



Partial C-fiber ablation modulates diphenylmethane-4,4'-diisocyanate (MDI)-induced respiratory allergy in Brown Norway rats

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Received 27 July 2006; received in revised form 21 August 2006; accepted 22 August 2006

Abstract

Brown Norway (BN) rats were topically sensitized to polymeric diphenylmethane-diisocyanate (MDI) and challenged with MDI-aerosol approximately every 2 weeks over a time period of 2 months. Half of the sensitized animals were pretreated with capsaicin for partial C-fiber defunctionalization. After the fourth challenge inflammatory and pro-inflammatory factors in bronchoalveolar lavage (BAL) fluid and cells and physiological delayed-onset breathing patterns were analyzed. The latter endpoint was examined in the capsaicin pretreated group before and after each challenge. Findings were compared against naïve but repeatedly MDI-challenged BN rats. BAL-neutrophils, -protein, and -LDH as well as lung weights were significantly increased in the MDI-sensitized and challenged rats relative to the naïve, challenged control rats. With regard to these endpoints, capsaicin pretreatment did not affect the responsiveness to MDI-aerosol. In contrast, pro-inflammatory cytokines, the Th2 cell cytokine IL-4, and the CC-chemokine MCP-1 were significantly increased in BAL-cells of capsaicin pretreated and MDI-sensitized rats, whilst in the normal MDI-sensitized rats markedly less pronounced changes (if any) occurred. In the former group, IL-4 and MCP-1 were also significantly increased in the lung draining lymph nodes. Time-related increased frequencies of delayed-onset responses were observed in MDI-sensitized rats after subsequent MDI-challenges, however, differences between capsaicin pretreated and normal rats were not found. Despite the remarkable differences between normal and capsaicin pre-treated rats in the concentrations of pro-inflammatory and Th1-/Th2-cell specific cytokines, the inflammatory endpoints in BAL as well as the physiological measurements did not identify appreciable differences amongst these groups. This study included an ancillary study addressing the analysis of the modulating effect of capsaicin pre-treatment of naïve Wistar rats exposed for single 6 h to MDI-aerosol. The results indicated more pronounced changes on endpoints in the BAL-fluid of capsaicin-pretreated rats as compared to rats with intact C-fibers. This complex picture appears to suggest that C-fibers may modulate the allergic inflammatory response elicited by MDI-challenge. It appears that tachykinergic sensory C-fibers modulate the protective pathways against irritant-related lung inflammation and, similarly, also pro-inflammatory immunological factors modulating allergic inflammation. Although difficult to disentangle unequivocally the mechanisms involved, neuro-immunological factors may be important in triggering and maintaining this complex disease and cytokine/chemokine patterns may not necessarily predict the functional outcome of test.

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Keywords: Diphenylmethane diisocyanate; MDI; Respiratory hypersensitivity; Lung function; Delayed responses; Penh; Neutrophilic inflammation; Asthma; Capsaicin; Cytokine fingerprinting

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1. Introduction

Diisocyanates are widely used in industry and are known to be involved as causative agents for occupational asthma (Chan-Yeung and Malo, 1994; Johnson et al., 2004). The pathogenesis associated with low-molecular-weight diisocyanates is not well understood, including the role of the dermal route of induction to initiate disease. The latency period in the reactions observed in specific challenge tests indicate immunological mechanisms, however, specific immunoglobulin E (IgE) antibodies have been identified only in 14–20% of the cases (Baur, 1983; Piirilä et al., 2000), suggesting that non-immunological factors may contribute to disease. Thus, the role of serum IgE in diisocyanate-induced allergic airway disease is still controversial. In this context, neural mechanisms and their interaction with inflammatory cells have been reported to be important modifying factors in the pathophysiology of asthma, commonly referred to as a neurogenic inflammation (Barnes, 1992; Bousquet et al., 2000; Joos et al., 1994). It has been suggested that C sensory fibers have a transient role in mediating neuroimmune effects on target cells in the respiratory tract (Fox, 1999; Scheerens et al., 1996; Kranefeld and Nijkamp, 2001; Hunter et al., 2000; Graham et al., 2001). Neurogenic inflammation is mediated by the release of neuropeptides subsumed as tachykinins, e.g. substance P, from capsaicin sensitive C-fibers in the rodent airway mucosa. Inflammation is characterized by increased vascular permeability, plasma extravasation, glandular secretion, and neutrophil chemotaxis. Stimulation of sensory nerves innervating the vascular bed of the tracheobronchial mucosa causes increased release of tachykinins resulting in vasodilation as well as to an enhanced production and release (pro)inflammatory mediators (Hunter et al., 2000; Kranefeld and Nijkamp, 2001). Many investigations of sensory nerve function have relied on the use of capsaicin, which acts through the vanilloid receptor. At the dose of capsaicin used in this study, defunctionalization of sensory nerves, including a partial depletion of several neuropeptides, that result in long-term loss of sensory nerve function have been described (Holzer, 1991; Chung et al., 1990; Lundblad, 1984; Lundblad et al., 1985; Morris et al., 1999; Stjarne et al., 1989). With regard to the ablation of C-fibers and responses to irritants appreciable differences across different rat strains were not observed (Takebayashi et al., 1998). Sterner-Kock et al. (1996) demonstrated that the capsaicin pretreatment of neonatal Wistar rats (examinations after 2 month) using a similar dosing regime as used in this study reduced the substance P content in lung tissue

homogenates by 77% compared to normal rats. However, not all C-fibers belong to the capsaicin-sensitive class (Hunter et al., 2000) so that localized tachykinergic innervation from C-fibers subclasses may still be operative, despite capsaicin pretreatment.

In previous studies in the Brown Norway (BN) rat with the benchmark low-molecular weight asthmagenic chemical agent TMA (trimellitic anhydride) remarkable pathophysiological changes in inflammatory and physiological (breathing patterns) endpoints have been observed during an inhalation challenge, independent of whether the induction was topical or by inhalation (Pauluhn et al., 2002; Pauluhn, 2003; Zhang et al., 2004). To the contrary, similar methodological approaches failed to identify MDI as an asthmagenic compound when using a single inhalation-challenge regime. However, when using a repeated inhalation challenge regime, MDI was unequivocally identified as an asthmagenic substance both with regard to transiently occurring respiratory responses delayed in onset as well as by a number of inflammatory endpoints in bronchoalveolar lavage (BAL), however, only following topical rather than inhalation induction (Pauluhn, 2005; Pauluhn et al., 2005). So far, only a limited number of studies have evaluated the sequence of inflammatory events taking place after repeated, chronic inhalation challenges (Holgate et al., 2000; Palmans et al., 2000, 2002; Tomkinson et al., 2001; Leigh et al., 2002; Kips et al., 2003). At face value, the data obtained with MDI in the repeated challenge BN rat model appear to support a hypothesis favoring an immunological rather than a non-immunological pathophysiology. However, multiple challenge exposures to MDI-aerosol are required to phenotypically manifest asthma-like effects as shown in previous studies (Pauluhn, 2005; Pauluhn et al., 2005).

