



Comparison of Airway Responses in Sheep of Different Age in Precision-Cut Lung Slices (PCLS)

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Abstract

Background: Animal models should display important characteristics of the human disease. Sheep have been considered particularly useful to study allergic airway responses to common natural antigens causing human asthma. A rationale of this study was to establish a model of ovine precision-cut lung slices (PCLS) for the *in vitro* measurement of airway responses in newborn and adult animals. We hypothesized that differences in airway reactivity in sheep are present at different ages.

Methods: Lambs were delivered spontaneously at term (147d) and adult sheep lived till 18 months. Viability of PCLS was confirmed by the MTT-test. To study airway provocations cumulative concentration-response curves were performed with different allergic response mediators and biogenic amines. In addition, electric field stimulation, passive sensitization with house dust mite (HDM) and mast cells staining were evaluated.

Results: PCLS from sheep were viable for at least three days. PCLS of newborn and adult sheep responded equally strong to methacholine and endothelin-1. The responses to serotonin, leukotriene D₄ and U46619 differed with age. No airway contraction was evoked by histamine, except after cimetidine pretreatment. In response to EFS, airways in PCLS from adult and newborn sheep strongly contracted and these contractions were atropine sensitive. Passive sensitization with HDM evoked a weak early allergic response in PCLS from adult and newborn sheep, which notably was prolonged in airways from adult sheep. Only few mast cells were found in the lungs of non-sensitized sheep at both ages.

Conclusion: PCLS from sheep lungs represent a useful tool to study pharmacological airway responses for at least three days. Sheep seem well suited to study mechanisms of cholinergic airway contraction. The notable differences between newborn and adult sheep demonstrate the importance of age in such studies.

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Introduction

The prevalence of asthma increased during the last decades [1,2] and may be related to Western lifestyle factors [2,3]. However, the causal reasons and underlying mechanisms are not well understood. Several studies have shown that in most cases of persistent asthma, the initial asthma-like symptoms occur during the first years of life [4,5]. It has been suggested that the child's environment plays an important role to develop asthma later in life. Children exposed to a farm environment had less asthma and atopy than children grown-up in a non-farming setting [6–9]. The mechanism associated with this protective effect is unknown. It is suggested that the child's immune system may be stimulated along a Th1 pathway by early exposure to increased concentrations of bacterial components present in stables such as endotoxin (LPS) [10]. This theory is known as the hygiene hypothesis [11]. For some of these children asthma symptoms seem to remit with time,

but many children develop asthmatic symptoms which persist throughout their life and are associated with more severe symptoms ending in the loss of lung function. About 15% of the wheezing infants develop persistent wheezing and clinical asthma later in life [12].

Animal models of asthma should display the pathology of the human disease and have carefully to be selected. Several studies indicate for instance that the innervation of the lung differs considerably between and within species [13,14]. Moreover, rodent airways do not or only weakly respond to leukotrienes [15], mediators that readily cause bronchoconstriction in humans [16] and guinea pigs [17]. Sheep show a greater resemblance to humans concerning lung development compared to rodents. Rodents and guinea pigs undergo the alveolar phase of lung development postnatally whereas sheep and humans undergo this phase in the uterus [18–20]. In addition sheep can, like rodents, be sensitized to house dust mite (HDM) antigen which is a common

human antigen in asthma, and have allergen-specific IgE responses and acute eosinophil responses to allergen challenge [21,22]. Therefore, sheep have been considered particularly useful as models to study allergic airway responses to common human natural antigens.

Airway responses can be visualized by precision-cut lung slices (PCLS), which are viable lung tissue slices of uniform thickness ($\approx 250 \mu\text{m}$). PCLS can easily be prepared from different species and are already established for many species including rat, mouse, guinea pig, non-human primates and humans [14,17,23–26]. PCLS represent a highly useful model to study bronchial and pulmonary vascular responses by videomicroscopy [23,27]. The responses in pulmonary vessels strips of newborn and adult sheep have shown interesting differences in reactivity [28]. The diameter of the pulmonary vessels increased from the newborn to the adult animals and the maximum velocity of shortening was in newborns much higher than in adult sheep. The responses of airways in newborn and adult sheep have not yet been investigated. We hypothesize that differences in airway smooth muscle reactivity in sheep are present at different ages.

In the present study the bronchoconstriction of newborn and adult sheep was studied in PCLS. A rationale of this study was to establish a model of ovine PCLS for the measurement of airway responses to early allergic response mediators and to allergens (after passive sensitization) in newborn and in adult animals.

Materials and Methods

Animal model

All animal procedures were approved by the Animal Ethics Committee of the University of Maastricht, The Netherlands. The newborns ($n = 7$) were delivered spontaneously at term (147 ± 2 days GA). The lambs were euthanized directly after surgical delivery with an intra-venous injection of pentobarbital. Adult sheep ($n = 5$) were euthanized at ~ 18 months with pentobarbital.

