

Brown Norway Rat Asthma Model of Diphenylmethane-4,4'-Diisocyanate (MDI): Analysis of the Elicitation Dose-Response Relationship

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The known human asthmagen polymeric diphenylmethane-diisocyanate (MDI) was investigated in the Brown Norway rat skin asthma model. Two types of dose-response relationships are addressed with the following focus: (1) does sensitization dose and surface area influence the subsequent elicitation response and (2) is the elicitation response more dependent on previous elicitation doses or more on skin sensitizing dose? These two aims are investigated in two elaborated experiments, using inflammatory (bronchoalveolar lavage, BAL) and physiologic (Penh) endpoints to characterize asthma-like responses in rats. Postchallenge measurements of Penh focused on responses delayed in onset. Inflammatory endpoints in BAL were performed one day after the fourth challenge. Both protocols utilized a dermal sensitization phase with two administrations on days 0 and 7 followed by four inhalation challenges with ≈ 38 mg MDI/m³ in intervals of 2 weeks. In the first protocol three groups of rats were topically dosed with 40, 10, and 2.5 μ l of MDI per rat. Each dose group consisted of three subgroups with dosed surface areas of 3.1–12.6 cm², 0.8–3.1 cm², and 0.4–0.8 cm², respectively. In the second protocol groups of rats were topically dosed with 40 μ l of MDI per rat followed by three challenges with 37 mg MDI/m³. At the fourth challenge subgroups of rats were either challenged with 8, 18, or 39 mg MDI/m³. Independent of the protocol used, response was characterized by increased influx of neutrophilic granulocytes in BAL and delayed respiratory response. All groups from the first study sensitized to and challenged with MDI elicited a distinct response relative to similarly challenged naïve rats. A sensitization dose dependence of the elicitation response was not found. The second protocol revealed that the elicitation dose correlates with increased neutrophils in BAL and delayed-onset respiratory responses. In summary, these data suggest that the vigor of asthma-like responses appear to be more dependent on the inhalation elicitation dose of previously challenged rats rather than the dermal induction dose.

Key Words: diphenylmethane-diisocyanate; MDI; respiratory allergy; diisocyanate asthma; lung function; delayed responses; Penh; neutrophilic inflammation; dose metric; dose-response.

Investigation of the dose-response relationships of occupational asthma caused by low molecular weight chemicals has been limited by the lack of satisfactory experimental animal models. Most of the currently applied bioassays focus on two distinct stages. The first is the induction or sensitization stage, whereas the second is elicitation, which results in the clinical manifestation of allergy. At a minimum it requires two encounters with the inciting agent; however, in many cases, it may require repeated exposures over weeks or months to model the more chronic type of airway inflammation. Current animal models use single to multiple exposures to the test compound for sensitization, either via the respiratory tract or by dermal contact, followed by a single or repeated challenges for elicitation (Boverhof *et al.*, 2008). For the identification of chemical-induced respiratory sensitization and allergy the inhalation route should be given preference (Pauluhn and Mohr, 2005). For any animal system to yield useful and valid insights into disease it should exhibit a phenotype of asthma similar to that occurring in humans. In this context, it has become apparent that the more persistent phenotype of allergic lung inflammation and remodeling requires chronic repeated inhalation challenges to haptens (or antigens) rather than an all-or-nothing, single sensitization excursion (Kips *et al.*, 2003; Kumar and Foster, 2002; Kumar *et al.*, 2000; Leigh *et al.*, 2002; Palmans *et al.*, 2000, 2002a, b; Tomkinson *et al.*, 2001).

The conflicting results of the role of Th1 cells in many of these models may indicate that the timing of Th1 cells in the setting of ongoing Th2-induced chronic airway inflammation is critical and determines the net outcome (Cohn *et al.*, 2004). Also the dose (irritant/nonirritant) and the site (skin/respiratory tract) of both the induction and elicitation exposures as well as their frequency may trigger a wide range of responses, compassing from costimulatory (adjuvant-like) activity to tolerance. Based on these variables, controversy surrounds the nature of antigen, the role of specific antibodies and the role of T-lymphocyte subsets in orchestrating the pathophysiological response leading to diisocyanate-related occupational asthma (Johnson *et al.*, 2004). Therefore, the focus of this animal model is on endpoints integrating the asthma-like respiratory response.