The objective of this study was to study the modifying impact of sensory nerves (C-fibers) that upon stimulation release neuropeptides through the axon reflex. Pro-inflammatory effects of these peptides also promote the recruitment, adherence, and activation of granulocytes that may further exacerbate neurogenic inflammation, i.e., plasma extravasation and vasodilation (Solway and Leff, 1991). The modifying role of capsaicin-induced sensory nerve defunctionalization on protein extravasation and the recruitment of inflammatory cells in BAL fluid was evaluated in a sensitization study using normal and capsaicin pretreated BN rats that were topical induced and repeatedly challenged with MDI-aerosol. This analysis included an evaluation of allergic respiratory responses delayed in onset. Moreover, pro-inflammatory cytokines/chemokines and inflammatory endpoints from bronchoalveolar lavage fluid and cells

and cytokines/chemokines from lung-associated-lymph nodes were compared to study whether the change in selected pro-inflammatory immunological factors match those of the more integrating inflammatory and physiological endpoints commonly addressed in this animal model.

2. Methods

2.1. Test material and chemicals

Polymeric methylenediphenyl-4,4'-diisocyanate (MDI) was from Bayer Material Science AG, Leverkusen, Germany. The free isocyanate (NCO) content was 31.05%. Tween®80, capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), terbutaline, and theophylline were from Sigma-Aldrich (Germany). All other reagents were obtained from local suppliers and were of the highest purity available.

2.2. Animals, diet, and housing conditions

Brown Norway (BN) rats of the strain BN/Crl BR and Wistar rats of the strain Hsd Cpb:WU (SPF) were purchased from Charles River, Sulzfeld, Germany and from Harlan Winkelmann GmbH, Borcheln, Germany, respectively. Animals were placed in polycarbonate cages (1 rat per cage), containing bedding material (lowdust wood shavings), and were provided with a standard fixed-formula diet (NAFAG No. 9439 W10 pellets maintenance diet for rats and mice) and municipality tap water (drinking bottles). Both feed and water were given *ad libitum* except during inhalation exposures. At the commencement of study, the mean body weights ($\pm$ S.D.) in the single 6 h study using Wistar rats were 236 ± 5 g and in the sensitization study using BN rats were 227 ± 6 g. For this study, only male rats were used and they were 2–3 months old. Animals were quarantined for at least 5 days prior to being placed on study. Animal rooms were maintained at approximately 22°C with relative humidity at 40–60% and a 12 h light cycle beginning at 0600 h. The studies described were conducted in accordance to the EU animal welfare regulations (European Community, Directive 86/609, 1986).

2.3. Experimental design and exposure protocols

2.3.1. Single exposure study

In an ancillary study, three groups of 12 male Wistar rats each were nose-only exposed to dry air (A1), 15.2 ± 0.7 mg MDI/m³ (A2) or 50.6 ± 4.0 mg MDI/m³ (A3) for 6 h (means and S.D.s of four adequately spaced filter analyses). Control rats were similarly exposed to dry air. For capsaicin pretreatment, from each group six rats/subgroup were treated as follows 7 days prior to the exposure to air or MDI: animals were first anesthetized with sodium pentobarbital (40 mg/kg, ip) and then treated with 10 mg/kg theophylline (sc, 10 mg/ml in 10:90 ethanol:distilled water) and 0.1 mg/kg terbutaline (ip, 0.1 mg/ml in saline). Animals then received 50 mg/kg

capsaicin (sc, 10 mg/ml in 1:1:8 ethanol:tween 80:saline). This is the dosing regime also used by other authors for similar study objectives (Lundblad, 1984; Stanek et al., 2001). Approximately 20 h after exposure the rats were necropsied and lungs were weighed and lavaged as previous time-course studies have shown that irritant concentrations of polyisocyanate aerosols cause maximum protein in BAL-fluid on the first postexposure day (Pauluhn, 2000, 2004b). Therefore, also in this study measurements were made on the first postexposure day. This ancillary study used Wistar rats to allow direct comparisons with previous data obtained in this strain (Pauluhn, 2000, 2004b; Sterner-Kock et al., 1996). The comparison of responses of Wistar and BN rats to MDI-aerosol utilizing similar endpoints revealed no appreciable strain differences (Pauluhn, 2004a).

2.3.2. Sensitization study

This study consisted of one naïve control group and two groups of BN rats that were sensitized topically using undiluted MDI directly applied to the shaved surface of the skin on days 0 and 7 (see Table 1). Each group consisted of eight male rats allocated to three groups by randomization. On day 0, the rats of the MDI-induction group received 40 μ l MDI on the left-dorsal area of the trunk. The same dose was administered to the contralateral flank on day 7 as booster. The doses were administered by using aluminum foil spots. After metering a predefined volume of MDI to each foil the weight of MDI was determined using a digital balance. The test substance was transferred to the skin by pressing the spot onto the skins' surface and the foil was then removed. Each foil spot was re-weighed to confirm the actual dose applied. Thus, the applied dose/rat/exposure session was as follows: 2 cm $\times$ 2 cm spots, each dosed with 20 μ l of MDI. The resultant cumulative dose/cumulative surface area was 94.2 ± 6.4 and 96.0 ± 2.6 mg MDI per 12.6 cm² in groups 2 and 3, respectively (for group allocation see Table 1). The cumulative dose to body burden relationship was 405 ± 30 and 417 ± 16 mg MDI/kg rat in groups 2 and 3, respectively. The skin was shaved 1 day prior to dosing. Rats were prevented from grooming or scratching by wearing an Elizabethan collar until the morning following topical administration (Buster Birdcollars; Kruuse, DK, Cat no.: 273375). All animals of the control group and the group 3 received capsaicin using the dosing regime described above, whilst no pretreatment was made in group 2 (for details see Table 1). Capsaicin pretreatment was on day 13, i.e., 6 days after the last topical induction (booster) and 7 days prior to the first inhalation challenge exposure.