Preparation of PCLS

PCLS were prepared from adult or newborn sheep lung as described in previous studies [14,17,23–25] with some modifications. Briefly, the lungs were filled via the lobular bronchus with 1.5% low-melting-point agarose solution and put onto ice until the agarose had solidified. Tissue cores (1 cm in diameter) with a penetrating airway were punched out. Those cylinders were then cut perpendicular to the airway by means of a Krumdieck tissue slicer (Alabama Research and Development, Munford, AL, USA) into approximately $250 \mu\text{m}$ thin PCLS. PCLS were transferred into a 10 cm cell culture dish and incubated under cell culture conditions (37°C , 5% CO_2 atmosphere) in minimal essential medium (MEM) that was frequently changed during the next 4 hours (h) and incubated over night. The medium exchange supported the removal of tissue released mediators as well as the wash out of agarose from airways. For measurements, only slices with airways free of agarose, with beating cilia and an intact and relaxed airway smooth muscle layer were used. We only studied PCLS with comparable airway size in parallel experiments to reduce interslice variations. Electric field stimulation (EFS) on PCLS and passive sensitization studies were performed within 24 h after preparation. All other physiological measurements were conducted within 48 h after preparation.

Viability of PCLS

Viability of PCLS over three day incubation was confirmed by intracellular reduction of a tetrazolium dye to its according purple formazan (MTT-test) and constriction responses. PCLS were

transferred into cavities of a standard 24-well plate (1 PCLS/well) and incubated with $900 \mu\text{L}$ MEM + $100 \mu\text{L}$ 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution (MTT, 7 mg/mL) for 15 min. The supernatant was discarded and the formazan was dissolved by incubation of PCLS with $200 \mu\text{L}$ formic acid/propanol (5%/95%) solution for 20 min. $100 \mu\text{L}$ of the purple supernatant were taken and transferred to 96-well plates to measure the extinction at 550 nm (Tecan GENios Microplate Reader). All reactions were carried out at room temperature and in the dark. For negative control measurements PCLS were digested with 0.2% (v/v) Triton-X100 ($300 \mu\text{L}$, 20 min, 37°C) before performing the MTT-assay.

Videomicroscopy

If not otherwise stated, PCLS were kept in cavities of standard 24-well plates and were immersed in 1 mL MEM during the experiment. The plate was then mounted on the stage of an inverted Leica DMIL microscope (Leica Microsystems, Wetzlar, Germany). The airways were imaged and digitized by videomicroscopy (SensiCam 365KL digital camera, Visitron Systems, Munich, Germany; Optimas 6.5 software, Optimas, Bothell, WA, USA). The airway area before any provocation was defined as 100%–initial airway area [%–IAA].

Airway provocations by early allergic response mediators and biogenic amines

Cumulative concentration-response curves were performed with methacholine (10^{-10} M– 10^{-4} M; 5 min/conc.; pictures every 5 s), serotonin (10^{-10} M– 10^{-4} M; 5 min/conc.; pictures every 5 s), histamine (10^{-9} M– 10^{-4} M; 5 min/conc.; pictures every 5 s), endothelin-1 (10^{-12} M– 10^{-6} M; 10 min/conc.; pictures every 10 s), leukotriene D₄ (LTD₄, 10^{-12} M– 10^{-6} M; 10 min/conc.; pictures each 5 s) and the thromboxane A₂ analogue U46619 (10^{-10} M– 10^{-5} M; 10 min/conc.; pictures every 5 s). To study a potential effect of the histamine H₂-receptor on bronchoconstriction, PCLS were pre-incubated with $10 \mu\text{M}$ cimetidine for 15 min prior to performing the histamine concentration-response curve.

Electric field stimulation (EFS)

As described before [29], EFS of PCLS was carried out in standard 12-well plates at a reaction volume of 1 mL standard MEM. The PCLS were placed in between two platinum electrodes of 12 mm distance and were mounted by a Teflon ring. The electric field was applied by a Hugo Sachs Electronics Stimulator II (Hugo Sachs Electronics, March Hugstetten, Germany). The electric stimuli were defined by a frequency of 50 Hz, pulse duration of 1 ms, a current amplitude of 200 mA, a train width of 2.5 s and a train rhythm of 60 s. Each train lasted 3.3 min. After the first control stimulation the muscarinic antagonist atropine ($10 \mu\text{M}$) was added and incubated for 15 min prior to the second stimulation. In an additional set of EFS experiments, frequency response curves were conducted on PCLS from adult and newborn sheep. In this, the frequency was steadily increased from 0.4 Hz–100 Hz, while the pulse duration and current amplitude were kept constant at 1 ms and 200 mA, respectively. Each frequency was applied once for 2.5 s and after a pause of one minute the next frequency was applied. The airway behavior of PCLS upon stimulation was monitored by videomicroscopy. Pictures were taken every 2.5 s. Airway area before the first stimulation was defined as 100% initial airway area (IAA).

Passive sensitization

Passive sensitization was performed by incubation with 1% serum from house dust mite (HDM)-sensitized sheep overnight. Medium was replaced by fresh serum-free MEM directly before provocation with 5000 Units of HDM from ALK-SHERAX (Wedel, Germany), which is normally used for intracutaneous testing of atopy. Airway responses were followed by videomicroscopy for 20 min. Pictures were taken every 10 s and pruned for clarity to each fifth data point.