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Importantly, although short-term exposures to very high mass concentrations are experimentally convenient, this is quite unlike the recurrent long-term exposure to low mass concentrations of allergen experienced by humans with asthma. Repeated challenge exposures may aggravate preceding events allowing more conclusive and robust assessments when compared with single-challenge protocols. Moreover, this type of protocol appears to be amenable to investigate the dose-response relationship of elicitation responses in “hypersensitive,” that is, “asthmatic” rats. In this bioassay the hallmarks of asthma, that is, airway inflammation and specific responses suggestive of airway obstruction, are addressed. Other hallmarks of asthma, such as immediate-onset respiratory responses and nonspecific airway hyperreactivity to methacholine, have not been addressed as the former two endpoints have been shown to be experimentally more expedient and robust to probe for MDI-related responses upon inhalation challenge exposures in this animal model (Pauluhn, 2005; Pauluhn *et al.*, 2005). For agents causing preferentially delayed asthmatic responses airway inflammation can occur without increased airway hyperresponsiveness (Lemière *et al.*, 2000).

Of considerable interest is the evidence that the induction response is often correlated with the sensitizing dose per skin unit surface area (Griem *et al.*, 2003; van Loveren *et al.*, 2008). However, empirical data demonstrating that this paradigm can also be applied for the response occurring following elicitation is scarce. In fact, several studies have either demonstrated no correlation or a reciprocal relationship for the latter (Blaikie *et al.*, 1995; Hayes *et al.*, 1992; Herrick *et al.*, 2002; Pauluhn and Mohr, 2005; Rattray *et al.*, 1994). So far, none of the models commonly used studied the dose-response relationship of inhalation elicitation response in “hypersensitive” animals. Previous studies investigating the sequence of inflammatory events after repeated MDI inhalation challenges have shown an unequivocal temporal association of the frequency of inhalation challenges with more severe delayed-onset respiratory responses and recruitment of neutrophilic granulocytes in bronchoalveolar lavage (BAL) (Pauluhn, 2005; Pauluhn *et al.*, 2005). There is increasing evidence that neutrophils play a role in the pathogenesis of occupational asthma (Jatakanon *et al.*, 1999; Jung and Park, 1999; Lemière *et al.*, 2002; Lindén and Adachi, 2002).

This paper describes induction- and elicitation-related dose-response relationships with several objectives. The first aim is whether the dermal sensitization dose and surface area influence the subsequent elicitation response; this included a second aim, *viz.* to analyze the most critical metric of the sensitizing dermal dose and whether the elicitation response is more dependent on either the dose per unit surface area of the exposed skin or the dose-to-body weight relationship. The third aim addressed whether the elicitation response is more dependent on previous inhalation elicitation doses or more on the skin sensitizing dose, using a dose-escalation challenge regime. Conceptually, the dose-response analysis following

inhalation challenge in “asthmatic” rats attempts to achieve a comparison of pulmonary irritant thresholds concentrations derived in “asthmatic” rats relative to naïve rats.

METHODS

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% 4,4'-MDI) with higher oligomeric MDI as balance. The free isocyanate (NCO) content was 31%. During handling and storage the headspace of MDI containing containments was purged with dry nitrogen to remove air and humidity to prevent its decomposition. Acetone (Seccosolv, max. 0.01% H₂O) and Olive oil were from Merck and Fluka, respectively.

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 (one 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 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 215 ± 15 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 0600 h. The studies described were conducted in accordance to the EU animal welfare regulations (European Community, Directive 86/609/EEC, 1986).

