite the presence airway reactivity, the OVA-sensitized/challenged mice similarly demonstrated decreased airway reactivity following repeated testing (Figure 2C). Methacholine-induced airway reactivity in OVA-sensitized/challenged mice after 2 tests was significantly lower than that seen after 1 test ($P < .05$); and significantly lower after 3 tests as compared to 2 tests ($P < .05$) (Figure 2C). All 3 groups of mice exhibited similar degrees of attenuation in their airway responses at the higher

TABLE 1 Attenuation of Airway Response to MCh Following Repeated Measurements in Untreated, Saline-Sensitized/Challenged, and OVA-Sensitized/Challenged Mice

		MCh (mg/mL)						
Test number		0	3.125	6.25	12.5	25	50	100
Untreated	1	0	0	0	0	0	0	0
	2	7.5	5.1*	10.2*	9.5*	12.1	18.0	24.5
	3	3.9	2.9*	13.0*	28.4	27.7	27.9	32.8
	4	2.0	-0.8*	20.3*	44.1	35.5	34.5	47.4
SAL	1	0	0	0	0	0	0	0
	2	-0.8	6.0*	11.6*	36.1	30.9	1.0	4.3
	3	5.6	7.8*	17.9*	31.6	27.5	21.9	34.4
	4	2.5	0.4*	16.0*	9.8*	10.6*	24.3	48.2
OVA	1	0	0	0	0	0	0	0
	2	4.2	34.4	43.0	37.0	36.1	9.3	4.3
	3	2.1	43.4	59.6	55.5	40.0	34.0	41.4
	4	-3.0	41.8	59.9	61.5	73.7	44.4	38.3

Note. Data represent the percentage decrease from the first measurement of MCh-induced airway response. * $P < .05$ Comparison to OVA-sensitized/challenged mice for the same test number and MCh dose.

doses of MCh (≥ 50 mg/mL) (Table 1). At the lower doses of MCh (3.125 and 6.25 mg/mL), the OVA mice showed greater attenuation in comparison to the saline-sensitized/challenged ($P < .05$) and the untreated ($P < .05$) mice (Table 1).

Effect of Airway Inflammation on Attenuation of Airway Reactivity Following Repeated Measurements

In order to determine if there is a direct relationship between airway inflammation and attenuation of airway reactivity, the relationship between airway inflammation and attenuation of airway reactivity was examined in OVA-sensitized/challenged, saline-sensitized/challenged, and untreated mice. The presence of airway inflammation was determined by the measurement of BAL total and differential cell counts. As expected, the OVA-sensitized/challenged mice demonstrated marked airway inflammation compared to saline-sensitized/challenged mice and untreated mice. The total BAL cell counts in OVA-sensitized/challenged mice ($9.2 \times 10^5 \pm 1.6 \times 10^5$) were significantly greater than BAL cell counts in saline-sensitized/challenged mice ($3.1 \times 10^4 \pm 5.1 \times 10^3$; $P < .05$) and the untreated control group ($2.7 \times 10^4 \pm 3.9 \times 10^3$; $P < .05$). The BAL cells in OVA-sensitized/challenged mice were predominantly eosinophils as compared to a largely macrophage dominant population in the saline-sensitized/challenged and untreated groups (Figure 3).

BAL cell counts were then examined in OVA-sensitized/challenged mice that had only received a single MCh-induced airway reactivity test. No significant differences were observed in BAL total or differential cell

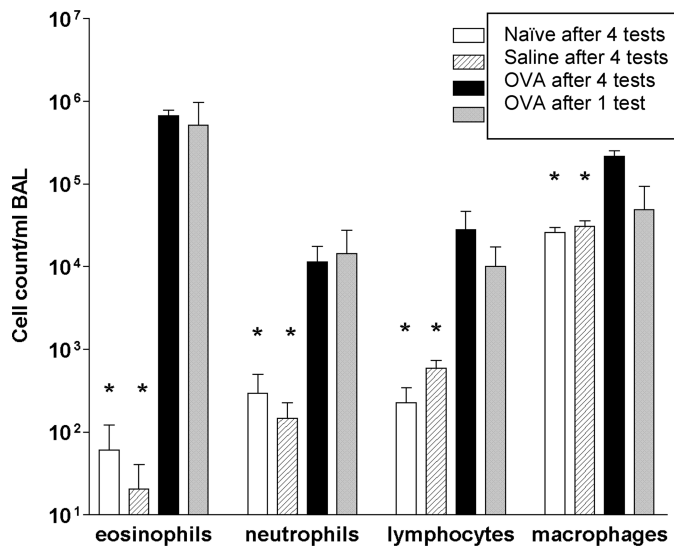


FIGURE 3 BAL differential cell counts following repeated MCh-induced airway reactivity measurements over 4 consecutive days. OVA-sensitized/challenged mice were exposed to a single MCh test or 4 MCh tests over 4 consecutive days. Saline-sensitized/challenged and untreated mice received 4 MCh tests over 4 consecutive days. * $P < .05$ as compared to OVA after 4 treatments. $n = 12$ mice/group.

