

INHIBITION OF MUCIN SECRETION WITH MARCKS RELATED PEPTIDE IMPROVES AIRWAY OBSTRUCTION IN A MOUSE MODEL OF ASTHMA

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ABSTRACT

Allergic asthma is associated with airway epithelial cell mucous metaplasia and mucin hypersecretion, but the consequences of mucin hypersecretion on airway function are unclear. Recently, a peptide derived from the MARCKS protein N-terminal sequence (MANS) was shown to inhibit methacholine (MCh) induced mucin secretion from airway mucous cells by more than 90%. We studied the effect of intranasal pre-treatment with this peptide on specific airway conductance (SGaw) during challenge with MCh in mice with allergen-induced mucous cell metaplasia. SGaw was non-invasively measured in spontaneously breathing restrained mice, using a double chamber plethysmograph. Pre-treatment with MANS peptide, but not a control peptide (RNS), resulted in partial inhibition of the fall in SGaw induced by 60 mM MCh (mean $\pm$ SE; baseline 1.15 ± 0.06 ; MANS/MCh 0.82 ± 0.05 ; RNS/MCh 0.55 ± 0.05 (cm H₂O·s)⁻¹). The protective effect of MANS was also seen in mice challenged with allergen for 3 consecutive days to increase airway hyperresponsiveness (AHR), though the degree of protection was less (baseline 1.1 ± 0.08 ; MANS/MCh, 0.65 ± 0.06 ; RNS/MCh 0.47 ± 0.03). Since routine SGaw measurement in mice includes nasal airways, the effectiveness of MANS was also confirmed in mice breathing through their mouths after nasal occlusion (baseline 0.92 ± 0.05 ; MANS/MCh 0.83 ± 0.06 ; RNS/MCh 0.61 ± 0.03). In all instances, SGaw in the MANS-pretreated group was about 35% higher than in RNS treated controls, and mucous obstruction accounted for about 50% of the MCh-induced fall in SGaw. In summary, mucin secretion has a significant role in airway obstruction in a mouse

model of allergic asthma, and strategies to inhibit mucin secretion merit further investigation.

INTRODUCTION

Mucins, the major glycoconjugates of mucus, are produced by airway epithelium in mammals. Mucus entraps inhaled pathogens and particles, and the mucus layer serves as a first line of defense of the lungs against foreign materials (16). In many chronic inflammatory diseases of the airways including asthma, cystic fibrosis and chronic obstructive pulmonary disease (COPD), excessive mucus is produced that may lead to anatomic airway obstruction (14;17). Expectoration of viscous mucus plugs and bronchial casts is commonly seen in patients with asthma, and extensive mucus plugging has been reported in cases of fatal asthma that failed to respond to conventional therapy (10;13;17). While the pathophysiologic role of mucus obstruction seems intuitively apparent, this hypothesis has neither been formally tested, nor has the contribution of airway luminal mucus to pulmonary airflow obstruction been quantified. Previous studies have been limited to reversing bronchoconstriction in the presence of mucin discharge (9), but this approach is inexact and does not separate the effects of airway wall edema and other factors besides mucin release that lead to luminal narrowing.

An alternative approach to dissecting the pathophysiologic role of mucus in airflow obstruction is selective inhibition of mucin secretion. This was not possible until recently, when a twenty-four amino acid peptide (MARCKS N-terminus sequence, MANS) corresponding to the N-terminal domain of the myristoylated alanine-rich C-kinase substrate (MARCKS) protein was found to inhibit mucin release from mouse airway epithelial cells by 90% (18). We used the MANS

peptide to selectively block MCh-induced mucin hypersecretion in mice with allergen induced airway epithelial cell mucous metaplasia. This allowed us to quantitatively resolve the contribution of mucin hypersecretion to pulmonary airflow obstruction and test the possible efficacy of specific inhibition of mucin secretion by the MANS peptide as a therapeutic approach in allergic asthma.

METHODS

This study was conducted in conformity with APS's *Guiding Principles in the Care and Use of Animals*.