All rats were repeatedly challenged with MDI-aerosol on days 20, 35, 50, and 62–64 for 30 min, except the last challenge, where each group was challenged on subsequent days rather than simultaneously. For all challenges, the concentration was 39 ± 1.8 mg MDI/m³ (mean $\pm$ S.D.). This concentration $\times$ time relationship of approximately 1170 mg/m³ min was considered to be minimally irritant based on the data shown in Fig. 1. In previous studies using essentially the same challenge regime, naïve BN rats elaborated a minimal increase in

Table 1
Group allocation and scheduling of induction to and challenge exposures

Group	Pre-treatment with capsaicin	MDI-topical (days 0 and 7)	MDI-challenge (days 20 ^a , 35 ^a , 50 ^a , 62–64 ^b)
Ancillary study in Wistar rats			
A1/–	No	No	single 6 h (day 0–0 mg/m ³)
A1/+	Yes (day-7)	No	single 6 h (day 0–0 mg/m ³)
A2/–	No	No	single 6 h (day 0–15.2 mg/m ³)
A2/+	Yes (day-7)	No	single 6 h (day 0–15.2 mg/m ³)
A3/–	No	No	single 6 h (day 0–50.6 mg/m ³)
A3/+	Yes (day-7)	No	single 6 h (day 0–50.6 mg/m ³)
Sensitization study in BN rats			
1	Yes (day 13)	No	Yes (0.5 h–39 mg/m ³)
2	No (day 13)	Yes	Yes (0.5 h–39 mg/m ³)
3	Yes (day 13)	Yes	Yes (0.5 h–39 mg/m ³)

A#/– or +: ancillary single 6 h exposure to MDI aerosol in normal (–) or capsaicin pre-treated (+) naïve Wistar rats.

^a All Brown Norway (BN) rats were simultaneously exposed to a mean ($\pm$ S.D.) 39 ± 1.8 mg MDI-aerosol/m³ for 30 min per challenge.

^b Rats were challenged subsequently, i.e., group-wise to the same mean concentration one group per day.

BAL lactate dehydrogenase (LDH) relative to BAL protein whereas at high exposure intensities the changes in BAL protein exceeded those of BAL LDH (Fig. 1). Thus, at an exposure intensity of approximately 1200 mg MDI/m³ min the relative increase in BAL-LDH in naïve BN rats is anticipated to be slightly higher than that of BAL protein. Four out of the eight rats of group 3 (see Table 1) were monitored the night before and after each MDI challenge for delayed onset for respiratory effects delayed in onset. Animals of groups 1 and 2 were challenged under identical conditions, however, measurements for

delayed-onset responses were made only on days 62–64 after MDI challenge. One day after the final challenge, rats were sacrificed, the weights of exsanguinated lungs and lung-associated lymph nodes were determined. Then the lungs were lavaged for the analysis of endpoints suggestive of an inflammatory response.

2.4. Exposure technique, aerosol generation and characterization

Details addressing the exposure technology and methods, including the lavage procedures used were published previously (Pauluhn, 2004a). Briefly, at the end of the acclimatization period rats were randomly assigned to the respective exposure group and then exposed by directed-flow nose-only inhalation to the targeted concentrations of aerosolized MDI. MDI was atomized under dynamic conditions using a digitally controlled Hamilton Microlab M pump (pump rate: 10 μ l MDI/min) and a modified Schlick-nozzle Type 970, form-S 3 (Schlick GmbH, Coburg, Germany) maintained at 40 °C. The stability of the test atmospheres was monitored continuously using a RAS-2 real-time aerosol photometer (MIE, Bedford, Massachusetts, USA). The exposure atmosphere was characterized by filter analyses, sampled from the vicinity of the breathing zone of the rats. For particle-size analyses, a low-pressure critical orifice AERAS stainless steel cascade impactor (HAUKE, 4810 Gmunden, Austria) as used. The mass median aerodynamic diameter (MMAD) and the geometric standard deviation (GSD) were calculated as described previously (Pauluhn, 1994). The MMAD was in a range of 1.6–2.2 μ m and the GSD was 1.6–1.9.

2.5. Bronchoalveolar lavage

Details of the lavage technique have been published elsewhere (Pauluhn, 2000). Briefly, rats were anesthetized with sodium pentobarbital (Narcoren®; 120 mg/kg bw, ip). After

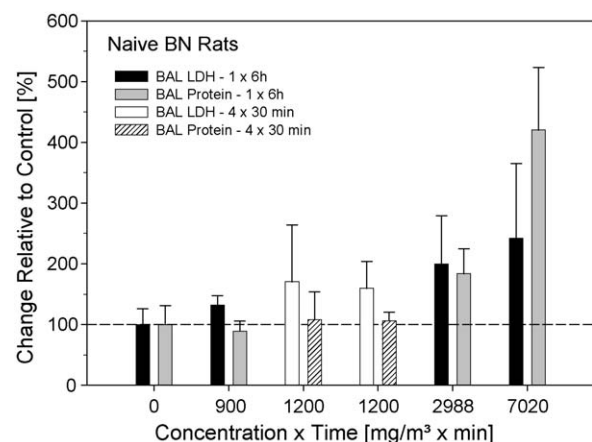


Fig. 1. Concentration dependence of lactate dehydrogenase (LDH) activity and total protein concentration in bronchoalveolar lavage fluid (BALF) of naïve Brown Norway (BN) rats either exposed for single 6 h to dry air (0), 2.5, 8.3, and 19.5 mg MDI/m³ (Pauluhn, 2004a), the respective $C \times t$ -products were 900, 2988, and 7020 mg/m³ min, or naïve control BN rats from previous sensitization studies challenged four-times to approximately 39 mg MDI/m³ for 30 min ($C \times t \approx 1200$ mg/m³ min) (Pauluhn, 2005). Rats were sacrificed 1 day after exposure (single 6 h) or the final challenge. Data were normalized relative to the mean of the air control group (single 6 h) and represent group means $\pm$ S.D. ($n=6-8$).

complete exsanguination (severing the *aorta abdominalis*), the excised wet lungs were weighed and then lavaged via a tracheal cannula with two volumes of 5 ml of physiological saline (at 37 °C), which was left in the lungs for 30 s, withdrawn, re-instilled for an additional 30 s. This procedure was repeated once. Then the pooled lavage fluid was centrifuged at $200 \times g$ for 10 min at 4 °C (GPKR-refrigerated centrifuge, Beckmann, Munich, Germany). The cell pellet was re-suspended in PBS-BSA (Dulbecco's phosphate buffered saline with Ca^{2+} and Mg^{2+} containing 0.1% bovine serum albumin; Sigma, Deisenhofen, Germany). The supernatant was analyzed for factors indicative of pulmonary capillary leakage, such total protein, LDH, phospholipids, and total cell counts. This included the determination of the concentration of phospholipids in BAL-cells (BALC). Cells (2×10^5 per cytospot) were centrifuged for 4 min onto slides using a cytocentrifuge (Shandon Cytospin 4, Life Science International, Frankfurt, Germany), air-dried on slides, fixed with a mixture of methanol:acetone (4:6), and stained according to Pappenheim. Cells were differentiated into macrophages, lymphocytes, granulocytes and eosinophils by light microscopy (300 cells were counted/cytospot).

