

CHRONIC AIRWAY DISEASE: DETERIORATING PULMONARY FUNCTION IN SHEEP ASSOCIATED WITH REPEATED CHALLENGES OF HOUSE DUST MITE

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□ *The aim of this work was to characterize lung function and cellular responses in a large animal model for chronic asthma. All sheep were sensitized to house dust mite (HDM) by subcutaneous injection of HDM before lung challenges. Groups of sheep were given weekly lung challenges with either HDM ($n = 12$) or saline (control, $n = 5$) for 3 months. Post challenge, there were significant increases in lung resistance in 7 out of 12 HDM-challenged sheep, compared to control sheep. In HDM-responding sheep, there was a progressive increase in the magnitude of HDM-induced resistance throughout the trial. All HDM-challenged sheep developed BAL eosinophilia and bronchial hyperresponsiveness. In conclusion, sheep chronically challenged intralung with HDM consistently develop airway hyperresponsiveness and eosinophilia, whereas allergen-specific bronchoconstriction is observed in just over half of these sheep.*

Keywords asthma, bronchial hyperresponsiveness, eosinophil, lung infection

Although asthma is a complex disease with many contributing disease components, the inflammatory process is the most common manifestation of the disease (extrinsic asthma) and is believed to be a result of inappropriate immune responses to common aeroallergens in genetically

Received 10 February 2006; accepted 7 July 2006.

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susceptible individuals [1]. The main characteristics of asthma are chronic inflammation of the airways, intermittent and reversible obstruction to airflow, and airway hypersensitivity [2]. The complexity of asthma in the human population has made the disease difficult to study and is often overlooked in animal models of asthma [3]. Most animal models of asthma are based on inbred rodent populations with genetically homogeneous individuals [4]. For example, there are a number of strains of mice that display distinct immunological, cellular, and physiological responses to allergen challenge. Although there are major differences in allergic responses between strains, there is considerably less variation in responses to allergen challenge between individuals within the strain [5].

Sheep have been used extensively as a model to investigate the disease mechanisms of asthma [6–9]. Outbred sheep display a wide range of atopic responses to allergen sensitisation [8] and display differing physiological, immunological, and structural responses to lung challenges with allergen [6, 7, 9]. Approximately, 50% to 60% of sheep peripherally immunized with house dust mite (HDM) respond with significantly elevated levels of immunoglobulin E (IgE) antibody in the serum [8]. Only atopic sheep with high IgE levels respond to HDM lung challenges with persistent recruitment of high numbers of eosinophils to bronchoalveolar lavage (BAL) fluid and airway tissues. Sheep responding to HDM with high serum IgE, airway eosinophilia, and airway mast cell hyperplasia, show varying degrees of airway remodeling following chronic lung challenge of HDM [9]. It is still not clear what mechanistic factors are responsible for airway remodeling, although it is likely that differing levels of repair/growth factors in individual sheep may account for the airway structural change observed. There is also diversity in the physiological responses of sheep exposed to aerosols of *Ascaris suum* extract. Although most sheep have an early phase response to *Ascaris* (i.e., an increase in lung resistance within the first hour after challenge), only a proportion of these sheep develop late phase responses (i.e., an increase in lung resistance at least 5 hours after challenge). Dual-responding *Ascaris*-exposed sheep (i.e., early- and late-phase responding sheep) are differentiated by predominant increases of neutrophils in the BAL and airway tissues in the early phase and by an increase in the number of eosinophils in the late phase period [7, 10]. This mimics the situation in the clinic where up to 60% of asthmatics who have early-phase also have late-phase reactions. The mechanism is believed to be related to the specific infiltrating cellular response in late phase asthmatics [11].

The focus of this study was to investigate the physiological responses to HDM allergen in the airways of chronically challenged sheep by monitoring the lung resistance (R_L) and identifying flow limitations and hyper-responsiveness to bronchoconstrictors. In addition, the pulmonary inflammatory response to HDM challenge was assessed.