Induction of airway mucous metaplasia

Six to eight week old BALB/c mice between 16 and 20 g were purchased from the Center of Cellular and Molecular Biology, India, and housed in accordance with institutional guidelines. To induce mucous metaplasia, mice were sensitized to ovalbumin (20 µg ovalbumin Grade V, 2.25 mg alum in saline, pH 7.4; Sigma, St. Louis, MO) administered by weekly intraperitoneal injection for four weeks. Sensitized mice were then challenged by exposure for 30 min to an aerosol of either 0.9% (wt/vol) saline (Ova / Sal) or 2.5% (wt/vol) ovalbumin in 0.9% saline (Ova / Ova) as described (4). Twenty mice were subjected to daily ovalbumin challenge for three days (Ova / Ova3) to increase airway inflammation and AHR. Experiments were conducted seven days after challenge because intracellular mucin reaches a maximum at that time (4).

Quantification of airflow obstruction

Specific airway conductance (SGaw) was measured as the time lag between flows recorded from the head and thoracoabdominal chambers in consciously breathing restrained mice, using the two-chambered body plethysmograph system devised by Buxco, USA (PLY-3351, Biosystem XA software) (5;15). Briefly, this equipment consists of two plastic cylinders used as the thoracoabdominal and head compartments that can be attached to one another with an orifice such that the head and neck of a mouse can pass through but not its shoulders. Mice were acclimatized to being restrained in the plethysmograph during the sensitization and challenge phase. To measure SGaw, mice were placed in the thoracoabdominal chamber, maneuvered to pass their head through the orifice, and gently wedged between the orifice and a piston posteriorly. Prior to attaching the head chamber, a suitably cut latex sheet was passed snugly over the neck and interposed between the two chambers to ensure that the chambers would be airtight. Phase lag between the airflow signals recorded from the two chambers was approximated using the time lag at zero flow and SGaw was calculated (5; 15). This technique has been extensively used in guinea pigs (15) and more recently mice (5). It is very sensitive to changes in airflow resistance in upper and central airways, but insensitive to peripheral and tissue resistance changes.

Experimental protocol

Following the sensitization, challenge, and an additional 7 days to allow for epithelial cell mucous metaplasia, baseline SGaw was recorded for 10 min. The head chamber was then removed and each mouse was administered 50 μ l of either 100 μ M MANS or a control Random N-terminal Sequence (RNS) peptide as small volumes into each nostril alternately over 2 min. After 15 min either a dose-response or a single challenge experiment was performed as follows: To obtain a dose-response curve, stepwise increasing concentrations of MCh (0 to 200 mM solution in normal saline (NS) were administered during 1.5 min aerosolizations using an aerogen micro-pump nebulizer (Buxco) connected to the plethysmograph, and were followed by measurement of SGaw for 3 minutes. In single challenge experiments, a single 3 min MCh aerosol challenge (60 mM solution in NS) was administered to mice pretreated with MANS or RNS, with/without an additional 3 min ATP aerosol challenge (100 mM solution in NS) immediately afterwards. This was followed by continuous measurement of SGaw. Stable SGaw values at the end of 3 min windows were recorded for analysis. In some experiments the nostrils of mice were occluded with adhesive tape prior to a single MCh challenge, thereby eliminating the effect of changes in nasal resistance on measured SGaw.

Histological analysis of mucus secretion

At the end of the experiment, mice were sacrificed and intracellular mucin remaining in the airways was assessed by routine and fluorescent PAS staining

(4). Serial sections of the main axial bronchus along with minor daughter branches that we refer to as penetrating bronchi were used for semi-quantitative scoring. We scored each airway from 1 to 4 using a retained mucin score as follows: 1 – near complete mucin secretion with many ghost cells (almost completely depleted of mucin granules creating an empty appearance with faint outlines, see Figure 1C) and most cells partially empty; 2 - semi-complete mucin secretion with many cells partially empty, few ghost cells, and few cells densely populated with mucin granules (full cells); 3 - limited mucin secretion with full cells and partially empty cells being equally predominant; 4 – minimal mucus secretion with mostly full cells. To eliminate bias, scoring was done while blinded to the treatment. Two to three sections were scored per lung, and specimens from at least 3 different experiments were used per group.

