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Extent of Allergic Inflammation Depends on Intermittent versus Continuous Sensitization to House Dust Mite

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Abstract

Objective: House Dust Mite (HDM) exposure is used to model experimental asthma in mice. However, a direct comparison of inflammatory responses following continuous versus intermittent HDM exposure has not been reported. Therefore, we investigated whether the HDM dose at sensitization or challenge affects extent of inflammation in mice that were either continuously or intermittently sensitized with HDM.

Materials and Methods: C57BL/6 mice received either 10 continuous exposures with 10 µg HDM per exposure or 2 intermittent HDM exposures over a period of two weeks and were subsequently challenged by three instillations with HDM during the third week. For the intermittent model, mice were sensitized with 1 or 10 µg HDM and challenged on three consecutive days with 1 or 10 µg HDM. Inflammatory cells in the bronchoalveolar lavage fluid and epithelial cell hyperplasia and mucous cell metaplasia were quantified.

Results: Significantly higher levels of inflammation and mucous cell metaplasia were observed when mice were sensitized intermittently compared to continuously. Intermittent sensitization and challenge with 10 µg HDM caused maximum inflammation, mucous cell metaplasia and epithelial cell hyperplasia. However, sensitization with 1 µg HDM only also showed increased inflammation when challenged with 10 µg HDM.

Discussion: These findings suggest major differences in adaptive immunity, depending on the sensitization protocol.

Conclusions: Because of significant differences, the HDM sensitization protocol should be carefully considered when designing studies to investigate the underlying mechanisms of immunity in mouse models of asthma.

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Keywords

House Dust Mite; Allergic Sensitization; Inflammation; Asthma; Mucous Cell Metaplasia

Introduction

Allergic asthma is a chronic disease characterized by inflammation, mucus production, and airway hyper-reactivity. It is the most common chronic disease affecting school children and is associated with mortality, particularly in inner city environments (Jackson et al. 2016). Children develop allergies when exposed to house dust mites (HDM) (Sporik et al. 1990), cockroaches (De Vera et al. 2003, Fishbein et al. 2016), pollen (Erbas et al. 2013), and fungi (Perzanowski et al. 1998, Tham et al. 2016, Vicencio et al. 2014). These allergies can be exacerbated by particulate matter consisting of wood smoke (Kumar et al. 2008), diesel exhaust (Braback and Forsberg 2009, Brauer et al. 2002, Weiland et al. 1994) and cigarette smoke (LeNoir 1999). In addition to genetic susceptibility factors, “sensitization” to an allergen in pre-natal and post-natal time periods can determine the development of allergic asthma (Sly et al. 2008). Therefore, it is crucial to understand exposure conditions that lead to sensitization as they determine extent of allergic inflammation and can help control incidence and course of disease.

Approximately 85% of asthmatics are allergic to HDM (Gregory and Lloyd 2011) and the dose of HDM exposure during infancy and early childhood is directly related to HDM allergy (Wahn et al. 1997). Development of allergy to HDM arises from the mite itself, as well as other associated components, such as fecal pellets, fungal spores (β -glucans) (Fahlbusch et al. 2003), HDM exoskeleton (chitin), and immunogenic epitopes, like Derp-1 (Gregory and Lloyd 2011).

In the classic mouse model of asthma, mice are sensitized to OVA by two intraperitoneal (IP) injections that are 7 days apart and then challenged by directly exposing the lungs to OVA. This regimen elicits a strong inflammation characterized by airway eosinophilia, lymphocytic infiltration, Th2 cytokines, and ovalbumin-specific IgEs in the serum (Ohkawara et al. 1997). More recently, sensitization by the mucosal rather than the systemic route is preferred to resemble the real-life immunization process. However, mucosal immunization with HDM has encompassed both intermittent and continuous sensitization. Numerous models in BALB/c mice have used continuous exposure regimens of 5 exposures per week for up to seven weeks leading to eosinophilia, airway hyper-responsiveness, and airway remodeling (Johnson et al. 2004, Tully et al. 2013). Other studies expose mice to 10 consecutive intranasal challenges with HDM doses ranging from 5–100 μ g (Cates et al. 2004), while others use two intermittent sensitizations separated by several days (Lewis et al. 2009, Lewkowich et al. 2005, Willart et al. 2012). To our knowledge, a direct comparison and the optimal HDM dose exposure regimen to elicit lung inflammation and mucous cell metaplasia has not been reported. This comparison is important because extent of inflammatory responses and the underlying mechanisms leading to asthmatic phenotype could be different and, therefore, should be emphasized when describing the sensitization and challenge regimen.

