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The effects of inhaled house dust mite on airway barrier function and sensitivity to inhaled methacholine in mice

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Turi GJ, Ellis R, Wattie JN, Labiris NR, Inman MD. The effects of inhaled house dust mite on airway barrier function and sensitivity to inhaled methacholine in mice. *Am J Physiol Lung Cell Mol Physiol* 300: L185–L190, 2011. First published November 5, 2010; doi:10.1152/ajplung.00271.2010.—Asthma is functionally characterized by increased airway sensitivity and reactivity. Multiple mechanisms are believed to underlie these functional disorders, including impairment of airway wall barrier function. One proposed mechanism of impaired barrier function is through the direct consequence of proteolytic properties of inhaled allergens, including house dust mite (HDM). Here, we have observed the direct effects of HDM on airway barrier function and response to nebulized or intravenous methacholine. HDM naïve BALB/c mice were anesthetized, exposed to intranasal or intratracheal HDM (15 or 100 μ g), and allowed to recover for 30 min or 2 h before methacholine challenge. A separate group of mice was exposed to intratracheal poly-L-lysine (PLL; 100 μ g) for a duration of 30 min. This group served as a positive control for the presence of impaired barrier function and airway hypersensitivity. Negative control mice received saline challenges. Outcomes included assessment of lung mechanics in response to nebulized or intravenous methacholine as well as clearance of intratracheally instilled technetium-labeled (^{99m} Tc) DTPA to evaluate airway epithelial barrier function. We found that PLL produced a leftward shift in the dose-response curve following nebulized but not intravenous methacholine challenge. This was associated with a significantly faster clearance of ^{99m} Tc-DTPA, indicating impairment in airway barrier function. However, HDM exposure did not produce changes in these outcomes when compared with saline-exposed mice. These findings suggest that direct impact on airway barrier function does not appear to be a mechanism by which HDM produces altered airway sensitivity in airway disease.

asthma; mouse; epithelium

ASTHMA IS A COMPLEX RESPIRATORY disease characterized by variable and reversible airway obstruction and associated with airway inflammation, airway remodeling, and airway hyper-responsiveness (AHR). AHR, common to all asthmatics, refers to the tendency of airways to constrict to a greater degree (airway reactivity) and to require less contractile stimulus (airway sensitivity) when compared with healthy airways (21, 24). AHR is thought to contribute significantly to asthma morbidity, and attempts to control this disorder result in improved patient management (22). Many possible contributors to AHR have been identified, including increases in smooth muscle mass (15), airway wall thickening (26), changes in smooth muscle sensitivity (6, 13), and changes in airway wall barrier function (10).

Airway wall barrier function as a determinant of airway sensitivity was illustrated by Mitchell and colleagues (18) when they demonstrated that removal of the epithelial border

markedly increased the sensitivity of dog bronchus to luminal, but not extraluminal, application of contractile agonists. This was extended by Bates et al. (2), who demonstrated that damage to mouse epithelium by intratracheal cationic protein poly-L-lysine (PLL) resulted in increased sensitivity to inhaled, but not intravenous, methacholine. Surprisingly, early observations did not demonstrate significant impairment of barrier function in asthmatic compared with healthy control subjects (7, 20). However, recent studies have demonstrated significant impairment of airway barrier function in poorly controlled pediatric and adult asthmatic patients (3, 19).

Impaired barrier function has been demonstrated in epithelial cells in coculture with activated human eosinophils (1) and as a result of IL-13-induced goblet cell metaplasia (14). In addition to pathology resulting from immune-based responses in the airway, it has been postulated that some allergens may have direct impact on the airway, contributing to impaired barrier function. Application of house dust mite (HDM) fecal pellets to cultured epithelial cells results in decreased intraepithelial staining for the zona occludens-1 adhesion molecule and increased rate of transepithelial movement of mannitol (25). In a later study, HDM extract was shown to have similar effects on E-cadherin and ZO-1 adhesion molecules, as well as produce impaired barrier function as assessed by transepithelial resistance in cultured human airway epithelial cells (8). As no sensitization had occurred in these models, these responses were independent of any preprogrammed immune response to the HDM.