Mast cell staining

Mast cells were stained with toluidine blue or with alcian blue and safranin. Briefly, PCLS were fixed with formaldehyde 4% (Roti-Histofix 4%, Carl Roth GMBH & Co. KG, Karlsruhe, Germany), dehydrated and embedded in paraffin. Then, 5 μ m thick sections were cut, deparaffinized, rehydrated and incubated in 1% toluidin blue for 30 min. After rinsing with water, sections were dehydrated and coverslipped. Alternatively after rehydration, sections were incubated in 0.05% Alcian Blue in 0.02 M acetate buffer, pH 5.8 with 0.2 M MgCl for 4 h. After rinsing in water, sections were incubated for 5 min in 0.25% safranin 0.02 M acetate buffer, pH 5.0. After a final rinsing step, sections were dehydrated and coverslipped.

Data analysis

Data are shown as means \pm standard error of the mean (SEM). Concentration-response curves were fitted by non-linear regression (4-parameter logistic equation). To analyze differences between curves, each parameter of the 4-parameter logistic equation was separately compared by the Extra sum-of-squares F-test. The 4-parameter logistic equation is $[Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{-(\text{LogEC}_{50} - X) * \text{HillSlope}})]$, with the bottom being the maximal response (i.e. fit values aim towards the minimum in the concentration response curves), the top being the initial situation (i.e. fit values aim towards 100%-IAA), the $\log EC_{50}$ being calculated to the half maximal response and the slope initially kept variable (i.e. unconstrained from a standard value of 1). A shared concentration-response curve was plotted for adult and newborn sheep, if no difference was found in any parameter of the 4-parameter logistic equation. Minimal airway areas in EFS before and after atropine treatment were compared by Student's *t*-test (due to homogeneity of variance). Mixed model analysis considering changes in airway area dependent on time and treatment was performed on time courses in the passive sensitization experiments. *P*-values < 0.05 were considered significant. The statistical analysis was performed by either GraphPad Prism 5 (GraphPad Software, La Jolla, CA) or SAS 9.1 (SAS Institute Inc., Cary, NC).

Results

Viability of PCLS

More than 50 slices were obtained from one lung lobe of either newborn or adult sheep. The viability of sheep PCLS was demonstrated by the MTT test and PCLS were viable for at least three days (figure 1). In figure 1A, high extinctions in this test indicate intact cellular reduction systems, which correlate to viability. PCLS of adult sheep had an extinction OD of 0.2 stable over 3 days compared to detergent control PCLS with an extinction OD of 0.05. PCLS of newborn sheep had also a robust extinction OD of up to 0.3 over 3 days. Functionality of airway smooth muscle contraction was shown by the application of 10^{-4} M methacholine, which evoked a strong bronchoconstriction in sheep PCLS (figure 1B and C).

Airway provocations by allergic response mediators or biogenic amines in adult and newborn sheep

In sheep PCLS all of the mediators of the early allergic response in humans, except for histamine, evoked a marked bronchoconstriction (figure 2A–F). In general, concentration-response curves are characterized by their efficacy and potency. Shared concentration-response curves in PCLS from adult and newborn sheep were found for methacholine and endothelin-1 with half-maximal responses ($\log EC_{50}$ [M]) of -7.0 ± 0.1 and -7.7 ± 0.1 , respectively (figure 2A and 2D). Age related differences in potency were found for serotonin and LTD₄. For serotonin the $\log EC_{50}$ [M] values were -6.3 ± 0.4 for newborn and -6.9 ± 0.2 for adult sheep (figure 2B), whereas for LTD₄ $\log EC_{50}$ values were smaller for newborn than for adult sheep (-9.7 ± 0.4 [M] vs. -8.0 ± 0.6 [M]) (figure 2E). Differences in efficacy were found for U46619, as airways of adult sheep responded markedly to U46619 ($\log EC_{50}$ [M] = -5.9 ± 1.0 ; max contraction to $64.6 \pm 8.4\%$ -IAA at 10^{-5} M), whereas airways of newborn sheep were mostly unresponsive (max. contraction to $93.4 \pm 6.5\%$ -IAA at 10^{-5} M) (figure 2F).

Airway provocation by histamine with or without cimetidine

Since in the preceding experiments no airway contraction was observed by histamine, PCLS were pretreated with or without cimetidine, which blocks the H₂-receptor. Again, airways in control PCLS, i.e. without cimetidine, did not contract in adult sheep (figure 3). In contrast, stimulation of airways in PCLS with histamine after cimetidine pretreatment resulted in a marked concentration-dependent contraction ($\log EC_{50} = -6.3$) (figure 3). No data were available for PCLS from newborn sheep.

Intrinsic activation of bronchoconstriction in sheep PCLS

In addition to the preceding exogenous activation of bronchoconstriction in PCLS by exogenous application of mediators, we studied whether an intrinsic activation of airway response is possible in sheep PCLS. In this regard, two approaches were chosen: EFS to study neutrally-induced bronchoconstriction and passive sensitization to evoke an early allergic response by mast cell degranulation after antigen challenge.

