

Ex Vivo Lung Function Measurements in Precision-Cut Lung Slices (PCLS) from Chemical Allergen–Sensitized Mice Represent a Suitable Alternative to *In Vivo* Studies

M. Henjakovic,^{*,2} C. Martin,^{†,2} H. G. Hoymann,^{*} K. Sewald,^{*} A. R. Ressmeyer,[†] C. Dassow,[†] G. Pohlmann,^{*} N. Krug,^{*} S. Uhlig,[†] and A. Braun^{*}

^{*}Department of Immunology, Allergology and Immunotoxicology, Fraunhofer Institute of Toxicology and Experimental Medicine, 30625 Hannover, Germany; and [†]Institute of Pharmacology and Toxicology, RWTH Aachen, Wendlingweg 2, 52074 Aachen, Germany and Division of Pulmonary Pharmacology, Research Center Borstel, 23845 Borstel, Germany

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A wide range of industrial chemicals can induce respiratory allergic reactions. Hence, there is an urgent need for methods identifying and characterizing the biological action of chemicals in the lung. Here, we present an easy, reliable alternative method to measure lung function changes *ex vivo* after exposure to chemical allergens and compare this to invasive *in vivo* measurements after sensitization with the industrial chemicals trimellitic anhydride (TMA) and 2,4-dinitrochlorobenzene (DNCB). Female BALB/c mice were sensitized epicutaneously with the respiratory allergen TMA and the contact sensitizer DNCB. The early allergic response to TMA and DNCB was registered *in vivo* and *ex vivo* on day 21 after inhalational challenge with dry standardized aerosols or after exposure of precision-cut lung slices (PCLS) to dissolved allergen. Airway hyperresponsiveness (AHR) to increasing doses of methacholine (MCh) was measured on the next day *in vivo* and *ex vivo*. Bronchoalveolar lavage (BAL) was performed for immunological characterization of local inflammation. TMA-sensitized mice showed AHR to MCh *in vivo* (ED₅₀: 0.06 μ g MCh vs. 0.21 μ g MCh in controls) and in PCLS (EC₅₀: 0.24 μ M MCh vs. 0.4 μ M MCh). TMA-treated animals showed increased numbers of eosinophils (12.8·10⁴ vs. 0.7·10⁴) and elevated eotaxin-2 concentrations (994 pg/ml vs. 167 pg/ml) in BAL fluid 24 h after allergen challenge. In contrast, none of these parameters differed after sensitization with DNCB. The present study suggests that the effects of low molecular weight allergens, like TMA and DNCB, on *ex vivo* lung functions tested in PCLS reflect the *in vivo* situation.

Key Words: lung function; bronchoconstriction; PCLS; TMA; DNCB; methacholine.

Occupational asthma is one of the most common types of occupational lung diseases in developed countries. It is characterized by reversible airway obstruction, remarkable eosinophilic airway inflammation, and increased airway hyper-responsiveness (AHR) to a variety of stimuli such as high molecular weight (HWM) and low molecular weight (LMW) agents (Mapp *et al.*, 2005). Industrial LMW organic chemicals, such as toluene isocyanate, reactive dyes, and acid anhydrides (e.g., trimellitic anhydride, TMA) in particular, are known or suspected to cause respiratory allergy and occupational asthma in humans (Baur and Czappon, 1995; Tee *et al.*, 1998; Zeiss *et al.*, 1977).

The mechanisms through which such chemicals may cause respiratory sensitization still await full elucidation. Nevertheless, there is growing evidence that chemical respiratory hypersensitivity is prevalently associated with the development of T lymphocytes with a type 2 (Th2) profile (Arts and Kuper, 2007; Dearman *et al.*, 2000, 2002). In contrast, well-known human skin sensitizers, e.g., 2,4-dinitrochlorobenzene (DNCB), induce a Th1 cytokine phenotype (Dearman *et al.*, 1998; Hayashi *et al.*, 2001; Vandebriel *et al.*, 2000). Some of the Th1-type-inducing chemicals, e.g., dinitrofluorobenzene and trinitrochlorobenzene, are able to induce IgE-independent airway hyperreactivity (Buckley and Nijkamp, 1994; Garssen *et al.*, 1989, 1991, 1993).

The best route of application for respiratory and skin sensitizers in animal models is still under discussion. Several investigations have shown that exposure of skin to respiratory sensitizers such as TMA may also be a relevant sensitization route for inhaled allergens (Arts *et al.*, 1998, 2003; Dearman *et al.*, 1997; Farraj *et al.*, 2006; Pauluhn *et al.*, 2002). In contrast, dermal exposure to the contact allergen DNCB did not result in any changes of pulmonary functions after DNCB or methacholine (MCh) challenge (Arts *et al.*, 1998; Vanoirbeek *et al.*, 2006).

In vivo invasive pulmonary tests are sensitive methods for detecting bronchoconstriction in animals (Glaab *et al.*, 2007;

¹ To whom correspondence should be addressed at Department of Immunology, Allergology and Immunotoxicology, Fraunhofer Institute of Toxicology and Experimental Medicine, Nikolai-Fuchs-Str. 1, 30625 Hannover, Germany. Fax: +49-511-5350-155. E-mail: armin.braun@item.fraunhofer.de.

² These authors contributed equally to the study.

Hoymann, 2006). These tests are suitable for pharmacological and toxicological characterization of industrial organic chemicals in animal models of allergic asthma, lung inflammation (acute respiratory distress syndrome), or pulmonary infection (Glaab *et al.*, 2007). Alternative *ex vivo* methods to test the allergic response for prediction of sensitizing potencies of chemicals in the respiratory tract are, as yet, missing.

Precision-cut lung slices (PCLS) represent an *ex vivo* alternative to *in vivo* respiratory measurements of bronchoconstriction for HMW allergens. For example, the immediate allergic response of the airways was assessed *ex vivo* in lung slices of ovalbumin-sensitized animals or in passively sensitized lung slices of naive animals (Dandurand *et al.*, 1994; Wohlsen *et al.*, 2001). Furthermore, lung tissue slices have been employed successfully in studies to investigate mediators involved in airway contraction and to study calcium signaling in smooth muscle cells of the airways (Bergner and Sanderson, 2002; Wohlsen *et al.*, 2001, 2003). Our previous findings have demonstrated that PCLS also provide a convenient and valuable method to gauge the local immune reactions of biologically active agents (Henjakovic *et al.*, 2008). The viability of PCLS can be examined to control local respiratory irritation induced after exposure of lung tissue to increasing concentrations of substances. This allows testing for cytotoxicity of multiple substances or multiple incubation times in one animal lung.