Here, we investigate the hypothesis that airway exposure to HDM in unsensitized mice will result in impaired airway barrier function, and that this will in turn result in hypersensitivity to inhaled but not intravenous smooth muscle agonists. To address this, we exposed allergen naïve mice to intranasal or intratracheal HDM extract and then 1) assessed airway barrier function based on single proton emission-computed tomography (SPECT) quantified clearance of intratracheally delivered ^{99m} Tc diethylene triamine pentaacetic acid (DTPA), 2) visualized distribution of intratracheally administered horseradish peroxidase (HRP) using scanning electron microscopy, and 3) assessed the impact on airway function by measuring responsiveness to both inhaled and intravenous methacholine. As a positive control, measurements were also made following exposure to PLL.

MATERIALS AND METHODS

Animals

Female BALB/c mice, aged 8–10 wk, were purchased from Charles River Laboratories (Saint-Constant, QC, Canada). They were housed in environmentally controlled ultraclean conditions and allowed standard laboratory chow and water ad libitum. All procedures

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were reviewed and approved by the Animal Research Ethics Board at McMaster University (Hamilton, ON, Canada).

Reagents

HDM *Dermatophagoides pteronyssinus* extract (Greer Laboratories, Lenoir, NC) was dissolved in sterile 1× Dulbecco's PBS (Invitrogen, Carlsbad, CA). PLL (Sigma-Aldrich Canada, Oakville, ON) was dissolved at a concentration of 0.6 mg/ml in 1× PBS for all experimental protocols.

All drugs were prepared daily. Rompun (xylazine hydrochloride; Bayer HealthCare, Ontario, Canada) was administered IP (10 mg/kg) followed 5 min later by pentobarbital sodium (Ceva Animal Health, Manchester, MO) also as an IP injection (30 mg/kg). Pancuronium bromide (Sandoz, Quebec, Canada) was administered as an IP injection (20 mg/kg) following anesthesia to prevent ventilatory efforts during physiological assessment.

Acetyl-β-methylcholine chloride solution (Sigma-Aldrich Canada, Oakville, ON), when nebulized, was prepared fresh on each experiment day by diluting in 1× PBS to the highest experimental concentration and performing serial dilutions down to the lowest concentration. The majority of experiments in this study used 3.125, 6.25, 12.5, 25, and 50 mg/ml nebulized MCh. However, this range of doses did not allow us to describe the full range of the dose-response curve in PLL-exposed mice. We therefore performed two separate dose-response assessments in these and control mice using the MCh ranges of 0.39, 0.78, 1.56, 3.125, and 6.25 mg/ml MCh in the first groups and 12.5, 25, 50, 100, and 200 mg/ml in the second. The reason for two separate ranges of doses is that we have observed that measurements become increasingly variable as mice are exposed to increased numbers of nebulizations. MCh solutions used for IV challenge were made fresh weekly and stored at 4°C when not in use. The concentrations used for IV challenge were 10, 33, 100, and 330 µg/kg.

Exposure Protocols

Assessment of airway responses was made 2 h following exposure to HDM as this was the time point where maximal barrier dysfunction was observed in vitro (25). Airway assessment was made 30 min following PLL exposure, according to the observations made by Bates and colleagues (2). To allow direct comparison between mouse groups, HDM-exposed mice were also assessed 30 min following exposure.

In the 2-h recovery groups, mice were lightly anesthetized with 2% isoflurane (Abbott Canada, Quebec, Canada) and then given one intranasal (IN) instillation of 15 µg of HDM protein in 25 µl of PBS. This dose of HDM is that which we have used to achieve maximal allergic responses in sensitized mice. Mice were allowed to recover from the anesthetic and assessed 2 h later. In all 30-min recovery groups, mice were given an IP injection of xylazine (10 mg/kg) followed 5–10 min later by an IP injection of pentobarbital sodium (30 mg/kg). Mice were then intubated via tracheostomy using an 18-gauge blunted needle as an endotracheal (ET) tube. The PLL exposure protocol was obtained from the methods of Homma and colleagues (12). In this protocol, 50 µl of PLL (2 mg/ml) was instilled into the trachea through the ET tube, followed by 3× 300-µl aliquots of air. The mouse was then immediately connected to a rodent ventilator (Scireq, Quebec, Canada) and ventilated at 150 breaths/min for 30 min. For the 30-min HDM exposure groups, mice were sedated and intubated in the same manner as above. HDM was then instilled through the ET tube either as 15 µg/50 µl or 100 µg/50 µl, depending on the treatment group, followed by 3× 300-µl aliquots of air. Mice were placed immediately on the rodent ventilator and ventilated as above for 30 min.