In response to EFS, PCLS from both adult and newborn sheep, strongly contracted to $43.7 \pm 13.9\%$ IAA and $34.3 \pm 6.5\%$ IAA (figure 4), respectively. This neutrally-induced response was potently blocked by atropine resulting in an airway contraction to only $84.0 \pm 7.6\%$ IAA for PCLS from adult sheep and 78.6 ± 6.8 for PCLS from newborn sheep. Moreover, to distinguish between a difference in sensitivity of PCLS from newborn and adult sheep to EFS, frequency response curves were conducted. However, a shared curve was found (figure 4 C), indicating equal sensitivity of PCLS from newborn and adult sheep to neural stimulation.

With respect to passive sensitization, provocation with 5000 U of HDM evoked a weak but significant early allergic response in PCLS from adult and newborn sheep, that had been incubated with serum from HDM sensitized sheep (figure 5A and 5B). Comparing the HDM-induced airway contractions in PCLS from adult and newborn sheep, a significant difference was found in the respective kinetics. The response to HDM was more sustained in adult than in newborn sheep. In order to study these responses, we stained for mast cells in the lung tissue since we consider them essential for the response. In accordance to the weak response, very few mast cells were found in PCLS from sheep at both ages as shown in figure 5C and 5D. We found on average less than one mast cell per 5 μ m thick section.

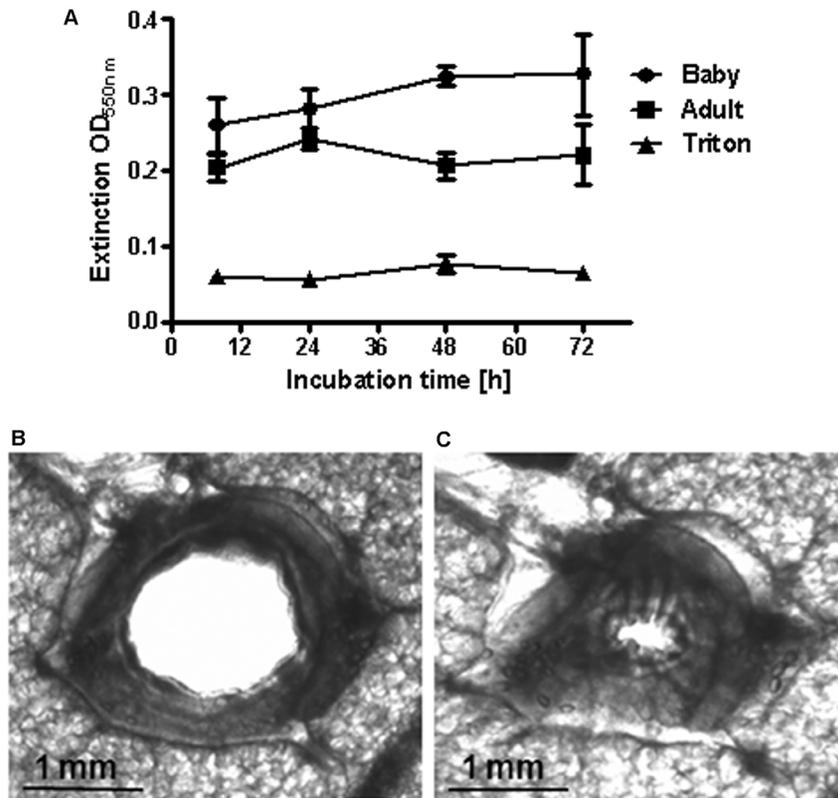


Figure 1. Viability of sheep PCLS. A) Viability was followed by the MTT test. High extinctions indicate high viability. Data are shown as mean \pm SEM, $n = 3$ PCLS from 3 animals per group. Exemplary photographs (in black and white) of a PCLS before (B) and after (C) provocation with 10^{-4} M metacholine.

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Discussion

To our knowledge this is the first time, the airway reactivity of newborn and adult sheep was compared in PCLS. Many of the responses were similar in newborn and adult sheep, such as the bronchoconstriction to EFS, and to the mediators methacholine, histamine and endothelin-1. On the other hand different responses in newborn and adult sheep were found for the eicosanoids LTD₄ and thromboxane as well as the biogenic amine serotonin. The response to allergen after passive sensitization was comparable in the extent of contraction, but prolonged in adult sheep.

PCLS are viable, can readily be obtained from different species and offer a great opportunity to study airway responses under cell culture conditions [14]. The microarchitecture of lung tissue, i.e. airways and vessels embedded in the parenchyma, remains intact and permit physiological measurements [23–26]. Even nerves remain functional and can be specifically activated [29]. In the past, PCLS from rat, mouse, guinea pig, non-human primate and human lung have been investigated [14,26]. Allergic responses in sheep have been suggested to be similar to humans [18,19,21,22], but the response of airways to early allergic response mediators in newborn compared to adult sheep had not been investigated yet. Recurrent wheezing is common in young infants and toddlers [30]. For some of these children asthma symptoms seem to remit with time, but many children develop asthmatic symptoms which persist throughout their life [12,31]. Therefore, it is important to compare the allergic responses in newborn to adult lung tissue.