2.6. Cytokine determination

Rat specific cytokines (interferon- γ (IFN- γ), IL-1 α , IL-4, TNF α , granulocyte-macrophage colony-stimulating factor (GM-CSF)) and the chemokine monocyte chemotactic protein-1 (MCP-1) were determined in BALF, BALC, and extracts of lung-associated lymph nodes of the hilus region (LALN). A multiplex fluorescent bead immunoassay (BMS725FF) of Bender MedSystems GmbH (Vienna, Austria) was used for analysis following the protocol provided by the manufacturer. Resuspended cells were adjusted to 3×10^6 cells/ml and analyzed using a flow cytometer (FACS-Canto, Becton-Dickinson (BD), Heidelberg, Germany).

2.7. Analysis of delayed-onset respiratory response

Selected subgroups of animals were monitored for delayed onset respiratory responses over a time period of approximately 20 h. The time delay between end of MDI-challenge and start of data collection was approximately 30 min. Data collection commenced shortly after placing the animals into the pre-calibrated barometric whole-body plethysmographs (Buxco Electronics, Troy, NY, USA; modified; software used for data-acquisition: BioSystem XA software Vers. 2.1.8., Buxco Electronics, Troy, NY, USA). Data analysis focused on 'enhanced pause' (Penh), tidal volume (TV) and respiratory rate. Data were collected every minute and digitally averaged over periods of 15 min. Details of this system have been published elsewhere (Pauluhn, 2004a).

2.8. Data analysis

Organ and body weights and BAL data were analyzed by one-way analysis of variance (ANOVA) followed by a

Tukey–Kramer post hoc test. Identical exposure groups of the ancillary study consisting of pretreated and normal rats were analyzed by a Pairwise Multiple Comparison procedure (Holm–Sidak method) using SigmaStat 3.1 for Windows (Systat Software, Inc., Point Richmond, CA). For all tests the criterion for statistical significance was set at $P < 0.05$. The area under the curve was calculated for the respiratory parameter Penh over the entire data collection period using a FORTRAN source code.

3. Results

3.1. Acute inhalation study

As illustrated in Fig. 2 lung weights, LDH, protein and phospholipids were significantly increased at both 15.2 and 50.6 mg MDI/m³. The most salient effect was related to changes in BALF protein. In these MDI exposure groups, capsaicin pretreated rats elaborated greater effects when compared to non-pretreated animals. At 15.2 mg/m³ this difference gained statistical significance. Cellular endpoints in the BAL of MDI exposed rats were also significantly different from the control, however, the changes did not show any appreciable concentration-dependence or impact of capsaicin pretreatment at this early time point (Fig. 3).

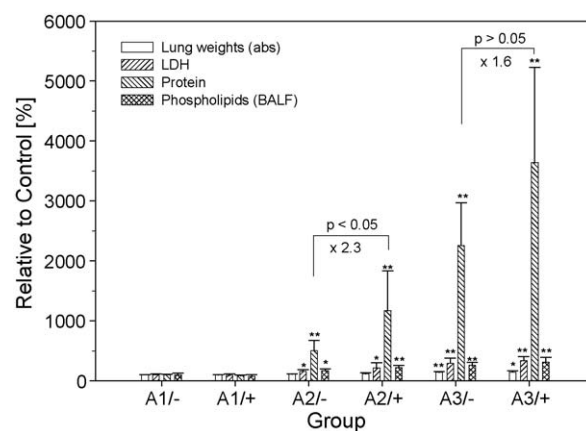


Fig. 2. Wet lung weights, lactate dehydrogenase (LDH) activity, total protein, and phospholipids concentrations in the bronchoalveolar lavage fluid (BALF) of Wistar rats from the ancillary study (see Table 1) exposed for single 6 h to dry air (A1), 15.2 (A2) or 50.6 mg MDI/m³ (A3). Suffixes '–' and '+' indicate subgroups not pre-treated and pre-treated with capsaicin, respectively. Rats were sacrificed on post-exposure day 1. Data were normalized relative to the mean of the control group (A1/–) and represent group means $\pm$ S.D. ($n = 6$). Asterisks denote significant differences to the control (A1/–) (* $P < 0.05$, ** $P < 0.01$) on log-transformed data. Subgroups without (A3/–) and with (A3/+) capsaicin pre-treatment were not found to be statistically significant different.

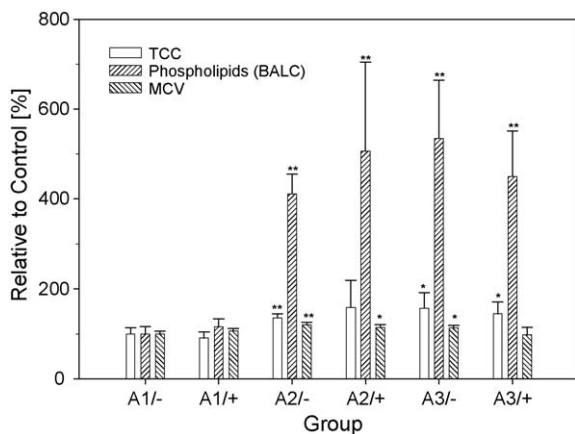


Fig. 3. Total cell counts (TCC), phospholipids concentrations in bronchoalveolar lavage cells (BALC), and mean corpuscular volumes of lavaged cells (MCV) in the bronchoalveolar lavage fluid (BALF) of Wistar rats from the ancillary study (see Table 1) exposed for single 6 h to dry air (A1), 15.2 (A2) or 50.6 mg MDI/m³ (A3). Suffices ‘-’ and ‘+’ indicate subgroups not pre-treated and pre-treated with capsaicin, respectively. Rats were sacrificed on postexposure day 1. Data were normalized relative to the mean of the control group (A1/-) and represent group means $\pm$ S.D. ($n = 6$). Asterisks denote significant differences to the control (A1/-) (* $P < 0.05$, ** $P < 0.01$) on log-transformed data. Subgroups without (A2/- and A3/-) and with (A2/+ and A3/+) capsaicin pre-treatment were not found to be statistically significant different.