Assessment of Airway Mechanics

Mechanics were assessed on a flexiVent v5.2 rodent ventilator (Scireq). Mice were ventilated at a rate of 150 breaths/min through an

18-gauge ET tube. Lung volume history was normalized before each dose of MCh by inflating the lungs to a pressure of 25 cmH₂O and maintaining this pressure for 3 s, followed by 30 s of normal ventilation. Total respiratory system resistance (R_{RS}) was measured using a single-compartment, standardized breath signal, which allows measurement of resistance, dynamic compliance, and elastance (Snap-Shot-150 perturbation). Measurements were obtained at baseline and following increasing concentrations of MCh. MCh was nebulized for 10 s through a micropump nebulizer (Aerogen, Galway, Ireland) during the inspiratory cycle at a flow rate of ~0.15 ml/min. Resistance measurements were obtained for 3 min following nebulization of each concentration of MCh. Airway responses to intravenous (IV) MCh challenge was also assessed in PLL- and saline (SAL)-treated mice using doses of 10, 33, 100, and 330 µg/kg. The ventilation rate and ET tube used were the same as described above for nebulized MCh. Lung volume history was also normalized as above. Due to the faster physiological response to IV MCh, resistance measurements were obtained for 1 min following injection of MCh. To ensure that animals were adequately ventilated and not experiencing distress, heart rate and oxygen saturation were monitored throughout the procedures using infrared pulse oxymetry (Biox 3700; Ohmeda, Boulder, CO) with a standard ear probe placed on the proximal portion of the mouse's hindlimb. After the last dose, mice were removed from the ventilator, killed via terminal exsanguination, and further processed for tissue collection. PC₂₀₀ values were calculated via interpolation to quantify the degree of leftward shift in the dose-response curve. This represents the dose of methacholine required to increase the R_{RS} 100% above baseline.

Assessment of Airway Barrier Function

^{99m}Tc-DTPA clearance. Three groups of four mice each were assessed: PLL (100 µg/50 µl IT), HDM (100 µg/50 µl IT), and SAL (50 µl IT). All groups were allowed to recover for 30 min with mechanical ventilation after treatment. After the 30-min ventilation period, 250 µCi ^{99m}Tc-DTPA was instilled in a 20-µl colloid solution through the ET tube with a microsyringe (Penn-Century, model FMJ-250). Each mouse was then placed in a GammaMedica-Ideas X-SPECT system (NorthRidge), and dynamic 2-D planar images of the entire body were acquired over a 20-min period at a rate of 1 frame/30 s (i.e., 40 frames total). The ^{99m}Tc-DTPA images over the 20-min period were analyzed using Amide medical image analysis software (Free Software Foundation). A standard region of interest was positioned on the whole lung area, and the amount of ^{99m}Tc-DTPA was measured for each 30-s period. The mean count from the lung region of interest was converted to percent of the highest (usually measured in the first or second frame) count for that mouse (and expressed as %max). To compare the control and exposure groups, the area above the time vs. %max curve for the 20-min period (area above the curve; AAC) was calculated for each mouse.

Horeseradish Peroxidase Uptake

This method was based on that used previously in guinea pigs (5). Mice were given an IP injection of xylazine (10 mg/kg) followed 5–10 min later by an IP injection of pentobarbital sodium (30 mg/kg). When the toe-pinch reflex was absent, the mouse was intubated and exposed to either saline (50 µl), PLL (50 µl of 2 mg/ml), or HDM (100 µg/50 µl). Each 50-µl treatment was instilled into the trachea through the ET tube, followed by 3× 300-µl aliquots of air. The mouse was then immediately connected to the flexiVent rodent ventilator and ventilated at 150 breaths/min for 30 min. After 15 min of ventilation, the mouse was temporarily removed from the ventilator, and HRP (0.25 mg dissolved in 50 µl of saline) was instilled into the trachea through the ET tube, followed by 3× 300-µl aliquots of air. The mouse was then immediately reconnected to the ventilator for the remaining 15 min of ventilation. After 30 min, mice were removed from the ventilator, and their lungs were removed and inflated with cold

