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THE EFFECTS OF ALLERGEN AND BRONCHOCONSTRICTOR EXPOSURE ON MUCUS BUILD-UP IN THE LUNGS OF MICE

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ABSTRACT

Background: The goal of this study is to examine the effects of a common allergen and a bronchoconstrictor drug on mucus build-up within the lungs of BALB/c mice. The allergen house dust mite (HDM) and the drug methacholine are both commonly used to model certain components of asthma in mice. HDM is commonly used to induce chronic airway inflammation. Methacholine is used to induce bronchoconstriction. Traditionally, airway inflammation has been proposed as the cause of a set of structural changes, termed airway remodeling, that often persist in the lungs of asthmatics. However, a study by Grainge et al. (2011) demonstrated that methacholine caused airway remodeling without inflammation in a group of asthmatic humans. Thus, this study assessed mucus build-up as a measure of chronic inflammation in the lungs of mice treated with methacholine.

Methods: Periadolescent mice (n=21) were subjected to 1 of 4 conditions: 1) Exposure to HDM, 2) Exposure to methacholine, 3) Exposure to both HDM and methacholine, and 4) Procedural Control. Periodic acid-Schiff (PAS) staining of the lungs was used to quantify mucus using an arbitrary scale termed mucus score.

Results: Although the procedural control group and the methacholine group did not have mucus build-up, both of the groups exposed to HDM had significant mucus build-up. There was no significant difference in mucus scores between the HDM group and the HDM with methacholine group. However, females had higher mucus scores than males.

Conclusion: Allergen (HDM) exposure causes mucus build-up in the lungs of BALB/c mice, but bronchoconstriction does not. Female mice appear to have greater sensitivity to the effects of allergen exposure, which leads to greater mucus build-up within the lungs.

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Chapter 1

Introduction

According to the CDC, about 18.7 million people in the US are currently suffering from asthma. Around 9.4% of all children in the US suffer from asthma (CDC, 2010). Asthma attacks can have many negative consequences such as missing school or work, inability to participate in sports, and even death (CDC, 2010). More recently, asthma attacks have even been suggested to increase a child's likelihood to exhibit anxious behavior and influence anxiety levels into adulthood (Richardson, et al., 2006). Although the mechanism is still unknown, it is possible that the physiological and psychological experience of stress could contribute to the development of certain mental disorders, such as anxiety and depression disorders (Afari et al., 2001). Thus, it is not only important to understand how to treat asthma, but also how to, if possible, prevent mental disorders among asthmatics. It is therefore necessary to identify which components of asthma are most related to the development of mental disorders.

Since there are many legal and ethical boundaries surrounding human studies, mice can be used to further investigate both the underlying mechanisms of asthma as well as the health outcomes that are associated with asthma. For example, taking lung tissue from live human subjects, especially from children, is normally not possible. Since many of the complications of asthma are believed to start developing in childhood, periadolescent mice provide a noteworthy model for studying chronic outcomes of asthma (de Graaf-Peters and Hadders-Algra, 2006). In addition, long-term consequences of treatments can be studied using a short-lived animal model.

Pathophysiological Components of Asthma

Asthma is a complex disorder which includes chronic symptoms related to airway inflammation, periodic acute symptoms associated with bronchoconstriction, and potentially irreversible restructuring of the airways (Vignola et al., 2003). Asthmatics are more prone to these symptoms because they have hyperresponsive airways. Severity of asthma symptoms and treatment needs are correlated with the degree of hyperresponsiveness (O'Byrne and Inman, 2003).

Chronic Airway Inflammation and Bronchoconstriction

Two important components of asthma are airway inflammation and bronchoconstriction. Chronic inflammation is known to lead to exacerbations of asthma symptoms as well as increased bronchial hyperresponsiveness (Bousquet et al., 2000). Bronchoconstriction is associated with acute inflammation and can cause shortness of breath and wheezing (Bousquet et al., 2000). Although these two components of asthma do not exist in isolation in asthmatic humans, they require different treatments. Furthermore, inflammation and bronchoconstriction could either together or independently account for the correlation between asthma and mental health outcomes. Thus, in order to prevent negative health outcomes, it is essential to understand the components of asthma both as a whole and as a combination of pathophysiological mechanisms.

Airway Remodeling and Implications for Treatment

A third important component of asthma is airway remodeling, which is characterized by structural changes such as goblet cell hyperplasia in the epithelium, increased subepithelial collagen, and smooth-muscle hypertrophy (Grainge et al., 2011).

Overall, airway remodeling causes increased vascularization of the airways due to an increase in the number and size of bronchial blood vessels (Vignola, 2003). Airway remodeling occurs in asthma as a response to tissue damage (Vignola et al., 2003). Remodeling is actually the body's attempt to heal the damaged tissue. However, remodeling occurs because the tissue repair process is pathological and is analogous to the formation of scar tissue (Bousquet et al., 2000). Currently, the common assumption is that this pathological repair process occurs in response to chronic inflammation. Acute inflammation is a normal and beneficial response in the body because it promotes wound repair and the return of tissues to normal functioning. However, asthmatics can experience chronic inflammation, which can lead to extensive damage. The healing response in the case of chronic inflammation therefore changes the structure of the airway tissues (Bousquet et al., 2000). Overall, both chronic inflammation and airway remodeling lead to decrease pulmonary functioning and exacerbation of asthma symptoms (Elias et al., 1999). In fact, airway remodeling can lead to airflow obstruction even when an asthmatic is asymptomatic.

The etiology of airway remodeling in asthma is not yet well understood. Traditionally, as previously mentioned, chronic inflammation experienced by asthmatics has been proposed as the cause of airway remodeling (Grainge et al., 2011). Nonetheless, airway remodeling is currently considered an irreversible process. Acute asthma attacks

involving bronchoconstriction can be reversed using bronchodilators. Chronic inflammation can be reversed (or prevented), although more slowly, by treatment with anti-inflammatory drugs. However, once airway remodeling has occurred, there is currently no treatment which has been clinically proven to reverse the changes in airway structure (Bousquet et al., 2000). Thus, it is critical to understand the mechanisms and etiology of airway remodeling because certain treatments and early interventions may be able to prevent remodeling. Not only would these treatments be able to prevent airway remodeling and exacerbations of asthma symptoms, but they may also have to potential to prevent other negative conditions associated with asthma, such as mental illness, if airway remodeling is a mediating factor.