METHODS

Animals

Ten-month-old female Merino-cross sheep used in these studies were immunized with HDM extract (*Dermatophagoides pteronyssinus*; CSL, Melbourne, Australia) as previously described [8]. Eighteen sheep with high HDM-specific serum IgE levels (atopic sheep) were selected for chronic challenges. The challenge protocol used in this study was modified from our previous study [9]. All animals were challenged weekly with 2×10 mL 200 μ g/mL HDM or SAL (normal saline) solution being infused directly into the left and right lung and every 4 weeks the animals were ventilated (BEAR 2; Bear Medical Systems, Palm Springs, USA) at 20 breaths per minute with a nebulized 1 mg/mL HDM or SAL solution for 15 minutes as per timeline in Figure 1 [4]. Twelve animals were challenged with HDM and 6 atopic control animals where challenged with normal saline. All experimental animal procedures and the collection of cells/tissues were approved by the Animal Experimentation Ethics Committee of the University of Melbourne.

Measurement of Lung Function

The measurement of lung function was performed by assessing esophageal and tracheal pressures and pulmonary airflows similar to earlier published methods [6, 15]. Briefly, pressures and flow were acquired from conscious sheep using a National Instruments Data Acquisition (DAQ) system with a Virtual Instrument programmed with LABView (National Instruments, Austin, USA). Unsedated sheep were appropriately restrained in a custom-made body sheath and head-restraining harness tethered in a

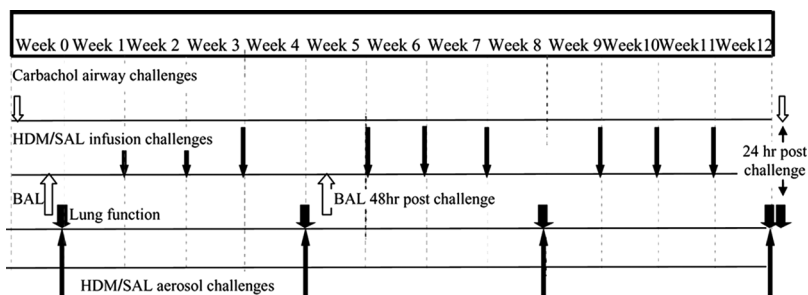


FIGURE 1 Outlines the timeline for all experimental procedures performed on sheep throughout the chronic challenge regime. Prior to week 0 all sheep were sensitized to house dust mite (HDM) with 3 fortnightly subcutaneous (SC) injections of HDM. Sheep that responded with high serum titres of HDM-specific IgE were selected for repeated lungs challenges. (SAL = normal saline).

metabolism cage. After topical anaesthesia of the nasal passages, a balloon catheter was advanced through one of the nostrils into the lower oesophagus. Through the other nostril, a bronchoscope was used to guide the insertion of a cuffed endotracheal tube. A side-hole catheter was then advanced 2 cm past the distal end of the endotracheal tube to measure tracheal pressures. The oesophageal and tracheal catheters were connected to differential transducers (Gaeltec 8T; Gaeltec, Dunvegan, Scotland) to measure oesophageal pressure (P_{oes}) and airway pressures (P_{aw}) respectively. Transpulmonary pressure (P_{l}) was obtained from P_{oes} with respect to P_{aw} . Flow was measured via a pneumotachograph (Hans Rudolph, Kansas City, USA) attached to the proximal end of the endotracheal tube. Lung dynamics were determined by a modified Mead-Whittenberger method [12] and measurements were taken at 28-day intervals. The breath by breath analyses of flow and P_{l} was used to determine R_{L} and dynamic compliance from an average of 5 breaths. Data from breaths that differed by more than 20% in volume and more than 10% of the breathing rate were rejected.