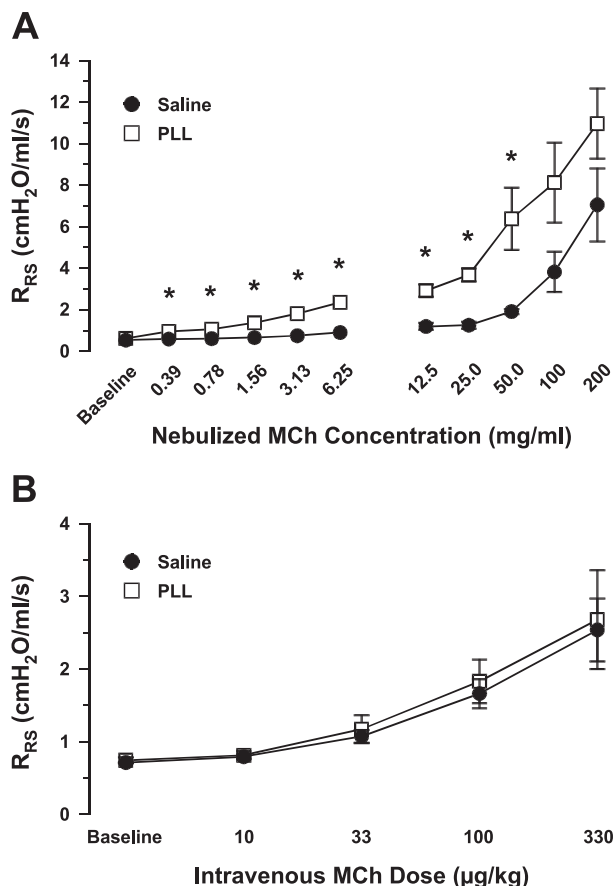


Fig. 1. Dose-response curves of total respiratory system resistance following increasing concentrations of nebulized (A) or intravenous (B) MCh in SAL and poly-L-lysine (PLL)-treated mice. The mice were treated with SAL (50 μ l) or PLL (100 μ g/50 μ l) intratracheally, ventilated for 30 min, and then subjected to a nebulized or IV MCh challenge. The break in the curve in A represents 2 separate experiments using 2 different ranges of MCh concentrations. Each point represents the mean and SE for SAL and PLL ($n = 5$ /group). * $P < 0.05$ compared with saline (SAL).

glutaraldehyde (20 cmH₂O pressure) and then fixed overnight in glutaraldehyde at 4°C. The following day, the second-generation airway of the left lung lobe was isolated by microdissection and stained in a solution of 3,3-diaminobenzidine tetrahydrochloride and imidazole. After washing in three changes of distilled water and postfixing for 90 min in osmium tetroxide, the tissues were dehydrated and embedded in Spurr resin. Thin sections were cut on an ultramicrotome and examined in a JEOL electron microscope (JEM -1200EX, Tokyo, Japan).

Statistical Analysis

All data are expressed as means $\pm$ SE of the mean. All PC₂₀₀ values were log transformed before statistical analysis. An unpaired Student's *t*-test was used for comparisons between groups. *P* values less than 0.05 were considered significant.

RESULTS

PLL and Airway Hypersensitivity

Mice were exposed to PLL or SAL and challenged with nebulized MCh to generate a dose-response curve over a range of MCh concentrations. Figure 1A illustrates the dose-response curves from two groups collectively challenged with MCh over

this range of concentrations. Mean peak respiratory system resistance (R_{RS}) was significantly greater ($P < 0.05$) in PLL-treated mice compared with SAL at all MCh concentrations including and above 0.78 mg/ml. The mean PC₂₀₀ MCh value for PLL-treated mice, 1.1 mg/ml, was significantly less ($P < 0.05$) than the value of 15.7 mg/ml calculated for SAL-treated mice.

Separate groups of SAL- or PLL-exposed mice were challenged with IV MCh. There were no significant differences in mean peak R_{RS} between PLL and SAL exposure at any concentration of IV MCh (Fig. 1B). There was also no significant difference between SAL (52.8 μ g/ml) and PLL (42.8 μ g/ml) PC₂₀₀ values.

