



Brown Norway rat asthma model of diphenylmethane-4,4'-diisocyanate (MDI): Impact of vehicle for topical induction

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Abstract

The development of this Brown Norway (BN) rat asthma model was focused on the duplication of at least some hallmarks of human diisocyanate asthma using the skin as the initial priming route of exposure. Equal total doses of polymeric diphenylmethane-diisocyanate (MDI) were applied to similar surface areas either dissolved in di-*n*-octyl sebacic acid ester (20%) (SEBA), in acetone:olive oil (20%) (AOO) or undiluted. The elicitation of respiratory allergy utilized four repeated nose-only inhalation challenges of 30 min with 39 mg/m³ MDI-aerosol approximately every 2 weeks. Emphasis was directed towards the analysis of respiratory responses delayed in onset. Endpoints suggestive of an allergic inflammatory response were examined by bronchoalveolar lavage (BAL) 1 day after the last inhalation challenge and comprised protein, LDH, cytodifferentiation of BAL cells, MCP-1, and some Th1 and Th2 cytokines. MCP-1 and cytokines were comparatively determined in three compartments: BAL fluid, BAL cells, and lung-associated lymph nodes (LALN). In all groups sensitized topically to MDI typical delayed-onset respiratory responses occurred. The lung and LALN weights, BAL-protein and -LDH were significantly increased as compared to the naïve control group challenged identically. There was compelling evidence of a neutrophilic rather than an eosinophilic inflammatory response. The patterns of interleukin (IL) IL-1 α , TNF- α , IFN- γ , GM-CSF, and IL-4 differed appreciably from one compartment to another and were essentially maximal in BAL cells. In contrast, MCP-1 was increased to the same extent in all compartments measured. Collectively, changes were slightly, although consistently more pronounced when using SEBA as vehicle when compared with the vehicle AOO or undiluted MDI. Notable was a discordance of cytokine profiles and respiratory responses. In conclusion, the priming potency of topically administered MDI and subsequent asthma-like responses following repeated inhalation exposures appear to be dependent on multiple factors, one of them appears to be associated with the type of matrix used to dissolve MDI. This animal model provides a versatile and robust experimental tool to evaluate and assess at least some features of MDI-related asthma.

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1. Introduction

When designing an *in vivo* model of respiratory allergy, it is important to clearly decide which aspects of this complex disease is the focus. In addition to expedience and practicability, the species and strain selection, the sensitization and challenge protocol as well as the endpoint(s) cho-

sen are paramount for the outcome of test. The relative abundance of biomarkers to probe responsiveness may change from one compartment to another and, for example, it may be different in serum, lung parenchyma and draining lymph nodes or bronchoalveolar lavage fluid (BALF) (Schuster et al., 2000; Pauluhn, 2006). However, how these tissue microenvironments shape the function of professional antigen-presenting cells remains elusive. In terms of endpoints, those that integrate independently a series of complex events might be most practical to probe for positive responses in animal models in order to detect

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and categorize lung-sensitizing agents. Once identified, dose (concentration $\times$ time)–response analyses may facilitate risk assessment. Suffice it to say, particularly for dose–response analyses, the inhalation route is considered to be superior to expedient ‘shortcut’ bolus dosing procedures as they may not adequately dose the bronchial airways, the site asthma prevails. Limitations of most of the currently applied bioassays are that they model the acute rather than the chronic manifestation of asthma. Apart from the difficulties in transposing rodent to human data, it has to be borne in mind that each animal model is a trait associated with asthma, rather than for modeling the entire asthma phenotype (Kips et al., 2003; Kumar and Foster, 2002; Redlich et al., 2002).

Allergic asthma is a pulmonary disease characterized by episodes of acute airway inflammation superimposed on a background of chronic inflammation and structural changes collectively referred to as airway wall remodeling and airway hyper-responsiveness to a variety of stimuli (Kumar, 2001; Maddox and Schwartz, 2002). It was sought to develop a Brown Norway (BN) rat model of chronic asthma in which topically sensitized animals were repeatedly challenged by inhalation to a carefully controlled, minimally irritant concentration $\times$ time relationship of MDI–aerosol, to minimize the likelihood of eliciting marked parenchymal inflammation. Currently, most rodent models are more correctly designated models of allergic bronchopulmonary inflammation rather than of a bronchial airway-specific inflammation. However, when using this repeated challenge protocol, BN rats exhibited many of the lesions that typify chronic human asthma (Pauluhn et al., 2005; Pauluhn, 2006). The focus of this animal model was to elicit an acute-on-chronic allergic inflammation of the airways and to consider the compartmentalization of pulmonary responses (Schuster et al., 2000).

The majority of current rodent models of asthma include a priming phase, when animals are sensitized by topical administration of the inciting agent. Sometimes this administration is together with a Th2-skewing adjuvant. The exact roles played by Th1 and Th2 cytokines during the initial phase of sensitization in the development of eosinophils-mediated and neutrophil-mediated asthma remain unclear (Fischer et al., 2007; Johnson et al., 2004). Although not typically considered to be a Th2 cytokine, TNF- α is prominent in asthmatic airways, and genotypes that correlate with increased TNF- α secretion are associated with an increased risk of asthma (Moffatt and Cookson, 1997), suggesting that TNF- α plays an important role in the development of asthma and allergic airways inflammation. Because of their unique profile of cytokine production, it was proposed to distinguish ‘inflammatory Th2’ cells (IL-4, IL-5, IL-13, IL-10) from ‘conventional Th2’ cells (IL-4, IL-5, IL-13, TNF- α) (Liu et al., 2007). However, it should be recognized that bronchial asthma is not the only isocyanate-induced disorder. Nonobstructive bronchitis, rhinitis, cutaneous hypersensitivity, and

hypersensitivity pneumonitis represent further diagnoses that may be associated with antibody responses (Baur, 2007).

Diisocyanates are widely used in industry and are known to be involved as causative agents for occupational asthma (Chan-Yeung and Malo, 1994). The pathogenesis associated with low-molecular-weight diisocyanates is not well understood and the current literature indicates that pathomechanisms involved in isocyanate-induced disorders are complex and heterogeneous. This includes the role of the dermal route of induction for priming and the inhalation route to elicit an asthma-like disease. More recently, concerns have been raised that skin exposure to isocyanates may prime exposed individuals to the subsequent development of asthma as a result of inhalation exposures (Bello et al., 2007). So far the sequence and frequency of exposure as well as the additional determining factors leading to disease remain poorly defined (Basketter et al., 2006; Boukhan and Maibach, 2001; Pauluhn, 2006). Among the issues contributing to controversy is the lack of knowledge whether the total dose, the concentration of the hapten (allergen), adjuvants (or irritation), absorption enhancers (vehicles) or skin injury, the exposed skin surface area, the frequency or repeated contact with the skin, and subsequent single or recurrent high-level inhalation excursions either alone or in combinations might be the critical determinant(s) leading to disease in susceptible individuals.