Bronchial Hyperresponsiveness (BHR)

BHR was assessed separately, before the start of the challenge period, and at the 3-month period, 24 hours after the last aerosol challenge (Figure 1). This was achieved by administering the bronchoconstrictor, carbachol, in a range of doubling aerosol doses (0.25% to 4% carbachol *w/v*) and measuring changes in R_{L} . The results were then expressed as the concentration of carbachol aerosol (1 breath unit [BU] is 1 breath of 1% *w/v* carbachol) needed to increase R_{L} by 200% from baseline.

Bronchoalveolar Lavage Analysis

BAL cells were collected from sheep directly before challenge, and again 48 hours after a HDM aerosol challenge, 4 weeks into the repetitive challenge regime (Figure 1). Ten mls of saline was introduced into the left lung of all sheep using a fibre-optic bronchoscope [8]. The recovered BAL cells were counted, centrifuged, dried onto a microscope slide as a cytospot and then differentially stained with Wright-Geisma stain (Sigma, Castle Hill, NSW, Australia).

Statistical Analysis

The change in resistances at each time point were examined by Kruskal-Wallis test and differences amongst groups for the chronic challenge were compared by Duncan's multiple range test. The pre- and post-HDM challenge values for breath units and BAL cell percentage counts were analysed

with the Wilcoxon matched-pairs test. All of the analyses were performed using Statistical Analysis System v9.1 (SAS Institute, Cary, NC, USA) and significant differences were determined when $P < .05$.

RESULTS

Early-Phase Allergen-Specific Airway Responses

Lung function data gathered after HDM challenge demonstrate that sheep clearly segregated into 2 distinct groups in terms of their early-phase allergen-specific responses to HDM aerosols (Figure 2). Sheep that responded to HDM (responders, $n = 7$) recorded significant increases in resistance over baseline for days 56 and 84 ($P < .05$). However, a proportion of sheep did not significantly respond to HDM (nonresponders, $n = 5$) at the 4 time points assessed. The airway responses to allergen challenge in nonresponder sheep were not significantly different from responses in control sheep challenged with saline. There was also a progressive increase in allergen-induced bronchoconstriction in responder sheep over the 4 time points assessed (Figure 2). There were no significant differences between responder and nonresponder sheep in dynamic compliance and tidal volumes (data not shown).

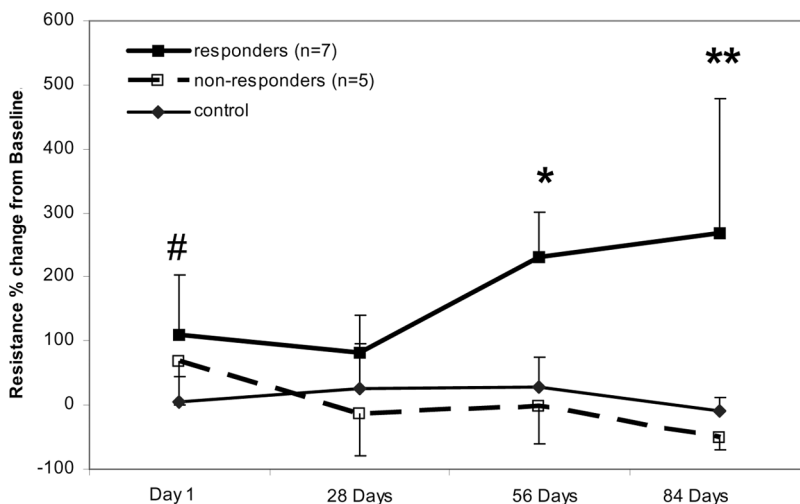


FIGURE 2 The mean percentage change from baseline in lung resistance in groups of sheep challenged with aerosols of either HDM or saline. The results for each time point represent a group mean of the maximum change in resistance for each sheep over the first hour after challenge. Sheep categorized as responders had significant increases in resistance after HDM challenge over baseline at days 56 and 85 (mean [SD]). # $P < .05$ between control and the responder group; * $P < .001$ between responders and others; ** $P < .001$ between all groups.