HDM and Airway Hypersensitivity

Figure 2A illustrates the dose-response curves of nebulized MCh from groups of mice treated with HDM (15 μ g/25 μ l IN) or SAL (25 μ l IN) and allowed to recover for 2 h, or PLL (100 μ g/50 μ l IT) and allowed to recover for 30 min. There were no significant differences between HDM and SAL peak R_{RS} values at any concentration of MCh. Consistent with this finding, there were no differences in the PC₂₀₀ values calcu-

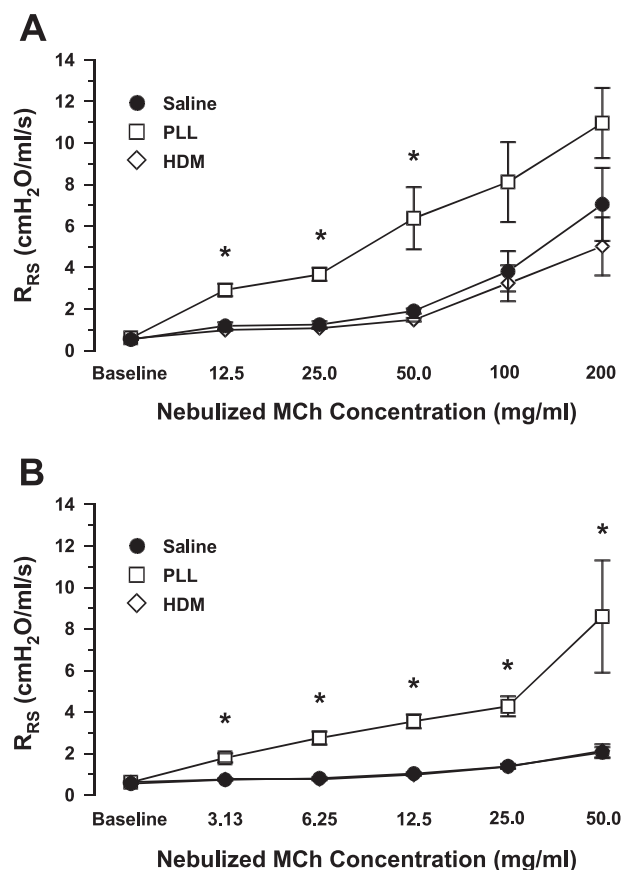


Fig. 2. Dose-response curve of changes in total respiratory system resistance following increasing concentrations of nebulized MCh in SAL-, house dust mite (HDM)-, or PLL-treated mice. The mice were treated with SAL (25 μ l IN) or HDM (15 μ g/25 μ l IN) and allowed to recover for 30 min (A) or 2 h (B). Comparison curves from mice exposed to PLL (100 μ g/50 μ l IT) and allowed to recover for 30 min are also shown. Where it is not visible in B, the HDM curve is located directly beneath the SAL curve. Each point represents the mean and SE for SAL, HDM, and PLL ($n = 5$ /group). * $P < 0.05$ compared with SAL.