With an increasing public demand to limit the number of animals used in research and to reduce the distress to the experimental animals (3R concept), PCLS represent a promising method with numerous advantages for pharmacological and toxicological studies. In contrast to other *in vitro* models, like isolated airways, tracheal, or bronchial rings, where contraction is either isotonic or isometric, in PCLS parenchymal tethering resulted in more auxotonic contractions like *in vivo*. Even the slicing procedure means some stress for the tissue, which is the same in all *in vitro* preparation; we have not found an induction of immediate early genes after an overnight incubation of the PCLS (Martin *et al.*, 2001).

One of the great advantages of the PCLS model is that up to 30 slices can be prepared from one mouse lung and much more from other species (rat, guinea pig, primate, or human), which offers the possibility to receive significant results using fewer animals where each of it is its own internal control. The measurements of airway contraction in PCLS of the same animal with ideal airway architecture vary about 10% from slice to slice, whereas differences between animals were clearly higher. The observed interanimal variances in PCLS lung function measurements are comparable to the levels seen in *in vivo* lung function tests.

The disadvantage of PCLS compared to *in vivo* methods is that migration of cells from the lung into the blood or lymph nodes and trafficking of cells from blood into the lungs during immune/inflammatory responses cannot be assessed in this model because of the lack of a circulatory system. However, all

cell types relevant to induction of immune responses like dendritic cells, macrophages, or mast cells are present, and the cells remain in their normal architectural relationship and interact with each other and with other resident cells relevant for bronchoconstriction like smooth muscle cells or nerves.

Our hypothesis of the present study is that the measurement of bronchoconstriction in PCLS from sensitized but not challenged animals represents a suitable alternative to *in vivo* studies. For this purpose, animals were epicutaneously sensitized with LMW allergens TMA and DNCB, and bronchoconstriction was measured *in vivo* using invasive lung function method and *ex vivo* in PCLS. Our findings showed that the respiratory allergen TMA, but not the contact sensitizer DNCB, induced AHR both *in vivo* and *ex vivo*. In addition, TMA, but not DNCB, induced pulmonary inflammation characterized by an influx of eosinophilic cells in the lung and increased eotaxin-2 levels in the bronchoalveolar lavage (BAL).

MATERIAL AND METHODS

Animals and House Keeping

Female BALB/c mice (Charles River, Sulzfeld, Germany), 8–10 weeks old at the time of first sensitization, were kept in a pathogen-free rodent facility (Fraunhofer ITEM, Hannover, Germany). Mice were maintained on laboratory food and tap water *ad libitum* in a regular 12-h dark/light cycle at an ambient temperature of 22°C. The study was approved by the responsible governmental authority (Bezirksregierung Hannover, Germany). The acclimatization period was at least 7 days before use.

Sensitization/Boost

Animals were exposed to TMA, DNCB, and vehicle as illustrated in Figure 1. The concentrations of TMA (Sigma Aldrich, Munich, Germany) and DNCB (Sigma Aldrich) were used for the sensitization based on results of prestudies at our laboratory (unpublished data). These data also showed clearly no difference between acetone/air and acetone/TMA animal groups in the early allergic response (EAR) and AHR. Therefore, in the present study we investigated only vehicle and allergen treated groups, both challenged with allergens what is a more stringent control.

For *in vivo* experiments, the animals were sensitized epicutaneously on each shaved flank with 50 μ l of 10% TMA or 1% DNCB in vehicle (acetone/olive oil 4:1 or dimethyl sulfoxide [DMSO]/olive oil 4:1 for further use in PCLS) on days 0, 1, and 2. Two different solvents were selected because the incubation of lung slices with lowest concentrations (0.01–0.05%) of acetone resulted in augmented cell damage and loss of lung function. On day 8, animals were boosted by epicutaneous application of 25 μ l of the same solutions of TMA or DNCB on each ear. The selected concentrations did not induce any dermal irritation during the sensitization phase. For *ex vivo* experiments, TMA and DNCB were dissolved in DMSO for further use in tissue culture.

Serum IgE Levels

Serum samples were prepared from blood by orbital puncture on day 13 and stored at -20°C . Serum IgE levels were measured using a commercially available enzyme-linked immunosorbent assay kit (BD Biosciences, Heidelberg, Germany). ELISA was performed in accordance with the manufacturer's specifications. Lower limits of detection were 1.6 ng/ml.

In Vivo Allergen Challenge and Characterization of EAR

On day 21, sensitized and vehicle-treated animals were challenged by inhalation exposure to dry aerosols of TMA and DNCB (Figure 1). TMA was

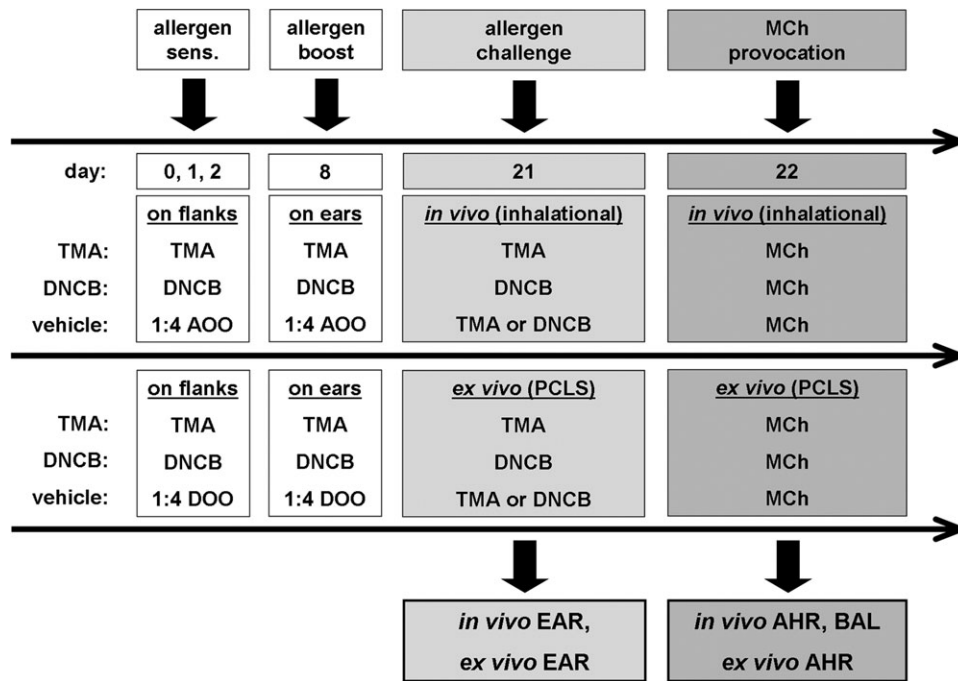


FIG. 1. Test scheme and groups. AOO = acetone:olive oil; DOO = dimethyl sulfoxide (DMSO):olive oil.