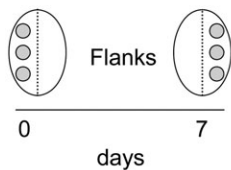
Experimental Design and Exposure Protocol

Dose and metric of dermal sensitization. The dependence of the topical sensitization dose and surface area upon the subsequent elicitation response was investigated as detailed in Figure 1 (protocol 1) and Table 1. The dermal sensitization phase consisted of two administrations on days 0 and 7 followed by four time-spaced inhalation challenges. Using this protocol three groups of rats were topically dosed with 40, 10, and 2.5 µl of MDI per rat. Each dose group consisted of three subgroups with dosed surface areas of 3.1, 6.3, and 12.6 cm², 0.79, 1.6, and 3.1 cm², and 0.39, 0.79, and 0.79 cm², respectively. Undiluted MDI was directly applied to the shaved skin (shaving was one day prior to administration) of the contralateral dorsal flanks on days 0 and 7. The different doses were administered by using aluminum foil disks of defined diameters. A defined volume of MDI was applied to each disk (Table 1). The dose transferred onto the skin was determined by the difference in weight of each disk before and after skin transfer. This determined mass of MDI per body weight or surface area is shown in Table 1. Rats were prevented from grooming or scratching by wearing an Elizabethan collar until the morning following each dosing session (Buster Birdcollars; Kruuse, DK, Cat no.: 273375). Details of this protocol have been presented elsewhere (Pauluhn, 2005, 2006). Animals were then challenged four times with 37.9 mg MDI/m³ aerosol (exposure duration 30 min) on target days 20, 35, 50, and 65. All groups were challenged simultaneously during the first three challenges whereas the last challenge was performed sequentially (one group per day) due to the elaborate procedures used. One naïve control group was challenged four times with 37.9 mg MDI/m³, as with the sensitized groups. The other naïve control group was not challenged at all. This allows a better appreciation of irritation-related changes in naïve animals as a result of the repeated inhalation challenges (groups 1 and 11, Table 1).

Dose of dermal sensitization and influence of elicitation dose. It is generally recognized that a higher dose of the same substance used for sensitization will require a lower dose for elicitation of a contact allergic

Protocol 1: Does sensitization dose and surface area influence the subsequent elicitation response?

Sensitization phase



Dosing on contralateral flanks. For disk number, surface area, and dose see Table 1.

Elicitation phase

30-min inhalation challenge with 37.9 mg MDI/m³

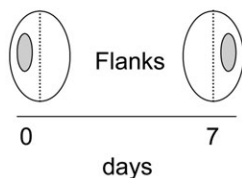


Physiological measurements after 4th challenge for approximately 20 hours

Sacrifice and bronchoalveolar lavage one after the 4th challenge

Protocol 2: Is the elicitation response more dependent on previous elicitation doses or more on skin sensitization dose?

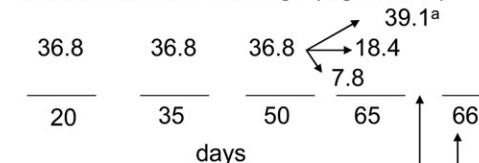
Sensitization phase



Dosing on contralateral flanks. For sensitization regime and dose see Table 2.

Elicitation phase

30-min inhalation challenge (mg MDI/m³)



Physiological measurements after 4th challenge for approximately 20 hours

Sacrifice and bronchoalveolar lavage one after the 4th challenge

a) Subgroups were challenged to either 7.8, 18.4 or 39.1 mg MDI/m³

FIG. 1. Two types of dose-response relationships were addressed. The first examined the influence of sensitization dose and surface area on the subsequent elicitation response, the second whether the elicitation response is more dependent on previous elicitation doses or more on the skin sensitizing dose.

reaction (Friedmann, 1990; Friedmann *et al.*, 1990; Hostynek and Maibach, 2004; van Loveren *et al.*, 2008). This aspect has been compared in a separate study (see Table 2) in which rats of group 1–5 (naïve controls) were not sensitized but subjected to a single or repeated inhalation challenge. Animals of group 6 were sensitized to MDI using a topical dose approximately four times as high as used in the high-level exposure groups 2–4 shown in Table 1. This group 6 was challenged four times with 15.7 mg MDI/m³, a concentration which is similar to the intermediate concentration used at the fourth challenge in the dose-escalation study (Fig. 2/protocol 2, Table 2). Data from groups 1, 2, and 6 (Table 2/protocol 2) were extracted from a previously published study (Pauluhn *et al.*, 2005).