It has been recognized for many years that the vehicle matrix in which a chemical allergen (hapten) is encountered on the skin may have profound effects on the response elicited and the extent to which skin sensitization will be acquired (Kligman, 1966; Basketter et al., 2001). Although not all chemical allergens are affected similarly, for certain substances a greater than 10-fold vehicle dependent change in potency is observed (Basketter et al., 2001). In the guinea pig, it has been shown that the vehicle can substantially affect the rate of sensitization, although this could not be related to the apparent bioavailability (Andersen et al., 1985). However, the view that the vehicle matrix is likely to have an important impact on the sensitizing effect of a chemical is also based on the underlying knowledge of skin penetration.

Especially for potentially irritant chemicals, such as MDI, skin damage-associated factors may be released by stressed tissue or necrotic cells which then modulate the functional maturation of antigen presenting cells (Langerhans’ cells) (Lambrecht and van den Toorn, 2007). Thus, selection of the vehicle solvent for delivery of MDI to the skin and the dose metric (total dose or skin surface area concentration) are important determinants of the outcome and deserve better appreciation during study design and execution. Chemical reactivity of MDI with skin proteins, water, sweat components, and self polymerization reactions, as well as limited understanding of the kinetics of these processes on the skin and the role of solvent on skin penetration and bioavailability of MDI, further emphasize the need to pay closer attention to these factors. For these

reasons, quantitative assessment of the vehicle effect and dose metric are time-consuming and technically challenging.

The objective of this study was to study the modifying impact on MDI-induced respiratory allergy (asthma) of three modes of topical skin exposure while maintaining an equivalent total dose and surface area. For this purpose groups of rats were topically sensitized to MDI dissolved in the two different vehicles, namely di-*n*-octyl sebacic acid ester (20%) (SEBA) and acetone:olive oil (20%) (AOO). Similarly dosed rats using undiluted MDI served as reference. The modifying role of these priming procedures on pro-inflammatory cytokines/chemokines and the recruitment of inflammatory cells in various lung compartments in repeatedly MDI-aerosol challenged BN rats were evaluated. SEBA was considered to be a nonreactive, nonvolatile lipophilic vehicle, whilst AOO (4:1) has been suggested as the vehicle of choice for other topical sensitization bioassays (Basketter and Kimber, 1996). Neither vehicle has any relevance to occupational exposure but may alter the stability, duration of topical exposure and thus the potential for skin penetration and bioavailability of this reactive chemical. Accordingly, the modifying properties caused by different formulation matrices were the focus of study.

2. Methods

2.1. Test material and chemicals

Polymeric methylenediphenyl-4,4'-diisocyanate (MDI) was from Bayer Material Science AG, Leverkusen, Germany. The content of monomeric MDI isomers was 43.9% (39.3% 4,4'-MDI) with higher oligomeric MDI as balance. The free isocyanate (NCO) content was 31.23%. Di-*n*-octyl sebacic acid ester (>95%), acetone (Seccosolv, max. 0.01% H₂O) and Olive oil were from Tokyo Chemical Industry (TCI) CO., Merck, and Fluka, respectively.

2.2. Animals, diet, and housing conditions

Brown Norway (BN) rats of the strain BN/Crl BR were purchased from Charles River, Sulzfeld, Germany. Animals were placed in polycarbonate cages (1 rat per cage), containing bedding material (low-dust wood shavings), and were provided with a standard fixed-formula diet (NAFAG No. 9439 W10 pellets maintenance diet for rats and mice) and municipal 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 were approximately 250 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 protocol

This study consisted of one naïve control group and two groups of BN rats that were sensitized topically using a ≈20% (w/v) solution of polymeric MDI either in SEBA or AOO (4:1). The stability of MDI (at 10%) in SEBA and AOO was examined at 22 and 32 °C over 24 h. Monomeric MDI was analyzed using the nitro-reagent derivatization method

according to Dunlap et al. (1976). This method was based on the chromatographic separation and quantification of the 2,2'- and 2,4'- and the 4,4'-MDI-urea derivatives. They were determined by high performance reverse phase liquid chromatography (HPLC) with UV-detection (injection volume: 25 µl; column-length 75 mm, -diameter 4.6 mm, stationary phase: Luna C18, 3 µm (Phenomenex); oven temperature: 40 °C, UV-detection at 246 nm; sample preparation: the samples (≈2 g) were allowed to react with nitro-reagent (*N*-(*n*-propyl)-4-nitrobenzylamine hydrochloride; CAS No.: 68133-98-2 from Fluka #00655; purum >99.0 % or Alfa Aesar #A19308; 99.0%) to form the corresponding urea derivative. These solutions were diluted with acetonitrile and 0.1% aqueous phosphoric acid (9:1); retention times: 9.0–9.2 min for the 2,2'-MDI-urea derivative, 8.1–8.4 min for the 2,4'-MDI-urea derivative, and 8.5–8.8 min for the 4,4'-MDI-urea derivative).

MDI was homogeneously dissolved in SEBA and AOO and there was no evidence of any decay of the more reactive monomeric species contained in the polymeric MDI in either vehicle system over this period.

This mixture was directly applied to the shaved flanks of the intact skin on days 0 and 7 (dosing was on contralateral flanks). Each group consisted of eight male rats allocated to three groups by randomization. On each day the rats were dosed with 200 µl solution. The dosed area was ≈10 cm². This topical dose of MDI (undiluted) induced respiratory allergy following repeated inhalation challenges to ≈40 mg/m³ in previous studies (Pauluhn, 2005; Pauluhn and Vohr, 2006). Different surface area doses of neat MDI were examined using the same protocol as described in this paper. However, a dependence on surface area was not evident (unpublished results). The fourth group was from a recent study where rats were dosed similarly on days 0 and 7 with undiluted polymeric MDI (Pauluhn and Vohr, 2006). Apart from the modified dosing regimen all other experimental variables were identical with the protocol used in the current study. The undiluted MDI was administered by using aluminum foil spots (two spots of 2 cm in diameter, each spot dosed with 20 µl of MDI). Thus, independent on the regimen used, in all groups the resultant cumulative dose was approximately 90 mg MDI/rat. The skin was shaved one day prior to dosing using a clipper (injury of skin did not occur). No skin wash was applied after dosing. Rats were prevented from grooming or scratching by wearing an Elizabethan collar for approximately 24 h after topical administration (Buster Birdcollars; Kruuse, DK, Cat. No.: 273375). This prevents also the nasal contract and/or uptake of liquid MDI from the skin.