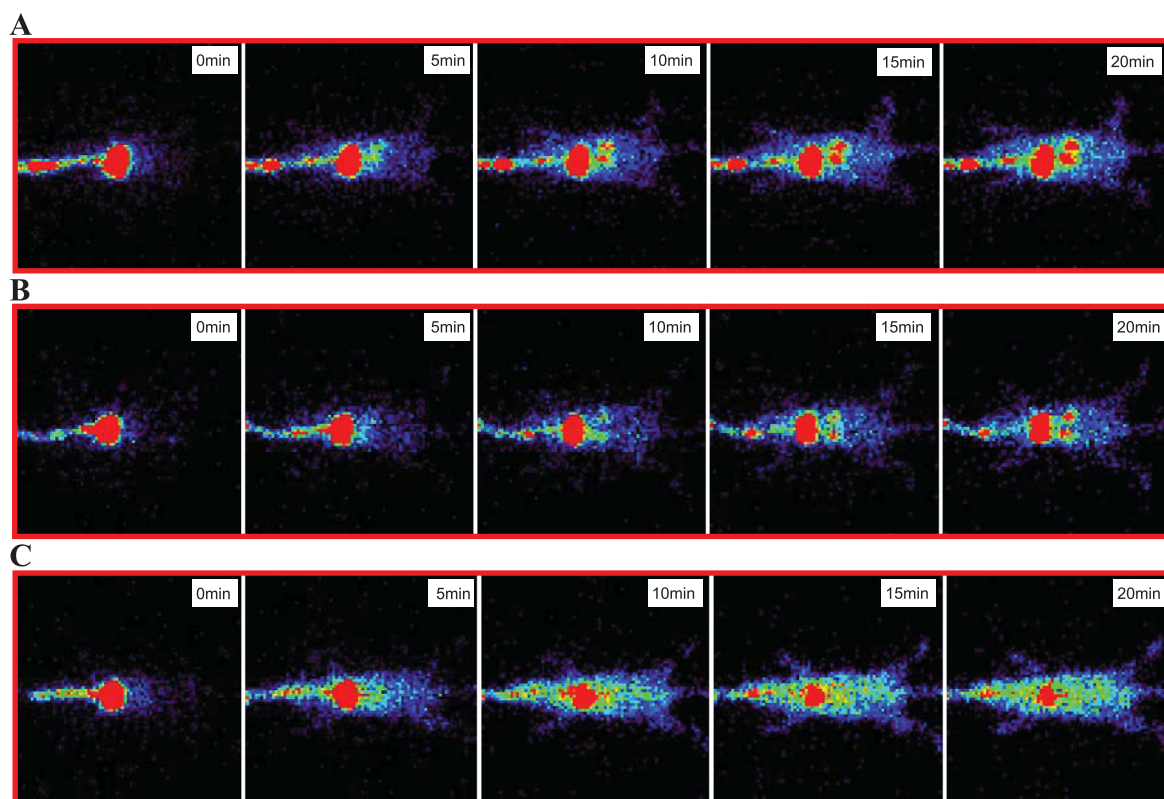


Fig. 3. SPECT images of mice instilled intratracheally with ^{99m}Tc -DTPA and imaged over 20 min. Images are representative of mice treated intratracheally with either SAL (A), HDM (B), or PLL (C) followed by 30 min of ventilation and then ^{99m}Tc -DTPA instillation. The color red indicates the highest ^{99m}Tc -DTPA counts followed by yellow, green, and then blue.

lated from HDM mice and SAL-exposed mice. In addition to a 2-h recovery period, airway responses were assessed following a 30-min recovery period after HDM (15 $\mu\text{g}/50\ \mu\text{l}$ IT) or SAL exposure (50 μl IT). These groups were again compared with a PLL-treated group with 30-min recovery as a positive control (Fig. 2B). Again, there was no difference in R_{RS} at any dose of nebulized MCh, nor was there an effect on the PC_{200} ($P > 0.05$). We also studied mice 30 min following a much higher concentration of HDM (100 $\mu\text{g}/50\ \mu\text{l}$) with a 30-min recovery period and again observed no influence on contractile responses to nebulized MCh (data not shown).

Airway Barrier Function Following PLL and HDM Exposure

The movement of ^{99m}Tc -DTPA out of the lungs was assessed in three mice in each group treated with either SAL, HDM, or PLL and allowed to recover for 30 min under mechanical ventilation before imaging. Figure 3 shows the SPECT images from representative mice in each group over the 20 min that images were collected. Lung radiation counts from these images were generated and then calculated as a percent of the highest count over time (Fig. 4). For statistical analysis, the area above the curve for the 20-min collection period was calculated and means compared (Table 1). This analysis confirmed that compared with control mice, clearance of ^{99m}Tc -DTPA was faster in the PLL-exposed mice but not the HDM-exposed mice.

Graphic demonstration of the impaired barrier function was also obtained from three mice per group by EM imaging of

HRP between epithelial cells and in association with smooth muscle. As shown in representative images (Fig. 5), HRP between epithelial cells was visualized in PLL- but not HDM-exposed mice (saline mice airways appeared identical to the HDM image shown in this figure).