Dose dependence of elicitation dose. Rats of groups 7–9 (Table 2) were sensitized to a topical dose that was similar to the high-level exposure groups 2–4 using protocol 1 (Table 1). In contrast to protocol 1, MDI was dissolved in 20% AOO (acetone:olive oil 4:1) and applied directly onto the contralateral flanks on days 0 and 7. Details addressing procedural aspects and the influence of the vehicle on elicitation-related responses are published elsewhere (Pauluhn, 2008). Akin to the above protocol 2, groups 3–5 (naïve controls) and MDI-sensitized groups 7–9 (Fig. 2/protocol 2, Table 2) were three times challenged as described above with 36.8 mg MDI/m³. At the fourth challenge

a dose-escalation-like regime was applied. Subgroups of six rats were either challenged with 7.8, 18.4, or 39.1 mg MDI/m³. The naïve control groups 3–5 was challenged using an identical regime. Based on previous experience, it can be assumed that at the time of the fourth challenge MDI-sensitized rats have developed “diisocyanate asthma”; thus, they are deemed to be hypersensitive to MDI (Pauluhn *et al.*, 2005).

Rationale for selection challenge concentrations. Diisocyanates have been shown to be potent respiratory tract irritants. However, little is known to which extent the pulmonary irritant threshold concentration characterized in naïve animals is lowered in “asthmatic” animals. Of note is that most potent (skin)sensitizers are also irritants and there is evidence that it is the irritant property that exerts the “adjuvant” effect of stimulating the innate immune response, hence potentiating the activation of the adaptive/acquired immune response (Friedmann, 2007).

Self-adjuvant effects due to respiratory tract irritation and stimulation of free nerve endings and their concentration \times time – dependence are commonly not well appreciated. For instance, rats exposed to a concentration \times time ($C \times t$) product of ≈ 1200 mg MDI/m³ \times min using exposure durations of 6, 3, 1.5 h, 45 min, and 23 min to MDI aerosol in concentrations of 3.4, 6.2, 12.7, 25.1, or 58.1 mg/m³, respectively, elaborated essentially identical levels of protein in

TABLE 1
Dermal Sensitizing Regimens to Study the Impact of Surface Area and Dose

Group	Disks/rat (no.)	Disk size (Ø cm)	SA/session (cm ²)	Dose/disk (µl)	Sensitizing dose/session (µl/rat)	Mean actual sensitizing dose/session (mg/rat)	Mean actual SA dose (mg/cm ²)	Mean actual body weight dose (mg/kg bw)	Challenges on day 20-35-50-65
1	—	—	0	0	0.0	0.0	0.0	—	1-1-1-1
2	4	2	12.6	10	40.0	45.6	3.6 ± 0.1	197.1 ± 10.4	1-1-1-1
3	2	2	6.3	20	40.0	41.5	6.6 ± 0.2	167.6 ± 3.9	1-1-1-1
4	4	1	3.1	10	40.0	44.8	14.3 ± 3.9	184.9 ± 3.9	1-1-1-1
5	1	2	3.1	10	10.0	11.1	3.6 ± 0.2	46.6 ± 2.2	1-1-1-1
6	2	1	1.6	5	10.0	11.7	7.5 ± 0.2	48.6 ± 1.8	1-1-1-1
7	1	1	0.79	10	10.0	11.3	14.4 ± 0.7	47.8 ± 3.2	1-1-1-1
8	1	1	0.79	2.5	2.5	3.0	3.8 ± 0.3	12.8 ± 0.8	1-1-1-1
9	2	0.5	0.79	1.25	2.5	3.0	3.8 ± 0.2	12.6 ± 0.9	1-1-1-1
10	1	0.5	0.39	2.5	2.5	2.9	7.3 ± 0.2	12.3 ± 1.2	1-1-1-1
11	—	—	0	0	0.0	0.0	0.0	—	0-0-0-0