The comparison of current data with past reference data of neat MDI may question the robustness of this bioassay and whether the differences of undiluted MDI and formulated MDI (SEBA or AOO) are incidental or not. This conflict has been resolved by the comparison of the reference data (neat MDI) with five independent control groups: CC (current control group), C+ naïve controls challenged 4-times with 38–40 mg MDI/m³ using exactly the same protocol as used in this study. The C-naïve controls not challenged with MDI but lavaged at the same time point after study initiation as described for this study. Cytokine/chemokine determinations were performed in two MDI-challenged control groups only. The key data potentially indicative of irritant-related lung injury were from studies performed during the past 2 years. The challenge concentration of ≈40 mg MDI/m³, a concentration × time relationship of ≈1200 mg/m³ min, is considered to be minimally irritant as detailed elsewhere (Pauluhn, 2002; Pauluhn and Vohr, 2006). This is substantiated further by the data presented in Fig. 1. Accordingly, this challenge dose was too small to affect relative lung weights or total cell counts in bronchoalveolar lavage (BAL) to any appreciable extent but was sufficient to mildly increase LDH, protein, and neutrophilic granulocytes in BAL fluid. The comparison of the pro-inflammatory factors in BAL cells revealed some variability across controls, especially for IL-4. The findings presented in Fig. 1 appear to reflect also some animal batch-to-batch variability. However, for most of the endpoints indicative of an allergic inflammation, neat MDI was unequivocally different to the current as well as past control groups.

The rats from all groups were repeatedly challenged with MDI-aerosol on days 21, 35, 50, and 64 for 30-min, except the last challenge, where each group was challenged on subsequent days (62–64)

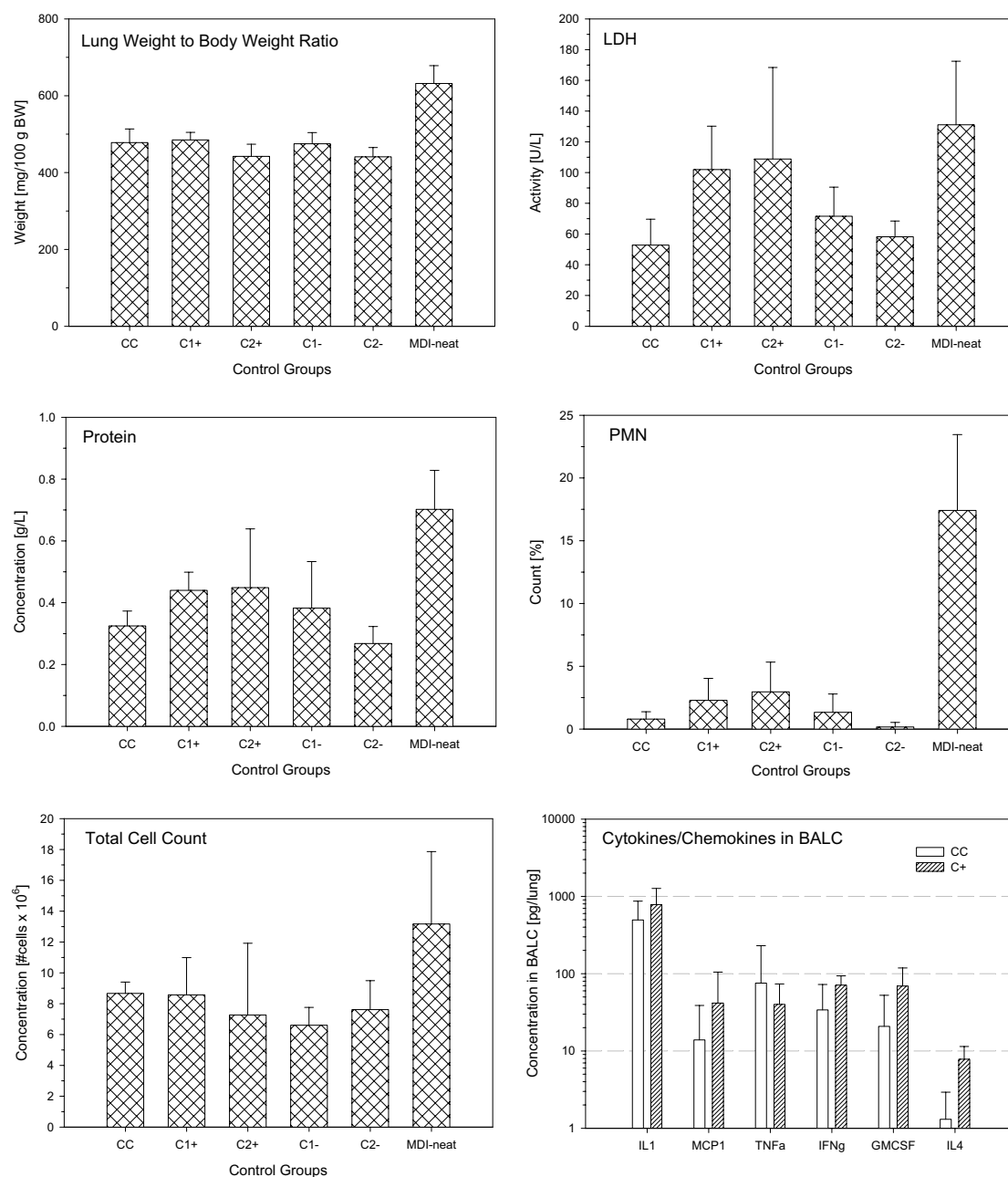


Fig. 1. Comparison of selected current control data (CC) with the respective data from independent studies in BN rats from 2005 to 2007. Rats were either challenged 4-times with ≈ 39 mg MDI/m³ (C1+, C2+) or not challenged (C1–, C2–). Protein, LDH, PMN, and total cell counts represent absolute data from bronchoalveolar lavage analyses. Control data of the pro-inflammatory cytokines/chemokines IL-1, MCP-1, TNF- α , IFN- γ , GM-CSF, and IL-4 in bronchoalveolar lavage cells (BALC) were from two recent studies only (C+, four MDI challenges). MDI-neat served as reference in this comparative study. All data were from studies utilizing essentially the same protocol as described in this study. Bars represent means $\pm$ SD ($n = 6-8$).