The present study was designed to compare HDM-challenged C57BL/6 mice that were exposed to either continuous sensitization or HDM or intermittent sensitization to HDM. Additionally, we also evaluated two doses of HDM at both sensitization and challenge and sought to determine how the dose used at sensitization affects inflammation, airway epithelial hyperplasia, and mucus cell metaplasia.

Methods

House dust mite exposure protocol

Male pathogen-free wild-type C57BL/6 mice were purchased from Charles River Laboratories. Mice were quarantined for 14 days under pathogen-free conditions and subsequently entered into protocol at 7–8 weeks old. All experiments were approved by the Institutional Animal Care and Use Committee and were conducted at Lovelace Respiratory Research Institute, a facility approved by the Association for the Assessment and Accreditation for Laboratory Animal Care International. For all HDM exposures, mice were anesthetized with isoflurane. The HDM, *Dermatophagoides Pteronyssinus* (lot #248041), was purchased as 21.5 mg protein per 103.3 mg dry weight and extracted in a freeze-dried form. It was resuspended in sterile phosphate buffered saline for a final concentration of 1 µg/µl and stored in frozen aliquots at –20°C. For the continuous sensitization protocol, mice were instilled intranasally to 10 µg HDM 5 d/wk over 2 wks or for comparison to 10 µg HDM only on days 1 and 8 (**Fig. 1**). For the intermittent sensitization protocol, mice were exposed to 1 µg or 10 µg HDM (Greer Laboratories, Lenoir, NC) intranasally on days 1 and 8 (**Fig. 4**). All mice were challenged via oropharyngeal aspiration on days 15–17 with PBS, 1 µg or 10 µg HDM as described (De Vooght et al. 2009, Lakatos et al. 2006). Briefly, mice were anesthetized with isoflurane for about 45 seconds (until they were observed breathing with deep gasps). The anesthetized mice were fixed on a 45 degree slanted surgery board and using forceps the tongue was gently pulled out of the mouth to prevent the swallowing reflex. The HDM or PBS suspension was pipetted onto the distal part of the oropharynx between deep gasps while the nose was gently closed, which forced the mouse to breathe through the mouth. The nose and tongue were released after at least two breaths had been completed. All mice were euthanized on day 19 by IP injection of euthasol (**Fig. 1**).

Broncho-alveolar lavage (BAL) and histology of lungs

After euthanasia, clamps were placed over left lungs to allow of lavaging the right lungs three times with 0.5 mL ice-cold PBS. The number of total cells in the lavage was determined using a hemocytometer. BAL samples were then centrifuged at 1000x g, re-suspended in 0.2% BSA in PBS, centrifuged onto cytopins, and stained with Giemsa. Percent cell populations of macrophages, neutrophils, lymphocytes, and eosinophils were determined by a person unaware of slide identity and experimental design in 300 cells per sample.

After removing the clamp, the left lung was inflated and fixed at 25 mm pressure with zinc formalin. Lung slices (4–5) prepared from the fixed lungs were embedded into paraffin and 5 µm tissue sections prepared for staining with Alcian Blue (AB) hematoxylin and eosin as described previously (Tesfaigzi et al. 2002). Digital images of the AB/H&E-stained slides

were captured using an Olympus NanoZoomer (Hamamatsu Photonics) slide scanner with a 20x objective lens. The Image analysis system VisioMorph system (Visiopharm A/S, Horsholm, Denmark) was used to quantify the number of AB-positive cells and total epithelial cell count per mm of basal lamina as described by the manufacturer of the software.

Statistical analysis

Data from at least 5 mice per group were presented as the mean $\pm$ standard error from the mean (SEM). Statistical analyses were performed using GraphPad Prism Software 5.0 (GraphPad Software, Inc., San Diego, CA). For all experiments, grouped results were analyzed using two-way analysis of variance (ANOVA). When significant main effects were detected ($p < 0.05$), a post test of multiple comparisons was performed (tukey) to determine differences between treatment groups. All groups were compared to the naïve, untreated group to determine significance.

Results

Comparison of intermittent and continuous sensitization

To mimic continuous sensitization with HDM, mice in Group 1 were given intranasal instillations of 10 μ g HDM daily over 5 d/wk for two weeks (**Fig. 1**). Following these two weeks of continuous exposure, mice were challenged once a day for three days with either 10 μ g HDM or PBS as a control. To mimic intermittent sensitization with HDM, mice in Group 2 were intranasally instilled with 10 μ g HDM on days 1 and 8 and subsequently challenged once a day for three days with either 10 μ g HDM or PBS as a control (**Fig. 1**). Analysis of BAL cells showed that intermittent compared to the continuous sensitization resulted in significantly higher numbers of total inflammatory cells (**Fig. 2A**), neutrophils (**Fig. 2B**), lymphocytes (**Fig. 2C**), and eosinophils (**Fig. 2D**). However, there was no significant difference in macrophage numbers between the two groups (**Fig. 2E**). Interestingly, in the continuous sensitization model, only a modest increase was observed in total cells and eosinophils in the HDM-challenged compared with the PBS-challenged group (**Fig. 2A and D**).