Note. Animals (eight rats per group) were dosed on days 0 and 7 on contralateral flanks. Animals of the control groups 1 and 11 were not sensitized. Dermal administrations were made on days 0 and 7 using undiluted MDI on the contralateral dorsal area of the trunk (groups 2–10). Each group consisted of eight male rats. Dosing utilized a well-defined disk of aluminum foil. The dose of MDI transferred to the skin was empirically determined (difference of weight before/after transfer to the skin). The column “mean actual sensitizing dose/session” represents the mean (± SD) administered dose from days 0 and 7. Group 1 rats were challenged with MDI aerosol in the same manner as the animals of groups 2–10, whereas group 11 rats were not challenged at any time point. The mean challenge concentration was 37.9 ± 3 mg MDI/m³ for groups 1–10. The duration of challenge was 30 min starting with day 21 every 2 weeks. SA: surface area. —: not applicable.

BAL as index of acute pulmonary irritation (Pauluhn, 2000, 2002). This exposure dose increased BAL-protein by ≈60% above nonexposed controls. Based on this, ≈1200 mg MDI/m³ × min, which is equivalent to ≈40 mg MDI/m³ × 30 min challenge duration, is considered to be minimally irritant to the respiratory tract. A conclusive concentration-dependent Instant stimulation of pulmonary irritant receptors began to occur at ≈16 mg MDI/m³ (Pauluhn, 2000). With regard to acute pulmonary irritation, ≈0.5 mg MDI/m³ × 360 min (≈180 mg MDI/m³ × min) was considered to represent the acute Integrated irritant threshold C × t product in naïve Wistar rats (Pauluhn, 2000). This threshold matched the calculated benchmark dose (data not shown) based on alveolar cell proliferation (increased pulmonary cell replication and BrdU labeling observed after 6 h/day, five times/week on 2 or 4 consecutive weeks; Kilgour *et al.*, 2002; Pauluhn *et al.*, 1999b). Hence, for the challenge periods of 30 min used in this study, the nonirritation threshold dose is considered to be ≈6 mg MDI/m³ whereas minimal irritation occurs at ≈40 mg MDI/m³. Concentration-dependent stimulation of nerve endings will occur above 16 mg MDI/m³. This matrix served as basis for the design of the dose-escalation protocol 2.

Timing of measurements. Four rats from groups 1–10 (Fig. 1/protocol 1, Table 1) and subgroups 3–5 and 7–9 (Fig. 2/protocol 2, Table 2) were monitored for delayed-onset respiratory responses in barometric plethysmographs after the fourth MDI inhalation challenge. Measurements commenced shortly after MDI-challenge and continued to the morning of the following day. The time delay between cessation of MDI-challenge and the start of data collection was approximately 0.5 h. All rats were sacrificed one day after the fourth challenge. Following exsanguination, the weights of the lung and lung-associated lymph nodes (LALN) were determined. The lungs were lavaged for the analysis of inflammatory endpoints, including cytodifferentiation. Details addressing the exposure technology and methods, including the lavage procedures used were published in detail previously (Pauluhn *et al.*, 2005, 2008).