Given the importance of small airways for asthma and COPD [24,25,32,33], the possibility to study these airways in PCLS is of clinical relevance. Small airways are defined by their inner

diameter being less than 2 mm in human (airway generations >11) and less than 780 μ m in rat airways [25,34]. Since sheep are comparable in weight to humans, lung/airway sizes and generations should correlate to the human structure. In this context of note, the current experiments were exclusively performed on small airways, since they were less than 2 mm in diameter. Therefore, our results reflect the situation only for the peripheral lung reducing interslice variations since airway responses can differ between large and small airways [23,29].

In the present study PCLS from sheep were viable for at least three days, not different from the viability of human PCLS [24], making it possible to study the effects of cytokines, growth factors and maybe even remodeling processes [35,36]. In addition, recent work by Behrsing and colleagues [37] has demonstrated that the process of slicing itself elicits a massive cytokine response, confounding early results of cytokine measurements in response to PCLS challenge. Moreover, in contrast to our study, they also used transcriptionally active agents like humulin, hydrocortisone and retinoic acid in their incubation medium. In the current study, we have performed frequent medium exchanges during the first 24 h after slicing to minimize the effect of endogenously released mediators. Nonetheless in general, the impact of mechanical damage conducted to create the PCLS should be noticed when cytokines and mediators are studied in PCLS.

PCLS of newborn and adult sheep responded very similar to methacholine and endothelin-1, mediators also effective in humans [17]. However, the response to serotonin, the thromboxane analogue U46619 and LTD₄ depended on the age of the sheep. In newborn sheep the response to serotonin and U46619

