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Brown Norway rat asthma model of diphenylmethane-4,4′-diisocyanate (MDI): Determination of the elicitation threshold concentration of after inhalation sensitization

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ABSTRACT

Occupational exposure to polymeric diphenylmethane-diisocyanate (MDI), a known human asthmagen, can be attributed to two potential routes: the skin and the respiratory tract. While the skin as the route of sensitization was the focus of a previous investigation (Pauluhn, 2008), this paper describes a modified sensitization protocol using a 5-day inhalation exposure (days 0–4) of Brown Norway (BN) rats to two concentration × exposure time ($C \times t$) relationships of 1000, 5000, and 10,000 mg MDI/m^3 × min at exposure durations of either 10 or 360-min. Apart from the differences in the induction protocol, all other experimental variables remained identical. This was followed by four 30-min inhalation challenges to 40 mg MDI/m^3 on target days 20, 25, 50, and 65. After the last challenge, changes in breathing patterns delayed in onset were recorded and allergic lung inflammation was probed by bronchoalveolar lavage (BAL). In a subsequent study groups of rats were sensitized using the 10-min $C \times t$ protocol and challenged 3-times at 40 mg MDI/m^3. At the fourth challenge a dose-escalation regimen was used to determine the elicitation threshold on 'asthmatic' rats. Consistent with the skin-sensitization protocol, the most sensitive endpoints characterizing an allergic pulmonary inflammation were again BAL-neutrophils and physiological measurements showing respiratory changes delayed in onset. The dose-escalation challenge yielded an elicitation threshold of 5 mg MDI-aerosol/m^3 at 30 min challenge duration. In topically sensitized rats this threshold was estimated to be 3 mg/m^3. In summary, these data suggest the $C \times t$ product of MDI-aerosol that triggers an elicitation response in 'asthmatic' rats is slightly below of that causing acute pulmonary irritation in naïve rats. The high concentration delivered to the respiratory tract during the 10-min exposure period elicited a more vigorous response than the similar $C \times t$ at 360 min. Therefore, short high-level exposure patterns appear to bear a higher sensitizing potency than equal $C \times t$ products at longer exposure periods. Taking into account the respective differences in exposure intensities, the comparison of elicitation thresholds of BN rats sensitized by inhalation or skin exposure did not demonstrate essential differences.

1. Introduction

While the immunological testing strategies are commonly focused on specific cytokine profiles and immunoglobulins or functional measurements immediate in onset (Dearman et al., 1996; Selgrade et al., 2006), the approach taken in this study is targeted on the dose–effect analysis of the manifested phenotypes of asthma, such as sustained lung inflammation or typical changes in lung function. Apart from the difficulties in transposing rodent data to humans, it has to be recognized 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). 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. Most chemicals that cause occupational asthma are also recognized respiratory tract irritants. Therefore, inhalation risk characterization attempts to associate the threshold dose triggering the allergic response with that of respiratory tract irritation. The latter is defined in conventional inhalation models on naïve rats.

Published evidence shows that several consecutive inhalation exposures of sufficiently high concentrations are required in animal models to produce an inflammation of the airway wall commonly observed in human asthma (Cohn et al., 2004; Kips et al., 2003; Kumar et al., 2000; Kumar and Foster, 2002). However, as long as no

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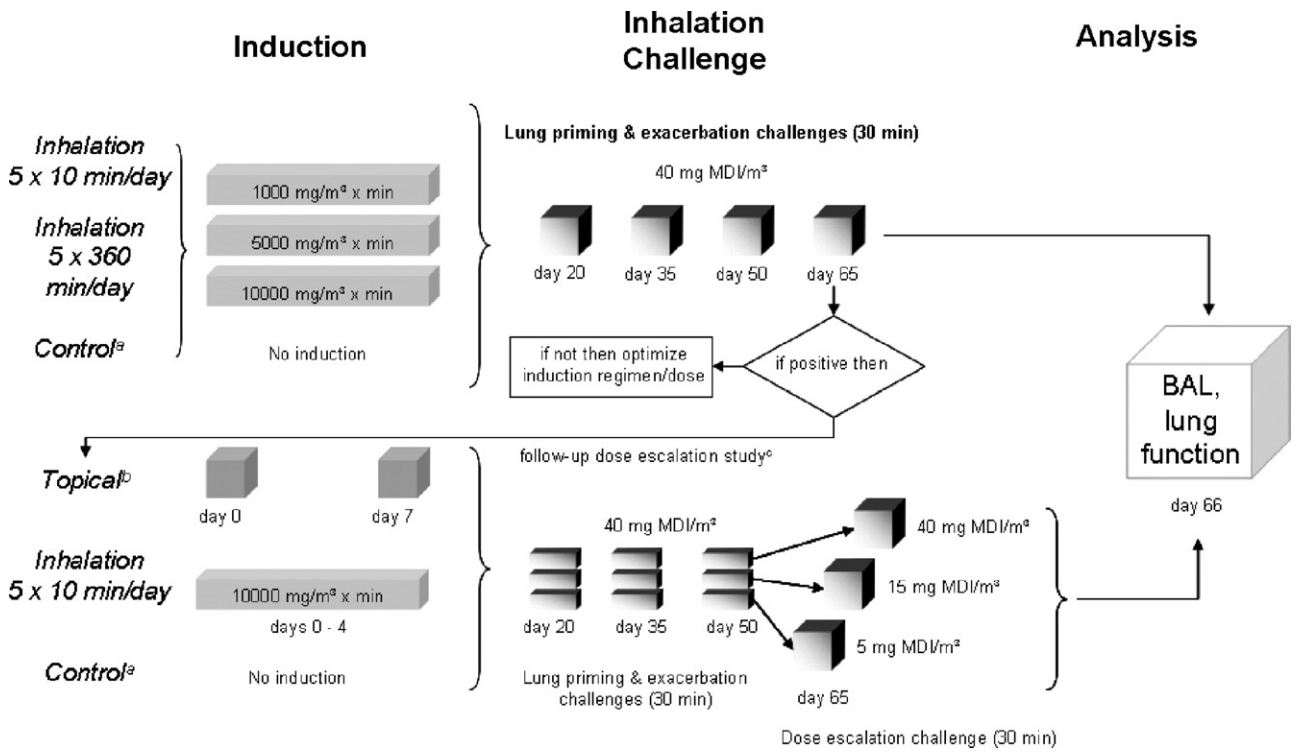