Analysis of AB/H&E stained lung tissues showed no significant differences in epithelial cell number per mm basal lamina (BL) between HDM groups that were sensitized continuously or intermittently over the PBS controls (**Fig. 3A**). However, while statistically not significant, the intermittently sensitized group showed an increase over the other groups (**Fig. 3A**). The mucous cells/mm BL was highest in mice sensitized intermittently, but was also increased in those sensitized continuously compared to the PBS challenged group. Interestingly, the PBS-challenged group also showed increased mucous cells/mm BL compared to naïve controls, suggesting minor inflammation was sufficient to cause mucous cell metaplasia when mice were continuously sensitized.

Comparison of different HDM doses for sensitization and challenge

Because intermittent sensitization was effective in increasing allergic reaction, we continued in this model to determine the minimal dose (1 or 10 μ g HDM) of sensitization and challenge

necessary to elicit an allergic reaction (**Fig 4**). Analysis of BAL collected from mice both sensitized and challenged with 10 µg HDM showed significant increases in total cells (**Fig. 5A**), lymphocytes (**Fig. 5B**), and eosinophils (**Fig. 5C**) compared with PBS and naïve controls. Mice that were sensitized with 10 µg HDM and challenged with 1 µg showed a trend towards an increase in total cells (**Fig. 5A**), total lymphocytes (**Fig. 5B**), and total eosinophils (**Fig. 5C**) compared to mice sensitized with 1 µg HDM. Additionally, mice that received 1 µg HDM sensitization and 10 µg challenge HDM also showed a trend towards an increase in total cells (**Fig. 5A**), lymphocytes (**Fig. 5B**), and eosinophils (**Fig. 5C**) and neutrophils (**Fig. 5D**), compared to mice sensitized with 1 µg HDM. However, mice both sensitized and challenged with 1 µg HDM did not show any significant increases in inflammatory cells compared to controls (**Fig. 5A-E**). For all groups, macrophage numbers showed not significant differences compared to controls (**Fig. 5E**). These data indicate that the dose of HDM at both sensitization and challenge are critical in initiating a strong HDM-induced inflammatory response.

No significant differences were observed in epithelial cell number among any of the groups (**Fig. 6A**). However, the number of mucous cells/mm BL were increased in mice sensitized with 10µg HDM and challenged with 1 or 10µg. Interestingly, those challenged with 1µg HDM did not show increased mucous cell metaplasia compared to naïve mice when challenged with 1µg but only when challenged with 10µg HDM (**Fig. 6B-C**). Unexpectedly, mice challenged with 10µg HDM showed significant mucous cell metaplasia when challenged with PBS. Collectively, these data demonstrate that higher doses of HDM at either sensitization or challenge aggravate inflammation and mucous cell metaplasia.

Discussion

The purpose of the present study was to compare inflammation, mucous cell metaplasia, and epithelial cell hyperplasia in C57BL/6 mice 1) following continuous and intermittent sensitization with HDM via the airway, and 2) and when different doses of HDM were used for intermittent sensitization and challenges. We demonstrate that intermittent compared to continuous sensitization results in higher inflammation for the same dose used at each HDM exposure. For continuous sensitization the total dose was 100 µg, whereas, for intermittent the total dose was only 20 µg, which could account for the differences observed in inflammation. Furthermore, results show that sensitization with a higher dose of HDM results in a slightly increased inflammatory response, even when challenged with a non-allergen and mild inflammatory insult, like PBS. Our data demonstrate that at least 10 µg HDM is required for either sensitization or challenge in order to elicit a strong HDM-induced inflammation, with maximum inflammation observed when 10 µg HDM is used for both sensitization and challenge. These studies provide critical information, suggesting that mechanisms underlying the immune responses may be different when continuous or intermittent sensitization protocols are used.