counts between the OVA-sensitized/challenged mice experiencing multiple airway reactivity tests and a group of OVA-sensitized/challenged mice receiving only a single airway reactivity measurement (Figure 3). Therefore, multiple airway response testing was associated with a progressive reduction in airway reactivity, in the absence of any change in airway inflammation.

DISCUSSION

Previous studies have suggested important roles for several key components of airway inflammation in preventing the development of attenuated airway reactivity with repeated testing in AAD [1, 8, 9, 17, 18]. Eosinophil major basic protein (MBP) [1, 10, 19], transforming growth factor $\beta 1$ (TGF- $\beta 1$) [8], and interferon γ [20] have all been shown to inhibit the negative-feedback processes within the lung that allow for attenuation of MCh response [10, 19, 21]. In this study, however, the OVA-sensitized/challenged mice demonstrated strong attenuation of airway reactivity with repeated testing despite the presence of marked airway inflammation. These findings indicate that attenuation of airway reactivity is not solely regulated by the presence or absence of airway inflammation and that the mere presence of airway inflammation alone is not sufficient to prevent the attenuation of airway reactivity with repeated testing. Furthermore, total and differential BAL cell counts were equivalent in OVA-sensitized/challenged mice that received multiple airway reactivity tests and OVA-sensitized/challenged

mice that only received a single test. This demonstrates that changes in airway responsiveness can occur independently of changes in airway inflammation. Taken together, these findings also suggest that the failure of patients with AAD to develop attenuated airway responsiveness with repeated measurements of MCh-induced airway reactivity does not solely relate to the presence of allergic airway inflammation *per se*. Other mechanisms present in humans with asthma but not mice may also be playing a role.

The observation that repeated testing does not affect airway inflammatory cell counts suggests that other mechanisms may be responsible for attenuation. In MCh challenge, contraction of airway smooth muscle is mediated by acetylcholine-induced activation of the M3 muscarinic receptors [22, 23]. Airway inflammation measured by eosinophils was not changed when MCh response was decreased with repeated challenges. Murine models of AAD exhibit poor eosinophil degranulation compared to human asthma. These mouse models also have low levels of extracellular major basic protein compared to humans [24, 25]. Major basic protein leads to M2 muscarinic dysfunction, increased acetylcholine levels, and increased M3 muscarinic receptor activity [19, 26–28]. Differences in major basic protein levels in humans and mouse models may contribute to disparities in attenuated bronchial response although additional studies in this area are required.

Mice sensitized and challenged with OVA demonstrate many of the inflammatory characteristics associated with human asthma, with increased airway reactivity, eosinophilic infiltrate in the BAL fluid, and elevated serum IgE levels. However, in contrast to human asthma, a condition in which airway responsiveness remains elevated despite repeated measurements [4–7], the OVA-sensitized/challenged mice demonstrated strong attenuation of MCh-induced airway reactivity with repeated measurements. Attenuation was also demonstrated in saline-sensitized/challenged and untreated mice, with all 3 groups of mice exhibiting similar degrees of attenuation with repeated testing. All 3 groups of mice responded in a similar manner to normal individuals [2–5]. Hence, although the murine model shares many of the characteristic hallmarks of asthma, there is a clear distinction between human and murine airway responses to repeated MCh provocation.

In our mouse model of allergic airways disease, we used Penh to measure airway response to methacholine. Penh is a measure of respiratory effort, which can be used without anesthesia with unrestrained mice. Bronchoconstrictors such as MCh cause increases in Penh, and this is higher in OVA-challenged animals [15, 16, 29]. Significant limitations of the technique have been identified theoretically and experimentally by a number of groups [30–33]. However, we and others have shown a good correlation between Penh and invasive measures of airway resistance [34, 35]. These

invasive techniques, although the gold standard, are not practicable for repeat testing.

In summary, our findings support the concept that airway inflammation and airway reactivity can be influenced independently. We have demonstrated that a commonly used mouse model of asthma has attenuation of airway reactivity that is not associated with inflammation. Furthermore, our findings highlight a potential pitfall of murine studies involving repeated measurements of MCh-induced airway reactivity. Unlike human asthma, consideration must be given to the potential impact of airway response attenuation with repeated measurements of MCh-induced airway reactivity in studies using murine models of AAD.

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