3.2. Sensitization study

From the three breathing parameters examined, Penh was clearly superior to respiratory rate and tidal volume (Figs. 4 and 5) for identifying differences across groups, as already observed in earlier studies (Pauluhn, 2005). Measurements of Penh made 1 day before each MDI challenge did not show any consistent changes in breathing patterns (Fig. 5), whilst distinct, delayed-onset respiratory changes occurred about 1–5 h after MDI-challenge in MDI-sensitized BN rats (Fig. 5). The exacerbation of respiratory changes correlated clearly with the time-related increase in challenge exposures. The most pronounced changes, including the highest incidence, were observed after the fourth challenge (Fig. 5). Identically challenged control rats did not elaborate any delayed-onset effects. Some rats gave high Penh values just after placement into the barometric plethysmographs, especially after the first challenge (Fig. 5). As this type of response waned during the course of study, this finding appears to be causally related to handling and habituation rather than specific substance-related effects.

When comparing the area under the curve (AUC_{20h}) of Penh calculated from the entire data collection period of approximately 20 h (Fig. 6) with the time-related changes in Penh (Fig. 5) it is apparent that respiratory

changes start with shallow, more subtle changes in Penh that become more vigorous upon repeated challenge exposures. With regard to the pre-challenge AUCs all groups were indistinguishable, whilst the post-challenge AUCs of MDI-sensitized rats responded to the MDI challenge appreciably different than naïve but MDI-challenged control rats. Capsaicin pretreatment did not affect the incidence and intensity of delayed-onset respiratory responses in MDI-sensitized rats.

In MDI-sensitized rats the increase in lung weights, BAL-protein, -LDH, -neutrophils, -lymphocytes, and -eosinophils gained significance when compared to naïve control (Figs. 7–9). Again, there were no differences between the capsaicin pretreated and non-pretreated rats.

With regard to immunological endpoints in bronchoalveolar lavage cells (BALC) and to some extent also in LALN remarkable differences within the MDI-sensitized groups of rats were found. In each compartment, the animal pretreated with capsaicin showed markedly stronger responses as compared to the non-pretreated group (Fig. 10). Thus, capsaicin pretreatment augmented the production of cytokines and MCP-1 in BALC. The pattern of changes in BALF (data not shown) was similar to that observed in BALC, however, the magnitude of changes was markedly less pronounced in BALF. The Th2 cell cytokine IL-4 was more pronounced in the lung draining lymph nodes (LALN) in MDI-sensitized rats when pretreated with capsaicin (Fig. 10). In addition, the CC-chemokine MCP-1, which is a chemoattractant protein mainly expressed by macrophages, was also produced to a higher extent.

4. Discussion

MDI-aerosol is known to cause pulmonary irritation and at high $C \times t$ products this reactive chemical may cause cytotoxicity and lung edema by direct interactions with cell surfaces. The interrelationship between transvascular fluid and protein fluxes may also be affected by interaction with surfactant components which then may cause an imbalance of the intricate relationship between hydrostatic and colloid osmotic pressures, the “Starling forces”, acting across the blood–air barrier (Parker and Townsley, 2004). Another plausible mechanism by which MDI in low exposure intensities may exert its effect on the lung is by interaction with sensory nerves innervating the airways. Stimulation of sensory afferent nerves causes the release of small bioactive peptides (tachykinins) that produce tachypnea and increased vascular permeability that may also mediate the increase of solutes and protein in bronchoalveolar

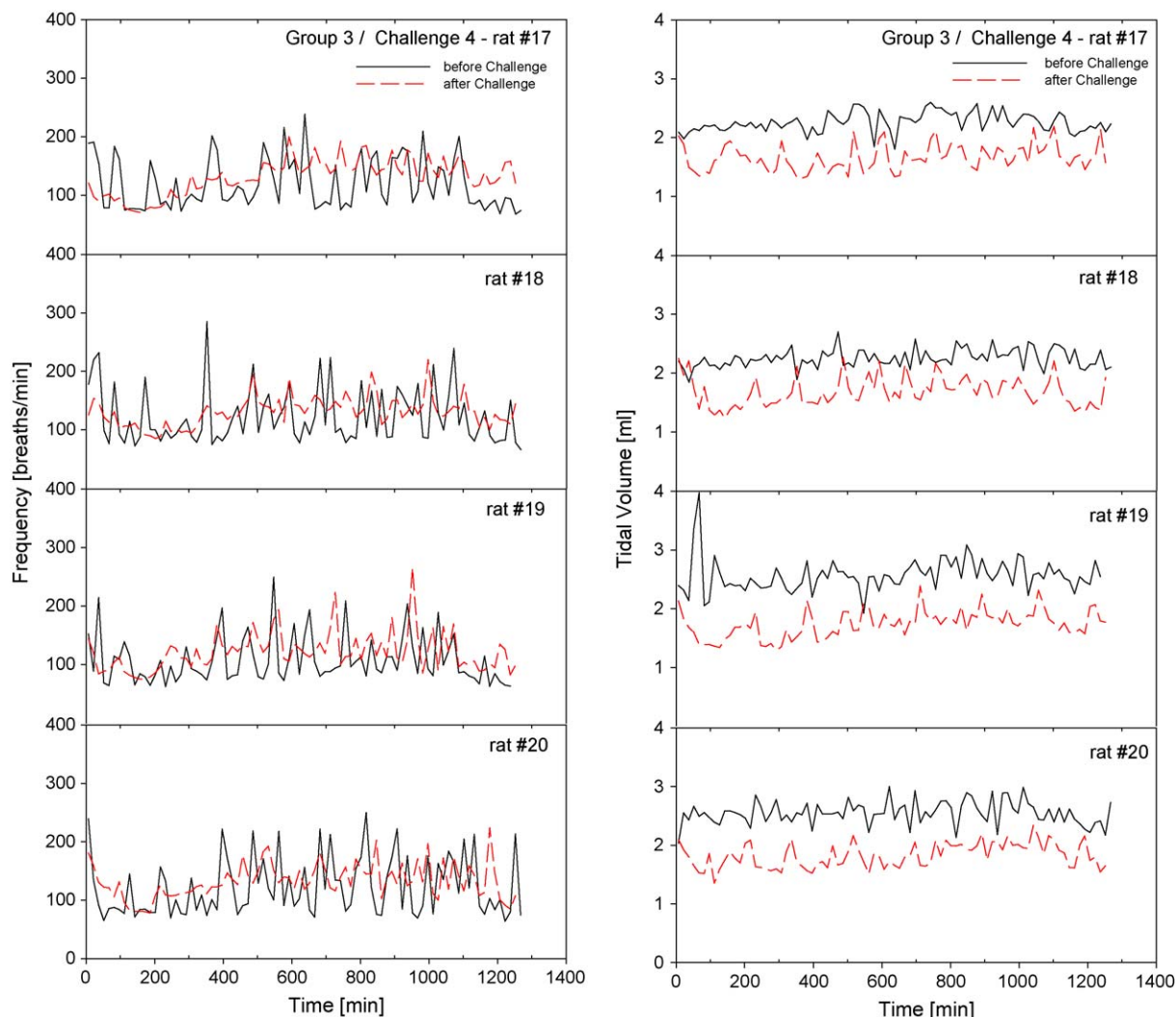


Fig. 4. Comparison of measurement of respiratory rate (left panel) and tidal volume (right panel) in individual Brown Norway rats pretreated with capsaicin, induced to and repeatedly challenged with MDI. Measurements took place before (solid line)/after (dashed line) the fourth challenge using data collection periods of approximately 20 h (for Penh data see Fig. 5). Respiratory patterns were monitored in four rats simultaneously using barometric plethysmographs.