Analysis

Standard MCh dose response curves were plotted for Ova/Sal, Ova/Ova and Ova/Ova3 mice receiving MANS or RNS. SGaw values recorded 3 min after MCh challenge were averaged for mice receiving either MANS or RNS (SGaw_{MANS} ; SGaw_{RNS}) for each of the following groups: 1) Ova/Sal, 2) Ova/Ova, 3) Ova/Ova3, 4) Ova/Ova with nasal occlusion. To ensure temporal comparability of the MCh response, SGaw values recorded 3 min after MCh/ATP challenge were compared with those recorded 6 min after MCh challenge. All values were expressed as means $\pm$ SE. Statistical significance of differences between groups was determined with unpaired *t*-tests. The criterion for significance was set at $p <$

0.05. For each group, the MANS-induced % improvement in SGaw [$100 \times (\text{SGaw}_{\text{MANS}} - \text{SGaw}_{\text{RNS}}) / \text{SGaw}_{\text{RNS}}$] was calculated along with the fall in SGaw attributable to mucous obstruction expressed as a percentage of the total fall [$100 \times (\text{SGaw}_{\text{MANS}} - \text{SGaw}_{\text{RNS}}) / \text{SGaw}_{\text{baseline}} - \text{SGaw}_{\text{RNS}}$]. Since the values used to derive these ratios were sample means of small magnitudes, variance could not be reliably estimated, nor could differences be tested statistically. To compare mucin scores, the non-parametric Kruskal-Wallis test followed by post hoc Mann-Whitney test was used, because the limited scoring scale resulted in a non-normal distribution of data.

RESULTS

MANS therapy is safe. Intranasal administration of 50 μl MANS or RNS peptide solutions in 104 mice was associated with no ill effects other than a transient fall in SGaw with recovery to baseline during the 15 minute observation period.

Intranasal MANS inhibits mucus secretion from the tracheo-bronchial airways. To determine whether intranasal MANS is effective in inhibiting tracheo-bronchial mucin secretion induced by MCh and/or ATP, retained intracellular mucin in axial and penetrating airways of MANS or RNS pre-treated mice was compared.

Representative sections showing mucin secretion in response to MCh and/or ATP challenge after MANS or RNS treatment are shown in Figure 1. Fluorescent PAS staining was found to be superior to conventional PAS staining for assessment of retained mucin since the traditional method has greater

background staining, which limits visualization of mucin granules (4). Pre-treatment with MANS but not RNS, blocked mucin release. Almost all airways (10 of 11) from RNS-pretreated mice were scored as 1 and 9 of these had nearly complete mucin secretion with almost no retained mucin (Figure 1C). In contrast, 14 of 20 airways from MANS-pretreated mice were scored as 4, of which 8 had negligible mucus secretion (Figure 1D). Thus, mucin secretion was significantly inhibited in MANS pre-treated mice (Figure 1E, retained mucin score: MANS/MCh, 3.4 ± 0.2 ; RNS/MCh, 1.1 ± 0.08 ; $p < 0.05$).

MANS therapy inhibits airflow obstruction induced by escalating doses of MCh.

To determine whether inhibition of mucin secretion by MANS can attenuate MCh-induced airflow obstruction, we studied the effect of MANS pre-treatment on the MCh dose-response curve in mice. AHR to MCh was observed in both Ova/Ova and Ova/Ova3 mice compared to control (Figure 2). As expected, repeated allergen challenge increased AHR as seen by a leftward shift of the dose-response curve i.e. lower doses of MCh were required for similar degree of fall in SGaw. In mice with mucous metaplasia, pre-treatment with MANS significantly improved SGaw in the mid-range of the dose response curve, and caused a rightward shift (Figure 2). No significant effect of MANS was found in control mice that lack mucous metaplasia, indicating that the mechanism of MANS action is by inhibiting mucus secretion. The MCh dose associated with a 35% fall in SGaw from baseline was >200 mM in Ova/Sal mice, 110 mM in Ova/Ova mice, and 75 mM in Ova/Ova3 mice. MANS-pretreatment increased this to 165 mM in

Ova/Ova mice and 100 mM in Ova/Ova3 mice. Thus MANS inhibits MCh-induced airway obstruction in mice with mucous metaplasia. However, it was not possible to precisely separate the effect of mucus and bronchoconstriction in an escalating MCh dose-response experiment, since the former is likely to get depleted while the latter increases progressively. Thus, to precisely study the role of mucus in MCh-induced airflow obstruction, single challenge experiments were performed.