rather than simultaneously. For all challenges, the concentration was 39 ± 1.8 mg MDI/m³ (mean $\pm$ SD). The mass median aerodynamic diameter (MMAD) was 1.4–1.7 μ m, the geometric standard deviation (GSD) was 1.6–1.9. Four out of the eight rats were monitored in barometric plethysmographs for approximately 20 h after the last MDI inhalation challenge. One day after the last challenge, the rats were sacrificed, the weights of exsanguinated lungs and lung-associated lymph nodes were determined and the excised lungs were lavaged. Details addressing the exposure technology and methods, including the lavage procedures used were published in details previously (Pauluhn et al., 2002, 2005; Pauluhn, 2002, 2005; Pauluhn and Vohr, 2006).

2.4. Bronchoalveolar lavage

Details of the lavage technique have been published elsewhere (Pauluhn, 2002). Briefly, the excised wet lungs were lavaged via a tracheal cannula with two volumes of 5-ml of physiological saline (kept at 37 °C), which was left in the lungs for 30 s, withdrawn, re-instilled for an additional 30 s. This cycle was repeated once. The lavage fluid was then centrifuged at 200g for 10 min at 4 °C (GPKR-refrigerated centrifuge, Beckmann, Munich, Germany). In the supernatant, BALF was analyzed for factors indicative of pulmonary capillary leakage, such as total protein, and lactate dehydrogenase (LDH) for detection of cytotoxicity. Protein was determined according to Layne (1957), for the determination of

LDH commonly applied procedures were used. All rats were sacrificed using sodium pentobarbital (Narcoren®; 120 mg/kg body weight, intraperitoneal injection). Complete exsanguination was achieved by severing the *aorta abdominalis*. After exsanguination, the excised wet lungs of the animals were weighed and then lavaged. The number of cells in BAL was determined in triplicates on re-suspended cells (Schärfe-System, Casy 1, Reutlingen, Germany). The cells were re-suspended in Dulbecco's calcium and magnesium containing phosphate buffered saline (PBS) substituted with bovine serum albumin (BSA). For cytology, cytoposits were stained according to Pappenheim and differentiated into macrophages, lymphocytes, neutrophilic granulocytes (PMN), and eosinophils by light microscopy (300 cells were counted/cytoposits). Rat specific cytokines (interferon- γ (IFN- γ), interleukin (IL) IL-1 α , IL-4, TNF- α , granulocyte-macrophage colony-stimulating factor (GM-CSF)) and the chemokine monocyte chemoattractant protein-1 (MCP-1) were determined in BALF, BALC, and homogenates of lung-associated lymph nodes from the hilus region (LALN). Re-suspended cells were adjusted to 3×10^6 cells/ml and analyzed using a flow cytometer (FACS-Canto, Becton-Dickinson, Heidelberg, Germany). BALC-related parameters were adjusted according to the total cell count retrieved by BAL and expressed as 'concentration/lung'.

2.5. 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 Version 2.1.8., Buxco Electronics, Troy, NY, USA). Data analysis focused on 'enhanced pause' (Penh). Measurement of Penh by unrestrained plethysmography does not provide a direct assessment of a specific physiologic variable, and its limitations are well documented (Pauluhn, 2004). However, Penh can suitably be used to monitor empirically functional changes delayed in onset. Data were collected every minute and digitally averaged over periods of 15 min. Details of this system have been published elsewhere (Pauluhn, 2004; Pauluhn and Vohr, 2006).

2.6. Data analysis

The objective of study focused on the relative comparison of the various parameters determined (especially those determined in BAL). This could be achieved best by presenting the data relative to the concurrent control group. The absolute values of this control group are presented in Fig. 1. All statistical comparisons were based on absolute data. BAL data were log-transformed. Data were analyzed by one-way analysis of variance (ANOVA) followed by a multiple comparison Tukey–Kramer *post hoc* test. Experimental data were compared with the concurrent control group (CC, naïve rats challenged 4-times with MDI-aerosol) as well as with the MDI-reference group (rats sensitized to undiluted MDI and challenged 4-times with MDI-aerosol). For all tests the criterion for statistical significance was set at $P < 0.05$.

3. Results

Lung weights, BAL-protein, -LDH, -total cell counts, -neutrophilic granulocytes, and -lymphocytes were significantly increased in all groups sensitized to and challenged with MDI (comparison with naïve control; Figs. 2–4). There was a consistent tendency that effects were slightly more pronounced in the group using the SEBA as vehicle.

With regard to the comparative evaluation of selected cytokines and chemokines in bronchoalveolar lavage fluid

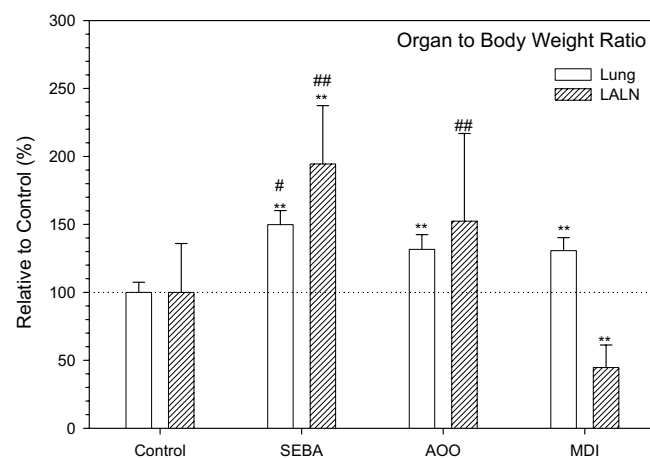


Fig. 2. Weights of the wet lung and lung-associated lymph nodes (LALN) from naïve control Brown Norway rats and rats sensitized by topical administration of MDI (days 0 and 7). Treatment groups received equal topical doses of MDI, either dissolved (20%) in the vehicle di-*n*-octyl sebacate (SEBA), 4:1 acetone:olive oil (AOO) or undiluted (MDI). The rats from all groups were challenged on days 21, 35, 50, and 64 to 39 mg/m³ MDI-aerosol for 30 min. Data were normalized relative to the mean of the control group (dotted line) and bars represent means $\pm$ SD ($n = 8$). Asterisks denote significant differences to the control group. Significant differences to the group using undiluted MDI are indicated by the hash sign (*, # $P < 0.05$, **, ## $P < 0.01$) on log-transformed data.