Fig. 1. Protocols used for topical and inhalation induction to and inhalation challenge with MDI in Brown Norway rats. (a) MDI-induced and challenged groups were compared with control groups of naïve rats using either four inhalation challenges without or with dose-escalation steps at the last challenge. The extent of challenge-induced respiratory irritation in naïve rats was additionally compared with rats not induced and not challenged (not shown); (b) data related to topical induction was previously published (Pauluhn, 2008), (c) separate groups of rats were used in the dose-escalation protocol using the most efficacious route-specific induction protocol. Inhalation induction utilized identical concentration × time products at two different exposure concentrations at exposure durations of either 10 or 360 min daily on five consecutive days. The three lung priming challenge exposures were identical in all protocols whereas the last elicitation challenge was performed either as single dose challenge for 'hazard identification' (or protocol optimization) or escalation dose challenge to determine the elicitation threshold. The time elapsed between challenges was considered adequate for irritant responses to wane and immunological responses to exacerbate. The rationale of these protocols was to examine the influence of the sensitization dose, dose rate (similar total dosages at different levels of acute lung irritation) and the route on the subsequent elicitation response and whether the elicitation threshold response shows any dependence on these induction exposure-related variables. Study days represent target days (for exact days see Table 1).

simple mechanistic explanation of human asthma can be provided, this evidence advocates that repeated exposure animal bioassays may be more suitable for risk characterization than models utilizing acute high-dose principles. Similarly, dose selection is most critical as repeated exposures to high doses may cause paradox phenomena such as 'immunologic tolerance' (Schramm et al., 2004) and the antigen presentation and processing may differ in a non-inflamed and inflamed microenvironment. Multiple protocols variations and endpoints have been studied to identify and characterize respiratory allergens in laboratory-specific models (Arts et al., 2001, 2008; Arts and Kuper, 2007; Dearman et al., 2003a,b; Herrick et al., 2002; Karol, 1983; Karol et al., 1985; Karol and Thorne, 1988; Selgrade et al., 2006; Zhang et al., 2004; Redlich et al., 2002; Tarkowski et al., 2007; Vanoirbeek et al., 2009). The benefits and limitations of these models relative to that described in this paper have been discussed elsewhere (Pauluhn and Mohr, 2005; Arts et al., 2006).

Polymeric diphenylmethane-diisocyanate (MDI) was investigated in a Brown Norway (BN) rat asthma model using topical (skin) sensitization and repeated inhalation challenges to MDI aerosol. The rats exhibited several of the lesions that typify chronic human asthma (Pauluhn et al., 2005; Pauluhn, 2005, 2008). From these studies the conclusions were drawn that the endpoint of 'allergic pulmonary inflammation' is appropriately characterized by neutrophils (PMN) in BAL. With each subsequent inhalation challenge BAL-PMNs increased further relative to the previous challenge in sensitized rats but not in equally challenged naïve rats. There is also increasing evidence that neutrophils play a role in the pathogenesis of human asthma (Jung and Park, 1999; Jatakanon et al., 1999; Lindén and Adachi, 2002; Lemière et al., 2002; Monteseirin, 2009). Physiological responses were characterized by changes in breathing patterns delayed in onset which corresponds with the delayed-onset manifestation of airway obstruction typically observed in human diisocyanate asthma (Lemière et al., 2000). The sensitivity of BAL-PMN to probe for differences in naïve and sensitized rats was higher than that of total IgE or any other endpoint examined (for details see Pauluhn et al., 2005; Pauluhn, 2005).

The objective of this study is to extend previous evidence obtained with MDI following skin sensitization to investigate as to whether repeated inhalation sensitization encounter have any impact on the elicitation threshold dose as defined previously in topically sensitized rats (Pauluhn, 2008). In order to address the modifying effect of respiratory tract irritation on sensitization, three different $C \times t$ inhalation induction doses at six different concentrations were examined under otherwise identical conditions.

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 was 38.1% with higher oligomeric MDI as balance. The free isocyanate (NCO) content was 31.1%. During handling and storage the headspace of MDI containers was purged with dry nitrogen to remove air and humidity to prevent its decomposition.

2.2. Animals, diet, and housing conditions

Male 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

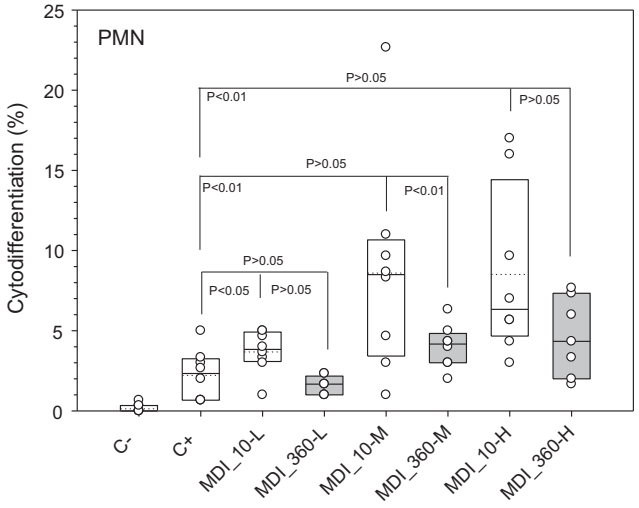

Fig. 2. Percentage of polymorphonuclear cells (PMNs) in bronchoalveolar lavage one day after the last challenge (day 65, details see Fig. 1). Empty and filled boxes represent data from inhalation induction protocols using 5 × 10 min/day and 5 × 360 min/day exposure periods, respectively. The group allocation, average induction and challenge concentrations are given in Table 1. Boxes represent Tukey Box Plots (dotted lines: mean, solid lines: median). Symbols indicate individual animal data of 8 rats per group. Statistical significances amongst groups and induction regimens were analyzed using a Mann–Whitney *U* statistics.

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 were approximately 230 g. For this study, only male rats were used and they were two to three 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 06:00 h.

All animal experiments were in accord with contemporary, internationally harmonized testing standards/guidelines (OECD, 2009) and utilized rigorously validated, controlled exposure methodologies as called for by current Good Laboratory Principles (GLP) (OECD, 1998). This study was performed in compliance with the OECD Principles of Good Laboratory Practice (1998) in an animal care-approved laboratory in accordance with the German Animal Welfare Act and the European Council Directive 86/609/EEC (1986).

2.3. Rationale of induction and challenge exposure regimens

The procedures utilized in this Brown Norway rat sensitization study was consistent with those described previously in lung sensitization studies with MDI-aerosol (Pauluhn et al., 2005; Pauluhn, 2005, 2008). Opposite to this study, previous studies utilized the topical (flank) route for sensitization at similar challenge conditions and endpoints. In this study six different concentrations (C) at two different exposure durations (t) were examined for the induction of 'respiratory allergy'. This approach served the purpose to investigate the modifying factor 'lung irritation' and whether the vigour of allergic inflammation depends more on the exposure dose ($C \times t$) or concentration used for inhalation sensitization. The respective C x t products were based on previous studies addressing this endpoint in naïve Wistar rats (Pauluhn, 2000, 2002, 2004a).