One possible mechanism for intermittent sensitization causing a stronger inflammatory response to HDM could be that the regulatory T cell response may not be as robust in this group as in the continuously sensitized group. Mice sensitized and challenged with HDM via the airway develop a regulatory T cell response and suppression of regulatory T cells

causes an increased capacity of dendritic cells to stimulate T cell proliferation and Th2 cytokine production (Lewkowich, Herman, Schleifer, Dance, Chen, Dienger, Sproles, Shah, Kohl, Belkaid and Wills-Karp 2005). It is possible that continuous exposure to HDM during the sensitization phase “overstimulates” the regulatory T cell response and thereby inhibits the establishment of a strong adaptive Th2 immune response. A recent study demonstrated that sublingual administration of higher HDM doses as tablet MK-8237 to patients with HDM allergy caused patients to display reduced rhinitis and ocular inflammation (Nolte et al. 2015). The results from these studies indicate that higher doses of HDM can suppress the immune response to HDM. The purpose of this study was to establish and describe whether significant differences are observed between the two exposure regimens by quantifying differences in inflammation, mucous cell metaplasia, and epithelial cell hyperplasia. Future studies are required to investigate the mechanisms underlying the differences in the possible accumulation and activation of regulatory T cells in the lungs of mice sensitized to HDM by the intermittent and continuous model.

Another possible mechanism for the suppressed response in the continuous model could be that epithelial cells develop tolerance to HDM exposure. This hypothesis is supported by the observation that children who grew up on dairy farms are protected against the development of allergic asthma (Ege et al. 2011, Illi et al. 2012, Stein et al. 2016). It is believed that this is at least in part due to lipopolysaccharide (LPS) exposure that causes a “tolerance” to inhaled allergens. A similar protective effect for LPS has also been shown in non-farming households that contain high levels of dust. LPS levels in samples of dust found in children’s mattresses were shown to be inversely related to allergic sensitization, asthma, and leukocyte-derived cytokines (Braun-Fahrlander et al. 2002). Previous studies also demonstrated that LPS administered in conjunction with ovalbumin and HDM leads to decreased lung Th2-inflammation (Daan de Boer et al. 2013, Watanabe et al. 2003). LPS, a known component of HDM (Gregory and Lloyd 2011), can cause a “tolerant” phenotype in the lung after continuous stimulations in multiple cell types, including epithelial cells, macrophages, and dendritic cells, and thus, affects downstream allergic inflammation (Albrecht et al. 2008, Hollingsworth et al. 2006, Kallapur et al. 2007). Thus, continuous exposure to HDM may induce a “tolerant” phenotype and reduced allergic inflammation. Future studies with inducible and conditional TLR4 deficient mice would need to be performed to verify this hypothesis.

When comparing continuous and intermittent sensitization, the mucous cell numbers did not demonstrate the same patterns as the inflammatory phenotype. While epithelial cell numbers were increased in intermittent versus continuous sensitization, both methods of sensitization increased mucous cell numbers in comparison to naïve and PBS controls. Previous studies have demonstrated that long term challenge with the OVA antigen facilitates IFN- γ -mediated resolution of mucous cell metaplasia, which is dependent on epithelial cell death (Mebratu et al. 2008). Interestingly, mechanisms governing allergic inflammation and mucous cell metaplasia are both somewhat dependent on epithelial cell activation (Rajavelu et al. 2015). Following allergen challenge, mucous cell numbers increase (Kung et al. 1994, Tesfagzi et al. 2000) and this increase is regulated by Th2 cytokines (Cohn et al. 1997). Allergen-mediated activation of Th2 cells leads to secretion of the Th2 cytokines, IL-13 and IL-4, which are both important in causing mucous cell metaplasia (Hirota et al. 2010,

Kuperman et al. 2005). It is possible that Th2 cytokine levels are different in the two sensitization models because of the regulatory T cells. Although we would hypothesize that IL-13 levels are higher in the intermittent-sensitization group, IL-13 levels in the continuous sensitization group may be sufficient to induce maximum mucous cell metaplasia. This may explain why no differences in mucous cell metaplasia were observed between the different sensitization groups.