MANS therapy inhibits mucus related airflow obstruction induced by a single dose of MCh. Baseline SGaw of Ova / Ova and Ova/Ova3 mice that had mucous metaplasia (1.15 ± 0.06 , 1.1 ± 0.08 [cm H₂O.s]⁻¹) was not different from control mice without mucous metaplasia (Ova / Sal, 1.3 ± 0.1). After a single challenge with 60 mM MCh that caused near complete mucin secretion, Ova/Ova mice pre-treated with the MANS peptide had a higher mean post-MCh SGaw than those given the control RNS peptide, (MANS/MCh, 0.82 ± 0.05 [-30% compared to baseline SGaw]; RNS/MCh, 0.55 ± 0.05 [-51%]; MANS vs. RNS, $+0.27 \pm 0.07$ [49% MANS-induced improvement], $p < 0.05$) (Figure 3). The protective effect of MANS peptide was also seen in Ova/Ova3 mice (baseline 1.1 ± 0.08 ; MANS/MCh, 0.65 ± 0.06 [-40%]; RNS/MCh 0.47 ± 0.03 [-57%]; MANS vs. RNS, $+0.18 \pm 0.07$ [+38%], $p < 0.05$), but the degree of protection was less than in Ova/Ova mice. However, MANS did not protect against the fall in SGaw in Ova/Sal mice that did not have mucous metaplasia confirming that the mechanism by which MANS attenuates the MCh effect is by inhibiting mucus

secretion (Baseline, 1.3 ± 0.15 ; MANS/MCh, 1.12 ± 0.08 [-14%]; RNS/MCh, 1.1 ± 0.1 [-15%]; MANS vs. RNS, $+0.02 \pm 0.13$ [+1%], $p = \text{NS}$). Thus, MANS inhibits mucus related airway obstruction during MCh challenge.

MANS-induced improvement in airflow obstruction is not limited to the nasal airways. To determine whether the effect of MANS on airflow obstruction was limited to the nasal airways, we studied the effect of MANS therapy in Ova/Ova mice that had their nostrils occluded by tape prior to MCh challenge, and were thus breathing from their mouths. In mice that had their nostrils occluded by tape, MANS pre-treatment continued to cause significant improvement in SGaw (baseline 0.92 ± 0.05 ; MANS/MCh, 0.83 ± 0.06 [-10%]; RNS/MCh 0.61 ± 0.03 [-32%]; MANS vs. RNS, $+0.22 \pm 0.08$ [+36%], $p < 0.05$) (Figure 4). Nasal occlusion was generally well tolerated by mice for the duration of the experiment. However, such recordings contained intermittent short stretches of erratic flow waveforms from the head chamber. These were marked by a sudden isolated fall in the amplitude of the flow tracing from the head chamber that did not correspond to the simultaneous flow tracing from the thoracoabdominal chamber. These are likely to be related to temporary closing of the mouth and were discarded.

MANS-induced improvement in airflow obstruction is not dependent on bronchoconstriction. To determine whether bronchoconstriction was essential for mucus related airflow obstruction, we administered ATP after MCh challenge. ATP has been previously reported to cause airway smooth muscle relaxation (6)

and is also a mucus secretagogue, unlike other bronchodilators. ATP challenge caused modest bronchodilation in Ova/Ova mice with MCh-induced airway obstruction (Baseline, 1.18 ± 0.09 ; MCh, 0.62 ± 0.04 ; MCh/ATP, 0.84 ± 0.06 ; MCh vs. MCh/ATP, -0.22 ± 0.07 , $p < 0.05$). In mice given MCh and ATP, MANS pre-treatment resulted in significant improvement in SGaw (MANS/MCh/ATP, 1.1 ± 0.1 [-3%]; RNS/MCh/ATP, 0.8 ± 0.05 [-30%]; MANS vs. RNS, $+0.3 \pm 0.11$ [+28%], $p < 0.05$) (Figure 5). Since SGaw of mice treated with MANS was similar to baseline, there was no significant bronchoconstriction, and the airflow obstruction in RNS treated mice was entirely mucus-related. Thus, mucus-related airflow obstruction is not dependent on bronchoconstriction.