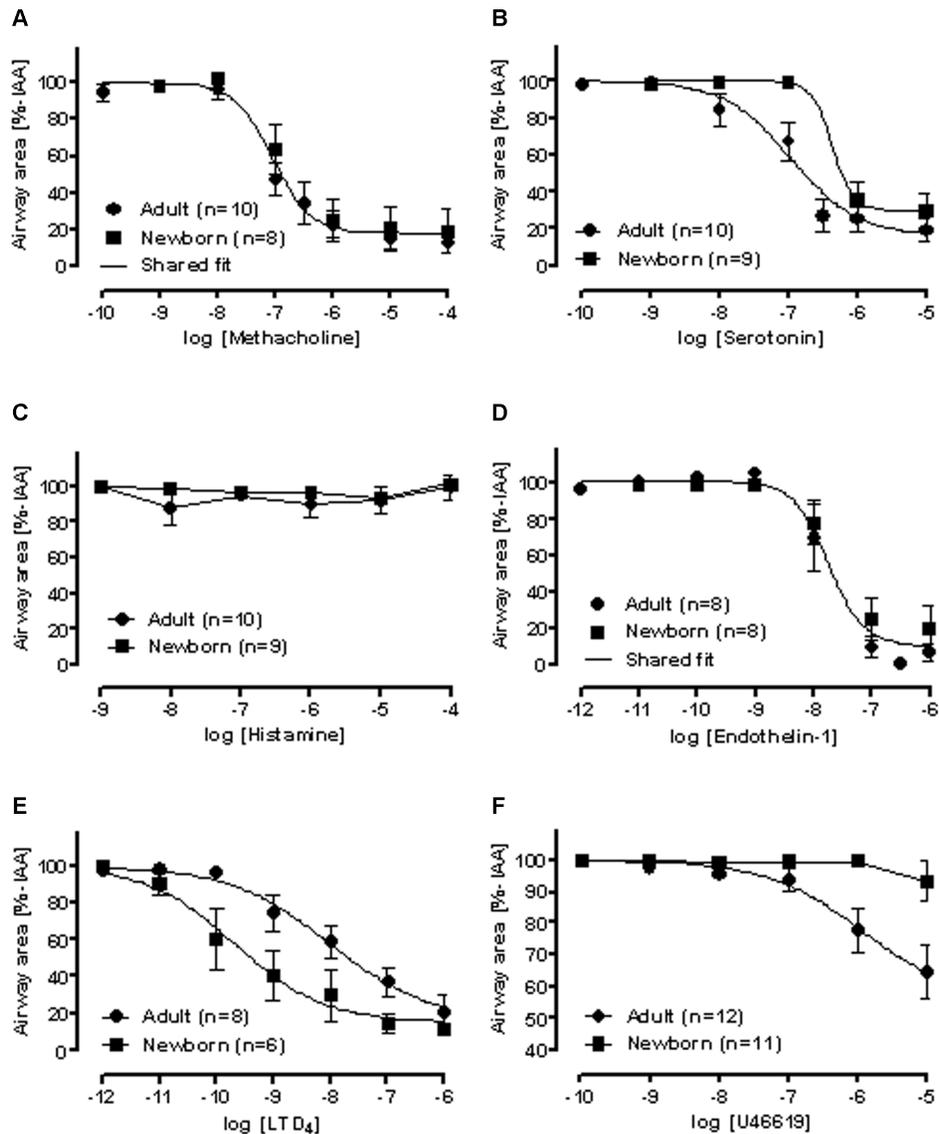


Figure 2. Concentration-response curves for common mediators of early allergic response in PCLS of adult (●) and newborn sheep (■). A) methacholine, B) serotonin, C) histamine, D) endothelin-1, E) leukotriene D₄ (LTD₄), and F) U46619. n = number of PCLS, whereby a total of 7 newborn and 5 adult sheep were examined. Data are shown as mean ± SEM. A shared concentration-response curve was plotted for adult and newborn sheep, if no difference was found in any parameter of the 4-parameter logistic equation.
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was weaker than in adult sheep, whereas the response to LTD₄ was stronger in newborn sheep. This is an important difference compared to rodent airways that do not or at best very weakly respond to LTD₄ [15]. Leukotrienes contribute to airway obstruction in many human asthmatics [29]. In this regard sheep PCLS, in particular newborn, may appear as a suitable animal model to study bronchoconstriction. From pharmacological and clinical point of view these results are very interesting. The strong bronchial response of newborn sheep to leukotrienes suggests that young animals will be more sensitive to the effects of montelukast, which is a leukotriene-receptor antagonist and used to prevent the wheezing and shortness of breath caused by asthma. On the other hand, in the present study it is demonstrated that adult sheep have a stronger bronchial response to serotonin. Therefore, adult sheep may be more sensitive to the effects of ketanserin, which is a highly selective antagonist for contractile serotonin 5-HT_{2A} receptors.

Our studies show a lifetime dependent response pattern to different antagonist, which suggest specific disease treatment with different antagonist depending on the age of the sheep.

Compared to human PCLS, there are also differences: sheep airways reacted to serotonin rather than to histamine, which is the opposite to what is observed in human and guinea pigs PCLS, in which histamine (EC₅₀ 2.7 mM) is effective but serotonin is not [17,24,25]. The reason for these species differences is not clear. It was suggested that it may, at least in part, be related to age differences in tissue as shown in young healthy guinea pigs versus middle-aged adults [17,24,25]. However, the present study demonstrated that the bronchoconstriction in both newborn and adult sheep was the same and that age differences may not play such a crucial role if one compares responses from animal tissue with that from human tissue.

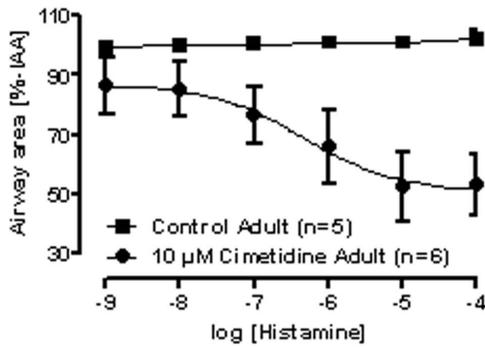


Figure 3. H_2 -receptor counteracts histamine-induced bronchoconstriction in adult sheep PCLS. PCLS from adult sheep were either pre-incubated with the H_2 -receptor antagonist cimetidine before histamine provocation (●) or kept untreated (control, ■). Data are shown as mean \pm SEM, n = number of PCLS of separate sheep. The concentration response curves were fitted by the 4-parameter logistic equation.

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Importantly, however, the lack of responsiveness to histamine was not due to the lack of H_1 -receptors. This was revealed by experiments with the H_2 -receptor antagonist cimetidine (figure 3), which has also minor affinity for H_1 -, H_3 - and H_4 -receptors [38,39]. Here, we consider effects of H_3 and H_4 activities unlikely, because H_3 is predominantly expressed in brain tissue and H_4 is attributed to immune cell responses [40,41]. Moreover, in literature there are no reports about H_3 and H_4 expression in sheep lung. Since no analysis of the different histamine receptor expression was performed in lung tissue in the present study the discussion about H_3 and H_4 remains speculative and is a clear limitation of this study. Normally, in the lungs histamine acts on H_1 - and H_2 -receptors and its effect is therefore balanced by the H_1 -mediated contraction and the H_2 -mediated relaxation of airway smooth muscles [42–45]. The observation that histamine contracts tracheal and bronchial airways, but relaxes smaller bronchi and bronchioles [46], indicates that there is a shift towards predominance of H_2 -receptors towards the peripheral airways. Thus, the present finding that histamine was able to contract ovine airways only after blockade of H_2 -receptors, may reflect the fact that the PCLS were derived from the lung periphery. In addition, the expression of histamine H_2 -receptors in the lungs may also differ depending on the type of lung sample, previous sensitization or infection, frequently leading to down-regulation of H_2 -receptors [47–49]. Therefore, H_2 -receptor deficiency in the airways may, at least in part, explain the commonly observed airway hyperreactivity to histamine in asthmatics.