Overproduction of Mucus as a Marker of Chronic Inflammation

Mucus is essential to the normal functioning and health of the airways, which come into contact with potentially harmful substances frequently through respiration. Mucus protects the lungs and prevents damage by trapping particles which enter the lungs so that they can be cleared easily from the airways by muciliary clearing (Kim et al., 1997). Mucus is also important because it contains antibiotics created by the body to kill harmful bacteria, such as *Helicobacter pylori*, which can cause stomach ulcers (Kawakubo et al, 2004). Thus, in many diseases of the airways, including asthma, abnormal quantity or quality of mucus can be fatal (Kim et al., 1997). In asthma, hypersecretion of mucus is caused by chronic inflammation of the airways (Bousquet et al., 2000).

Goblet Cell Hyperplasia as a Marker of Airway Remodeling

Mucus in the airways comes from 2 different types of cells, which each stain positive with Periodic acid-Schiff stain: goblet cells on the surface of the airway epithelium and gland cells in the submucosal layer (Kim et al., 1997). Along with chronic inflammation, abnormal mucus production can also occur when there is hyperplasia of the mucus-secreting cells (Humbles et al., 2004). Hyperplasia of mucus-secreting cells, particularly goblet cells, is a change in the structure of the airway and is therefore considered a component of airway remodeling (Grainge et al., 2011).

The Mouse Model of Asthma

Airway inflammation is used in this mouse model, established by the August laboratory to represent the chronic physiological aspects of asthma (Ferrera et al, 2006). Bronchoconstriction, which causes labored breathing, in contrast, are used to represent the acute psychological aspects of an asthma attack. In this model, the physiological response is modeled using house dust mite (HDM) . In previous studies, methacholine has been used to assess airway reactivity (Walsh et al., 2008). However, in the model used in this study, methacholine is used to model the psychological experience of labored breathing, called dyspnea, which causes bronchoconstriction.

Chronic Inflammation: House Dust Mite Extract

Although ovalbumin has been used to model allergic response in the past, it is not used in this model because mice develop ovalbumin tolerance (Cates et al., 2004). In

order to model the chronic inflammation that asthmatics experience, house dust mite (HDM) extract can be given to mice over a long period of time without the mice developing a tolerance (Saglani et al., 2009). Furthermore, HDM is the most common airborne allergen and therefore provides a relevant method for modeling asthma in mice (Warner et al., 1993).

Bronchoconstriction: Methacholine

Methacholine acts as an agonist of muscarinic acetylcholine receptor M_3, which is a G-protein-coupled receptor, and increases release of acetylcholine to smooth muscle cells (Billington and Penn, 2002). Currently, the principle use of methacholine is to provide a measurement that distinguishes asthmatics from non-asthmatics in the population. Histamine is another drug used for this purpose. Objective measures are necessary in order to be able to properly diagnose asthmatics, as well as determine proper treatments, since its symptoms are experienced somewhat subjectively by each individual patient (Dixon, 1983). The bronchial challenge test involves exposing the patient to methacholine or histamine and measuring bronchoconstriction. Because the airways of asthmatics are hyperresponsive, bronchoconstriction will occur at a lower dose for asthmatics than for nonasthmatics. Accordingly, the test is carried out by giving patients controlled doses of either methacholine or histamine until they reach the dose which provokes the standard level of bronchial constriction or until they have taken the maximal dose (Dixon, 1983).

Additionally, methacholine has implications in providing a model of dyspnea in mice. In a study which evaluated the use of bronchoconstrictors in mice, Martin *et al.*

compared the effectiveness of methacholine and histamine. They found that methacholine administration had reproducible responses in the mice but that histamine did not (Martin et al., 1988). These findings suggest that the methacholine challenge test is applicable not only to humans but also to mice. If methacholine acts only to induce bronchoconstriction and not allergic reaction and chronic airway inflammation, it can be used to separate labored breathing, and more specifically dyspnea, as separate variables from the entire allergic response.

Methacholine as a Potential Cause of Airway Remodeling

Since HDM causes airway inflammation, it would follow, due to our current understandings, that HDM causes airway remodeling. Since methacholine, however, is believed to cause only bronchoconstriction and minimal inflammation, it would follow that methacholine would not cause airway remodeling. However, in a recent study, Grainge et al. (2011) suggested that methacholine does cause airway remodeling without inflammation in asthmatic humans. This finding indicates not only that methacholine could cause airway remodeling, but also that the mechanical stress of bronchoconstriction, without inflammation, can cause remodeling. This finding also challenges the model of asthma used in this study and suggests that it may not be isolating the psychological (dyspnea caused by labored breathing) and physical (airway inflammation and remodeling) components. If methacholine can cause permanent changes in the structure of the airways through bronchoconstriction, then certain behavioral outcomes, such as anxiety, could be due to physiological factors.

Purpose and Hypotheses

Thus, the purpose of this study is to assess whether the methacholine and HDM treatments cause mucus build-up in the lungs, an indication of chronic inflammation and, potentially, airway remodeling (specifically, goblet cell hyperplasia). Since HDM causes inflammation, it is hypothesized that nonasthmatic mice treated with HDM will have mucus build-up (Cates et al., 2004). Since methacholine does not cause inflammation, it is hypothesized that nonasthmatic mice, treated with methacholine will not have mucus build-up because they do not have hyperresponsive airways (Grainge et al., 2011). However, when nonasthmatic mice are treated with both HDM and methacholine, they could develop hyperresponsive airways and be more comparable to the asthmatic humans in the study by Grainge et al. (2011). In this sense, mice treated with both HDM and methacholine could be a model for how the pathological healing mechanisms of airway remodeling develop in asthmatics. Thus, it is hypothesized that mice treated with both HDM and methacholine will have more mucus build-up than mice treated with only HDM.