Late-Phase Allergen-Specific Responses

Two of the 7 early phase responders challenged with HDM showed late-phase asthmatic responses. The data in Figure 3 show that the peak late-phase response was 5 hours post challenge in 1 sheep and 24 hours in another sheep. In both cases the magnitude of their early-phase response was the highest (560% increase in resistance from baseline) of all the responders and the magnitude of the late-phase response was less than half the magnitude of the early phase response.

Bronchial Hyperresponsiveness

Airway responsiveness to the bronchoconstrictor carbachol was increased in all sheep chronically challenged with HDM. There was a significant increase in airway sensitivity to carbachol over baseline 24 hours after a single HDM challenge ($P < .05$ for both responder and nonresponder sheep; Figure 4). In contrast, control sheep challenged with aerosols of saline did not show increased responsiveness to carbachol.

BAL Cell Responses

In responder and nonresponder sheep, there were increases in the group means for the percentage of eosinophils detected in the BAL 48

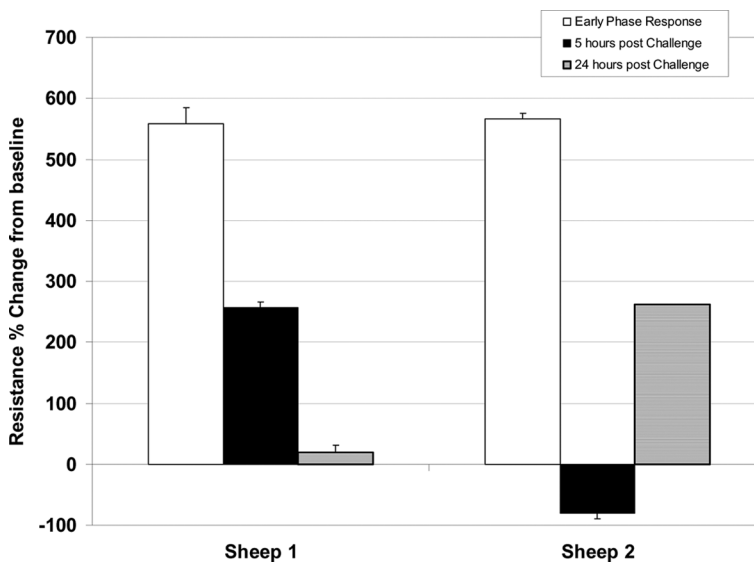


FIGURE 3 The percentage change from baseline in lung resistance of 2 sheep, after an aerosol challenge with HDM. Note that sheep 1 had a late phase response at 5 hours and sheep 2 had a late phase response at 24 hours (mean [SD]).