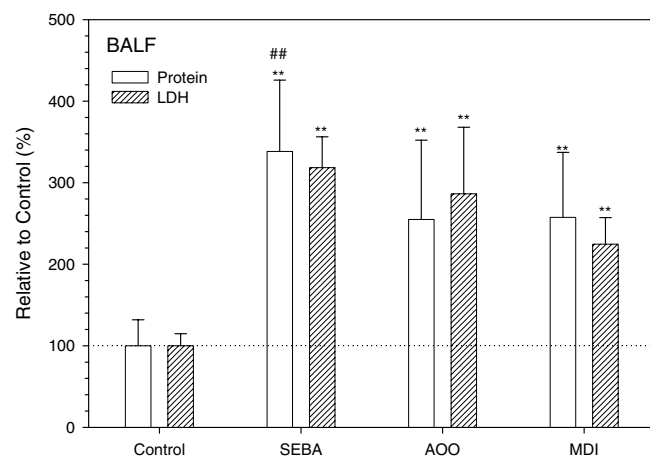


Fig. 3. Protein and LDH bronchoalveolar lavage fluid of rats challenged repeatedly with MDI (for details see Fig. 1). Data were normalized relative to the mean of the control group (dotted line) and bars represent means $\pm$ SD ($n = 8$). Asterisks and hashed-bars denote significant differences to the control and MDI group, respectively (*, # $P < 0.05$, **, ## $P < 0.01$) on log-transformed data.

(BALF), -cells (BALC), and LALNs remarkable differences amongst each compartment existed (Fig. 5). In the groups sensitized with MDI, dissolved in a vehicle, the magnitude of changes in BALC exceeded those in LALNs and especially BALF. With regard to undiluted MDI, the increase of IL-1, TNF- α and GM-CSF in LALNs were more pronounced than those in BALC or BALF. In spite the higher variability of these endpoints (Fig. 1), it was notable that most of the pro-inflammatory endpoints were maximal when using the SEBA as vehicle. The most

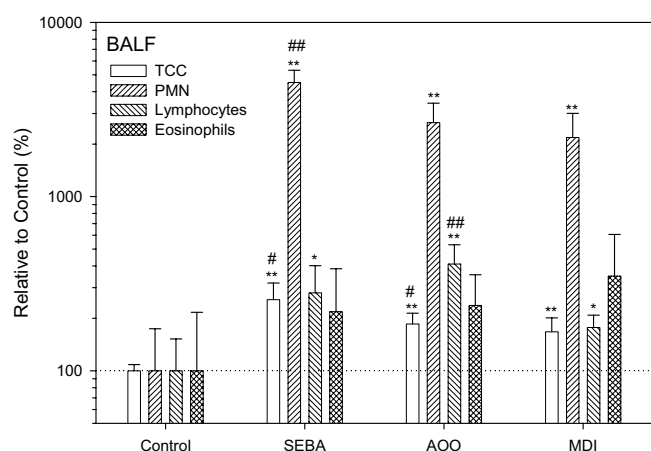


Fig. 4. Cytodifferentiation of cells in the bronchoalveolar lavage of rats challenged repeatedly with MDI. TCC: total cell count, PMN: neutrophilic granulocytes (for details see Fig. 1). Data were normalized relative to the mean of the control group (dotted line) and bars represent means $\pm$ SD ($n = 8$). Asterisks denote significant differences to the control group. Significant differences to the group using undiluted MDI are indicated by the hash sign (*, # $P < 0.05$, **, ## $P < 0.01$) on log-transformed data.

remarkable differences between the SEBA and AOO groups were characterized by IL-1 α .

Measurements of Penh made overnight after the last MDI-aerosol challenge revealed distinct delayed-onset respiratory changes which reached their maximum within 2–3 h after MDI-challenge in MDI-sensitized BN rats (Fig. 6). When comparing the area under the curve (AUC_{20 h}) of Penh calculated from the entire data collection period of approximately 20 h it is apparent that respiratory changes were significantly more pronounced in the groups sensitized to MDI (Fig. 7). Although limited by the small group size and variability across the different groups, distinct differences related to the contrarities of the sensitization regimen were not apparent.

4. Discussion

Previous sensitization studies with MDI in BN rats have demonstrated stereotypical changes in breathing patterns delayed in onset following topical induction to and repeated inhalation challenges with MDI (Pauluhn, 2005; Pauluhn et al., 2005; Pauluhn and Vohr, 2006). Furthermore, those studies demonstrated a progressive increase in the respiratory response with increasing number of inhalation challenge exposures to mildly irritant concentrations of MDI-aerosol. In contrast, repeated inhalation challenge exposures at nonirritant concentrations (≈ 15 mg MDI/m³) did not elicit specific responses (Pauluhn et al., 2005). This demonstrates one basic prerequisite of this animal model, *viz.* for each compound the respiratory tract irritation potency must be adequately characterized and defined in order to meet the targeted minimally irritant concentration $\times$ time product for challenge exposures. As demonstrated with the benchmark sensitizer trimellitic

anhydride (TMA), this animal model was suitable to establish a dose–response relationship (constant surface area dosed with different concentrations of TMA in AOO) based on variable induction-doses and inhalation challenge with an efficacious elicitation dose (Pauluhn, 2003).

For both TMA (Pauluhn, 2003) and MDI (Pauluhn, 2005; Pauluhn et al., 2005; Pauluhn and Vohr, 2006) it was demonstrated that this experimental model replicates several essential features of chronic asthma with high fidelity. Although it is reasonable to assume that the mechanisms of sensitization via the skin are similar in humans and animal models, an inherent problem of animal models is associated with the interpretation of potency. To a large extent, this uncertainty appears to be also related to both protocol-dependent and test condition-dependent experimental variables (Pauluhn, 2006). Commonly, bioassays use variable concentrations and constant surface areas for dose–response analyses. While the matrix (vehicle) system may affect sensitization dose–response relationships due to changes in penetration, stability, duration of effective skin contact and superimposed irritation, from such studies it is difficult to conclude unequivocally whether the total dose or the concentration per surface area is the leading metric. Evidence in humans suggests that the area over which a chemical is applied becomes a critical determinant of effectiveness of sensitization if it gets too small (Boukhan and Maibach, 2001; Rees et al., 1990).