Chapter 2

Methods

Study Subjects

The BALB/c mouse strain was chosen for this study because it has previously been used to model asthma. This strain of mice is known to have hyperresponsive airways. Specifically, periadolescent BALB/c mice have been shown to present asthma-like inflammation in response to house dust mite (HDM) exposure (Saglani et al., 2009). The 21 mice came from 3 different litters (a litter of 3 mice, a litter of 5 mice, and a litter of 13 mice). The mice were housed in the same room of the facility, which was kept on a reverse 12:12 light schedule (lights off at 6:00 am EST). The facility was pathogen-free and temperature, humidity, and ventilation were all controlled.

Study Design

HDM and Methacholine Treatments

The 21 mice were divided into 4 groups: 1) Exposure to HDM (*HDM*), 2) Exposure to methacholine (*Meth*), 3) Exposure to both HDM and methacholine (*HDM+Meth*), and 4) Procedural Control (*Con*). The protocols for HDM and methacholine administration are presented in Appendix A. Animals in groups 1 and 3 were exposed to HDM through nasal administration. In order to control for the administration process itself, animals that did not receive HDM (groups 2 and 4) were

given the same exact treatment except instead of HDM, they were given saline. Animals were place in a sealed chamber and methacholine was administered to groups 2 and 3 with a nebulizer at increasing concentrations until the animal experienced significant bronchoconstriction. Significant bronchoconstriction was indicated by labored breathing, inactivity, hunching over, and drooling. To adjust for increased airway sensitivity in allergen-exposed mice, the range of methacholine concentrations was half of as much for animals exposed to both HDM and methacholine (group 3; 0, 3, 6, 12, 25 mg/ml) as compared to animals exposed to only methacholine (group 2; 0, 6, 12, 25, 50 mg/ml) Animals that did not receive methacholine (groups 1 and 4) were given the same exact treatment except instead of methacholine, they were given saline. Group 3 received lower dosages of methacholine to accommodate for their increased sensitivity to the drug due to HDM exposure.

Timetable

HDM and methacholine treatments were given to neonatal and periadolescent mice in order to mimic symptoms of human childhood asthma (Saglani, 2009). Mice were marked with a permanent marker to maintain individual identities until they were old enough to be ear-marked. HDM or saline treatments were administered 24 times between postnatal day (PD) 3 and 56. Methacholine or saline treatments were administered 6 times between PD 22 and 57. On PD 139, the mice were sacrificed and lungs samples of each mouse were obtained from both the left and right lobes. The lung samples were stored in formaldehyde (10% w/v) before they were stained.

During PD 2, 3, 4, and 9 the rates of ultrasonic vocalization (USV) of each mouse were measured to obtain a basal measure of internalizing behaviors. After HDM and methacholine treatments, behavioral outcome measures were obtained. However these measures will not discussed in this study.

Histological Analysis

Samples of both right and left lungs from each mouse were examined. Three cross-sections of each lung were stained with Periodic acid-Schiff (PAS). A positive PAS stain (bright magenta) indicates the presence of mucus (Kuperman et al., 2002). The lungs were observed under 4x magnification. Mucus build-up was measured using an arbitrary numbering system that I developed (0-6) based on previous systems for quantifying goblet cells and mucus using PAS (Townsend, 2000). Goblet cells (filled with mucus) surrounding the airway and mucus within the airway were both taken into account. Each lung was given a mucus score and then each animal was given a mean mucus score, each in arbitrary units. Mucus score was determined as a blind measurement by a single observer using the standardized numerical system (0: no mucus build-up; 1: thin layer of goblet cells around 1 or more airways; 2: lining of goblet cells around 1-2 airways; 3: lining of goblet cells around 3 or more airways; 4: lining of goblet cells around 0-1 airways and complete coverage of 1 airway with mucus; 5: lining of goblet cells around 2-3 airways and complete coverage of 1 airway with mucus; 6: lining of goblet cells around 2 or more airways and complete coverage of 2 or more airways with mucus).

Statistical Analyses

To determine if HDM and/or methacholine influenced mucus buildup in the lungs, I used an ANOVA with 2 factors: experimental condition and sex. Significance was set at $\alpha = 0.05$. To determine variance between the 2 lung samples of the same animal, a difference value between mean mucus scores of left and right lungs was also calculated for each animal.

Chapter 3

Results

The mucus scores for each lung cross-section, each lung, and each animal are presented in Appendix B. To determine the differences between the 4 treatments and the differences between males and females, the mean mucus scores for each animal were analyzed. To determine the variance between left and right lungs within each animal, the mean mucus scores for individual lungs were analyzed.

HDM vs. Methacholine

All HDM-treated animals (the HDM and HDM+Meth groups; n=10) had a mean mucus score of 1.5 or above. In contrast, all animals that did not receive HDM treatment had a mean mucus score of 0 (the Meth and Control groups; n=11). In fact, for every animal of the 2 conditions without HDM, each cross-section of both the right and left lung received a mucus score of 0. Because there was no variance within the Meth and Control groups mucus scores, statistical analyses were restricted to the HDM and HDM+Meth groups. The mucus score for each animal within the HDM and HDM+Meth groups (separated by experimental condition and sex) are presented in Figure 3-1. Although there was an effect of HDM on mucus score, there was no significant difference between the mucus scores of the HDM and HDM+Meth groups ($F_{(1,6)} = 0.34$, $p = 0.582$).