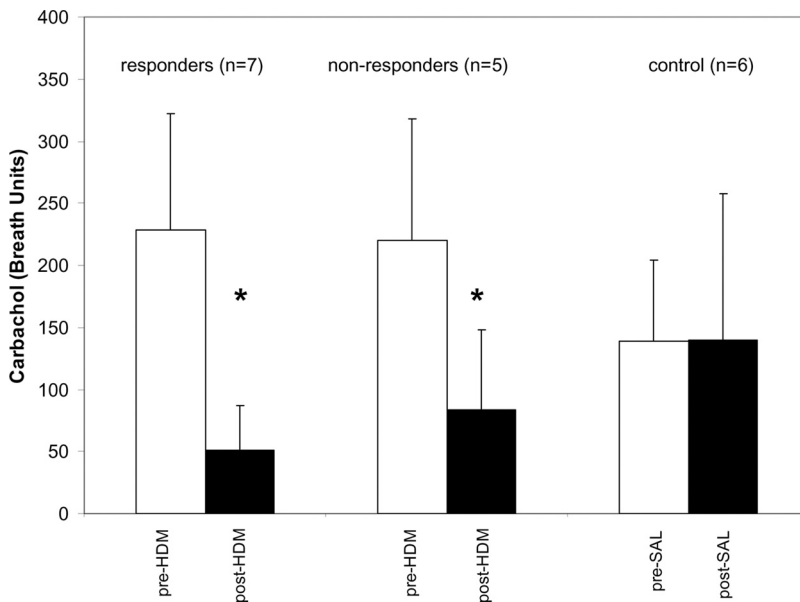


FIGURE 4 Bronchial responsiveness to aerosolised carbachol as assessed before the first challenge (*open bars*), and 12 weeks into the repeated challenge regime, 24 hours after the previous HDM/saline challenge (*closed bars*). Responder and nonresponder sheep refer to early phase responses as shown in Figure 2. One breath unit is equivalent to one inhaled breath of 1% (*w/v*) carbachol. The columns represent the number of breath units of carbachol needed to increase R_L by 200% from baseline (mean $\pm$ SD; * $P < .05$).

hours post HDM challenge (Figure 5). The percentage increase in BAL eosinophils was significant for responder sheep ($P < .05$). In contrast to that found for HDM-challenged responder sheep, there were essentially no eosinophils in the BAL of control sheep 48 hours after saline challenge. For the responder, nonresponder, and control sheep, there were no significant differences between the pre- and 48-hour post-challenge percentages of BAL lymphocytes, monocytes, and neutrophils.

DISCUSSION

This report describes a sheep model for chronic asthma that displays key characteristics of the human disease. We show that sheep repetitively exposed to house dust mite over a 3-month period develop bronchial hyperresponsiveness and a proportion of HDM-challenged sheep have pronounced early-phase asthmatic responses. The current study utilizes repeated administration of HDM, an allergen considered clinically relevant, and more likely to contribute to major components of the asthma pathogenesis compared to experimental allergens utilized in many animal

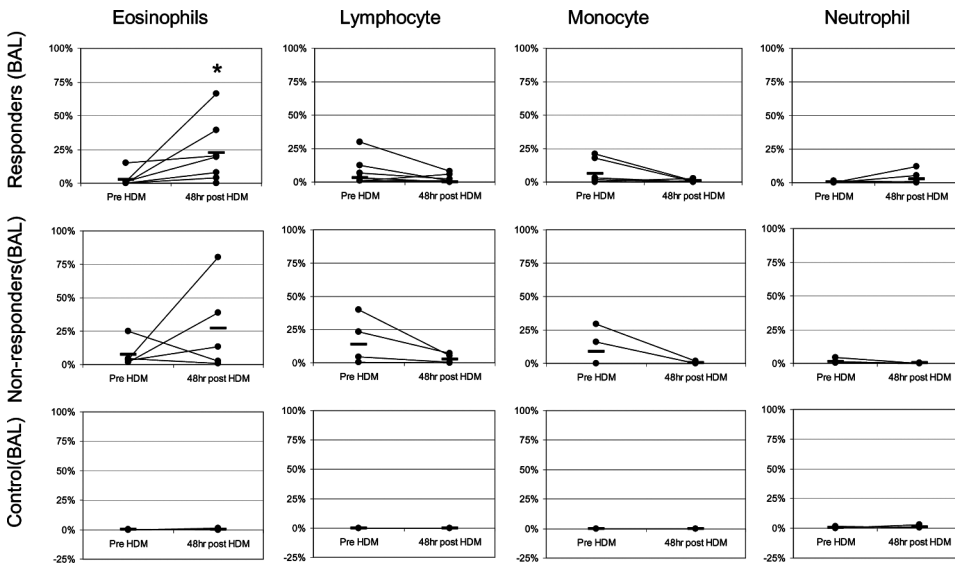


FIGURE 5 The data show individual BAL cell types expressed as a percentage of all BAL cells collected from sheep directly before challenge, and again 48 hours after a HDM/saline aerosol challenge, 4 weeks into the repetitive challenges. The responding, nonresponding, and control groups are defined in Figure 2 (dots represent individual sheep, bars represent group means; * $P < .05$).

models of asthma [13]. Evidence supporting this notion is that allergic sheep, which have been experimentally challenged in the lungs with HDM, respond with significant recruitment of eosinophils to the BAL and lung tissues [8, 9] and pronounced airway wall remodeling after chronic HDM challenges. The eosinophil is known to be an important cell in asthma and thought to be involved in airway remodeling and associated morbidity in asthmatics [14].