Mucus related obstruction is an important component of the MCh response. The difference in post-MCh SGaw between MANS and RNS treated mice is attributable to inhibition of mucin secretion, while the difference between the baseline and post-MCh SGaw of MANS-treated mice is attributable to airway narrowing due to bronchoconstriction, edema, and residual mucin secretion. Table 1 shows the percentage of total airflow obstruction after a single 60 mM MCh challenge that is attributable to mucus, as well as the improvement in SGaw attributable to MANS treatment. As expected, mice without mucous metaplasia had no mucus-related obstruction or MANS-induced improvement. Mucus-related obstruction was maximal relative to bronchoconstriction when ATP was used since ATP reversed MCh-induced bronchoconstriction. Mucus related obstruction was least relative to bronchoconstriction in Ova/Ova3 mice that had greater AHR.

Importantly, about half of the total MCh response in Ova/Ova mice appeared to be due to mucus. After eliminating the nasal changes, mucous obstruction appeared to be even larger relative to bronchoconstriction. It is important to note that this is specific to airway challenge with 60 mM MCh. At higher MCh doses mucous obstruction is a smaller fraction of the total airway obstruction as can be seen from Figure 2. MANS-induced improvement in SGaw, which reflects absolute mucus related obstruction, was relatively constant in mice with mucous metaplasia

DISCUSSION

In this study we found that intranasal instillation of a MARCKS related peptide, MANS, inhibited mucin secretion in the lung and improved airflow obstruction after MCh challenge. This extends the previous work by Singer *et al* describing the inhibitory effect of intratracheal (i.t.) MANS on mucin secretion *in vivo* (18), and provides a functional basis for potential use of MARCKS related peptides as therapy in diseases characterized by mucus related obstruction.

While we histologically confirmed the inhibition of mucin secretion from bronchial mucous cells, we did not directly measure the amount of mucus secreted into the lumen. Singer *et al* have previously found that MANS therapy caused near complete inhibition of secreted mucin (Muc5AC). Importantly, this was by ELISA based measurements in bronchoalveolar lavage specimens and was different from histological bronchial mucin measurements. In their study, post-MCh intracellular bronchial mucin content was reduced by 20% in MANS-treated mice

and 97% in RNS-treated mice i.e. MANS treatment resulted in about 80% inhibition of secretion. That is comparable to the changes seen by us.

To determine the functional consequences of MANS treatment, we utilized restrained double-chamber plethysmography that has been shown to be more sensitive to MCh-induced airflow obstruction than classical invasive mechanics (5). However, non-invasive methods are unable to adequately resolve changes in peripheral airway resistance and lung parenchymal mechanics. Additional investigations based on partitioning of respiratory system impedance may therefore be useful in future studies.

We found that mucus-related obstruction accounted for about half of the total airflow obstruction induced by an intermediate dose of MCh that induced maximal mucus secretion but not maximal bronchoconstriction (Figure 2).

Mucus-related obstruction was a significant component of MCh-induced obstruction seen in dose-response experiments used to quantify AHR (Figure 3).

It is therefore important that changes in AHR to MCh should not be directly equated with changes in airway smooth muscle contractility, especially when using non-invasive methods.

Since changes in nasal resistance can affect SGaw, it was necessary to eliminate the possibility that MANS was effecting changes by altering the nasal airway architecture. We occluded the nostrils of spontaneously breathing mice, forcing them to breathe from their mouths, thereby nullifying the effect of any changes in nasal resistance. We found that MANS-induced improvement in SGaw was not significantly affected by nasal occlusion (open, 0.27 ± 0.07 ;

occluded, 0.22 ± 0.08) and is thus unrelated to changes in nasal resistance. It was not possible to quantitatively isolate the nasal component of the MCh or MANS response in our experiments because oropharyngeal resistance in orally breathing mice was an unknown additional variable. The limited effect of MANS on the nasal airways is probably related to differences in mucin secretion between the lung and the nose. The nose contains mucin secreting submucosal glands, connected to the lumen by ducts (2;12). In contrast, the respiratory epithelium lining the tracheo-bronchial airways of mice lacks such glands or mucous cells, but is rich in Clara cells, similar to the peripheral small airways of humans that are of approximately the same size. During mucous metaplasia, Clara cells increase mucin production resulting in the development of a mucous cell phenotype. Since the mechanism of action of MANS involves contact with the target cell membrane, it is much more likely to affect mucous cells lining the airway lumen than sub-mucosal glands. Newer peptides related to MANS that are more soluble and suitable for aerosol delivery may be more effective and should be studied further.