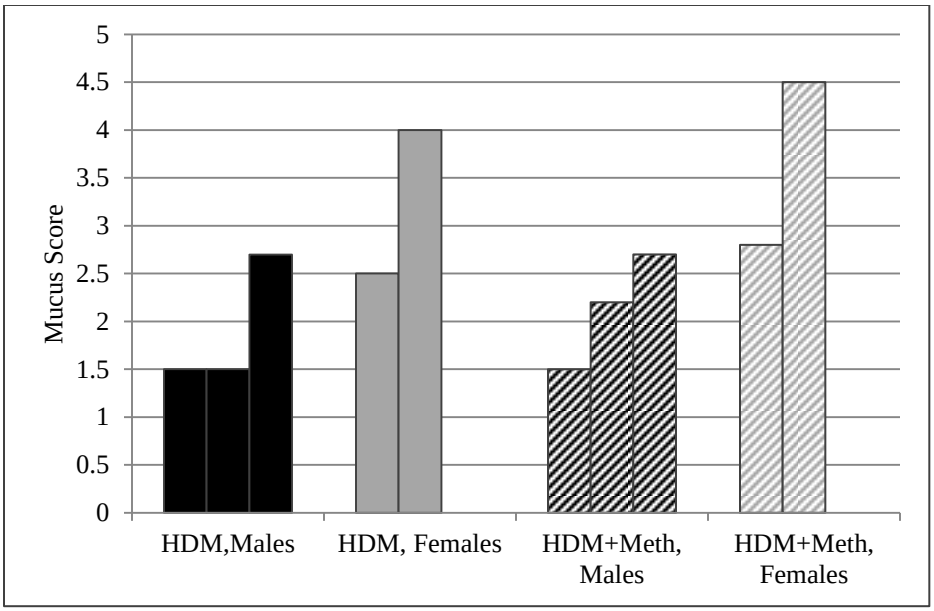

Figure 3-1 The mucus scores within the HDM-treated groups (HDM and HDM+Meth), categorized by treatment group and sex; each column represents a single mouse.

Male vs. Female Mice

There was, a significant difference between the males and females of the HDM-treated groups (HDM and HDM+Meth) for mucus score ($F_{(1,6)} = 6.95$, $p = 0.0387$). There was not an interaction effect of sex and treatment group ($F_{(1,6)} = 0.023$, $p = 0.883$). The effect of sex on mucus score is shown in Figure 3-2. The sex difference in the HDM-treated groups is also apparent in Figure 3-1.

All of the females of the HDM and HDM+Meth scored above 4 for at least 1 lung (Appendix B). In contrast, only 1 male mouse (a member of the HDM treatment group had a single lung score above 3 (4), and its mean mucus score was 2.7 (Appendix B).

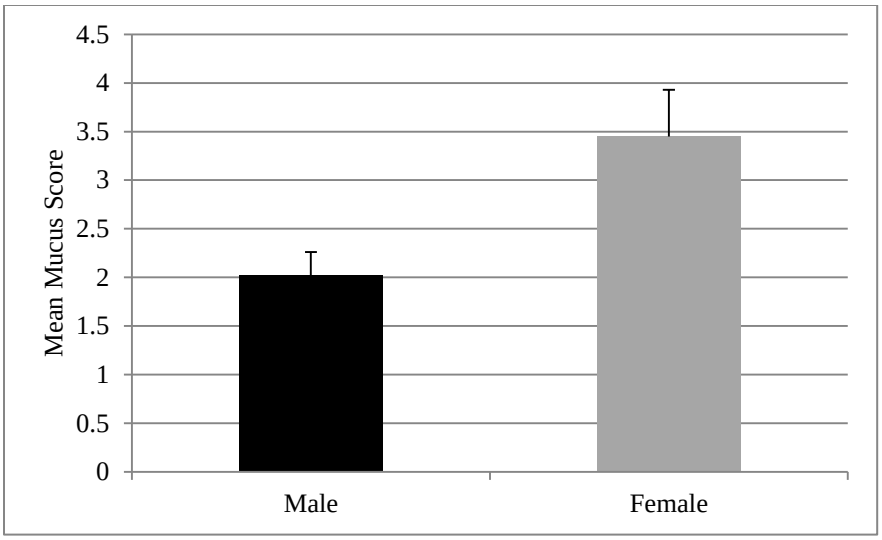

Figure 3-2 The effect of sex on mean mucus score within the HDM-treated groups (HDM and HDM+Meth) (error bars ± 1 standard error).

Discrepancies between Right and Left Lungs

For each animal, within each lung, the 3 cross-sections had relatively consistent mucus scores. Thus, the mean mucus scores of the 2 lungs were compared for each animal using a difference value. The lungs were not identified as left or right. Since all of the non-HDM-treated animals (the Meth and Con groups) had a score of 0 for every cross-section of each lung, their difference values were all 0. In the HDM-treated animals (the HDM and HDM+Meth groups), all of the differences are presented in Table 3-1. All of the animals, except 1, that received HDM treatments had variability between the left and right lung mucus scores. Since the range of the mucus score was only 0-6, most of animals in these groups show a considerable variability between lung mucus scores.

Table 3-1 The difference values between right and left lungs of each animal within the HDM-treated groups (HDM and HDM+Meth).

	Male	**Female**
HDM, Difference Values	1 2.7 3	3 2
HDM+Meth, Difference Values	0 1 0.3	3.7 3

Chapter 4

Discussion

HDM, but not Methacholine, Causes Mucus Build-up

The major finding of this study is that methacholine does not have a significant effect on mucus score in nonasthmatic mice and mice exposed to an allergen. When methacholine was administered on its own, the mice did not have mucus scores which differed from the control group. When methacholine was administered in addition to house dust mite (HDM), the mucus score was greater than the control group but was not significantly greater than the group which only received HDM treatments. Thus, the following hypotheses were supported: HDM on its own causes mucus build-up but methacholine on its own does not. However, the hypothesis that together HDM and methacholine would have a cumulative or increased effect on mucus build-up was not supported.

These findings indicate 2 important features of modeling asthma in BALB/c mice: 1) bronchoconstriction (induced by methacholine) itself does not cause mucus build-up and, 2) even when airways are already chronically inflamed due to allergen (HDM) exposure, bronchoconstriction does not exacerbate the effect of the allergen on mucus build-up. In this study, I interpret mucus score as a measurement of chronic airway inflammation because it quantifies both mucus build-up within the airways and mucus within the mucus-secreting goblet cells surrounding the airways a few weeks after the end of the treatments. Although the presence of goblet cells may indicate airway

remodeling in the mice treated with HDM, it cannot be concluded that airway remodeling occurred since goblet cell hyperplasia was not defined (see "Validity of Mucus Score"). These findings are weakened by the small sample size of HDM-treated mice (n=10) and the fact that litter effects were not taken into account.