It is interesting that the sheep challenged with HDM could be divided into 2 clearly discernable groups after assessing the magnitude of R_L during the early-phase response. One group, the nonresponding HDM-challenged sheep, had not increased their R_L more than 100% from baseline during the early-phase at the 4 time points assessed throughout the trial period. Sheep designated as responders, on the other hand, had clear increases in R_L of a mean of 200% from baseline during their early phase responses after challenge at the 56- and 84-day time points. This increase in resistance was not observed in either control or non-responding animals.

At this stage it is not known why sheep segregate into responder and nonresponder groups. It appears to be a characteristic of allergic responses in sheep, as an earlier study that utilized repeated lung challenges with extracts from the pig parasite *Ascaris suum* also found a similar division of physiologically responsive and nonresponsive sheep [15]. However, unlike

Bosse and colleagues who found differences in the cellular responses between responding and nonresponding sheep, the present study shows a similar mean increase in eosinophils into the BAL fluid of both responder and nonresponder animals. In addition, both responder and nonresponder sheep in the present study were atopic for HDM in terms of their high level of HDM-specific serum IgE. The mechanism underlying the segregation of the 2 groups of sheep may therefore be related to the signals that activate constriction of airways after allergen exposure.

One possibility is that the difference may be related to mast cell activity in the airway wall. Mast cells are key cells in allergic reactions, which are known to be important in early asthmatic responses by releasing mediators that induce bronchospasms. Previous studies have demonstrated an increase in mast cells in the vicinity of the airway walls of sheep chronically exposed to HDM, but there was a large variation in the number of these mast cells [9]. It is possible that these variations may account for the segregation into responder and nonresponder groups identified in this study and current studies are examining the airway walls, mast cell numbers, and inflammatory mediators in these sheep.

Sensitization and continual exposure to HDM are causally related to both development and exacerbation of asthma in humans [16]. We demonstrate here that the early-phase response in responding sheep showed progressively increase in magnitude of R_L throughout the repeated challenge period. These responses indicate that the airways in responder sheep with continual challenges become more reactive to HDM aerosols and display increasingly greater airflow limitations. A progressive increase in the number of mast cells in and around the airway walls in response to repeated HDM exposure may account for the exacerbation of the early asthmatic response observed in the HDM chronic asthma model [9]. It is known that mast cell density is higher in lung tissues of fatal asthmatics and mild asthmatics compared to nonasthmatic controls, with mast cells concentrated particularly around airway smooth muscle bundles in asthmatics [17, 18].

In the present study, it was noteworthy that all responding and nonresponding sheep demonstrated BHR after challenge with HDM. All sheep also showed recruitment of eosinophils into the BAL, which is consistent with the known association between cell infiltration and BHR in asthma [19]. Longitudinal cohort studies have shown that children with atopic diseases have a higher risk of developing asthma [20, 21]. However, not all atopic patients that develop sensitization to allergens and have early wheeze go on to develop asthma [20]. The divergent responses in this sheep model of chronic asthma may be useful for examining the link between BHR and allergen induced bronchoconstriction.

Data presented here reveal that sheep have pronounced but diverse physiological responses to HDM, an important human allergen. The ability

to compare the differing biological parameters of responding and non-responding sheep makes the sheep model for chronic asthma a potentially useful one for studying the underlying mechanisms of asthma.

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