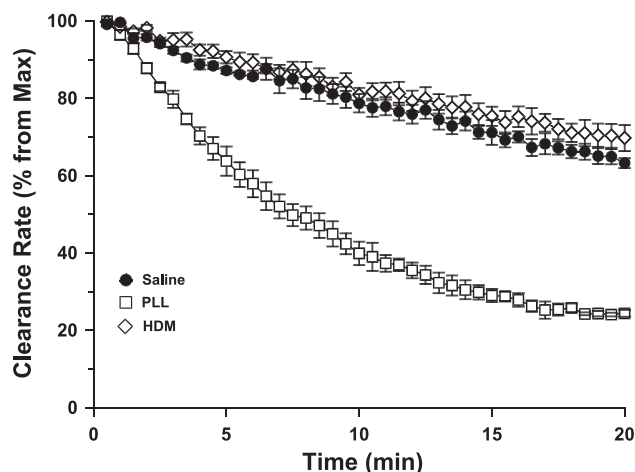


Fig. 4. Change in ^{99m}Tc -DTPA mean voxel lung counts as a percent of highest count over 20 min in SAL-, HDM-, and PLL-treated mice. The mice were treated with SAL (50 μl IT), HDM (100 $\mu\text{g}/50\ \mu\text{l}$ IT), or PLL (100 $\mu\text{g}/50\ \mu\text{l}$ IT) and ventilated for 30 min before ^{99m}Tc -DTPA was instilled through the endotracheal tube. Each point is the mean $\pm$ SE for SAL, HDM, and PLL ($n = 4/\text{group}$).

Table 1. Mean area above curve ($\pm$ SE) and *P* values of ^{99m}Tc -DTPA lung counts from mice treated with SAL, HDM, or PLL

Treatment	Area Above Curve	<i>P</i> value (compared with SAL)
SAL	20.4 $\pm$ 1.7	—
HDM	16.9 $\pm$ 2.2	>0.05
PLL	52.3 $\pm$ 1.9	<0.001

DISCUSSION

We hypothesized that direct impact on barrier function was one of the mechanisms by which HDM exposure produced airway hypersensitivity in exposed individuals. To address this, we exposed HDM naïve mice to HDM extract and assessed for changes in both airway barrier function and airway sensitivity. While both of these outcomes were influenced as expected by the positive control exposure (PLL), neither outcome was affected by exposure to HDM. We conclude from these studies that it is unlikely that direct effects of HDM play a significant role in the airway hyperresponsiveness observed in asthma.

Impairment of barrier function has been proposed as one of several mechanisms underlying AHR (10, 11). This is supported by observations of impaired barrier function in poorly controlled asthma (3, 19). Further support was obtained in studies of direct exposure of cultured epithelial cells to HDM fecal pellets or extract. Wan and colleagues (25) observed a time-dependent decrease in barrier function, which peaked at $\sim$ 2 h and was still present at 6 h, whereas Heijink et al. (8) observed decreased transepithelial resistance at 15 min which had resolved by 60 min. The fact that we did not observe any *in vivo* effect of direct exposure to HDM on either barrier function or responsiveness to MCh after either 30 min or 2 h suggests that any such effect must be minor and would seem unlikely to play a significant role in severity of disease. This would imply rather that impairment of barrier function in asthma is more likely a consequence of the disease itself, including such factors as eosinophil granule products (12) or IL-13-associated goblet cell metaplasia (14).

One possible explanation for the discrepancy between previous *in vitro* findings and our findings in live mice is that our exposure method failed to deliver HDM in sufficient quantities to the airways where allergen-induced damage would have an impact on measurements of barrier function or airway sensi-

tivity. We have previously characterized the intranasal delivery method, finding that $\sim$ 50% of instillates in the range of 25 to 75 μl becomes dispersed throughout the lungs (23). We have also used this method of delivery in sensitized animals, observing widespread inflammation and remodeling changes in both large and small airways, again suggesting widespread distribution (9). The lowest dose of HDM that we exposed mice to was 15 μg in 50 μl . This is the dose that we routinely use both for sensitization and in recall challenges of sensitized mice (16). This dose has also been shown to be sufficient to produce maximal recall responses, in terms of inflammation and AHR in sensitized animals (17). As we were concerned, however, that the dose of allergen may not have been sufficient to achieve damage to airway barrier, we also exposed mice to 100 μg in 50 μl , again observing no impact on either barrier function or airway sensitivity.