nebulized as a dry powder using a screw feeder. DNCB was melted at 70°C and then aerosolized by means of a Pari LC Star nebulizer. The invasive repetitive lung function measurements were performed essentially as described before (Glaab *et al.*, 2004, 2005). Lung resistance (R_L) and dynamic lung compliance (C_{dyn}) were calculated from transpulmonary pressure (P_{TP}), tidal flow, and volume signals over an entire breath cycle. Respiratory parameters were continuously recorded using HEM 3.5 software (Notocord, Croissy, France). Maximum R_L values were taken and expressed as delta percent changes from corresponding baseline values.

After recording baseline values, animals were challenged for 15 min with an inhalational dose of 20 μ g TMA or 0.3 μ g DNCB aerosols (mass median aerodynamic diameters [MMAD]: 3.2 μ m for TMA and 2.2 μ m for DNCB) (Table 1). Aerosol concentrations were determined by a gravimetrically calibrated photometer. The total inhaled doses were calculated and controlled by a computerized feedback dose control system based on the continuously

measured respiratory minute volume and aerosol concentration signals for each animal (Hoymann, 2006). To prevent hypoxia, the animals were provided with 30% oxygen. Registration of lung function was continued for 15 min after the end of exposure. After the measurements, mice were extubated as soon as they began recovering from anesthesia.

In Vivo Characterization of AHR

AHR to inhaled MCh was assessed 24 h after inhalational allergen challenge. Mice were anesthetized with 67-mg/kg propofol injected ip and 1.5% halothane, and lung function measurements were performed as described above (Glaab *et al.*, 2004, 2005). After recording baseline values, airway responsiveness to aerosolized MCh chloride (calculated inhaled doses of 0.063, 0.125, 0.25, 0.5, 1.0, 2.0, and 4.0 μ g MCh; Sigma, Deisenhofen, Germany) was assessed. Dried MCh aerosols (MMAD 2.4 μ m) were generated from 5% MCh solution by a computer-controlled, jet-driven aerosol generator system

TABLE 1
TMA and DNCB Doses Used in Different *In Vivo*, *Ex Vivo*, and *In Vitro* Studies

	<i>In vivo</i> methods		<i>Ex vivo</i> and <i>in vitro</i> methods		
	Invasive lung function measurement (present study)	Noninvasive "Penh system"	Testing of PCLS viability (irritative/toxic effects, present study)	Bronchoconstriction of PCLS (present study)	Cytokine secretion of macrophages
TMA	20 μ g aerosol (inhalational dose)	200 μ g intranasal dose ^a	>3 μ g/ml	1.2 μ g/ml	1.9 μ g/ml and 19 μ g/ml ^b
DNCB	0.3 μ g aerosol (inhalational dose)/mouse	4 μ g intranasal dose ^a ; 600 μ g intranasal dose ^c	>1 μ g/ml	1 μ g/ml	—

^aVanoirbeek *et al.* (2006).

^bValstar *et al.* (2006).

^cFarraj *et al.* (2007).

(Bronchy type III, Fraunhofer ITEM, licensed by Buxco, NY) as previously described (Hoymann, 2006). To administer defined inhalation doses independent of the respiratory minute volume of the individual animal, a feedback dose control system was used which has been successful in generating constant dosing in mice (Glaab *et al.*, 2004). The total inhalation doses were calculated based on the inspiratory aerosol concentrations continuously measured by a gravimetrically calibrated photometer and the respiratory volume per min (MV). The software allows to preselect a dose in microgram, and an exposure time and the system controls the aerosol concentration in dependence of the MV of the animal by controlling a dilution air stream via mass flow controllers (constant dose/time, e.g., 0.25 μg MCh in 36 s in each mouse). Three minutes were allowed between doses to return to baseline values. Increasing doses of MCh were applied until resistance reached at least 200% of the baseline value. The effective dose of MCh that elicited an increase of R_L above baseline of 50% (ED_{50}) was calculated from the individual dose-response curve.

Ex Vivo Characterization of EAR and AHR

Preparation of PCLS. Preparation of PCLS was performed as previously described (Held *et al.*, 1999; Martin *et al.*, 1996). Briefly, mice were anesthetized through ip injection of 60 mg/kg pentobarbital sodium (Narcoren, Pharmazeutische Handelsgesellschaft GmbH, Garbsen, Germany). The trachea was cannulated, and the animals were exsanguinated by cutting the vena cava inferior. Lungs were filled *in situ* with 0.75% low-melting agarose (Sigma Aldrich) in minimal essential medium (MEM, pH 7.2) and covered with ice for 10 min. Lungs were removed from the thoracic cavity and cooled on ice for an additional 10 min. Lung lobes were separated, embedded in 3% agarose solution, and cooled on ice for 5 min. Embedded lobes were cut with a Krumdieck tissue slicer (Alabama Research and Development, Munford, AL) into 220- μm thin slices. Lung slices were incubated in MEM at 37°C, 5% CO_2 , and 100% air humidity under cell culture conditions. Incubation medium was changed every 30 min for 2 h after slicing, followed by a change every hour for the next 2 h, in order to remove agarose residues and cell debris from the tissue. Medium for overnight incubation contained penicillin and streptomycin (100 U and 100 $\mu\text{g}/\text{ml}$, respectively).