Nevertheless, in a study of eosinophil-deficient mice, Humbles et al. (2005) found that eosinophilic inflammation does play a key role in some aspects of airway remodeling. Eosinophils are cells involved in the immune response which are not normally present in the lungs. Presence of eosinophils in the lungs indicates some type of pathology, such as allergy or asthma (Pizzichini et al., 1997). Mice with a mutation that caused them to be eosinophil-deficient did exhibit the following aspects of airway remodeling after allergen exposure: peribronchiolar collagen deposition and increase of airway smooth muscle. However, lack of eosinophils did not diminish airway hyperresponsiveness to methacholine (lung function as measured by a plethysmograph) or goblet cell hyperplasia (after both acute and chronic allergen challenges) (Humbles et al., 2005). Thus, although eosinophilic inflammation plays a role in airway remodeling, the mechanisms and extent of its role are still unclear.

The recent study by Grainge et al. (2011), suggests that methacholine can cause airway remodeling through mechanical stress alone (without increased eosinophilic inflammation) in asthmatic humans (n=48). Airway remodeling, measured by subepithelial collagen-band thickness and positive PAS-staining (mucus), occurred to the same degree in both the group which was exposed to an allergen and the group which was exposed to methacholine. However, the methacholine group did not show increased eosinophilic inflammation (Grainge et al., 2011). Unlike Grainge et al., my study of

BALB/c mice also contained a group of subjects exposed to both allergen and methacholine. However, the hypothesis of this study was that the HDM+Meth mice would be a good model of the methacholine-exposed asthmatic humans, who are likely to have underlying chronic airway inflammation. In one way, the findings of the study agree with the findings by Grainge et al.; the allergen (HDM) caused airway inflammation, but bronchoconstriction (methacholine) did not. However, since airway remodeling cannot be assessed through mucus score, conclusions cannot be drawn about whether or not methacholine caused airway remodeling.

Nonetheless, for the purposes of this laboratory, the findings of this study are essential since, at the very least, they indicate that mucus build-up is only caused by HDM, not be methacholine. In addition, since the Meth group had the same results as the control group (i.e. no mucus build-up) and the HDM+Meth group results did not differ significantly from the HDM group's results, it can be concluded that any mucus build-up that did occur in the mice was due to allergen-induced chronic airway inflammation and not bronchoconstriction. By extension, if mucus score is a valid measure of the effects of chronic inflammation, it is clear that methacholine does not cause chronic inflammation in BALB/c mice. Thus, it is a valid model to use methacholine to account for some of the effects of asthma that are not associated with chronic inflammation.

Sex Differences in Airway Responsiveness

Although it is limited by our sample size within the HDM-treated animals (n=10), another important finding is that the female mice (n=4) appeared to be more sensitive (as

measured by mucus score) to HDM than male mice (n=6). That is, HDM caused more mucus build-up in the lungs of the female mice than the lungs of the male mice. The female mice all had higher mean mucus scores because they had mucus both in the goblet cells and within the airways (i.e. a score of 4-6) for at least 1 lung (Appendix B). The inherent assumption of the mucus score is that mucus within the goblet cells precedes the secretion into the airways. In fact, although in certain mice mucus was seen in the goblet cells but not within the airways (i.e. the male HDM-exposed mice), mucus within the airways was never present without mucus in the goblet cells (i.e. the female HDM-exposed mice). Thus, mucus within both the goblet cells and the airways was considered more severe and was given a higher score than mucus only within the goblet cells. This aspect of the numbering system must be taken into account when analyzing the sex differences in this study. What the sex difference in this study truly indicates is that along with having mucus within the goblet cells, females also had mucus build-up within their airways. Overall, this finding suggests that the female BALB/c mice were more responsive to the allergen (HDM) than the male mice. Another possible explanation is that, since females weigh less, giving females the same dosage of HDM as males will have a greater physiological effect. This study did not take body weight into consideration for dosages of HDM due to prior consultation with Dr. Avery August.

Several previous studies have investigated adult male and female differences in airway responsiveness, mostly to ovalbumin, in the murine model. Matheu et al. (2010) concluded that although sex differences in airway responsiveness exist in certain strains of mice, they do not exist in others. Using ovalbumin as the antigen, they found that B10.RIII mice did not show a sex difference in responsiveness (Matheu et al., 2010).

However, other researchers have found a sex difference in responsiveness in the BALB/c strain of mice. Studies by Melgert et al. (2005) and Hayashi et al. (2003) both demonstrated that female BALB/c mice had greater airway sensitivity to exposure to ovalbumin than male BALB/c mice. Melgert et al. (2005) found that females had a larger number of eosinophils, which is a marker of airway inflammation. Hayashi et al. (2003) also found that females had a greater infiltration of eosinophils along with lymphocytes. Interestingly, Hayashi et al. (2003) also found that castrated males, with lower levels of testosterone than standard males, had the same sensitivity to ovalbumin as females. These findings suggest that testosterone may have a protective affect against airway inflammation in BALB/c mice (Hayashi et al., 2003).

Blacquièrea et al. (2010) also studied airway responsiveness to allergen in adult male and female BALB/c mice. However, they compared the effects of ovalbumin exposure to the effects of HDM exposure. In both the ovalbumin model and the HDM model, female BALB/c mice had increased airway inflammation and increased responsiveness to methacholine as compared to male BALB/c mice. Airway inflammation was measured by quantifying eosonophils in lungs and antigen-specific IgE (a class of antibody) in serum. However, interestingly, the increased airway sensitivity of females to allergen exposure did not correlate with an increase in airway remodeling, as measured by goblet hyperplasia, collagen deposition, and increased smooth muscle in the airways. That is, males and females had a similar level of airway remodeling, despite different inflammatory responses to the allergen. This finding is particularly pertinent because it suggests that increased airway inflammation does not cause more severe airway remodeling (Blacquièrea et al., 2010). When compared to the results of this

study, the findings of Blacquièrea et al. (2010) suggest that the higher mucus scores of the female mice may not have caused airway remodeling. Rather, the higher scores were most likely indicating a greater severity of airway inflammation in response to HDM exposure.

Sex differences in airway responsiveness have also been demonstrated in asthmatic humans. The prevalence of asthma in childhood is higher among boys than girls. Once children reach adolescence, the prevalence is equal for boys and girls. In adulthood, however, the prevalence is higher for than women (Bjornson and Mitchell, 2000). The most likely explanation for this switch in prevalence is a combination of hormonal and genetic factors. Overall, a higher risk for developing asthma in general is linked to increased sensitivity of the airways (Postma, 2007).