This study consisted of two phases; the first was designed to identify which type of $C \times t$ protocol is most efficient to sensitize the rats by repeated inhalation exposures. The focus of the second phase was to determine the elicitation threshold using the most efficient inhalation sensitization regimen as identified in the first phase of study. The results were compared with previously published data (Pauluhn, 2008) using the same inhalation challenge protocol but topical (skin) sensitization (Fig. 1). Self-adjuvant effects due to respiratory tract irritation or tolerogenesis have been considered in previous studies (Pauluhn et al., 2005; Pauluhn, 2005). These data showed that both neutrophils (PMNs) in BAL as well as respiratory responses delayed in onset increased with increasing numbers of inhalation-challenge exposures. Based on these findings it was concluded that this type of challenge protocol does not produce tolerogenesis and that the most vigorous response occurs at the last (fourth) challenge exposure. Previous single exposure studies using a $C \times t$ product of ≈1200 mg MDI/m^3 × min, which is equivalent to ≈40 mg MDI/m^3 – 30 min challenge duration, provides evidence that this $C \times t$ product causes mildly increased BAL-protein as evidence of acute pulmonary irritation. Based on the concentration-effect and time-course analyses in Wistar rats exposed for 360 min to graded concentrations of MDI an acute pulmonary irritation threshold of ≈0.5 mg MDI/m^3 (≈180 mg MDI/m^3 × min) was derived (Pauluhn, 2000). Marked strain differences between Wistar and BN rats were not apparent (Pauluhn, 2004b). Hence, for the challenge period of 30-min used in this study, the acute irritation threshold concentration is in the range of ≈6 mg MDI/m^3 whereas minimal but definite pulmonary irritation occurs at ≈40 mg MDI/m^3. The applied challenge regimen utilized 2-week periods between each 30-min challenge exposure in order to minimize carry-over of irritation-related effects from one challenge to another. This time period has been demonstrated to be sufficient by time-course analyses of Wistar rats acutely exposed to MDI aerosol (Pauluhn, 2000; Pauluhn, 2004a). Thus, the devised protocol appears to be conducive to booster 'immunologic memory' rather than to exacerbate the irritant response.

2.4. Study design

This first phase of study consisted of two naïve control groups (one not challenged, the other one challenged) and six groups of BN rats that were sensitized by inhalation exposure (see Table 1). Each group consisted of eight male rats. The inhalation induction to MDI-aerosol was by daily directed-low nose-only inhalation using either a 5 × 10-min/day (breathing zone concentrations: 97.1, 475, and 936 mg/m^3) or a 5 × 360-min/day (breathing zone concentrations: 2.9, 14.9, and 29.9 mg/m^3). This was followed by four inhalation challenge exposures on target days 20, 35, 50, and 65 (tolerance 61–66 days due to multiple examinations at the last challenge). Each challenge exposure was for 30-min. After the last challenge, all rats were monitored for changes in lung function delayed in onset for ≈20 h. Measurements commenced shortly after challenge. One day thereafter, the weights of exsanguinated lungs and lung-associated lymph nodes (LALN) of the lung hilus region were determined followed by bronchoalveolar lavage of the excised lungs. The second phase utilized the high-dose $C \times t$ protocol at 10 min exposure dura-

Table 1
Inhalation sensitization of BN rats using three graded concentration × time relationships followed by four identical challenge exposures (groups 2–8) or dose escalation challenge exposure (groups 9 and 10).

Group	Induction duration (min)	Induction (days × mg/m^3)	Target exposure intensity (mg/m^3-min)	Concentration (mg/m^3) mean ± SD	Challenge I–IV (mg/m^3) mean ± SD
Inhalation induction/elicitation regimen					
1[a]	–	–	–	–	–
2	360	–	–	–	43.8 ± 5.3
3-L	10	5 × 100	1000	97.1 ± 8.1	44.1 ± 5.1
4-M	10	5 × 500	5000	474.9 ± 26.2	43.6 ± 5.5
5-H	10	5 × 1000	10,000	936.4 ± 41.9	43.3 ± 5.8
6-L	360	5 × 3	1080	2.9 ± 0.2	40.5 ± 2.8
7-M	360	5 × 15	5400	14.9 ± 1.5	40.4 ± 2.8
8-H	360	5 × 30	10,800	29.9 ± 2.3	40.4 ± 2.8
Optimized inhalation induction/dose escalation elicitation regimen					
9[b]	10	–	–	–	41.0 ± 2.0/5.0–12.8–39.4[c]
10	10	5 × 1000	10,000	965.9 ± 33.9	39.8 ± 3.1/5.2–16.2–39.6

Inhalation induction exposures on 5 consecutive days (Fig. 1).
[a] Eight Brown Norway rats per group.
[b] Twenty-four Brown Norway rats per group.
[c] Average concentration of MDI during challenges I–III on days 20, 35, and 50/dose escalation challenge (IV) steps on day 65. L, M, H: low, intermediate, high induction group.