Analysis of Delayed-Onset Respiratory Response

Measurements took place in 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 four chambers. Data

TABLE 2
Dose-Response Challenge Protocol

Group	Dermal sensitization	Challenge 1-2-3, MDI (mg/m ³)	Challenge 4, MDI (mg/m ³)
1	—	15.7 ± 1.4	15.7 ± 1.4
2	—	—	15.7 ± 1.4
3	—	36.8 ± 6.0 ^a	7.8 ± 0.2
4	—	36.8 ± 6.0	18.4 ± 0.1
5	—	36.8 ± 6.0	39.1 ± 0.9
6	2 × 150 µl—100%	15.7 ± 1.4	15.7 ± 1.4
7	2 × 200 µl—20% AOO	36.8 ± 6.0	7.8 ± 0.2
8	2 × 200 µl—20% AOO	36.8 ± 6.0	18.4 ± 0.1
9	2 × 200 µl—20% AOO	36.8 ± 6.0	39.1 ± 0.9

^aInstability of aerosol generation system during the second challenge (31.6 mg/m³). Animals of the control groups 1, 2, and 5 were not sensitized. In group 6 rats were sensitized by dermal administrations as follows: day 0: 150 µl of MDI (undiluted) on the dorsal area of the trunk (treated area approximately 5–8 cm²), day 7: booster administration to the skin of the dorsum of both ears using 75 µl of undiluted MDI per ear. Rats of groups 7–9 were sensitized using by dermal administrations of 200 µl of MDI 20% in AOO on the contralateral dorsal flanks on days 0 and 7 (treated area approximately 10 cm²). With the exception of the rats of group 2 all remaining rats were challenged by inhalation with MDI aerosol for a duration of 30 min starting with day 21 every 2 weeks. The mass median aerodynamic diameter was ≈1.7 µm, the geometric standard deviation was ≈1.9. Data represent means ± SD.

collection commenced shortly after placing the animals into the precalibrated barometric whole-body plethysmographs (Buxco Electronics, Troy, NY; modified; software used for data acquisition: BioSystem XA software Vers. 2.1.8., Buxco Electronics). Based on previous experience, data analysis focused on “enhanced pause” (Penh) (Pauluhn, 2004, 2005). Measurement of Penh by unrestrained plethysmography does not provide a direct assessment of a specific

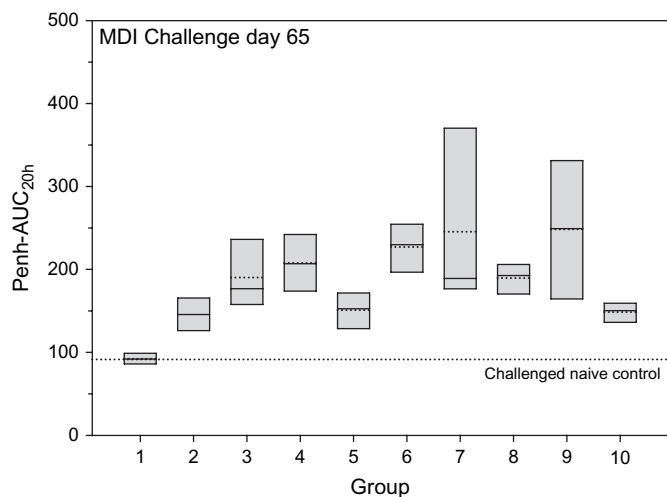


FIG. 2. Area under the curve (Penh-AUC_{20h}) in BN rats ($n = 4$) after the fourth challenge using protocol 1 (for details see Fig. 1 and Table 1). At all time point the mean challenge was to 37.9 ± 3 mg MDI/m³. Measurements commenced within ½ h in whole-body barometric plethysmographs and continued during a data collection period of approximately 20 h (overnight). Boxes represent Tukey Box Plots (dotted lines: mean, solid lines: median).

physiologic variable, and its limitations are well documented (Pauluhn, 2004). However, Penh can suitably be used to monitor functional changes delayed in onset. Data were collected every minute and digitally averaged over periods of 15 min. Details have been published elsewhere (Pauluhn, 2005).