Validity of Mucus Score

"Mucus score" is an arbitrary scale created for the purpose of evaluating accumulation of mucus build-up in the airways of the mice in this study. However, this measurement evaluates both mucus within the lungs and mucus in the goblet cells. Given the results of this thesis work, this laboratory will use a modified system that evaluates these 2 components of mucus build-up separately and, further, will quantify epithelial collagen build-up to assess airway remodeling in the future (see "Future Recommendations").

As a Measure of Chronic Airway Inflammation

Hypersecretion of mucus in the airways is an indication of chronic inflammation (Bousquet et al., 2000). Since the lung samples were taken a few weeks after the treatments ended, it is clear that the inflammatory effects of HDM were persistent, even after the allergen exposure stopped. Since mucus score takes into account the relative amount of mucus in the lungs, it can be considered a valid measure of mucus build-up. Indeed, using the control group as a baseline measure, the presence of mucus build-up and, therefore, chronic inflammation, can be quantified using this method. However, since mucus score is an arbitrary scale, it has low validity as a method of quantifying mucus build-up. In this sense, strong conclusions can be drawn about the presence of chronic airway inflammation. However, conclusions about the quantity of mucus build-up, the degree of chronic inflammation, or the responsiveness of the airways are weaker since mucus score is such an imprecise measure.

The number of airways affected could also vary depending on the sample of the lung that was taken. Furthermore, the number of airways could vary depending on which lung lobe was taken. As shown in Table 3-1, in the HDM-treated animals, there was a relatively large discrepancy between right and left lungs. Since the identity of right or left was not retained throughout the process, it unknown whether the left or right lobe tended to have a higher mucus score or whether it was due to the random piece of lung tissue that was taken.

As a Measure of Airway Remodeling

Other researchers have used PAS staining techniques to quantify goblet cells and diagnose goblet cell hyperplasia (Towsend et al., 2000). Goblet cell hyperplasia is one of several structural changes, termed remodeling, which often occur over time in the airways of asthmatics (Kim, et al., 1997). If airway remodeling occurred in the mice in this study, it would be expected that their lungs would have an increased number of goblet cells. Although I originally created the mucus score to assess both chronic inflammation and airway remodeling, it is not clear whether I succeeded in assessing the latter.

When attempting to assess goblet cell hyperplasia using the mucus score created for this study, there were several inherent problems in the system. The first problem with this method is that it presents no definition for a pathological amount (i.e. hyperplasia) of goblet cells (as opposed to a physiologically normal amount). The second problem is that the control group and methacholine group animals did not have any goblet cells in their lungs which stained positively (bright magenta) with PAS. Since some quantity of goblet cells is normally present in the airways, this finding suggests that only goblet cells which are filled with mucus at the time of death stained positively. The third problem is that goblet cells were not "counted", as in other researchers' methods (Townsend et al., 2000). Rather, the number of airways which were surrounded by goblet cells (along with mucus build-up within the airways) was counted. As stated before, the number of airways affected could vary depending on the sample of the lung that was taken and which lobe (left or right) was taken.

Conclusions and Future Recommendations

Although mucus score was a valid measure of chronic airway inflammation, it was not a valid measure of airway remodeling. However, there are several ways that future studies can be modified in order to gather a more accurate picture of mechanisms involved in asthma symptoms and severity.

Modification of Outcome Measures

First, the mucus score itself will be modified. Since the mucus score currently takes into account 2 variables of mucus build-up (within goblet cells surrounding the airways and within the airways themselves), these variables will be split into 2 measures. In addition, the number of airways with goblet cells filled with mucus will not be counted. Instead, based on previous methods for assessing goblet cell hyperplasia with PAS staining, the number of positively stained (bright magenta) goblet cells per airway will be counted. Goblet cell number will then be presented as a percentage of goblet cells per airway (Townsend et al., 2000). This method will make it possible to define goblet cell hyperplasia and, therefore, airway remodeling. Thus, both chronic inflammation of the airways and airway remodeling will can be assessed.

Second, additional outcome measures can be added to the study in order to better assess chronic airway inflammation, bronchoconstriction, and airway remodeling. For example, subepithelial collagen-band thickness, as described by Grainge et al. (2011), can be used as a measure of remodeling. An increase in smooth muscle of the airways, as described by Humbles et al., could also be used as a measure of remodeling (2004). Originally, this study also included an evaluation of expiratory capacity

(bronchoconstriction and labored breathing) using a plethysmograph during the methacholine administration. However, due to technical problems, those values are not available. Future studies should include these values in order to measure the degree of bronchoconstriction and also to establish the appropriate dosages. For example, females may require a lower provocative dose than males due to lower body weight and/or increased airway sensitivity to methacholine. Overall, a larger group of mice must be used and litter effects must be accounted for in order to establish more concrete conclusions on the effects of HDM and methacholine on both chronic airway inflammation and airway remodeling.

The Potential Effect of Bronchoconstriction on Airway Remodeling

Grainge et al. found that methacholine caused remodeling without inflammation in their study population of asthmatic humans (2011). This study goes against the current understanding of airway remodeling as being caused by chronic inflammation. Overall, more research, both in animal models and in asthmatic and nonasthmatic humans, needs to be completed in order to provide conclusive evidence that both inflammation and bronchoconstriction can, independently, cause airway remodeling.

There is a possibility that the methacholine drug itself somehow caused the airway remodeling. That is, there could be some unintended effect of the methacholine challenge test that involves airway remodeling. In this case, the mechanical stress of bronchoconstriction itself would not be the cause of remodeling. If methacholine does, in fact, cause airway remodeling, it may be necessary to find other bronchoconstrictors for the bronchial challenge test or to find other diagnostic methods altogether for asthma.