lavage fluid. Several authors demonstrated the protective role for C-fibers in the lower respiratory tract airway inflammation and injury induced by ozone or phosgene (Bauer et al., 1997; Graham et al., 2001; Sterner-Kock et al., 1996; Takebayashi et al., 1998; Tepper et al., 1993). To investigate the role of tachykinins in MDI-induced acute lung injury, capsaicin was used to deplete lung nerve terminals of capsaicin-sensitive C-fibers from these types of neuropeptides.

As shown in previous acute inhalation studies with MDI-aerosol, a hallmark of acute exposure in rats is the accumulation of total protein in the BAL fluid (Pauluhn, 2000, 2004a,b). At low exposure intensities (252 mg/m³ min; single 6 h) BAL protein declined to the

level of the control up to the first postexposure day, whilst for higher exposure intensities its climax was on the first postexposure day. Lavagable total protein commonly is considered to be a sensitive and early indicator of the disruption of the epithelial permeability barrier and it is detectable at early stages of injury even before cellular breakdown occurs. However, stimulatory effects on sensory C-fibers in airways may produce a thickened protective mucoid lining layer, which is comprised of proteins and components capable to react chemically with MDI. As a result the amount of MDI reaching the underlying tissue is decreased, i.e., small changes in lavagable total protein may indicate 'defense and protection' and not necessarily 'adversity'. The relationship shown in Fig. 2

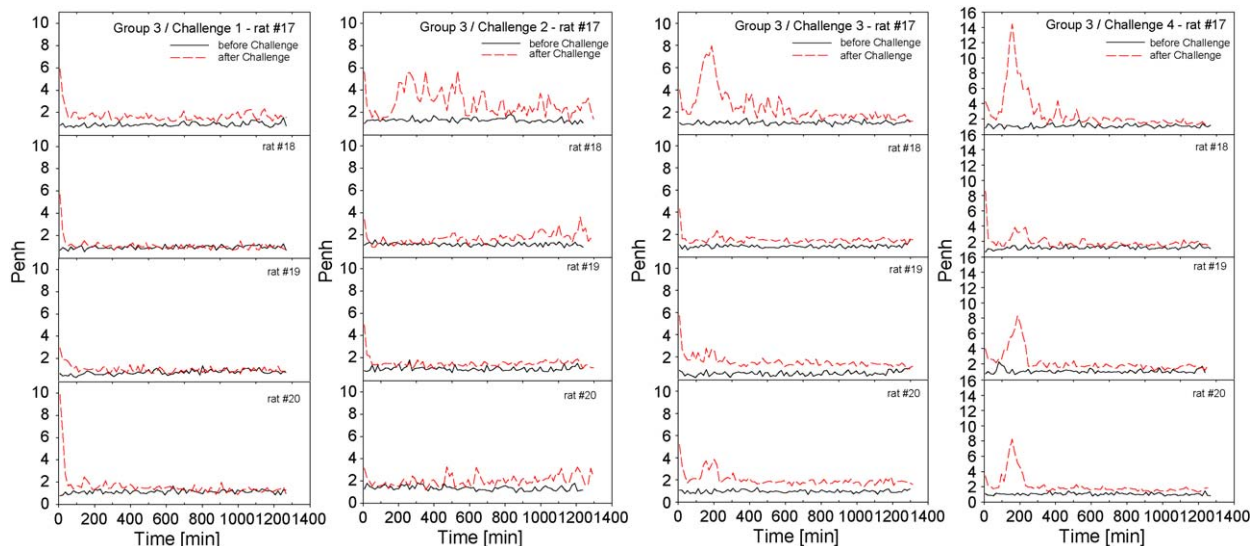


Fig. 5. Change of Penh in individual Brown Norway rats pretreated with capsaicin, induced to and challenged four times with MDI (for more details see legend of Fig. 4). Measurements took place using data collection periods of approximately 20 h. Respiratory patterns were monitored in four rats simultaneously using barometric plethysmographs.

demonstrates that total lavagable protein was a more sensitive indicator for MDI-related pulmonary irritation than all other markers determined. However, at minimally irritant concentrations of MDI aerosol, BAL-LDH

appears to precede that of BAL-protein (Fig. 1). The higher molecular weight of LDH, relative to albumin, would support a hypothesis that discharge from glandular structures of the airways is more likely than increased transudation via a disrupted air–blood barrier. In a previous study it was shown (Pauluhn et al., 1999) that MDI-aerosol elicited pulmonary chemoreflexes, characterized by apnea and decreased tidal volume, which is typical for C-fiber stimulation (Lee and Widdicombe, 2001). This may further indicate that the stimulation of capillary C-fiber receptors (J receptors) with subsequent liberation of tachykinins (Widdicombe, 1998) may account for the changes observed. Despite these unclear etiopathologies, bronchopulmonary C-fibers are known to be primarily responsible for eliciting the airway reflexes which protect the lung against inhaled irritants (Graham et al., 2001; Lee and Widdicombe, 2001).

The results from acutely exposed naïve Wistar rats indicate that the acute response to MDI aerosol was augmented by pretreatment with capsaicin. The data shown in Fig. 2 support the hypothesis that partial C-fiber ablation intensifies MDI-induced changes in BAL. The magnitude of differences between similarly exposed groups of animals pretreated or non-pretreated with capsaicin was not at variance with data obtained with ozone (Sterner-Kock et al., 1996; Takebayashi et al., 1998; Tepper et al., 1993). It could be speculated that the residual sensory nerve activity from non-capsaicin sensitive C-fibers may have contributed to this unexpected result. The single high dose of capsaicin used in this study