In the present study, the early allergic response in passively sensitized sheep PCLS was weak. One explanation might be that after degranulation mast cells mainly release histamine, which – as seen before – is ineffective unless H_2 -receptors are blocked. Alternatively, this finding can be explained by the small number of mast cells found in the airways of PCLS from non-sensitized sheep. It was demonstrated that exposure to HDM (active sensitization) doubled the number of mast cells mostly in alveolar septa and in airway walls [22,50]. Notably, these responses required at least 16 weekly HDM challenges [50], while in the present study the sheep lungs were unchallenged and PCLS were only passively sensitized with serum from actively sensitized sheep. This might explain the small numbers of mast cells in the present study. Another interesting observation in the allergen-induced bronchoconstriction after passive sensitization was the prolonged airway

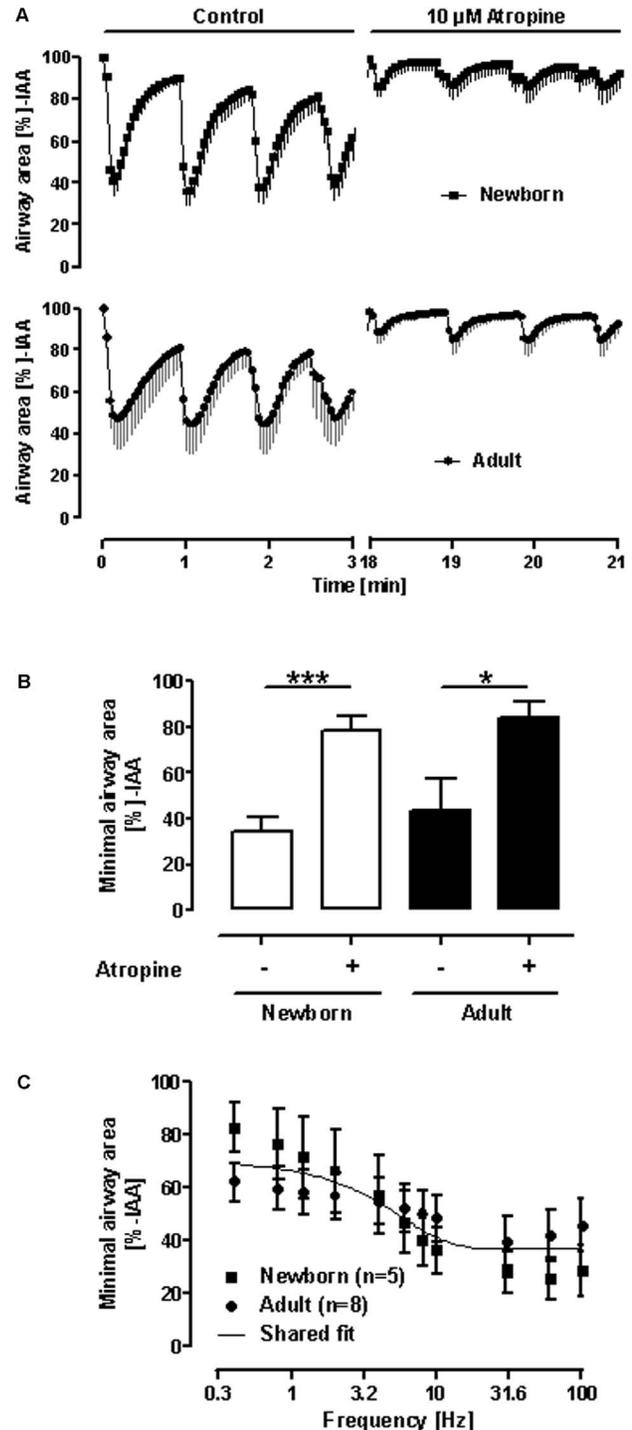


Figure 4. Electric field stimulation (EFS) of PCLS from newborn and adult sheep. **A)** Course of airway area changes during repeated EFS in absence or presence of atropine. **B)** Statistical analysis on minimal airway area during EFS as obtained in **A**. Data are shown as mean \pm SEM, $n=7$ for newborn sheep and $n=4$ for adult sheep, whereby each PCLS was taken from an independent sheep; * $p<0.05$; *** $p<0.001$ in Student's t -test. **C)** Frequency-response curves of EFS-induced airway contractions. Data are shown as mean \pm SEM, $n=5$ PCLS from three newborn sheep and $n=8$ PCLS from eight adult sheep. A shared frequency-response curve was plotted for adult and newborn sheep, since there was no difference in any parameter of the 4-parameter logistic equation assuming that the top is equal to 100%-IAA and EF_{50} is larger than zero Hz.