Viability of PCLS. Viability of tissue slices was checked by calcein acetoxymethyl/ethidium homodimer-1 (calcein AM/EthD-1) staining (Invitrogen, Karlsruhe, Germany) using confocal microscopy (Henjakovic *et al.*, 2008). Live cells were distinguished by enzymatic conversion of calcein AM to intensely green fluorescent calcein. EthD-1 produces intracellular red fluorescence in nuclei of dead cells. Lung slices were incubated with 4 μM calcein AM and 4 μM EthD-1 for 45 min at room temperature. PCLS were washed in Dulbecco's Modified Eagle Media and investigated by confocal microscopy (40 $\times$ water immersion objective, excitation wavelengths 488 nm and 543 nm, emission filters BP 505–550 nm and LP 560 nm, thickness 20 μm). Image stacks of a defined volume were analyzed with Bitplane IMARIS 4.5.2. In a defined volume, the ratio of numbers of EthD-1-labeled cell nuclei to volume of calcein in cytoplasm of live cells was determined. Cell nuclei of dead cells were counted as spots ≥ 5 μm diameters, and volumes of cytoplasm of live cells stained with calcein were determined. Thresholds were set once for each channel and used for all datasets. Viability of PCLS is expressed as quantity of spots in $10^5 \mu\text{m}^3$ green tissue volume.

Measurement of bronchoconstriction in PCLS. For measurement of bronchoconstriction, lung slices with comparable airway sizes were selected. Lung slices were placed on 24-well plates and fixed with a nylon thread attached to platinum wire. Slices were continuously covered with 1 ml incubation medium. The 24-well plates were positioned on the stage of an inverted microscope. Airways were imaged and digitized with an analog video camera (JAI 2040, JAI PULNiX, Alzenau, Germany). The airway area before addition of the mediators was defined as 100% (Figure 2A). For *ex vivo* investigation of the EAR on day 21, PCLS of TMA- and DNCB-sensitized or DMSO/olive oil control mice were exposed with 1.2 $\mu\text{g}/\text{ml}$ TMA and 1 $\mu\text{g}/\text{ml}$ DNCB, respectively. The airways were imaged at least every 30 s during an incubation time of 20 min. Bronchoconstriction was determined as the

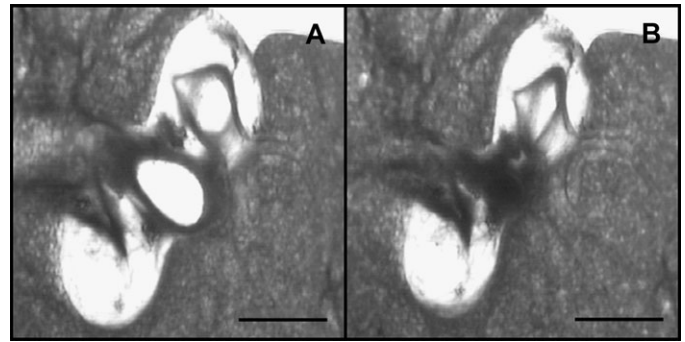


FIG. 2. Methacholine-induced contraction in TMA-sensitized mouse PCLS. Frames were taken of the same slice before (A) and after (B) methacholine (100 μM) application. The black bar represents a length of 250 μm .

percentage decrease in airway area compared with the control airway area, but presented inversely as percentage airway contraction. After measurement of allergen-induced bronchoconstriction, these TMA- and DNCB-exposed lung slices were cultivated under standard cell culture conditions. After an incubation period of 16 h, bronchoconstriction (AHR) was investigated after exposure of the same slices with increasing concentrations of MCh (0.01–100 μM) (Figure 2B).

Cell Counts in BAL Fluid

Lungs of animals were lavaged twice, each with 0.8 ml ice-cold PBS on day 22. BALs were pooled and centrifuged immediately after collection, and supernatants were stored at -80°C for cytokine analysis. The cells were resuspended in 0.5 ml PBS and counted by the standard method in a using a Beckman Coulter AcT8 hematology analyzer. Cytospins were prepared (Shandon, Midland, Canada) and stained with May Grünwald and Gimsa staining (Sigma Aldrich). Cells were counted using light microscopy and categorized as macrophages, eosinophils, neutrophils, or lymphocytes, as determined by their morphology.

Quantification of IL-5 and Eotaxin-2 in BAL Fluid

Cellular proteins IL-5 and eotaxin-2 were measured in BAL fluid using commercially available ELISA kits (ELISA DuoSet, R&D, Wiesbaden-Nordenstadt, Germany). ELISA was performed in accordance with the manufacturer's specifications. Lower limits of detection were 30 pg/ml for IL-5 and 15 pg/ml for eotaxin-2.

Statistical Analysis

Data in the figures are given as means $\pm$ SEM. Comparison of animal groups was performed with the two-sided unpaired *t*-tests (software: GraphPad Prism 4, version 4.03). Differences between sensitized and control animal groups were considered as statistically significant at the level of $p < 0.05$. The analysis was performed using a four-parameter logistic regression algorithm (sigmoidal curve fit) to fit the bronchoconstriction (*in vivo* R_L or *ex vivo* airway area) of each animal versus MCh concentration (software: GraphPad Prism 4, version 4.03).

RESULTS

Dose Finding/Sensitization

In this study, irritant effects of TMA and DNCB were excluded beforehand by *in vivo* and *ex vivo* investigations. For this purpose, we characterized the *in vivo* lung functions and the local inflammation in BAL after inhalation exposure of naive animals to TMA and DNCB. Furthermore, the viability

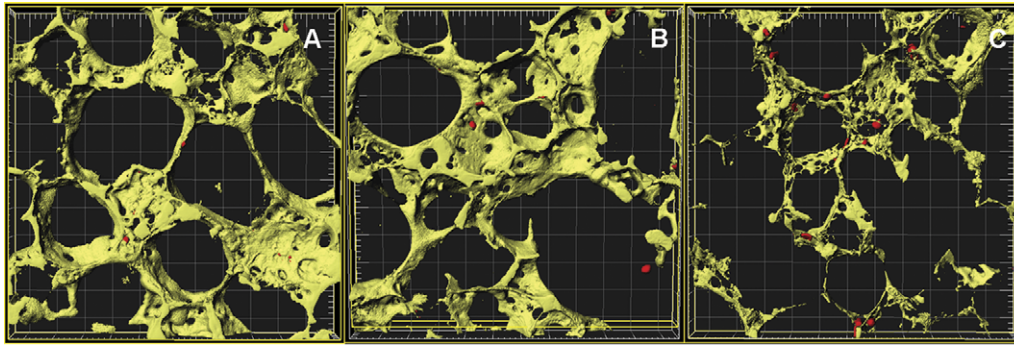


FIG. 3. Image analysis of PCLS viability. Tissue slices were stained with 4 μ M calcein AM and 4 μ M EthD-1 after 16 h cultivation with 0.05 % DMSO (A) or 3 μ g/ml (B) and 12.5 μ g/ml TMA (C). The images were examined by two-color immunofluorescence confocal microscopy (40 $\times$ water immersion objective fold, excitation wavelengths 488 nm and 543 nm, emission filters BP 505–550 nm and LP 560 nm, thickness 20 μ m, grid spacing = 20 μ m) and analyzed with IMARIS 4.5.2. Red color shows cell nuclei (ϕ 5 μ m) of dead cells and green color the cytoplasm of viable cells.