The mechanisms of mucus-related airflow obstruction are likely to be primarily related to changes in airway caliber. This can be divided into two components. Firstly, airway epithelial height increases by up to 5 μm during mucous metaplasia (4). This should slightly reduce the diameter of the proximal airways and increase airway resistance (R_{aw}) that varies to the fourth power of the radius during laminar flow. In our experiments, development of mucous metaplasia (Ova/Ova and Ova/Ova3 mice) was associated with a trend towards increased

airway resistance (15% rise in R_{aw} assuming constant lung volume) that failed to reach statistical significance ($p=0.065$). Secondly, during triggered mucin secretion, intracellular mucin that is stored in a condensed phase is secreted into the lumen where it gets hydrated and expands up to five hundred times (20). Since this expansion is far greater than the associated loss of epithelial height during mucin secretion, it is likely to cause airway narrowing and consequent airway obstruction. From the differences in post-MCh $SGaw$ in MANS and RNS treated mice that were breathing orally, it can be estimated that tracheo-bronchial airway resistance increased by about one-third (assuming constant lung volume) due to mucin secretion. This requires an airway narrowing of slightly less than 10% on average or about 15-20% in the proximal and upper airways that are the sites of mucus production and the major contributors to resistance to airflow (cross-sectional area increases with increasing generations). The potential mucus volume (conservatively assuming hundred fold expansion (20)) stored in the mucin granules of mucous cells is about $0.1 \mu\text{l} / \text{mm}^2$ [$\sim 10 \text{ nl} / \text{mm}^2$ (mucin volume density (4)) $\times 100$]. Thus a 1 mm segment of a large proximal airway with mucous metaplasia (0.5-1 mm diameter) has potential mucus content of 0.18-0.35 μl and a volume of 0.2-0.8 μl . Even if half were secreted, the airway would narrow by more than 20%. These numbers are only representative of the potential for mucus related narrowing since the actual dynamics of mucus accumulation into the lumen are complex and include the function of the mucociliary escalator that clears mucus. However, it can be seen that the

magnitude of changes in Raw reported by us are well within the potential for mucus related airway narrowing.

The time course of mucus related obstruction in our study was similar to the kinetics of mucin secretion. In recent studies, mucin exocytosis was 100% complete at 3 min when measured by capacitance changes during granule fusion (3); and 50% complete by 3.7 min, when measured as morphometric apical volume loss (8). Differences between MANS and RNS treated mice were seen within the first set of stable measurements (~ 6 min from start of MCh exposure), and lasted for about 10-15 min, by which time both recovered to baseline. This correlates well with the expected time frame of mucus secretion and implies that mucus is being effectively cleared since no lasting effect is seen.

The mouse model of mucus hypersecretion we used was designed to mimic mucus related obstruction in human asthma. While the model provides an excellent means of analyzing the secretory event, there are some differences between this model and human asthma. Unlike severe human asthma where extensive mucus plugging can be seen, the effect of mucus secretion on airway conductance in our model was modest and transient (13). The reasons for this could be manifold. Firstly, the airways of mice constitute a large percentage of the lung and have a relatively large airway lumen compared to humans. This large airway caliber is speculated to reduce the flow-resistive load that would otherwise result from the rapid respiratory rate (250–350 breaths per minute) required by the mouse to maintain body temperature (11). Secondly, occlusion of airways by mucus probably requires either excessive mucus and/or extremely

narrow airways. Since murine airways have relatively large caliber and mucous metaplasia is limited to the first five generations of airways (5), it is likely that significant airway plugging is uncommon in murine models. While there were no plugged airways in our histological specimens, displacement during inflation with formalin cannot be excluded. A theoretical model by James and Carroll (1995) predicted that bronchoconstriction would amplify the effect of mucus discharge on airway obstruction due to critical narrowing and plugging. We did not find any greater MANS-induced improvement in Ova/Ova3 mice than Ova/Ova mice despite greater AHR and presumably greater bronchoconstriction. Thirdly, marked airway inflammation as in severe asthma is associated with compromised epithelial integrity and reduced mucociliary clearance. Exuded plasma proteins and cellular debris may promote the production of viscous mucus and the formation of luminal mucus plugs (7). These features may not be sufficiently recapitulated in our model since our experiments were performed one week after allergen challenge, by which time mucous metaplasia is maximal, but inflammation may have partially subsided. Airway closure has been detected in response to MCh challenge in allergically inflamed mice lungs shortly after allergen exposure (21). Thus, further evaluation of the MANS peptide in alternative models of chronic or severe asthma would be helpful (19).