Previous studies in BN rats with MDI have shown that repeated inhalation challenge exposures are required to elicit delayed-onset respiratory responses in topically sensitized BN rats (Pauluhn, 2005; Pauluhn et al., 2005). In other animal models (guinea pigs) the focus was on immediate-onset respiratory responses following a single inhalation challenge with MDI-aerosol. In contrast to the benchmark compound trimellitic anhydride, MDI did not elicit conclusive effects using a single-challenge regimen, with the exception of a mildly increased eosinophilic inflammation of airways (Pauluhn et al., 1999). This can be taken as indirect evidence that several consecutive inhalation exposures of sufficiently high $C \times t$ products are required to produce an acute-on-chronic inflammation of the airway wall commonly observed in human asthma (Cohn, 2001; Kips et al., 2003; Kumar and Foster, 2002).

In accordance with previous studies, the lung and LALN weights, BAL protein, LDH, and neutrophilic granulocytes in this study were significantly increased in topically sensitized and inhalation challenged animals (Pauluhn, 2005; Pauluhn et al., 2005). The cell counts of lymphocytes and eosinophilic granulocytes were greater in sensitized groups than in the naïve but challenged control (Fig. 4). It is of note that histology findings from previous studies were characterized by an increased peribronchial/perivascular influx of eosinophils rather than of neutrophils, despite the clear evidence of the neutrophilic type of allergic bronchopulmonary inflammation evidenced by the cytodifferentiation of BAL cells. Most of the currently published animal models using BAL to probe

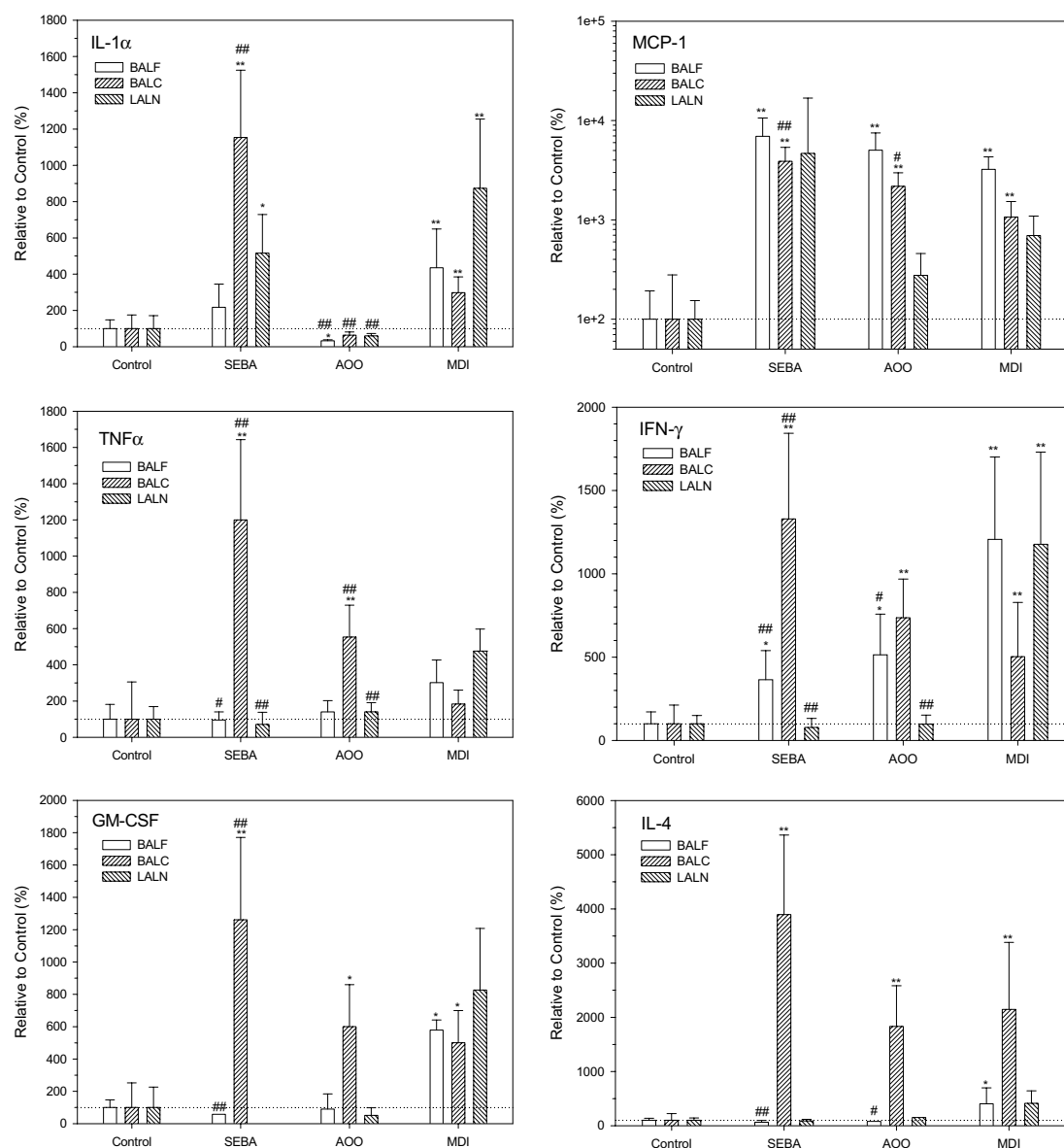


Fig. 5. Concentrations of the pro-inflammatory cytokines/chemokines IL-1, MCP-1, TNF- α , IFN- γ , GM-CSF, and IL-4 in bronchoalveolar lavage fluid (BALF), BAL-cells (BALC) and lung-associated-lymph nodes (LALN) of rats challenged repeatedly with MDI (for details see Fig. 1). Data were normalized relative to the mean of the control group (dotted line) and bars represent means $\pm$ SD ($n = 8$). Asterisks denote significant differences to the control group. Significant differences to the group using undiluted MDI are indicated by the hash sign (*, $P < 0.05$, **, $P < 0.01$) on log-transformed data.

for inflammatory changes appear to be associated with an allergic bronchopulmonary inflammation. Accordingly, as the BAL technique preferentially probes for changes at the alveolar level, the inflammatory lesions of the airways may have been overshadowed. Thus, the findings observed corroborate the notion that each diagnostic tool chosen for probing a specific response within the heterogeneous lung may preordain a particular outcome. Finally, also the inhalation challenge exposure *per se* may contribute to differences in the phenotypic manifestation of disease. Especially for MDI, the occupational exposure of humans is more likely attributed to the vapor of MDI rather than high concentration $\times$ time products of respirable aerosols

as used in experimental studies. By their nature, respirable experimental aerosols preferentially deposit at the pulmonary location of respiratory tract. Similar experimental shortcomings might be associated with alternative 'short-cut' dosing procedures, such as intratracheal and intranasal bolus instillations. Thus, it appears to be difficult to disentangle unequivocally whether the extensive neutrophilic inflammation observed in rodent bioassays is truly rodent-specific, mode of challenge-dependent or related to the method to analyze the disease.