of lung slices was determined by quantitative image analysis of calcein AM/EthD-1 staining after incubation with increasing concentrations of TMA and DNCB (Figure 3). The invasive measurement of respiratory irritation in naive mice revealed that the maximum inhalation dose showing no effect were about 20 μ g TMA and 0.3 μ g DNCB aerosols. Incubation with 1.2 μ g/ml TMA or 1 μ g/ml DNCB did not affect the viability of PCLS. Concentrations of >3 μ g/ml TMA (Figure 3) and >1 μ g/ml DNCB induced an increase in the number of dead cells, loss of tissue architecture, and nuclear degeneration in PCLS under same experimental conditions. In addition, the results of the pilot study showed strongly elevated serum IgE levels in TMA-sensitized animals compared to control and DNCB groups on day 13 after the start of sensitization (Figure 4). DNCB sensitization also induced an increase in serum IgE levels, but in definitely lower manner than TMA (Figure 4).

Lung Function Measurements

The effect on R_L and C_{dyn} *in vivo* was assessed during and after exposure of the animals to 20 μ g TMA or 0.3 μ g DNCB dry aerosols. In this early phase, respiratory airway responses obtained for sensitized animals were comparable to control animals and were not altered by the sensitization. In sensitized animals, neither TMA nor DNCB challenge had a significant effect on R_L (Figure 5A) or C_{dyn} (data not shown).

The unspecific *in vivo* AHR was assessed 24 h after the inhalational allergen challenge. Dose-dependent increases in R_L in response to inhaled MCh were determined in TMA- and DNCB-sensitized and in control mice. Of both tested chemicals, only TMA-sensitized mice showed a significantly decreased ED₅₀ in response to MCh, indicating a marked AHR (0.21 μ g MCh vs. 0.06 μ g MCh) (Figure 6A).

PCLS are a new and reliable *ex vivo* method to study airway and vascular response. *Ex vivo* allergen-induced changes of bronchoconstriction were assessed by exposure of PCLS from sensitized and control animals to 1.2 μ g/ml TMA or 1 μ g/ml DNCB. TMA and DNCB themselves did not contract

individual airways in PCLS from sensitized animals (Figure 5B).

Sixteen hours after incubation of PCLS with TMA and DNCB, we assessed the airway responsiveness toward MCh. While DNCB sensitization had no effect, TMA significantly decreased the EC₅₀ for MCh-induced bronchoconstriction (EC₅₀: 0.41 μ M MCh vs. 0.24 μ M MCh), indicating AHR (Figure 6B).

Pulmonary Inflammation

Pulmonary inflammation was checked on day 22, i.e., 24 h after allergen (TMA, DNCB) challenge. To this end, we determined the influx of eosinophils and the increase in Th2 cytokines in the BAL. Topical exposure and subsequent inhalation challenge with TMA resulted in a significant increase in eosinophils (Figure 7). This result correlates well with increased levels of eotaxin-2 in BAL fluids of TMA-sensitized animals (Figure 8). In DNCB-treated animals, eosinophils and eotaxin-2 concentrations were not elevated

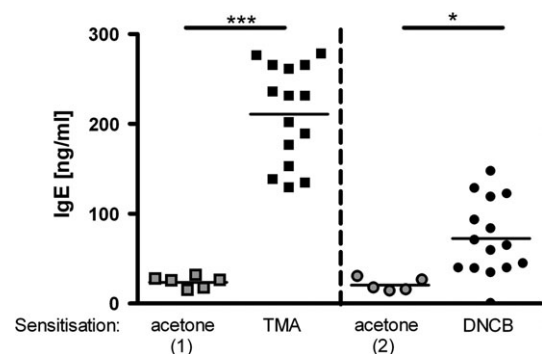


FIG. 4. Total IgE serum levels were determined on day 13 after the start of sensitization. Blood samples were collected from mice by retroorbital bleeding. Mice were sensitized with TMA, DNCB, and acetone. Data are represented as mean $\pm$ SEM. * p < 0.05, *** p < 0.001. $n_{\text{acetone}(1)}$ = 6; n_{TMA} = 15; $n_{\text{acetone}(2)}$ = 5; n_{DNCB} = 15.