In summary, mucin secretion has a significant role in airway obstruction. Administration of a MARCKS-related peptide blocks mucin hypersecretion, and improves mucus obstruction.

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TABLE 1.

	Mucus-related obstruction (% total obstruction)	MANS-induced improvement in SGaw (%)
Ova/Sal, MCh	10	1
Ova/Ova, MCh	45	48
Ova/Ova, MCh (nasal occlusion)	78	36
Ova/Ova, MCh/ATP	80	28
Ova/Ova3, MCh	29	28

LEGENDS

Table 1. *Mucus related obstruction is an important component of the MCh response.* The percentage of total airflow obstruction after a single 60 mM MCh challenge attributable to mucus and the MANS-induced improvement in SGaw are summarized. Ratios were derived from group means for each category and therefore variance could not be calculated and statistical tests of difference were not performed. Ova/Sal, allergen sensitized but not challenged; Ova/Ova, single allergen challenge; Ova/Ova3, daily allergen challenge for three days.

Figure 1. *Intranasal MANS inhibits mucin secretion from the tracheo-bronchial airways.* Fluorescent PAS staining of MCh and MCh/ATP challenged airways from mice with mucous metaplasia is shown. Mucin appears red. Intracellular mucin depletion can be seen in RNS pre-treated mice (*left: A, C*) compared to MANS treated mice (*right: B, D*). At low magnification (*top*) adjoining blood vessels (*arrows*) can be seen. At higher magnification (*bottom*), intracellular mucin can be seen clearly (*arrowheads*). (*C*) An example of near-complete mucin exocytosis with numerous hollow looking ghost cells (*curved arrow*). Scale bar (*bottom left*) is 10 μm in *c, d* and 40 μm in *a, b*. (*E*) Averaged retained mucus scores are shown (mean $\pm$ SE). Number of airways scored (*n*) is shown below each bar. Controls are mice with mucous metaplasia (Ova/Ova) that were not challenged. MANS / RNS denotes respective peptide pre-treatment prior to

challenge with MCh or MCh/ATP. * – significantly different from control ($p < 0.05$);
 ** – significantly different from MCh, RNS/MCh.

Figure 2. *MANS therapy inhibits airflow obstruction induced by escalating doses of MCh.* Averaged results of graded MCh challenge in Ova/Sal (squares) Ova/Ova (circles, dotted lines) and Ova/Ova3 (daily ova challenge x 3d, triangles, dashed lines) mice with/without MANS-pre treatment is shown ($n=5$ each). Repeated allergen challenge (Ova/Ova3) increased AHR compared to single allergen challenge (Ova/Ova). MANS pre-treatment (open symbols) shifted the curve to the right and significantly improved SGaw (*, $p < 0.05$).

Figure 3. *MANS therapy inhibits mucus related airflow obstruction induced by a single dose of MCh.* Averaged results of a single 60 mM MCh challenge in mice with/without mucous metaplasia treated with MANS or a control peptide (RNS) are shown. Number of animals is shown below each bar (n). Baseline SGaw (no pattern) is reduced by MCh treatment (patterned bars). MANS treatment (horizontal lines) is associated with improved SGaw compared to RNS (vertical lines) (*, $p < 0.05$) in mice with airway mucous metaplasia but does not significantly affect Ova/Sal mice.

Figure 4. *MANS-induced improvement in airflow obstruction is not limited to the nasal airways.* Averaged results of single 60 mM MCh challenge during nasal occlusion in Ova/Ova mice pre-treated with MANS or a control peptide (RNS) are

shown. Number of animals is shown below each bar (n). MANS treatment is associated with improved SGaw compared to RNS (*, $p < 0.05$)

Figure 5. *MANS-induced improvement in airflow obstruction is not related to bronchoconstriction.* Averaged results of ATP (100 mM solution) challenge in Ova/Ova mice are shown. Number of animals is shown below each bar (n). ATP challenge had no effect on SGaw by itself but improved SGaw when administered after MCh. MANS treatment is associated with improved SGaw compared to RNS in mice treated with both MCh and ATP, that have negligible bronchoconstriction. (*, $p < 0.05$)