Though it is unknown whether either methacholine or HDM causes airway remodeling, based on my thesis work, it seems that HDM causes airway inflammation and methacholine does not. Although studies which compare well-controlled cases of asthma to uncontrolled cases (i.e. frequent asthma attacks) may provide some insight into the process of airway remodeling, it is difficult to separate the components of chronic airway inflammation and bronchoconstriction in asthmatics. It may be possible to induce chronic inflammation and bronchoconstriction separately in nonasthmatics. However, inducing asthmatic symptoms in otherwise healthy subjects poses many ethical issues and thus may not be plausible in human subjects at this time.

Implications for Treatment and Prevention

Since airway remodeling is a set of persistent structural changes which cause exacerbations of asthma symptoms, it could be a factor in the association of asthma with other negative outcomes. For example, airway remodeling may account for the correlation of asthma with anxiety and depression. If this is the case, it is imperative to understand the etiology of both chronic airway inflammation, which is already thought to cause remodeling, and airway remodeling itself. The latter is not as well understood and thus further research should be dedicated to it. If bronchoconstriction and mechanical stress do lead to remodeling of the airways (without chronic inflammation as a mediating factor) more attention must be put on treatments which stop and prevent bronchoconstriction and airway remodeling in asthmatics. Overall, individuals with severe asthma are known to have a greater degree of airway remodeling than asthmatics

whose symptoms are well-controlled (Chetta et al., 1997). Thus, control of asthma symptoms should always be a priority both in research and in clinical practice.

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Appendix A

HDM and Methacholine Treatment Protocols

Written by: Sonia A. Cavigelli

Last Updated: April 4, 2011

Intranasal HDM Administration

On Tuesday (01 June 2010), we visited Avery August's lab to learn the intranasal administration technique. This method was be used to administer house dust mite extract (allergen) to mice in the 'PSIN Asthma-Anxiety' study. We worked with Fei Huang (post-doc) & Arun Kumar (2nd year grad student). Present from our lab: Sonia Cavigelli, Kerry Michael, Danielle Cardell, Sumi Gnanarajah, Hashim Chaudhry. Neonatal House Dust Mite extract administration protocol used in PSIN Asthma-Anxiety study was taken from Saglani et al. (2009).

Supplies/Equipment Needed:

- Properly diluted allergen solution – there are 100 µl aliquots of correctly diluted solution in small tubes in CBL Rm 188 freezer. Remove/thaw one vial from freezer for each 5-6 mice that you need to treat.
- Sterile saline – also in 100 µl aliquots in freezer
- Pipettor and pipette tips (10 or 100 µl tips)
- Clean (disinfected) container to hold mouse that can be attached to anesthesia machine (e.g. glass 1-litter flask)

- Paper towels
- Gloves
- Isofluorane
- Code sheet indicating which mice to treat with allergen and which to treat with saline

Procedure:

1) For all mice older than 3 weeks of age, set up anesthesia machine. Make sure there is enough isofluorane in tank. Place a clean container (i.e. uninfected) from our lab onto the anesthesia machine vent table, place a paper towel in container, put the output tube of anesthesia machine into container, and cover with a lid. Turn anesthesia machine on and 'fill' container with isofluorane fumes. (For mice less than 3 weeks, there is no need for isofluorane.)
2) Place mouse into container and watch until its heartbeat has reduced to 1 beat/second. During this time, you can load your pipette tip with the proper volume of either allergen solution or saline – consult code sheet. Use a new pipette tip for each mouse. Volume for mice less than 3 weeks = 10µl, volume for mice older than 3 weeks = 15µl. At the appropriate time, remove mouse from container and hold it on its back in your non-dominant hand, pinching the scruff of the neck between thumb and forefinger and allowing its body to lie horizontal in your hand.
3) With dominant hand, hold pipettor at a 30 degree angle from horizontal and release a little of the allergen solution so that it forms a drop on the outer edge of

the pipette tip. Touch the drop of solution to the mouse's nares and allow them to breathe it in. Keep working like this until the full volume of allergen has been administered. Be careful to allow the mouse to take a breath between each drop administered, but work quickly so that you do not need to give the mouse another dose of isofluorane.

4) Once the full dose has been administered, return mouse to home cage and observe it to make sure it recovers from anesthesia. Return to cage rack after full recovery.

Methacholine Administration

Setting Up an Experiment

NOTE: You may skip this part of the procedure by loading a pre-existing setup. Select File>Open>"Mouse Asthma and Anxiety"

1) Right-click on Experiment node and choose Add> Site
2) Right-click on Site node and choose Add> WBP (for whole-body plethysmograph)

3) Right click on WBP>setup to set up analyzer
    a. Logging type should be "Event Based"
    b. On Input tab, set Connection to Lead 1
4) Right-click on WBP1>Show Views
    a. Select Add> Strip Chart

i. Right-click on table and select Select Table

ii. Choose the bottom-most icon (WBP1)

b. Select Add> Fast Graph

i. Right-click on graph and select Add Trace

ii. Click on Outputs

iii. Select Penh from that list

5) Right-click Site 1 and rename to Mouse 1
6) The whole previous bit sets up for one mouse. To copy all of this to have one site for each mouse:
    a. right-click on WBP1>Copy Items and select Analysis, Views, and Settings
    b. Right-click Experiment>Add> Site
    c. Right-click Site> Paste WBP1

Calibration

1) Set silver switch on pre-amp to OFF
2) Click System>Calibrate
3) Pull 1mL of air into the attached syringe
4) Turn the zero knob until the signal is at zero (for large adjustments, use the Balance screw with the tiny screwdriver)
5) Flip the silver switch to DC and re-zero
6) Push 1mL of air from the syringe into the chamber and hit F7
    a. Accept the settings the computer gives you
7) Flip the silver switch to AC

8) Save and close
9) Open “Asthma and Anxiety Study”

Loading the Animal

1) Holding the mouse by the tail, place the front end of it on the grate in the chamber. Pull gently to make the mouse hold on to the grate.
2) Let the mouse go – it will circle inside the chamber and pull its tail in behind it.
3) Close the chamber. Make sure the THING on top is open (parallel to tubing)