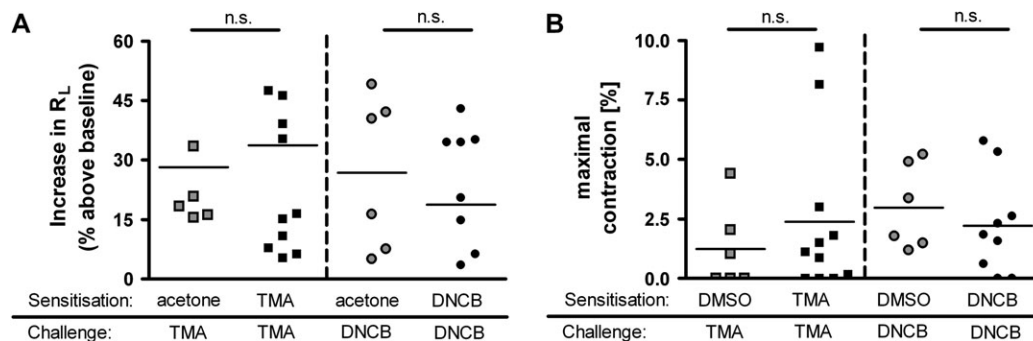


FIG. 5. *In vivo* and *ex vivo* EAR measured after exposure to TMA and DNCB using invasive lung function measurements *in vivo* and PCLS *ex vivo*. TMA- and DNCB-sensitized mice (A) and lung slices of sensitized animals (B) were challenged with TMA or DNCB. Control animals were sham sensitized with acetone or DMSO and challenged with TMA or DNCB. Bronchoconstriction *in vivo* was determined as the percentage increase in pulmonary resistance (RL) and *ex vivo* as the percentage increase in maximal airway contraction. Data are represented as mean $\pm$ SEM. n.s., nonsignificant. $n_{A(\text{acetone/TMA})} = 6$, $n_{A(\text{TMA/TMA})} = 12$, $n_{A(\text{acetone/DNCB})} = 6$, $n_{A(\text{DNCB/DNCB})} = 10$; $n_{B(\text{DMSO/TMA})} = 6$, $n_{B(\text{TMA/TMA})} = 11$, $n_{B(\text{DMSO/DNCB})} = 6$, $n_{B(\text{DNCB/DNCB})} = 9$.

(Figure 7 and 8A). IL-5 in BAL fluid did not differ among the four groups (Figure 8B). However, neutrophilic lung inflammation was induced by both compounds (Table 2). The number of macrophages and lymphocytes remained unaffected (Table 2).

DISCUSSION

The aim of this study was to examine the use of PCLS as an *ex vivo* tool for assessing the respiratory sensitizing potential of LMW chemicals. To this end, using a murine model of allergic airway inflammation, mice were sensitized with two relevant industrial LMW organic chemicals, and both EAR and AHR were determined *in vivo* and *ex vivo*. The correlation between EAR and AHR measurements *in vivo* and *ex vivo* indicates that PCLS from sensitized animals are suitable for monitoring the airway-sensitizing potential of organic chemicals.

The LMW chemical TMA was used as a prototype for respiratory sensitizers and compared to the dermal sensitizer

DNCB. TMA and DNCB had been used as reference substances in various studies before, e.g., to investigate the activation of lymph node cells and their cytokine secretion profile (Arts *et al.*, 1998; Dearman *et al.*, 2002; Hayashi *et al.*, 2001; Vandebriel *et al.*, 2003). They induced IL-4 expression and a mixed Th1 and Th2 cytokine profile in lymph node (auricular and cervical) cells (Dearman *et al.*, 1996; Vanoirbeek *et al.*, 2006).

In case of acid anhydrides, IgE plays an important pathogenic role in the development of respiratory allergy, which is indicative of an activation of Th2 cells (Vanoirbeek *et al.*, 2006; Zhang *et al.*, 2006). The results of the pilot study are in line with several publications where topical TMA sensitization increased serum IgE levels (Arts *et al.*, 1998; Vanoirbeek *et al.*, 2006). Our results are also in the line with several publications that dermal DNCB sensitization induces an increase in serum IgE levels in BALB/c mice (Ban and Hettich, 2001; Guo *et al.*, 2002). However, similar to our results, both chemicals TMA and DNCB induced greater IgE production than vehicle control, but the serum IgE induced by

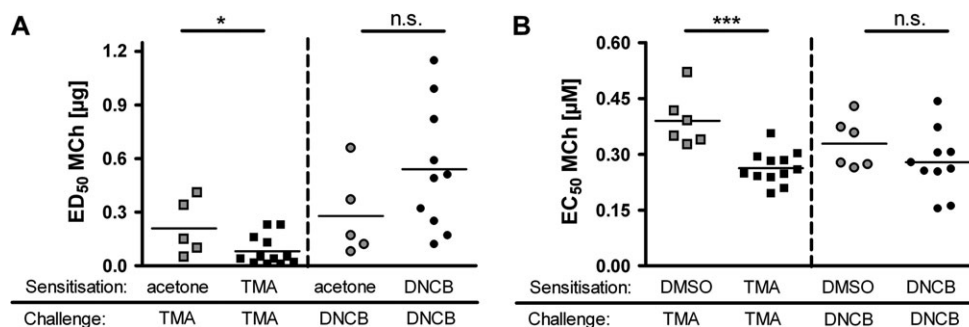


FIG. 6. *In vivo* and *ex vivo* AHR to MCh 24 h after exposure of mice to TMA and DNCB aerosols or 16 h after incubation of PCLS with TMA and DNCB. ED₅₀ (*in vivo*) and EC₅₀ (*ex vivo*) were determined from the application of different doses/concentrations of MCh (*in vivo*: 0.063–2 μ g MCh; *ex vivo*: 10^{-8} to 10^{-4} M MCh). TMA- and DNCB-sensitized mice (A) and PCLS obtained from sensitized animals (B) were challenged with TMA or DNCB. Control groups were sensitized with acetone or DMSO and challenged with TMA or DNCB. ED₅₀: inhalational MCh doses causing a 50% increase in RL. EC₅₀: MCh concentration causing a half maximal bronchoconstriction in lung slices. Data are represented as mean $\pm$ SEM. n.s., nonsignificant, * $p < 0.05$, ** $p < 0.01$. $n_{A(\text{acetone/TMA})} = 5$, $n_{A(\text{TMA/TMA})} = 12$, $n_{A(\text{acetone/DNCB})} = 5$, $n_{A(\text{DNCB/DNCB})} = 10$; $n_{B(\text{DMSO/TMA})} = 6$, $n_{B(\text{TMA/TMA})} = 12$, $n_{B(\text{DMSO/DNCB})} = 6$, $n_{B(\text{DNCB/DNCB})} = 12$.

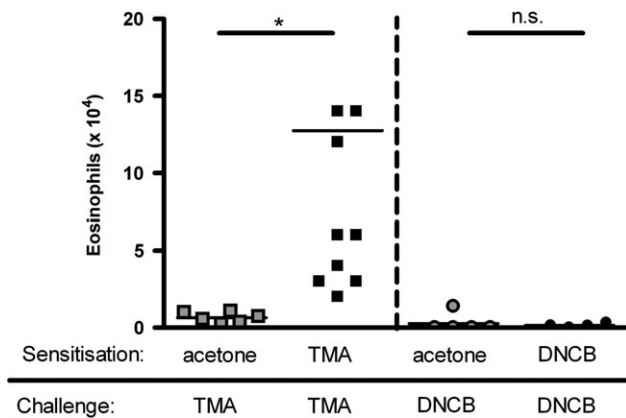


FIG. 7. The number of eosinophil cells in BAL was determined 24 h after allergen challenge. TMA- and DNCB-sensitized mice were challenged with TMA or DNCB aerosols. Control animal groups were sensitized with acetone and challenged with TMA or DNCB. Data are represented as mean $\pm$ SEM. n.s., nonsignificant, $*p < 0.05$. $n_{(\text{acetone/TMA})} = 6$, $n_{(\text{TMA/TMA})} = 12$, $n_{(\text{acetone/DNCB})} = 5$, $n_{(\text{DNCB/DNCB})} = 4$.