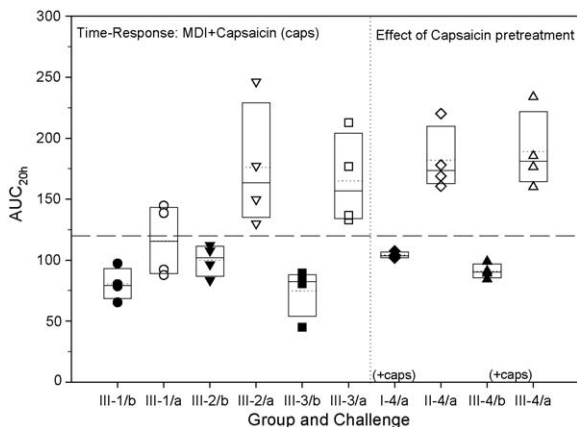


Fig. 6. Area under the curve (AUC) of Brown Norway rats based on changes of Penh (enhanced pause) before ('/b') and after ('/a') challenge. Rats were pretreated with capsaicin, induced to and challenged four times ('–1' to '–4') with MDI as follows (left panel): III: rats of pretreated with capsaicin, induced to and challenged with MDI. Right panel: rats of group I were pretreated with capsaicin, not typically induced to MDI and challenged four times with MDI ('–1' to '–4'). Rats of groups II were not pretreated with capsaicin but induced and challenged as the rats of group III. Rats of group III were pretreated with capsaicin, induced to and challenged four times with MDI. AUCs above the dashed line are considered positive. Data represent the area under the curve of individual rats'. Boxes represent Tukey box plots (dotted line: mean, solid line: median).

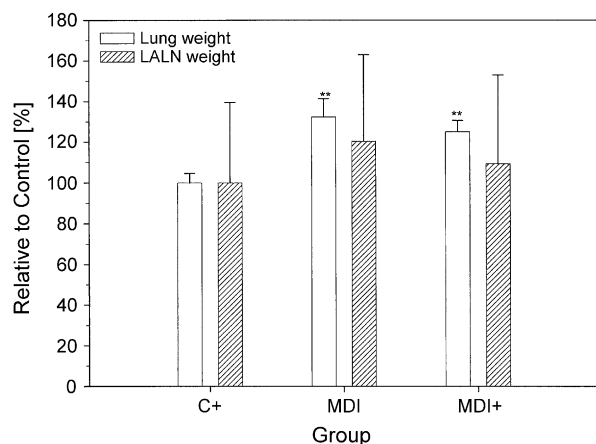


Fig. 7. Weights of the wet lung und lung-associated lymph nodes (LALN) from naïve control Brown Norway rats and rats sensitized by topical administration of MDI (days 0 and 7). The rats of the control (C+) group and 50% of the MDI-induction group were either pre-treated with capsaicin (MDI+) or normal rats (MDI). All animals were challenged as shown in Table 1. Data were normalized relative to the mean of the control group (C+) and bars represent group means $\pm$ S.D. ($n=8$). Asterisks denote significant differences from the control (C+) (* $P<0.05$, ** $P<0.01$) on log-transformed data.

had been shown in previous studies to damage sensory neurons with resulting long-term loss of sensory nerve function (Lundblad, 1984; Lundblad et al., 1985; Morris et al., 1999; Stjarne et al., 1989; Stanek et al., 2001; Sterner-Kock et al., 1996; Bauer et al., 1997). Thus, the defunctionalization of sensory nerves might have reduced the acute irritation-induced protective responses commonly associated with the stimulation of C-fiber

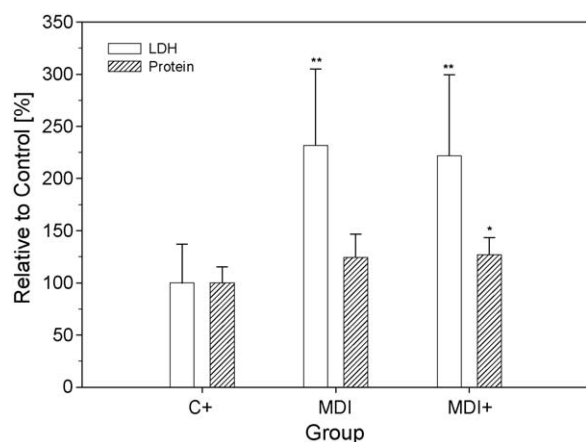


Fig. 8. Protein and LDH bronchoalveolar lavage of rats challenged with MDI (for details see Fig. 7). Data were normalized relative to the mean of the control group (C+) and bars represent group means $\pm$ S.D. ($n=8$). Asterisks denote significant differences from the control (C+) (* $P<0.05$, ** $P<0.01$) on log-transformed data.

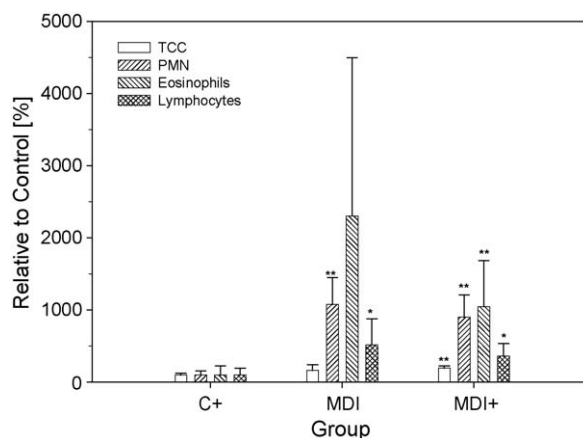


Fig. 9. Cyto differentiation of cells in the bronchoalveolar lavage (for details see Fig. 7). Data were normalized relative to the mean of the control group (C+) and bars represent group means $\pm$ S.D. ($n=8$). Asterisks denote significant differences from the control (C+) (* $P<0.05$, ** $P<0.01$).

receptors, e.g., by diminished discharge of mucins and proteins into the airways which may interact with electrophilic chemicals, such as MDI.

In previous sensitization studies with MDI in BN rats stereotypical changes in breathing patterns delayed in onset were observed in rats sensitized topically to and challenged repeatedly by inhalation with MDI-aerosol. Opposite to similar physiological responses described to occur in BN rats sensitized with TMA (Zhang et al., 2004), the delayed respiratory responses observed in MDI-sensitized rats were transient and occurred only after repeated inhalation challenges with MDI-aerosol. Although this pattern of response appears to be supportive of an allergic pathogenesis, sensory C-fibers could also have contributed to this response. Therefore, in order to elucidate the role of tachykinins for this response to occur in MDI-sensitized and challenged BN rats, MDI-sensitized and capsaicin-pretreated rats were compared with non-pretreated rats. This included an analysis of the cytokines and chemokines considered to be produced locally in the airways and involved in asthma (Kips, 2001; Bernstein et al., 2002).