The patterns of the cytokines IL-1 α , TNF- α , IFN- γ , GM-CSF, and IL-4 differed appreciably from one compartment to another, unlike the chemokine MCP-1 which

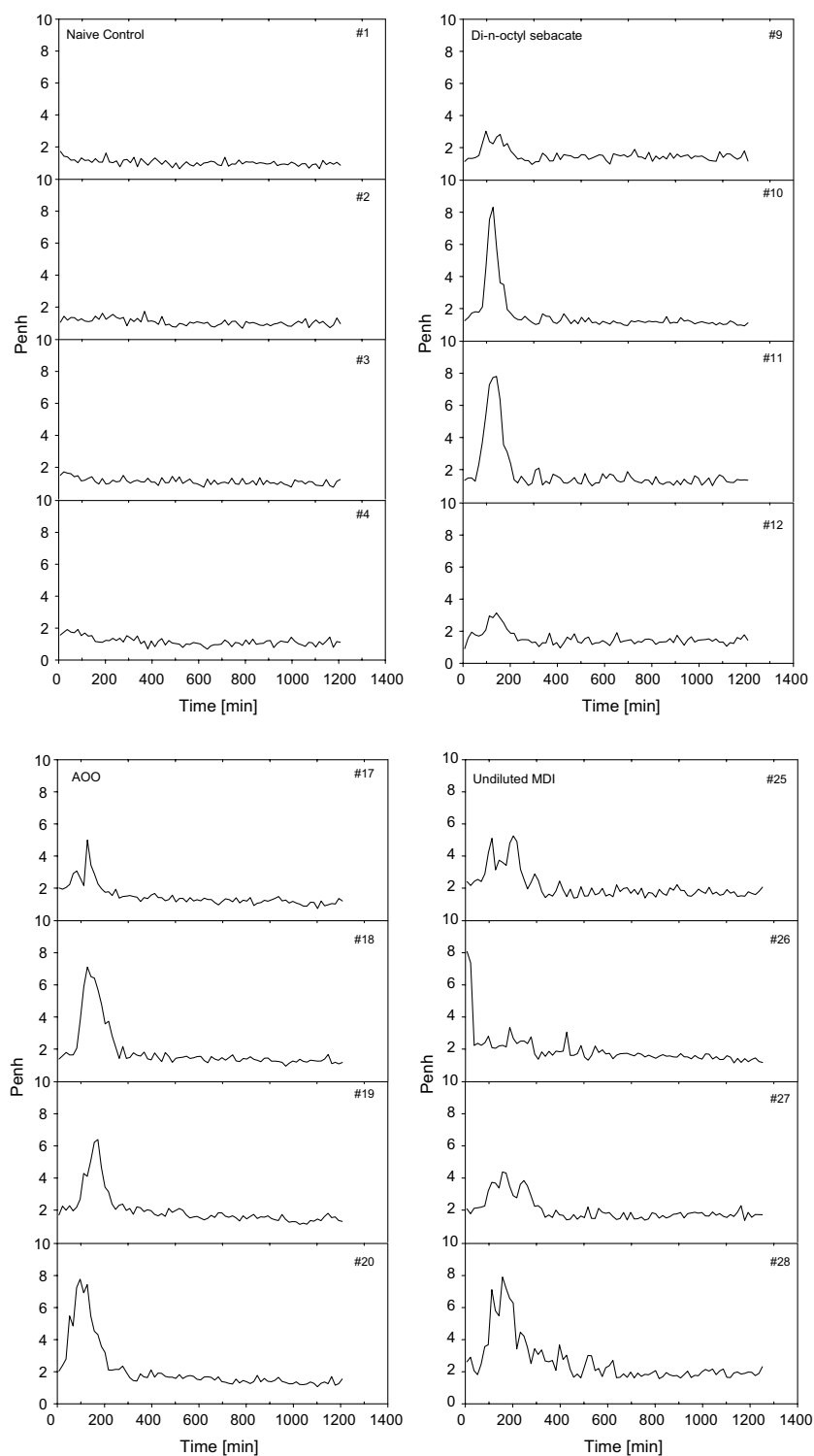


Fig. 6. Change of Penh in individual Brown Norway rats from naïve control Brown Norway rats and rats sensitized by topical administration of MDI (days 0 and 7). Groups sensitized with MDI received equal doses of MDI: dissolved (20%) in the vehicle di-*n*-octyl sebacate (SEBA) or 4:1 acetone:olive oil (AOO) and undiluted (MDI). The rats from all groups were challenged on days 21, 35, 50, and 64 to 39 mg/m³ MDI-aerosol for 30 min. Measurements commenced within 1/2 h after the fourth challenge (day ≈65) and was followed by a data collection period of approximately 20 h (overnight). Respiratory patterns were monitored in four rats simultaneously using barometric plethysmographs.

was equally elevated in all the compartments measured. In most instances, the changes in BAL cells were diagnostically superior to determinations in LALNs and particularly

BAL fluid. For all endpoints determined, the magnitude of changes was greater when using a 20% solution of MDI in the vehicle SEBA as compared to the vehicle AOO or undi-