TMA was at least twice as high as that induced by DNCB (Ban and Hettich, 2001).

In contrast to previously published results in mouse (Vanoirbeek *et al.*, 2006), rat, and guinea pig allergy models (Arts *et al.*, 1998; Larsen and Regal, 2002; Zhang *et al.*, 2006), in the present work TMA- or DNCB-sensitized mice and also lung slices from sensitized animals did not exhibit an EAR after challenge with TMA or DNCB. Possible explanations for these differences are as follows. Firstly, in some of these studies, the allergic airway response was measured in mice using a critically discussed (Kirschvink *et al.*, 2005) non-invasive technique (PenH system) (Vanoirbeek *et al.*, 2006), whereas we used a very precise invasive method (Glaab *et al.*, 2004). The results of the noninvasive PenH technique are difficult to correlate with direct airway resistance (Kirschvink *et al.*, 2005). PenH may also respond to alterations of the upper airways such as allergic rhinitis, which was reported in humans inhaling TMA dust (Grammer *et al.*, 1993), and this may have

biased the results found by Vanoirbeek (Vanoirbeek *et al.*, 2006). However, invasive measurements of resistance and compliance in guinea pig have noted an EAR using challenge of TMA-sensitized animals with TMA dust (Larsen and Regal, 2002). The possible explanation is that airways of guinea pig reacted more sensitive to provocation with TMA than airways of the mouse. In PCLS, our previous studies demonstrated that airways of mice and rats showed only diminished response to immunoreactive mediators such as leukotrienes and histamine in comparison to guinea pig (Held *et al.*, 1999; Ressmeyer *et al.*, 2006; Wohlsen *et al.*, 2001), which may simply be due to the fact that guinea pigs are no rodents (D'Erchia *et al.*, 1996). In principle, the invasive pulmonary function test we used in the present study is capable for detecting EAR in mice which were sensitized to *Aspergillus fumigatus* antigens or ovalbumin (Erpenbeck *et al.*, 2006; Glaab *et al.*, 2004; F. Hoymann, unpublished data). The results of the present study suggested that a single TMA provocation was not sufficient to induce an allergen-induced bronchoconstriction. Similar results were published for single-ovalbumin aerosol challenge in a mouse asthma model (Neuhaus-Steinmetz *et al.*, 2000; Path *et al.*, 2002; Zosky *et al.*, 2008). Based on our extensive experience with this models, we know that EAR in mice occurs only if there is a preexisting inflammation in the lung leading to a severe AHR in combination with high IgE levels in blood. In the setup used in the current study, no preexisting lung inflammation is present.

Secondly, we delivered the allergen in a natural occurring way by inhalation exposure to dry aerosols, rather than intranasal challenge. For the *in vivo* provocation studies, we used particles of a defined size, consisting only of allergens, to avoid any secondary solvent effects. Irritation by the solvent and easier penetration of the epithelial border is likely to be a problem with typical solvents such as DMSO or acetone. Thirdly, the allergen doses we used to sensitize and provoke airway responses were below the toxicity level inducing *per se* irritation or bronchoconstriction, while the doses used by Vanoirbeek (Vanoirbeek *et al.*, 2006) were 10-fold higher.

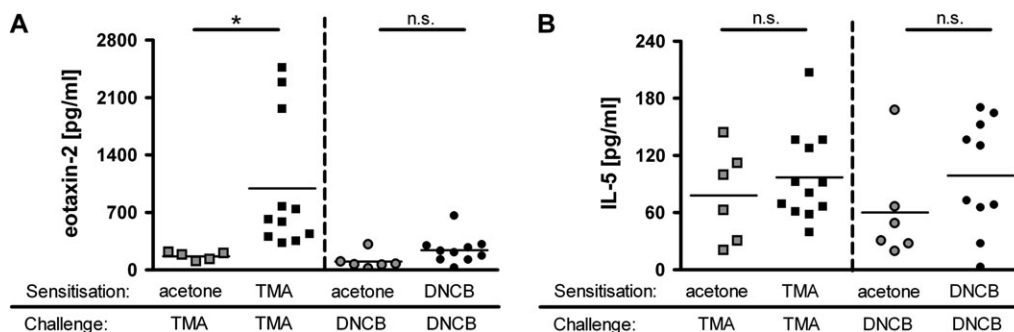


FIG. 8. Levels of eotaxin-2 (A) and IL-5 (B) in BAL fluid 24 h after allergen challenge. TMA- and DNCB-sensitized mice were challenged with TMA or DNCB aerosols. Control animal groups were sensitized with acetone and challenged with TMA or DNCB. Data are represented as mean $\pm$ SEM. n.s., nonsignificant, $*p < 0.05$. $n_{A(\text{acetone/TMA})} = 6$, $n_{A(\text{TMA/TMA})} = 12$, $n_{A(\text{acetone/DNCB})} = 6$, $n_{A(\text{DNCB/DNCB})} = 10$; $n_{B(\text{acetone/TMA})} = 5$, $n_{B(\text{TMA/TMA})} = 11$, $n_{B(\text{acetone/DNCB})} = 6$, $n_{B(\text{DNCB/DNCB})} = 10$.