Collecting Data

1) Click **Run** button (little man running)
    a. Select the Mouse Asthma and Anxiety study
    b. Under Session Description, enter which litter you are running and the date
    c. Enter the appropriate mouse IDs under “subject ID” for each site. Make sure to note down which mouse ID is at which site on a separate paper or document for later reference.
    d. Click Go!
2) Select **Mouse1>Protocol** on the **Command Menu**, the box on the left side of the screen. Below the Command Menu, a box displaying the protocol task list should be observed along with several command options (i.e. ‘Go On’, ‘Restart’, ‘Stop’). Protocol Status should be displayed atop the task list, at this point counting down from 3 minutes for the mouse’s acclimation period.

a. If the protocol task list is not displayed, select the **Mouse1** tab on the lower box to display it. Data will be automatically collected after the acclimation period ends.

b. When the protocol reaches **task 9**, add PBS to the nebulizer and click the **'Go On'** Command below the task list. You are given 10 minutes for this step, before the protocol will continue on its own.

c. When the protocol reaches **task 14**, add Methacholine (or saline for controls) to the nebulizer and click the **'Go On'** Command below the task list. You are given 10 minutes for this step, before the protocol will continue on its own.

d. When the protocol reaches **task 18**, data collection for the first mouse ID is complete. Move on to Step 3.

3) Load the next animal and select **Mouse2>Protocol** to view the protocol task list for the next mouse ID. The protocol should be paused, counting down from a time in the 900 minutes range.

a. If the task list is not displayed, see step 2a.

b. Select **'Go On'** to begin the 3 minute acclimation period and following data collection task.

c. On **task 10**, add PBS and click **'Go On'**.

d. On **task 15**, add Methacholine (or saline for controls) and click **'Go On'**.

e. At **task 19**, data collection for the second mouse ID is complete. Move on to Step 4.

4) Follow Step 3 for the remaining animals, with appropriate protocols selected per trial (**Mouse3>Protocol**, and so on) until all the animals have undergone the procedure.

Click the **Stop** button (To the right of the **Run** button).

Appendix B

Mucus Score Data Sheet

Animal #:	1	
Tx/Sex:	HDM+Meth/F	
	Side 1	Side 2
Slice 1	4	1
Slice 2	5	1
Slice 3	5	1
Mean =	4.666667	1
Total Mean=	2.833333333	

Animal #:	4	
Tx/Sex:	HDM+Meth/M	
	Side 1	Side 2
Slice 1	2	3
Slice 2	3	3
Slice 3	3	2
Mean =	2.66667	2.66667
Total Mean=	2.666666667	

Animal #:	2	
Tx/Sex:	Meth/M	
	Side 1	Side 2
Slice 1	0	0
Slice 2	0	0
Slice 3	0	0
Mean =	0	0
Total Mean=	0	

Animal #:	5	
Tx/Sex:	Meth/M	
	Side 1	Side 2
Slice 1	0	0
Slice 2	0	0
Slice 3	0	0
Mean =	0	0
Total Mean=	0	

Animal #:	3	
Tx/Sex:	HDM/M	
	Side 1	Side 2
Slice 1	1	2
Slice 2	2	2
Slice 3	0	2
Mean =	1	2
Total Mean=	1.5	

Animal #:	6	
Tx/Sex:	Con/F	
	Side 1	Side 2
Slice 1	0	0
Slice 2	0	0
Slice 3	0	0
Mean =	0	0
Total Mean=	0	

Animal #:	7	
Tx/Sex:	Con/F	
	Side 1	Side 2
Slice 1	0	0
Slice 2	0	0
Slice 3	0	0
Mean =	0	0
Total Mean=	0	

Animal #:	11	
Tx/Sex:	Con/M	
	Side 1	Side 2
Slice 1	0	0
Slice 2	0	0
Slice 3	0	0
Mean =	0	0
Total Mean=	0	

Animal #:	8	
Tx/Sex:	HDM+Meth/M	
	Side 1	Side 2
Slice 1	1	2
Slice 2	1	2
Slice 3	1	2
Mean =	1	2
Total Mean=	1.5	

Animal #:	12	
Tx/Sex:	Meth/M	
	Side 1	Side 2
Slice 1	0	0
Slice 2	0	0
Slice 3	0	0
Mean =	0	0
Total Mean=	0	

Animal #:	9	
Tx/Sex:	Con/M	
	Side 1	Side 2
Slice 1	0	0
Slice 2	0	0
Slice 3	0	0
Mean =	0	0
Total Mean=	0	

Animal #:	13	
Tx/Sex:	HDM/M	
	Side 1	Side 2
Slice 1	4	1
Slice 2	4	1
Slice 3	4	2
Mean =	4	1.333333
Total Mean=	2.666666667	

Animal #:	10	
Tx/Sex:	HDM+Meth/M	
	Side 1	Side 2
Slice 1	2	3
Slice 2	2	2
Slice 3	2	2
Mean =	2	2.333333
Total Mean=	2.166666667	

Animal #:	14	
Tx/Sex:	HDM/F	
	Side 1	Side 2
Slice 1	4	1
Slice 2	5	1
Slice 3	3	1
Mean =	4	1
Total Mean=	2.5	

Animal #:	15	
Tx/Sex:	Meth/F	
	Side 1	Side 2
Slice 1	0	0
Slice 2	0	0
Slice 3	0	0
Mean =	0	0
Total Mean=	0	

Animal #:	18	
Tx/Sex:	Meth/F	
	Side 1	Side 2
Slice 1	0	0
Slice 2	0	0
Slice 3	0	0
Mean =	0	0
Total Mean=	0	

Animal #:	16	
Tx/Sex:	HDM/F	
	Side 1	Side 2
Slice 1	3	5
Slice 2	3	5
Slice 3	3	5
Mean =	3	5
Total Mean=	4	

Animal #:	19	
Tx/Sex:	Meth/F	
	Side 1	Side 2
Slice 1	0	0
Slice 2	0	0
Slice 3	0	0
Mean =	0	0
Total Mean=	0	

Animal #:	17	
Tx/Sex:	HDM/Male	
	Side 1	Side 2
Slice 1	0	3
Slice 2	0	3
Slice 3	0	3
Mean =	0	3
Total Mean=	1.5	

Animal #:	20	
Tx/Sex:	HDM+Meth/F	
	Side 1	Side 2
Slice 1	3	6
Slice 2	3	6
Slice 3	3	6
Mean =	3	6
Total Mean=	4.5	

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