Another key finding from our studies is that C57BL/6 mice exposed to HDM demonstrate a dose-dependent increase in both inflammation and mucous cell metaplasia. Our data show that while 1 µg HDM at both sensitization and challenge are not sufficient, 10 µg HDM at sensitization shows a modest increase for both airway eosinophilia and mucous cell metaplasia when challenged with only 1 µg HDM, and vice versa. However, a 10 µg HDM dose for both sensitization and challenge results in the highest increase in eosinophilic inflammation and mucous cell metaplasia. Interestingly, neutrophils are significantly increased following 1 µg HDM sensitization when mice were challenged with 10 µg HDM. While this finding may suggest an alternate inflammatory response, this effect can be considered minimal, as the neutrophils only accounted for approximately 5% of the total cells. Additionally, it also appears that mice both sensitized and challenged with only 1 µg HDM developed an inhibitory response, because eosinophilia and mucous cell metaplasia were less compared to mice challenged with PBS only (**Figs 5E and 6B**). Interestingly, mice sensitized with 10 µg HDM and challenged with PBS showed significantly less inflammation and mucous cell metaplasia, but some eosinophils were observed that likely would not be present in response to the two HDM sensitizations alone. These data would indicate that sensitization to HDM primes the airway to an inflammatory response, regardless of whether the challenge is an allergen or any inflammatory stimulus. Because the purpose of this study was to compare intermittent to continuous sensitization we chose to sensitize with HDM either intermittently or continuously and challenge with PBS, as control. These controls did not demonstrate a significant increase in eosinophils compared to naïve controls. We chose not to include sensitization with PBS as control group, because numerous studies have established that continuous exposure to HDM (as in our protocol) elicits an allergic response, including increased IgE levels and eosinophilia (Botelho et al. 2011, Johnson, Wiley, Fattouh, Swirski, Gajewska, Coyle, Gutierrez-Ramos, Ellis, Inman and Jordana 2004, Piyadasa et al. 2016, Tully, Hoffman, Lahue, Nolin, Anathy, Lundblad, Daphtary, Aliyeva, Black, Dixon, Poynter, Irvin and Janssen-Heininger 2013, Yu et al. 1999).

Conclusion:

Collectively, these results demonstrate that the method of sensitization and the dose of HDM used are critical in determining the extent of inflammation and mucous cell metaplasia in an HDM-induced model of allergic asthma. These results provide critical information for designing experiments to generate HDM-induced inflammation in lungs of C57BL/6 mice.

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Figure 1: Exposure Regimen for continuous and intermittent sensitization.

C57BL/6 mice were exposed via intranasal instillation to 10µg HDM either on days 1–5 and 8–12 (continuous sensitization, Group 1) or on days 1 and 8 only (intermittent sensitization, Group 2). Subsequently, both groups of mice were exposed via oropharyngeal aspiration to either PBS or 10µg HDM once a day on days 15–17. Mice were euthanized on day 19 and lungs were taken for bronchoalveolar lavage fluid (BALF) and histology.

Figure 2: Intermittent sensitization causes extensive inflammation.

C57BL/6 mice were exposed to HDM as described in Figure 1. BALF was collected and assessed for numbers of (A) total cells, (B) neutrophils, (C) lymphocytes, and (D) eosinophils, (E) macrophages. * indicates significant differences compared to the naive control group (p < 0.05).

Figure 3: Extent of epithelial cell hyperplasia and mucous cell metaplasia after continuous and intermittent sensitization.

C57BL/6 mice were exposed to HDM as described in Figure 1. Left lungs were inflated, fixed with zinc formalin, and processed for staining with Alcian Blue and hematoxylin and eosin. Representative histological images depicting epithelial and mucous cells were quantified for (A) total epithelial cells per mm BL and (B) total mucous cells per mm BL. * indicates significant differences compared to the naive control group ($p < 0.05$). (C) Photomicrographs of lung sections were taken at 20x magnification and stained for epithelial and mucous cells (purple). Scale bar=25 μ m

Figure 4: Varying the dose of HDM for intermittent sensitization and challenge.

C57BL/6 mice were intranasally instilled with 1 or 10 μ g HDM on days 1 and 8.

Subsequently, mice were exposed via oropharyngeal aspiration with PBS, 1 or 10 μ g HDM on days 15–17. Mice were euthanized on day 19 and lungs were taken for bronchoalveolar lavage fluid (BALF) and histology.

Figure 5: Extent of inflammation depends on the dose of HDM used at sensitization and challenge.

C57BL/6 mice were exposed to HDM as described in Figure 4. BALF collected was assessed for total (A) cells, (B) lymphocytes, (C) eosinophils, and (D) neutrophils, (E) macrophages. * indicates significant differences compared to the naive control group (p < 0.05).

Figure 6: Extent of epithelial cell hyperplasia and mucous cell metaplasia depends on the dose of HDM used at sensitization and challenge.

C57BL/6 mice were exposed to HDM as described in Figure 4. Left lungs were inflated, fixed with zinc formalin, and processed for staining with Alcian Blue and hematoxylin and eosin. Representative histological images depicting epithelial and mucous cells were quantified for (A) total epithelial cells per mm BL and (B) total mucous cells per mm BL. * indicates significant differences compared to the naive control group ($p < 0.05$). (C) Photomicrographs of lung sections were taken at 20x magnification and stained for epithelial and mucous cells (purple). Scale bar=25 μ m