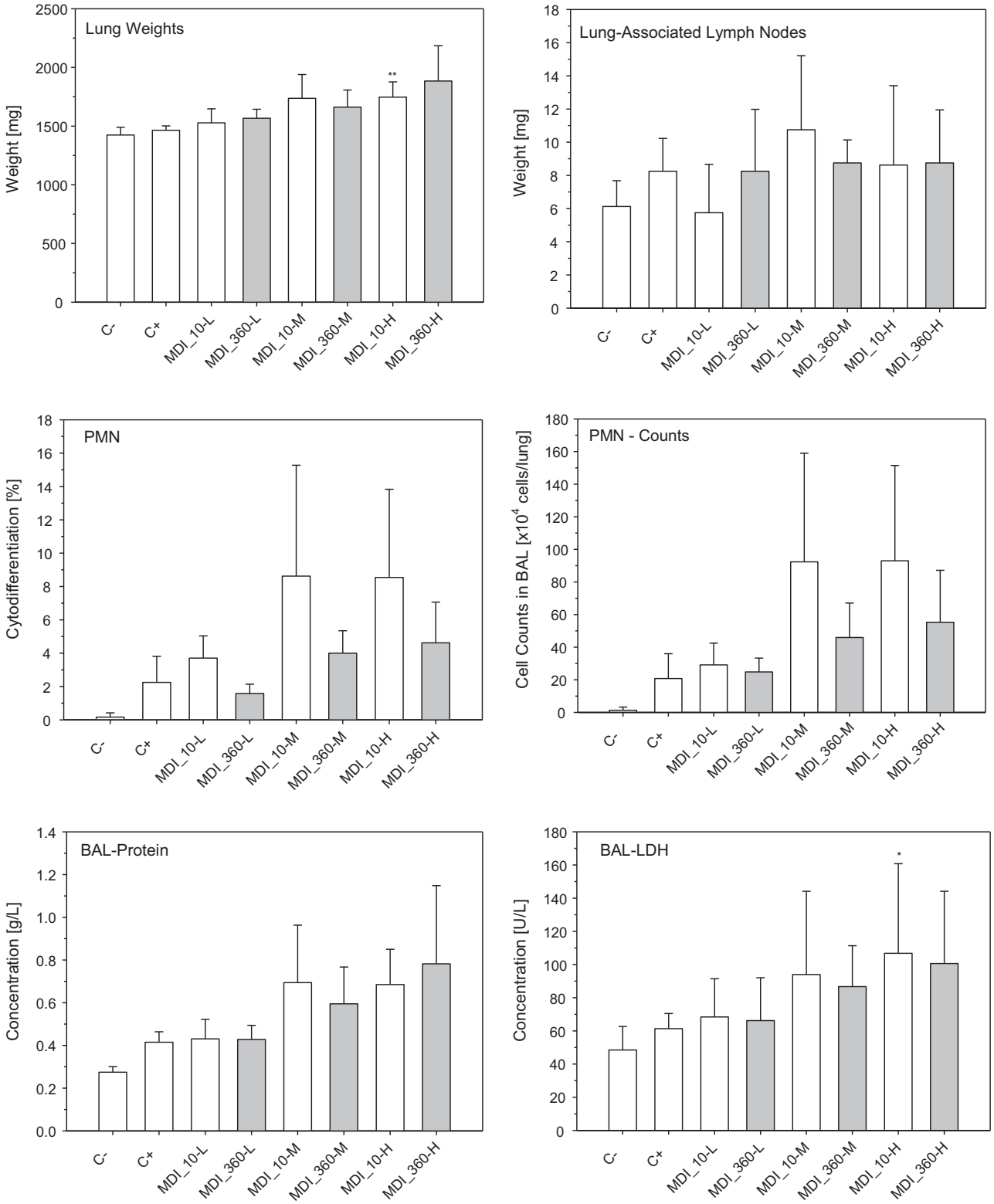

Fig. 3. Lung and lung-associated lymph node (LALN) weights and endpoints determined in bronchoalveolar lavage (BAL) 1 day after the last challenge (day 65, details see Fig. 1). Empty and filled boxes represent data from inhalation induction protocols using 5 × 10 min/day and 5 × 360 min/day exposure periods, respectively. The group allocation, average induction and challenge concentrations are given in Table 1. PMNs: neutrophilic granulocytes (cell differentials), PMN-counts: PMNs adjusted to the total cell count retrieved by BAL, LDH: lactate dehydrogenase. Bars represent means ± SD ($n = 8$). Asterisks denote significant differences to the challenged naïve control (group 2, see Table 1). Significant differences to this group are indicated by asterisks ($^*P < 0.05$, $^{**}P < 0.01$).

tion because of the more vigorous response observed in this group. This first three booster challenges used in study phase II were similar to that of phase I. A stepped dose-escalation challenges was applied at the fourth challenge. Naïve control and sensitized rats, each consisting 8 male rats per challenge step, were challenged under similar conditions.

2.5. Generation and characterization of exposure atmospheres

Atmospheres of MDI for inhalation exposures were generated under dynamic conditions using a digitally controlled Harvard PHD 2000 pump and a modified Schlick-nozzle Type 970, form-S 3 (Schlick GmbH, Coburg, Germany). MDI was

atomized using conditioned (dry, oil-free) compressed air (dispersion pressure approximately 600 kPa, 15 L/min and inhalation chamber segment). The nozzle was maintained at approximately 40 °C using a water jacket connected to a digitally controlled JULABO thermostat. The increase of temperature within the nozzle resulted in a marked decrease in viscosity and increased the respirability of aerosol. Targeted concentrations were achieved by pull/push dilution cascades. The equilibrium concentration (t_{95}) was attained in less than 1 min with air flow rates of 0.75 L/min at each exposure port. The test atmospheres were characterized by gravimetric analyses (filter: Glass-Fibre-Filter, Sartorius, Göttingen, Germany). The temporal stability of the aerosol generation and exposure system was measured by using real-time aerosol photometers (RAS-2 MIE, Bedford, MA, USA). The mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) was determined using an 8-stage critical orifice cascade impactor. Throughout all exposures the aerosols size was in a similar range (MMAD 1.5–1.9 μm, GSD 1.6–1.9). For both filter and particle size analyses air samples were collected from the breathing area. Details of the validation of this exposure system have been published elsewhere (Pauluhn and Thiel, 2007; Pauluhn, 2008). The exposure methodology used is consistent with that called for by OECD-GD39 (2009). The inhaled deposited dose was estimated by using MPPD2 software (Anjilvel and Asgharian, 1995; RIVM, 2002).

2.6. Analysis of delayed-onset respiratory response

Measurements were on unrestrained, spontaneously breathing rats in a calibrated barometric whole-body plethysmograph (volume: 4.2 L, air flow rate: 2 L/min). Food and water were available *ad libitum* during the course of measurement. Measurements were made simultaneously in eight chambers. Data collection commenced shortly after challenge by placing the animals into the pre-calibrated barometric whole-body plethysmographs (equipment used for data-acquisition: ACQ7700XE, software: Ponemah, version: 4.80, DSI Ponemah, Valley View, OH). Based on previous experience, data analysis focused on 'enhanced pause' (Penh) (Pauluhn, 2005; Pauluhn and Mohr, 2005). Measurement of Penh by unrestrained plethysmography does not provide any direct assessment of a specific physiologic variable, and its limitations in context with MDI-induced lung irritation are well documented (Pauluhn, 2004b). These data appear to show that Penh parallel those of pulmonary parenchymal changes rather than 'airway resistance' as commonly believed (Hamelmann et al., 1997). Despite of these published shortcomings (Mitzner et al., 2003), Penh appears to integrate several physiological endpoints in a wholly non-invasive and non-disturbing manner so that non-specific changes in lung function can readily be identified with time-point of onset. However, caution is advised to link these changes to specific pathophysiological effects. Data were collected every minute and were digitally averaged over periods of 15 min. Although data were collected over approximately 20 h, the Penh-AUC was integrated during the first 8 h after challenge (Penh-AUC_{8h}). Previous studies have shown that MDI-related changes in Penh typically occur within this time period (Pauluhn, 2005, 2008) and differences amongst groups can be better differentiated by restricting the analysis to this time period.