In previous repeated challenge studies in BN rats using MDI, it was shown that respiratory allergy was characterized by delayed onset responses, inflammatory changes in BAL, and without appreciable elevations in serum IgE (Pauluhn et al., 2005; Pauluhn et al., 2005). It was shown that challenge concentrations have to be high enough to be effective for the elicitation of an allergic phenotype. Based on the metrics used in Fig. 1, the $C \times t$ -product of approximately 450 mg MDI/m³ min was apparently too low to elicit conclusive changes

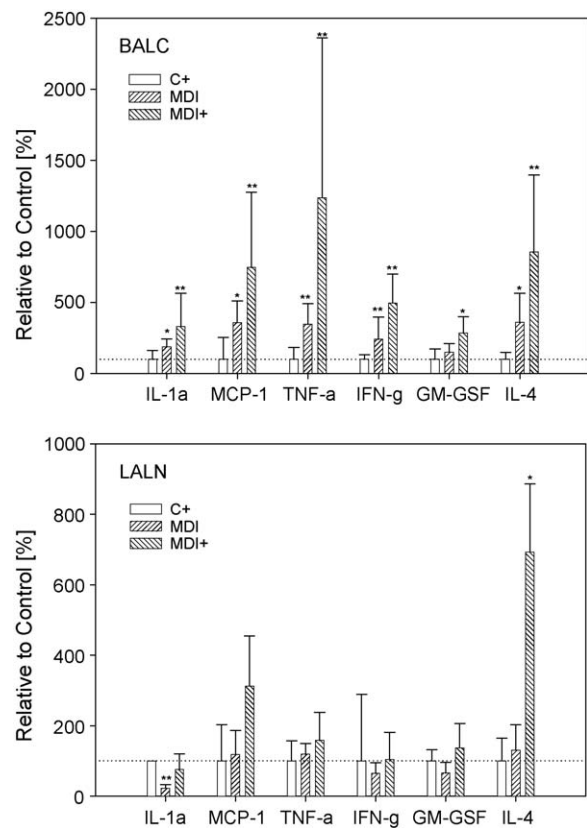


Fig. 10. Pro-inflammatory cytokine concentrations in bronchoalveolar lavage cells (BALC) and lung-associated-lymph nodes (LALN) of naïve and topically sensitized BN rats pretreated with capsaicin (suffix '+') repeatedly challenged with MDI-aerosol. Rats were examined 1 day after the fourth challenge. Data were normalized relative to the mean of the control group (dotted line, C+) and represent group means $\pm$ S.D. ($n=6$; IL-1- α and IL-4 were in below the limit of quantification in two thirds of the rats of the control). Asterisks denote significant differences from the control (C+) (* $P<0.05$, ** $P<0.01$) on log-transformed data.

in BAL, whilst the slightly irritant $C \times t$ product of approximately $1200 \text{ mg/m}^3 \text{ min}$ (Pauluhn, 2005) produced changes indicative of an allergic airway response. Indeed, this exposure intensity is much higher than experienced in episodes of human exposure to high concentrations of MDI. However, the major focus of this bioassay is to investigate systematically at least some traits of human asthma in a robust bioassay rather than attempting to simulate potential human exposure conditions.

Amongst the most prominent and consistent endpoints in BAL fluid was the increase in neutrophilic granulocytes (PMN). Neutrophils are known to be involved also in human asthma (Maghni et al., 2004; Jatakanon et al., 1999; Lemi re et al., 2002; Sun and Hon, 2004) and they may also play an important role in airway

remodeling via the production of proteolytic activities as surmised by Yong-chang and Chu (2004). In humans exposed to toluene diisocyanate, neutrophils and related mediators also appear to be involved in the development of physiological responses delayed in onset (Fabbri et al., 1987).

The cardinal feature of atopic disease is exuberant CD4 Th2-cell activation. This is evidenced by the predominance of Th2 cytokines, such as IL-4, measured in BALC and LALNs. Some remnant activity of the Th1 cytokine IFN- γ was still detectable in BALC but not in LALNs. Collectively, the data shown in Fig. 10 demonstrate that the site of tissue sampling is critical for the outcome of test. The apparent up-regulation of neuro-immunological pathways in capsaicin pretreated rats matches the higher inflammatory response observed following acute inhalation exposure (Fig. 2). Although difficult to understand mechanistically, the partial ablation of protective C-fibers appears to have caused marked immunostimulatory effects towards a Th2-mediated response, however, this elevation in pro-inflammatory factors was not associated with increased acute inflammatory endpoints in BAL-fluid or delayed-onset physiological responses. Thus, the outcome of immunological parameters cannot be taken as predictive surrogate for the toxicological or functional outcome of test. This is not a surprise, because the functional outcome is generally not paralleled by immunological changes in occupational asthma or asthma models (Vanoirbeek et al., 2006).

The cytokine/chemokine fingerprinting clearly shows an inflammatory state in BAL-cells. In contrast to the cells of the lung draining lymph nodes there is no definite dissociation in Th1 or Th2 reaction. A dominant Th2 activation would have inhibited the TNF α and IL-1 expression via IL-4 production. It could argued that in the milieu represented by the BAL-fluid there is still activation of Th0 cells (IFN- γ + IL-4 expression) attracted from the blood into the inflamed tissue. However, from the data generated it can not be unequivocally deduced whether the cytokine/chemokine pattern observed reflects a snapshot of the shift from Th2-mediated to Th1-mediated chronic asthma or whether it is still be influenced by Th0 cell activity. Accordingly, aspects addressing the compartmentalization and time-course of changes require further research. In contrast to BALC, only Th2 cell activity was detected in the LALNs. This is, however, exclusively true only for MDI-sensitized rats receiving capsaicin post-induction and pre-challenge. In the absence of capsaicin pretreatment, the expression of cytokines and the monocyte chemoattractant protein-1 (MCP-1) did not exceed the

background levels measured in the naïve control rats pre-treated with capsaicin and challenged with MDI. Interesting to note, that in diisocyanate-related occupational asthma, MCP-1 was shown to have a better sensitivity and specificity than specific IgE and IgG (Bernstein et al., 2002; Malo et al., 2006).

In summary, results from the acute exposure study in naïve rats indicate that MDI-aerosol elicited lower respiratory tract irritation and ensuing increased protein extravasation and that partial ablation of C-fibers amplified the response. With regard to MDI-induced respiratory allergy, neither the delayed-onset respiratory response nor the inflammatory endpoints measured in BAL were appreciably different in normal and in C-fiber ablated rats. In contrast, the determination of selected pro-inflammatory and immunological signals revealed a conspicuous difference across these otherwise equally treated groups. This appears to support the hypothesis that in this animal model the elicitation of MDI-induced respiratory allergy is dependent on a number of factors to elicit an asthma-like phenotype. Although difficult to disentangle unequivocally, neuro-immunological factors may be important in triggering and maintaining this complex disease, however, the outcome of immunological parameters do not predict the functional outcome of test.

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