TABLE 2
The Number of Macrophages, Neutrophils, and Lymphocytes in BAL 24 h after Allergen Challenge

Animal groups	Acetone/TMA ($\times 10^5$)	TMA/TMA ($\times 10^5$)	Acetone/DNCB ($\times 10^5$)	DNCB/DNCB ($\times 10^5$)
Cells in BAL				
Macrophages	20.8 $\pm$ 0.24	2.78 $\pm$ 0.51	8.08 $\pm$ 1.9	6.67 $\pm$ 0.90
Neutrophils	0.51 $\pm$ 0.15	1.97 $\pm$ 0.39*	0.27 $\pm$ 0.08	1.69 $\pm$ 0.46*
Lymphocytes	0.01 $\pm$ 0.01	0.30 $\pm$ 0.13	0.11 $\pm$ 0.10	0.12 $\pm$ 0.08

TMA- and DNCB-sensitized mice were challenged with TMA or DNCB. Control animal groups were sensitized with acetone and challenged with TMA or DNCB. Data are represented as mean $\pm$ SEM. $n_{\text{acetone/TMA}} = 6$; $n_{\text{TMA/TMA}} = 12$; $n_{\text{acetone/DNCB}} = 5$; $n_{\text{DNCB/DNCB}} = 4$.

* $p < 0.05$, compared with vehicle group (acetone/TMA or acetone/DNCB).

Dermal exposure is a clinically relevant route of sensitization to cause respiratory hypersensitivity. It is known that dermal exposure to and respiratory challenge with, for example, acid anhydrides (e.g., TMA) causes respiratory allergy with Th2 immune response in mice (Dearman *et al.*, 2000; Sailstad *et al.*, 2003). Moreover, there is evidence that dermal sensitization to TMA occurs in humans (Beck and Leung, 2000; Karol, 1986; Kimber *et al.*, 1992, 2001).

The *in vivo* and *ex vivo* responsiveness to MCh 24 h or 16 h after allergen challenge was significantly increased in TMA-sensitized mice and in PCLS from those animals, demonstrating that TMA caused a marked AHR. On the other hand, DNCB-sensitized animals and lung slices from DNCB-treated mice did not show clear signs of AHR. Although the nominal differences between TMA and DNCB groups to their vehicle control groups were similar and TMA and DNCB groups did not differ significantly from each other, only the differences between TMA and control reached statistical significance. Therefore, this method is not suitable to differentiate between respiratory and skin sensitizers at this stage of development.

The AHR toward MCh is thought to be dependent on airway inflammation characterized by accumulation of Th2 cytokines and chemokines, activation of mast cells, and the infiltration of inflammatory cells, e.g., eosinophils (MacLean *et al.*, 1999). As cause for the development of AHR *in vitro*, influx of inflammatory cells can be excluded in our study. Therefore, we believe that allergen is captured by IgE bound to resident mast cells or eosinophils within the tissue and this leads to mediator release, e.g., mast cell mediators *in vitro*. Although the mediators are not potent enough to lead to direct airway contraction (EAR), they can prime the smooth muscle cells and other resident cells (unpublished results) and induce hypersensitivity to unspecific stimuli like MCh (AHR). One of the mediators involved in this smooth muscle cell priming is thromboxane as shown previously (Held and Uhlig, 2000).

The present *in vivo* results are in line with several publications reporting the effect of dermal TMA sensitization on MCh responsiveness (Arts *et al.*, 1998; Vanoirbeek *et al.*, 2006; Zhang *et al.*, 2006). The findings reported here agree also with published data showing that DNCB sensitization does not induce any or much less AHR compared to TMA treatment

(Vanoirbeek *et al.*, 2006). In our study, measurements of AHR *in vivo* after dermal treatment with chemicals proved to be a very sensitive indication of airway allergenicity. In this study, we show for the first time that the presence of inflammatory cells (e.g., eosinophils) and accumulation of Th2 cytokines are not essential for the detection of *ex vivo* AHR in PCLS. Other studies have also suggested that the respiratory hypersensitivity effects of some LMW allergens are not characterized by a selective stimulation of Th2 lymphocytes and that other unknown mechanisms may be involved (Farraj *et al.*, 2007; Tarkowski *et al.*, 2007).

Our and other findings (Greene *et al.*, 2005; Regal *et al.*, 2001; Vanoirbeek *et al.*, 2006; Zhang *et al.*, 2004, 2006) support the view that TMA induces an allergic response with expression of at least some typical mediators such as eotaxin-2 and pulmonary infiltration of eosinophils and neutrophils. In contrast, dermal exposure and respiratory challenge to DNCB did not induce immune-mediated inflammation of the lung, and eotaxin-2 expression was less enhanced compared to the TMA-sensitized group ($p_{(\text{DNCB/DNCB vs. TMA/TMA})} = 0.0104$). These results agree with several publications showing that DNCB does not induce respiratory allergenicity and causes predominantly a Th1 immune response (Dearman *et al.*, 2002; Farraj *et al.*, 2007). In our study, the IL-5 levels in BALF were not increased 24 h after inhalational challenge with TMA as usually seen in asthma models with protein antigens like ovalbumin. IL-5 and the eotaxins are believed to control eosinophil trafficking in the lung. The elevated eotaxin-2 levels in absence of elevated IL-5 levels point to a direct effect of TMA mainly on resident lung cells like epithelial cells, smooth muscle cells, and fibroblasts as main producers of eotaxin rather than to a direct effect on Th2 cells.

As previously shown (Held *et al.*, 1999; Henjakovic *et al.*, 2008; Martin *et al.*, 1996; Wohlsen *et al.*, 2003), we obtained from one mouse lung 10–30 ideal cut slices. This allows evaluation of full dose-response curves of one pharmaceutical agent or ranking of different substances in tissue of the same animal. When using PCLS in triplicate for our experiments, the number of animals used can be reduced by a factor of 3–10 what means an enormous reduction as compared to full *in vivo* studies for those chemicals. Another advantage of this method

is the existence of internal controls and the multiple measurement of one mediator in one lung.

In summary, we have developed an *in vivo* model, where dermally sensitized animals were exposed by inhalation to solvent-free defined particles, representing a close approximation to the human exposure situation. The *in vivo* findings for EAR and AHR correlated well with those in PCLS obtained from sensitized animals. This study has shown that TMA but not DNCB induces (1) allergic sensitization, (2) inflammation of the respiratory tract, and (3) airway responsiveness to MCh *in vivo* and *ex vivo*. Our findings show that the airway-sensitizing effects of LMW allergens may be studied by measuring lung function in PCLS *ex vivo*. However, sensitization still occurs *in vivo* by dermal application. According to the 3R concept, this technique allows to reduce animal numbers by using many lung slices from one animal and avoiding the stress for the individual animal since lung function measurements are performed *ex vivo*. With an increasing public demand to reduce the number of animals used in research, PCLS represent a promising approach for assessing potentially allergic agents in the lungs.

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