2.7. Bronchoalveolar lavage

All rats were anaesthetized using sodium pentobarbital (Narcoren®; 120 mg/kg body weight, intraperitoneal injection). Complete exsanguination was achieved by severing the *aorta abdominalis*. After exsanguination, the excised lung was weighed and then lavaged via a tracheal cannula with two volumes of 5-ml of physiological saline (kept at 37 °C), each withdrawn, re-instilled once. In the supernatant, BAL-fluid was analyzed for total protein and lactate dehydrogenase (LDH). Prior to centrifuging the samples were kept on ice. Pooled BAL-fluid was centrifuged at 200 × *g* for 10 min at <10 °C (Sigma 4K15C-centrifuge). The cell pellet was re-suspended in PBS-BSA and centrifuged (2×10^5 per cytospot) onto slides using a cytocentrifuge (Shandon Cytospin 4). Air-dried slides were fixed with a mixture of methanol:acetone, stained according to Pappenheim, and differentiated by light microscopy (300 cells were counted/cytospot). Cell counts were determined in triplicates after 1:1000 dilution using a CASY cell counter + analyzer (Innovatis, Reutlingen, Germany). Absolute counts of PMNs were calculated based on the total number of cells in BAL and the respective percentage obtained by cytodifferentiation.

2.8. Data analysis

Organ weights and BAL data were analyzed by one-way analysis of variance (ANOVA) followed by a multiple comparison Tukey–Kramer *post hoc* test. Statistical differences of Penh-AUC and PMN differentials between the challenged control and MDI groups were evaluated by a Mann–Whitney Rank Sum Test (SigmaPlot 11, Systat Software, Point Richmond, CA). For all tests the criterion for statistical significance was set at $P < 0.05$.

3. Results

3.1. Characterization of exposure atmospheres

Details of the $C \times t$ exposure regimens that were used for sensitization and challenge are summarized in Fig. 1. The actually measured breathing zone concentrations are given in Table 1. Based on the measured aerosol characteristics of MDI-aerosol the estimated pulmonary, thoracic, and total deposition of aerosol was 8, 13, and 34%, respectively.

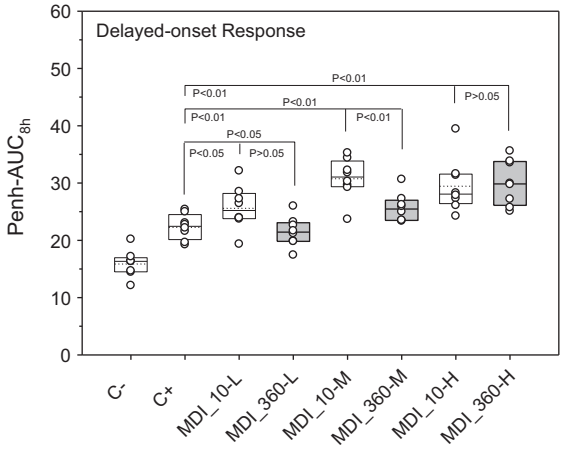

Fig. 4. Integration of measurement of enhanced pause (Penh) in whole body barometric plethysmographs over a post-challenge time period of 8 h (Penh-AUC_{8h}) in eight Brown Norway rats after the fourth challenge (see Fig. 1 and Table 1 for challenge concentration and group allocation). Empty and filled boxes represent data from inhalation induction protocols using 5 × 10 min/day and 5 × 360 min/day exposure periods, respectively. Boxes represent Tukey Box Plots (dotted lines: mean, solid lines: median). Symbols indicate individual animal data of 8 rats per group. Statistical significances amongst groups and induction regimens were analyzed using a Mann–Whitney *U* statistics.

3.2. Sensitization efficiency of differing $C \times t$ inhalation exposure protocols

Animals were sensitized and challenged as illustrated in Fig. 1 and detailed in Table 1. All endpoints were determined after the fourth challenge (day 65). The most sensitive endpoint to probe for induction-dependent differences upon challenge were PMNs in BAL (Figs. 2 and 3). The average intensity of the PMN influx was higher at 5000 mg MDI/m^3 × min at 10 min/day relative to 10,800 mg MDI/m^3 × min at 360 min/day. Based on the analysis depicted in Fig. 2, at the four identical challenge exposures of

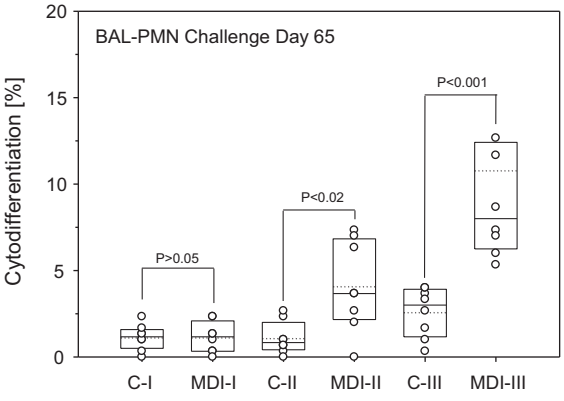

Fig. 5. Percentage of polymorphonuclear cells (PMNs) in BAL one day after the last escalation challenge steps I to III (see Fig. 1). Naïve control rats (C) and rats repetitively induced by inhalation to 966 mg MDI/m^3 using a 5 × 10 min/day exposure protocol were challenged on days 20, 35, and 50 to 40 mg/m^3 followed by a dose-escalation challenge on day 65 (eight rats per escalation step, average induction and challenge concentrations are given in Table 1). Boxes represent Tukey Box Plots (dotted lines: mean, solid lines: median). Symbols indicate individual animal data of 8 rats per group. Significant differences between groups with equal escalation challenge concentrations were compared using a Mann–Whitney *U* statistics.