In our studies, we have confirmed observations made by Bates and colleagues (2, 12) that cationic proteins will increase airway sensitivity to inhaled, but not intravenously delivered, agonists. This increase in sensitivity is illustrated in Fig. 1, where the dose-response curve is clearly shifted to the left (lower concentrations of MCh), and by the lower PC₂₀₀ measured in the PLL-exposed mice. We were not able to determine whether PLL exposure increased the maximal bronchoconstricting effect of MCh, as plateaus in the dose-response curves could not be achieved in either PLL- or control-exposed mice. The fact that there was no difference in the dose-response curves established with IV delivery suggests that neither contractile machinery, nor its linking to the airway lumen, were affected by PLL exposure. We agree with Bates and colleagues, therefore, that the most likely explanation for the observed effects of PLL is enhanced delivery as a result of impaired barrier function. In fact, we have extended their observations by confirming that barrier function was impaired in these mice. PLL exposure resulted in increased clearance of ^{99m}Tc -DTPA and movement of HRP between adjacent epithelial cells.

By including PLL-exposed mice in this study, we demonstrated that imaging the clearance rates of ^{99m}Tc -DTPA was a sensitive tool with which to detect impairment of airway wall barrier function. It is possible that this tool is not sensitive enough to detect smaller, but biologically relevant impairments to barrier function, as may have been the case with HDM

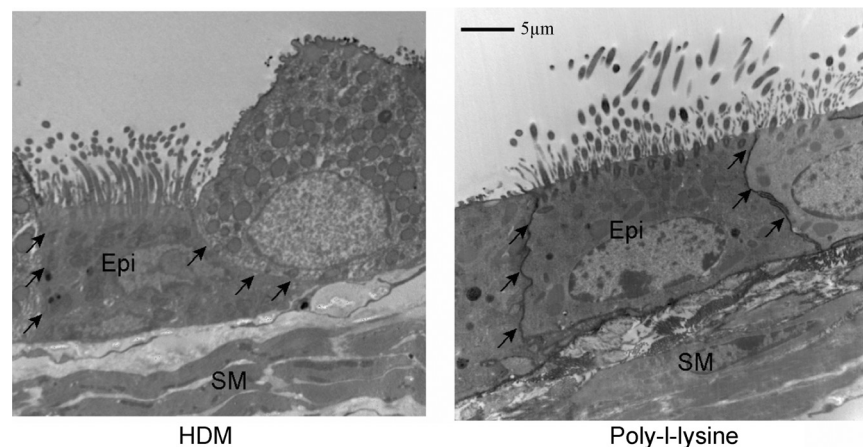


Fig. 5. EM images of HRP distribution in the airways of PLL- and HDM-exposed mice. The mice were exposed to HDM (100 $\mu\text{g}/50 \mu\text{l}$ IT) or PLL (100 $\mu\text{g}/50 \mu\text{l}$ IT) and ventilated for 15 min before instilling HRP (0.25 mg/50 μl IT) and ventilated for a further 15 min before death. Epi, epithelial cell; SM, smooth muscle; arrows point to intraepithelial cell junctions. HRP is visualized as black.

exposure. However, this method has been used to detect difference in barrier function between mice strains, as well as changes in barrier function in response to subtle changes in positive end-expired pressure in mice (4). We have also used this method to detect transient changes in barrier function following ozone exposure (unpublished observation). We are therefore confident that this method is a sensitive tool with which to assess barrier function in mice. Furthermore, the fact that HDM exposure also failed to induce any change in airway sensitivity to MCh supports the conclusion that if there was in fact a subtle change in airway barrier function, this was small enough not to have altered responses to inhaled agonists.

The HDM extract we studied has been shown to be proteolytically active and is the same extract that has induced transient impairment of barrier function in cultured human airway epithelial cells (8). It is possible that continued exposure to the proteolytic effects of HDM may result in impaired barrier function over time. This would not have been detected in our design, where the longest duration of observation was 2 h after a single instillation. However, in vitro, near maximal effects were observed after only 15–30 min of exposure (8, 25). Moreover, a design with repeated exposure over several days would not have been feasible to address our hypothesis. Such exposure protocols result in sensitization, and it would not have been possible to separate immune-mediated effects on barrier function from direct effects of the allergen extract.

In conclusion, using a model of in vivo exposure of HDM to mice, we were not able to detect a direct impact of such exposure on either airway barrier function or airway function. However, the observation of significant hypersensitivity associated with impaired barrier function in PLL-exposed mice strengthens the hypothesis that such impairment may have significant functional consequences in airway disease.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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