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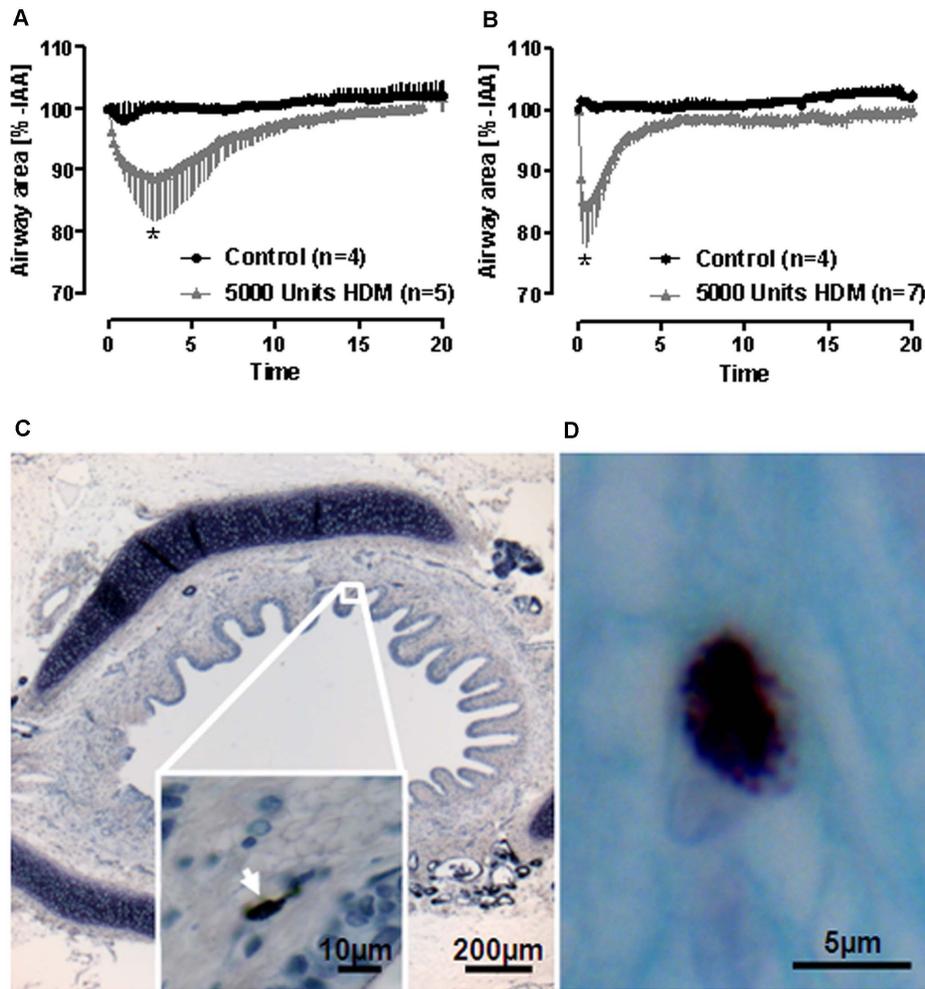


Figure 5. Early allergic response in sheep PCLS after passive sensitization. PCLS from newborn (A) or adult (B) sheep were passively sensitized with serum from actively sensitized sheep against HDM and provoked with 5000 U HDM. Data are shown as mean \pm SEM, n as indicated on PCLS from different sheep. Time courses were statistically compared by mixed model analysis; * $p < 0.05$. If one compares HDM groups in A and B, courses are also significant different, which points out differences in the kinetics of allergen-induced bronchoconstriction in adult and newborn sheep. C, D) Less than one mast cell per 5 μ m thick section were found in PCLS from sheep. PCLS were stained for mast cells with toluidine blue (C, arrow) or with alcian blue and safranin (D).
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contraction in PCLS from adult sheep compared to newborn sheep. To our knowledge this is the first study examining the early allergic response of adult and newborn animals in parallel. Reasons, such as a sustained mediator release, diminished degradation of bronchoconstrictors or different receptor density in adult sheep, are therefore speculative. Nonetheless, this example confirms, that extreme caution is mandated selecting the appropriate animal model, when studying childhood asthma or asthma in adults as responses may differ tremendously.

We further demonstrated that ovine PCLS respond to electric field stimulation. Airway responses to electric field stimulation differ largely between species [14]. PCLS from rats and marmoset airways contract by about 20%, whereas guinea pigs, sheep and humans contract at maximum by about 40–60% [14]. The present study adds that the maximum contraction ($>50\%$) in both newborn and adult sheep did not differ. Moreover, since the sensitivity of sheep PCLS from newborn and adult sheep did not differ, one may speculate that the neural network in sheep lung is widely established by birth. The shared frequency-response curve is also in line with the exogenous application of methacholine, in

which also a shared curve, i.e. same sensitivity to the agonist, was found. These findings are rounded off by the high atropine-sensitivity of EFS-induced airway contractions pointing out that lung innervation in sheep is mostly cholinergic. Hence, airway responses in sheep PCLS are a reasonable proxy for human airways, if cholinergic airway contractions are intended to study.

In our model we focused on the acute response to exogenously added inflammatory mediators and passive HDM sensitization to demonstrate that ovine PCLS are a reliable tool for in vitro measurement of airway responses. It was beyond the scope of this study to examine the role of cytokine and mediator secretion in response to a physiologically relevant stimulus or active sensitization. In future research it will be relevant to compare acute versus chronic responses after HDM sensitization and also to compare HDM to sensitization with other allergens. In conclusion, PCLS from sheep lungs represent a useful tool to study pharmacological airway responses for at least three days. Sheep seem well suited to study mechanisms of cholinergic airway contraction. Their airway pharmacology differs in some respects to that observed in humans. Bronchoconstriction is similar in newborn and adult sheep, except

for the lipid mediators (LTD₄, U46619) and serotonin. Early allergic response is weak which is probably based on a small number of mast cells.

Author Contributions

Conceived and designed the experiments: LJZ SU BWK CM. Performed the experiments: VAL MS CD PK. Analyzed the data: VAL MS CD PK LJZ. Contributed reagents/materials/analysis tools: LJZ SU BWK CM. Wrote the paper: VAL MS.

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