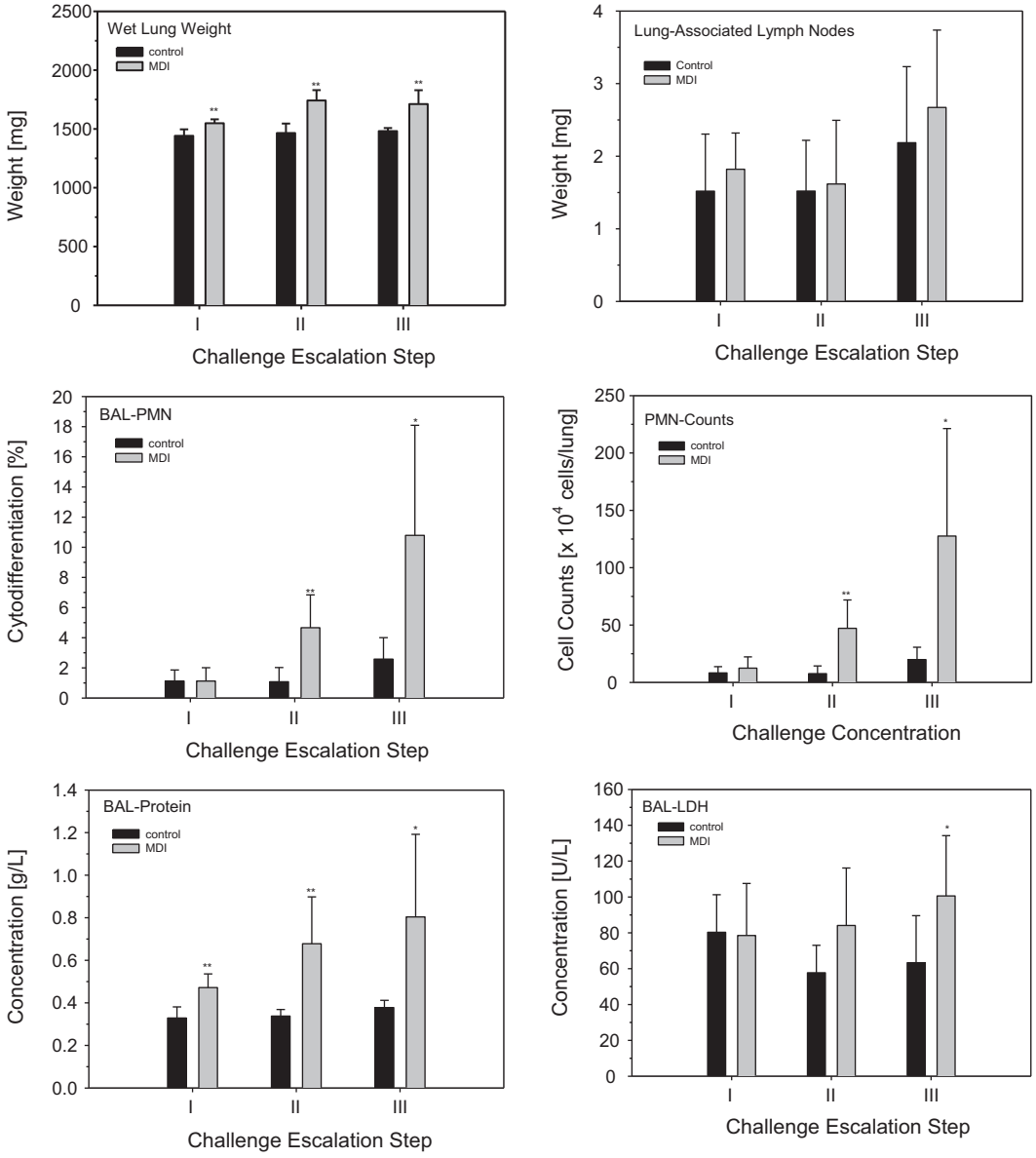

Fig. 6. Lung and lung-associated lymph node (LALN) weights and endpoints determined in (BAL) one day after the last escalation challenge steps I to III (see Fig. 1). Naïve control rats (C) and rats repetitively induced by inhalation to 966 mg MDI/m^3 using a 5 × 10 min/day exposure protocol were challenged on days 20, 35, and 50 to 40 mg/m^3 followed by a dose-escalation challenge on day 65 (eight rats per escalation step, average induction and challenge concentrations are given in Table 1). Bars represent means ± SD (n = 8). Asterisks denote significant differences to the equally challenged naïve control (see Table 1). Significant differences between groups with equal escalation challenge concentrations are indicated by asterisks (*P < 0.05, **P < 0.01).

the first phase of study, the high-concentration short-exposure duration sensitization protocol produced a significant and more vigorous elicitation response on BAL-PMNs as compared to the low-concentration 360-min exposure induction protocol. Relative to BAL-PMNs, the differences amongst 10-min and 360-min sensitization regimens were less pronounced based on lung weights, BAL-protein and BAL-LDH. Conclusive changes in LALN weights did not occur.

For Penh, an endpoint suggestive of changes in breathing patterns, for each group the area-under-the-curve of post-challenge measurements of Penh over a time period of approximately 8 h (Penh-AUC$_{8\,h}$) is illustrated in Fig. 4. This analysis revealed $C \times t$-dependent AUCs independent on the exposure duration. The endpoints examined in the non-challenged ($C-$) and challenged ($C+$) naïve control groups were minimally elevated in the challenged $C+$ group relative to the $C-$ group (Figs. 2–4). This finding provides supporting evidence that the $C \times t$ used for challenge exposures is minimally irritant and its extent does not interfere with the interpretation of study. Due to the limited numbers of animal per group and inter-animal variability the outcome of the non-parametric statistical analyses is given preference to the parametric ANOVA method. The Mann–Whitney Rank Sum Test was applied to the key endpoints PMN and Penh only.

3.3. Dose escalation challenge – determination of elicitation threshold

Based on the analysis shown in Figs. 3 and 4, the high-concentration short-exposure duration induction protocol using a $C \times t$ product of 10,000 mg MDI/m^3 × min was selected for this ancillary study. After three inhalation challenges with 40 mg MDI/m^3 subgroups of rats were re-challenged using a dose-escalation regimen in steps of 5 (I), 15 (II), and 40 (III) mg MDI/m^3 (for actual breathing zone concentrations see Table 1).

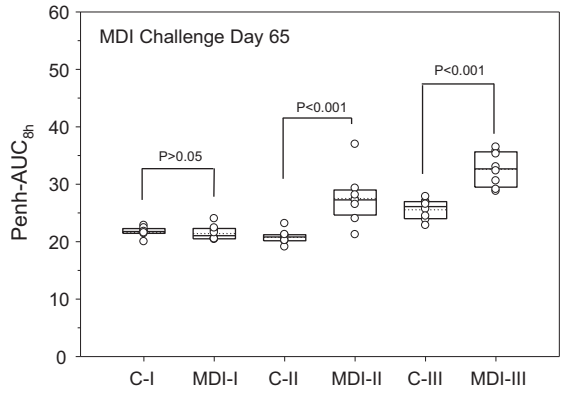

Fig. 7. Integration of measurement of enhanced pause (Penh) in whole body barometric plethysmographs over a post-challenge time period of 8 h (Penh-AUC_{8h}) in eight Brown Norway rats after the last escalation challenge steps I to III (see Fig. 1). Naïve control rats (C) and rats repetitively induced by inhalation to 966 mg MDI/m^3 using a 5 × 10 min/day exposure protocol were challenged on days 20, 35, and 50 to 40 mg/m^3 followed by a dose-escalation challenge on day 65 (eight rats per escalation step, average induction and challenge concentrations are given in Table 1). Boxes represent Tukey Box Plots (dotted lines: mean, solid lines: median). Symbols indicate individual animal data of 8 rats per group. Significant differences between groups with equal escalation challenge concentrations were compared using a Mann–Whitney *U* statistics.