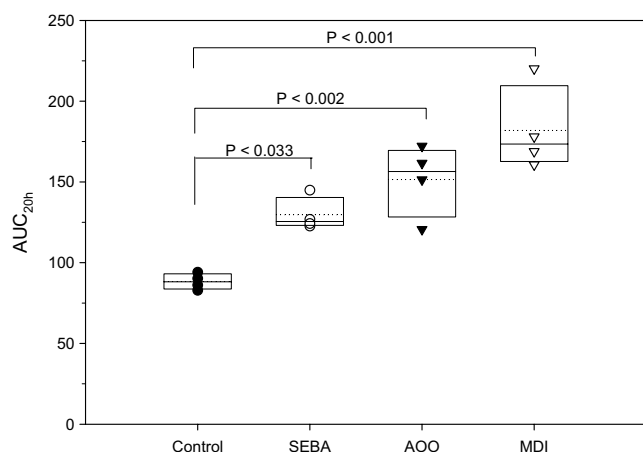


Fig. 7. Area under the curve (AUC) of Brown Norway rats based on changes of Penh after the fourth challenge exposure (for details see Fig. 1). Data represent the area under the curve of individual rats'. Boxes represent Tukey box plots (dotted line, mean; solid line, median). Statistical differences amongst groups were evaluated by a Kruskal–Wallis one-way analysis of variances on ranks and a pairwise multiple comparison Tukey post hoc test.

luted MDI. This difference was indicated especially by the pro-inflammatory endpoints examined (Fig. 5). These findings appear to suggest that formulation-specific factors at the site of initial skin contact may potentially modulate a subsequent allergic inflammation in the lung upon repeated inhalation exposures. It appears to be difficult to disentangle the relationship of pro-inflammatory and inflammatory endpoints and the discordance of these biomarkers with respiratory responses. However, these data support the notion that certain co-stimulatory factors may be associated with the mobile cell populations found in BAL and these may act to amplify the existing pathology at the site of inflammation. This may challenge existing paradigms focusing on draining lymph nodes or serum measurements only.

In human beings, as in rats, two distinct patterns of cytokine secretion have been defined among CD4⁺ helper T-cell clones. Human type 1 helper (Th1), but not type 2 helper (Th2), cells produce *inter alia* gamma-interferon (IFN- γ), whereas Th2, but not Th1, cells secrete IL-4, but not IFN- γ . IL-4 is not the original trigger of Th2 responses but rather the autocrine stabilizer and enhancer of Th2 development (Liu et al., 2007). It is well established that IL-4 is a major inducer of IgE and IgG₁ producing cells in mice (Fischer et al., 2007). Other cytokines, such as GM-CSF, or TNF- α , are produced by both Th1 and Th2 cells. In this context it appears to be important to distinguish conventional from inflammatory Th2 cells (see Section 1). Th0 cells, a third Th subset, show combined production of Th1- and Th2-type cytokines. The different cytokine patterns are associated with different functions. In general, Th2 cells provide an excellent helper function for B-cell antibody production, particularly of the IgE class. On the other hand, Th1 cells are responsible for

delayed type hypersensitivity reactions and are cytolytic for autologous antigen-presenting cells, including B cells. More recently, the important role of chemokines in the inflammatory network responsible for allergy and asthma has also been discovered. Chemokines can directly cause cellular activation and inflammatory mediator release by eosinophils and basophils, and promote the selective recruitment of different effector cell subsets, including Th2 cells, in the inflamed tissue (Romagnani, 2001). These polarized cytokine profiles have been used to categorize chemicals into 'skin' and 'respiratory' allergens (Dearman et al., 2003a,b). Although cytokines play a central and integral role in the initiation, propagation, and persistence of inflammatory and immunologic process in asthma (Chung and Barnes, 1999), due to their pleiotropic nature and compartmentalization, an unequivocal distinction with regard to irritation- and allergy-mediated inflammatory processes (in the bronchial airways) appears to be difficult. Thus, they do not necessarily portend respiratory allergy. Therefore, also in this study, it was not possible to demonstrate an unequivocal polarization of Th1 and Th2 cells. These thoughts are not at variance with those expressed by other authors (Plitnick et al., 2005; Selgrade et al., 2006).

Many currently used test protocols appear to be biased to produce discordant findings between profiles of pro-inflammatory factors collected from extrapulmonary tissues and respiratory responses either immediate and/or delayed in onset. In this animal model, respiratory responses may either occur immediate or delayed, transient or prolonged (Pauluhn, 2003, 2006; Zhang et al., 2004). Therefore, challenge-related time–response relationships need to be measured. An intranasal bolus instillation is often considered to be a feasible shortcut to inhalation challenges. This is doubted to be true for highly reactive, locally irritant chemicals, such as isocyanates. Depending on experimental variables, the nasal or pulmonary dose may vary substantially and the actual target structures, i.e., the bronchial airways, may be dosed in a quite unpredictable manner. These limitations make it difficult to extrapolate findings to occupational exposure patterns.

In summary, the results obtained in this topical-induction, repeated-challenge MDI BN rat bioassay support the conclusion that concentration- and matrix-related exposure variables appear to be important factors for quantitative dose–response analyses and risk assessment. The principle results obtained with MDI match those obtained with TMA as long as the different patterns and quality of responses (early vs. late respiratory response) are appreciated (Pauluhn, 2003, 2006; Zhang et al., 2004). The differences in outcome due to the vehicle-matrix used for topical induction were preferentially expressed at the pro-inflammatory level, suggesting that additional triggers (e.g., adjuvant effects through localized irritation) are required to become phenotypically manifested. Thus, for reactive mixtures containing MDI, the matrix, the composition, and the concentration of MDI present in the mixture may be contributing factors that modulate the

sensitizing and asthmagenic potency of final products as already hypothesized by other authors (Baur, 2007; Bello et al., 2007). In turn, the exposed surface area does not appear to be a critical variable (Pauluhn, 2000).

To conclude, at this state of knowledge it does not appear that the relative asthmagenic potency of volatile and/or nonvolatile diisocyanates as pure compounds or as formulated mixtures can reliably be predicted from simple and acute experimental models analyzing selected (pro-)inflammatory endpoints. Similarly, the collection of biological specimens distant to the respiratory tract may not necessarily mirror the processes taking place at the specific microenvironments/anatomic locations within this organ. As long as no simple mechanistic explanation of human asthma can be provided, it appears to be premature to advocate one simple 'acute' animal bioassay for risk assessment. Moreover, apart from genetic predisposition, exposure factors, the associated metric, and the dose (concentration $\times$ exposure time)–response relationship of irritation and allergy need to be characterized. Despite this complexity, repeated challenge protocols using endpoints known to integrate the biological response (BAL, histopathology, lung function measurements) appear to be most promising to define dose–effect relationships both for sensitization and asthma-like respiratory responses. Consequently, it remains elusive to search for a single, universal biomarker that predicts this complex disease.

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