Again, PMNs appeared to be most sensitive for elicitation-dose related differences between the control and MDI challenged groups (Figs. 5 and 7). Minimal, although significant, differences between challenged control and MDI-induction groups were evidenced by increased BAL-protein and lung weights (Fig. 6). Rats challenged at the highest step III had elevated LALN weights in both the control and MDI-sensitized rats (Fig. 6). However, the pair-wise comparison of naïve with sensitized rats did not demonstrate significant differences in LALN-weights.

Elicitation response-dependent changes on BAL-PMNs are summarized in Fig. 8, those of Penh-AUC_{8h} in Fig. 9. PMNs were consistently increased with increasing challenge concentration following topical (data duplicated from Pauluhn, 2008) and inhalation induction. At the dose-levels selected for inhalation induction the no-observed-adverse effect level (NO(A)EL) or NEL (ECHA, 2008) was 5 mg MDI/m^3 at 30 min exposure duration. The respective threshold of PMNs was lower and than of Penh-AUC_{8h}. Therefore, the BAL-PMN endpoint was taken as lowest dose descriptor. This concentration–response relationship was additionally analyzed using the US-EPA benchmark (BMD) approach (U.S. EPA, 2010). Based on the curve-fitting shown in Fig. 8 (lower panel) the BMD lower bound was 8.6 mg/m^3 (confidence level: 0.95). This demonstrates that the applied analysis is implicitly conservative.

4. Discussion

Skin exposures to isocyanates and the subsequent development of diisocyanate asthma is receiving increased attention (Beck and Leung, 2000; Bello et al., 2007; Liljelind et al., 2010; Liu et al., 2009; Redlich and Herrick, 2008; Redlich, 2010). The systematic investigation of the dose–response relationships of occupational asthma caused by skin and/or inhalation exposure to reactive, low molecular weight chemicals, such as diisocyanates, is limited by the lack of both harmonized and internationally recognized animal bioassays of respiratory allergy and asthma (Kimber and Dearman, 2005; Kimber et al., 2007, 2010). This is complicated further as intermittent peak exposures, which could entail both respiratory and skin exposure (Tarlo et al., 1997; Liljelind et al., 2010), are believed to be critical in the development of lung sensitization (Bernstein et al., 1993). Typically, such exposures are unpredictable

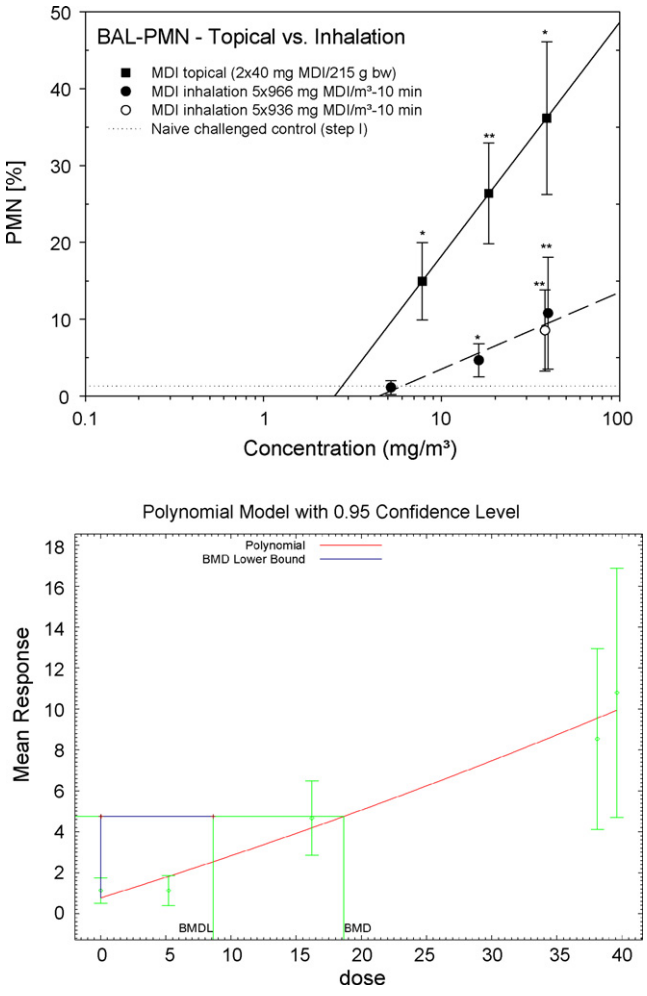

Fig. 8. Analysis of escalation challenge-concentration and elevations of neutrophilic granulocytes (PMN) in BAL (day 65, see Fig. 1) by linear regression (upper panel) and benchmark calculation by US-EPA-BMDS software (lower panel). MDI-topical: sensitization of Brown Norway by dermal administrations of 200 μL MDI 20% in acetone:olive oil (4:1) on the contralateral dorsal flanks on days 0 and 7 using the same pre-challenge and dose-escalation challenge protocol as used in this study (data duplicated from Pauluhn, 2008). MDI-inhalation 966 mg/m^3: Brown Norway were rats repetitively induced by inhalation to 966 mg MDI/m^3 using a 5 × 10 min/day exposure protocol were challenged on days 20, 35, and 50 to 40 mg/m^3 followed by a dose-escalation challenge on day 65. MDI-inhalation 966 mg/m^3: similar as previous protocol but challenge with 40 mg MDI/m^3 at all four challenges (group 5-H, Table 1). Symbols represent means ± SD (n = 8). The mean of the control challenged at step I of the dose-escalation protocol is indicated by dotted line.

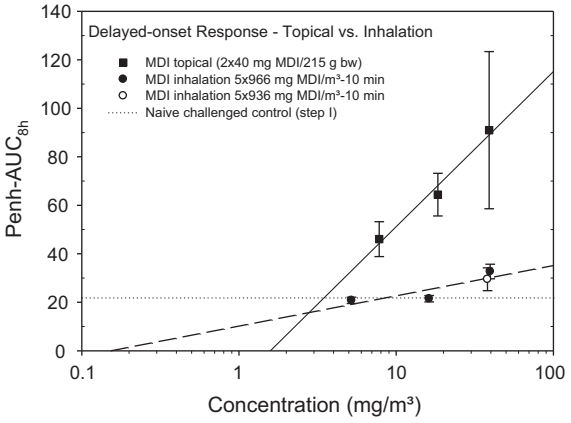

Fig. 9. Analysis of escalation challenge-concentration and the integrated changes in Penh over a time period of 8 h data collection period (Penh-AUC_{8h}) by linear regression. For further details see legend of Fig. 8.

and frequently accidental, making them difficult to systematically investigate and quantify (Bello et al., 2007). The approach described in this paper attempts to address whether sensitization via the skin and inhalation exposure lead to differences in the elicitation thresholds characterizing respiratory allergy.

The inter-animal variability increased when using the 10-min instead of the 360-min exposure sensitization protocol which appears to be caused by changes in both breathing patterns in rats not yet fully adapted to the nose-only mode of inhalation exposure and irritation-related changes in breathing patterns (Pauluhn, 2000). Reflexively induced changes in breathing patterns affect the inhaled dose which appears to be the primary source of variability. Such a route-of-exposure inherent inter-animal variability does not occur following dermal sensitization (Pauluhn, 2008). Therefore, bioassays using topical rather than inhalation induction methods appear to be more expedient, independent on any $C \times t$-related variability, and are technically less demanding. These factors make bioassays using the dermal sensitization route inherently more robust than those using inhalation as the route for induction. Nonetheless, the inhalation challenge protocol is considered mandatory and is superior to any short-cuts thereof, e.g., intranasal instillation. One major advantage of the inhalation route is that the regional deposition of aerosol can be estimated by MPPD2 modeling and that vehicle-free systems can be used for both sensitization and challenge exposures. At any rate, the repeated exposure to vehicles such as olive oil is considered critical, as it may contain protein impurities that could be conjugated by the reactive isocyanate moieties to antigens. Moreover, oil instillation into the respiratory tract is likely to be conducive to alveolitis whereas inhalation exposure results also in a reasonable direct exposure of the bronchial airways, the target structure of asthma.

When comparing the outcome of elicitation thresholds of animals sensitized by different routes, it appears to be of paramount importance to take into account the differences dosages used for sensitization and challenge because the threshold of elicitation is not an inherent property of the allergen (hapten) *per se*, but is also a consequence of the severity of the induction regimen (Hostynek and Maibach, 2004).

This study used a 5×10-min aerosol exposure of 965.9 mg MDI/m^3. Based on previously published data (Pauluhn and Thiel, 2007), the inhaled total dose is 48 mg MDI/kg-bw with a total deposited dose of 16.4 mg MDI-aerosol/kg-bw. At 40 mg/m^3, the 30-min challenge exposure results in a total deposited dose of 0.4 mg MDI-aerosol/kg-bw. In the dermal reference study (Pauluhn, 2008) the dose applied was 2×200 μL/rat (20% MDI in AOO) which is equivalent to approximately 240 mg MDI/kg-bw. This induction dose is somewhat similar to that used by other authors in mice focusing on immunological endpoints (Farraj et al., 2007). These authors applied monomeric MDI (2×100 μL/mouse; 2% mMDI in AOO) which is roughly equivalent to 370 mg MDI/kg-bw (adjusted to the content of monomeric MDI contained in polymeric MDI). Mice were challenged by the intranasal instillation of 60 μL/mouse (1% mMDI in acetic ester-olive oil). Adjusted to the type of MDI used in this study this dose is equivalent to approximately 55 mg MDI/kg-bw. Thus, from a dosimetric perspective, the results presented in Figs. 8 and 9 are plausible and the differences in elicitation responses appear to be more dependent on the total induction dose rather than the route chosen. The estimated dosages used for challenge were markedly lower in this study than those focusing on immunological endpoints (in mice). Hence, the lack of any changes in LALN weights appears to be plausible. Previous studies in BN rats with MDI (Pauluhn et al., 2005; Pauluhn, 2005) and BALB/c mice (Farraj et al., 2007) arrive at similar conclusions, namely that Th2 cytokines in the skin and serum IgE do not portend the respiratory allergy effects of MDI. Farraj et al. (2007) conclude that immunological biomarkers of exposure that would universally predict respiratory sensitization remain elusive. Therefore, the focus of this study was solely on endpoints integrating the target structure-specific inflammatory and physiological changes delayed in onset. As shown by this analysis, it appears as if MDI-induced asthma is dependent on both immunological (dose-dependent) and neurogenic (concentration-dependent) components.

Accordingly, the bioassay devised focuses solely on the threshold dose which prevents the manifestation of the elicitation response characterizing respiratory allergy. BN rats that were sensitized using a matrix of six different concentrations and three graded $C \times t$ products followed by four inhalation challenges. The chosen $C \times t$ products cannot be translated to human workplace concentrations without species-specific adjustments in aerosol deposition patterns and exposure durations. The retained dose in the rat tracheobronchial region (TB) is never greater than 7% of the total deposited dose, whereas in the case of humans, the maximum retained TB dose reaches as high as 30% of the total deposited dose (Brown et al., 2005). From a conservative perspective 3 mg MDI/m^3 $\times$ 30 min (90 mg/m^3 $\times$ min) constitutes the NO(A)EL for both skin and respiratory tract sensitization (Fig. 8, top panel). Acute pulmonary irritation in naïve Wistar rats has been reported to occur at ≈0.5 mg MDI/m^3 $\times$ 360 min (180 mg/m^3 $\times$ min; Pauluhn, 2000). Accordingly, two entirely different testing approaches appear converge into an almost identical threshold $C \times t$ which supports a conclusion that the elicitation response is likely to be interrelated with acute lung irritation. An occupational challenge test with MDI (0.15 mg/m^3; 4, 30, 60 min) demonstrates a late asthmatic response to MDI at the 60 min challenge (9 mg MDI/m^3 $\times$ min) with a NO(A)EL (4.5 mg MDI/m^3 $\times$ min) at the 30 minute challenge (Leroyer et al., 1998). Hence, the above estimate of the human equivalent NO(A)EL in rats (90 mg/m^3 $\times$ min) is in concordance with human evidence when using a total adjustment factor of 20 on the threshold $C \times t$ of 3 mg MDI/m^3 $\times$ 30 min.

In summary, this BN rat MDI respiratory allergy bioassay demonstrates the existence of a threshold for the elicitation of respiratory sensitization following both skin and inhalation sensitization encounters. The derived elicitation threshold $C \times t$ appears to be plausible relative to human evidence and provides a concept to prevent asthma from occurring after both skin and inhalation priming exposures. The close association of $C \times t$ products triggering an elicitation response in 'asthmatic' rats with the acute pulmonary irritation threshold $C \times t$ is intriguing and supports the view that for this class of chemicals portal of entry related allergic responses appear to be linked with pulmonary and/or lower airway irritation. Accordingly, high concentrations delivered to the respiratory tract during short exposure periods appear to bear a higher sensitizing potency than equal $C \times t$ products during longer exposure periods.

Conflict of interest

Bayer Material Science and Dow Chemicals are producers of MDI and are also member companies of the International Isocyanate Institute (III), USA. The conclusions are those of the authors and not those of the III.

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