Bronchoalveolar Lavage

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 lung was weighed and then 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, reinstalled for an additional 30 s. This cycle was repeated once. The BAL fluid was then centrifuged at $200 \times g$ for 10 min at 4°C (GPKR-refrigerated centrifuge, Beckmann, Munich, Germany). The supernatant was analyzed for total protein and lactate dehydrogenase (LDH). Cell counts in BAL were determined in re-suspended cells (Schärfe-System, Casy 1, Reutlingen, Germany). Cytospots were stained according to Pappenheim and differentiated into macrophages, lymphocytes, polymorphonuclear neutrophilic granulocytes (PMN), and eosinophils by light microscopy (300 cells were counted/cytospot).

Data Analysis

Data were analyzed by one-way ANOVA followed by a multiple comparison Tukey-Kramer *post hoc* test. Statistical differences of Penh between control and MDI-challenged groups (protocol 2) were evaluated by a Mann-Whitney Rank Sum Test one-way analysis of variances on ranks and a pairwise multiple comparison Tukey *post hoc* test (SigmaStat 3.1, Systat Software, Point Richmond, CA). For all tests the criterion for statistical significance was set at $p < 0.05$.

RESULTS

Metric of Dermal Sensitization

The mean dose-to-body weight relationships ranged from 12 to 197 mg MDI/kg bw whereas the dose-to-surface area

relationships ranged from 3.5 to 14.2 mg MDI/cm² with dosed surface areas from 0.4 to 13 cm² (Table 1).

Animals were challenged four times by inhalation to a mean concentration of 37.9 ± 3 mg MDI/m³ for a duration of 30 min starting as illustrated in Figure 1 (protocol 1). All endpoints were determined after the fourth challenge. For each group the area under the curve of postchallenge measurements of Penh over a time period of approximately 20 h (Penh-AUC_{20h}) is illustrated in Figure 2. This analysis revealed AUCs of variable intensity without any conclusive dependence on the sensitization regime (groups 2–10). The Penh-AUC_{20h}s of these groups were higher than the AUC of the nonsensitized but challenged control group.

Endpoints determined in BAL were significantly increased in all groups sensitized to and challenged with MDI in comparison with the equally challenged naïve control. Independently of the sensitizing dose and associated metric, consistent differences across sensitized and challenged groups did not emerge (Fig. 3). In turn, a trend of increased eosinophilic granulocytes in BAL was observed at lower doses. The slight difference between naïve controls either challenged four times (group 1) or singly (group 10; fourth challenge only) did not gain statistical significance. The diagnostic power of total cell differential counts instead of relative differential counts was compared in Figure 4. This comparison revealed no particular advantage of absolute counts over relative counts. Due to the higher variability of the former, data analysis on relative counts was given preference.

The most pronounced difference between naïve and sensitized animals was demonstrated by significantly increased counts of neutrophilic granulocytes (PMNs) in BAL. The comparison of the variable induction regimens show that neither the total dose per body weight nor the total dose per surface area appears to be critical for the outcome observed upon elicitation.

Dose of Dermal Sensitization and Influence of Elicitation Dose

The protocols compared in this study segment are detailed in Table 2 and Figure 2. In fact, two protocols were compared: the first utilized a high dermal sensitization dose regime (2×150 µl of MDI per rat) followed by four borderline-irritant MDI-aerosol challenges (4×15.7 mg/m³, see Table 2). The protocol addressing the dose dependence of elicitation responses utilized a lower dermal sensitization dose regime (2×40 mg MDI/rat dissolved in AOO) followed by three minimally irritant MDI-aerosol challenges of 36.8 mg/m³ and one additional 30-min challenge to either 7.8, 18.4, or 39.1 mg/m³ (Fig. 1/protocol 2; Table 2). Groups 1 and 2 are the control groups for group 6 whereas groups 3–5 are the respective controls for groups 7–9.

Distinct differences in breathing patterns (Penh) between equally challenged controls (groups 3–5) and MDI-sensitized rats (groups 7–9) occurred. Also the vigor of the delayed

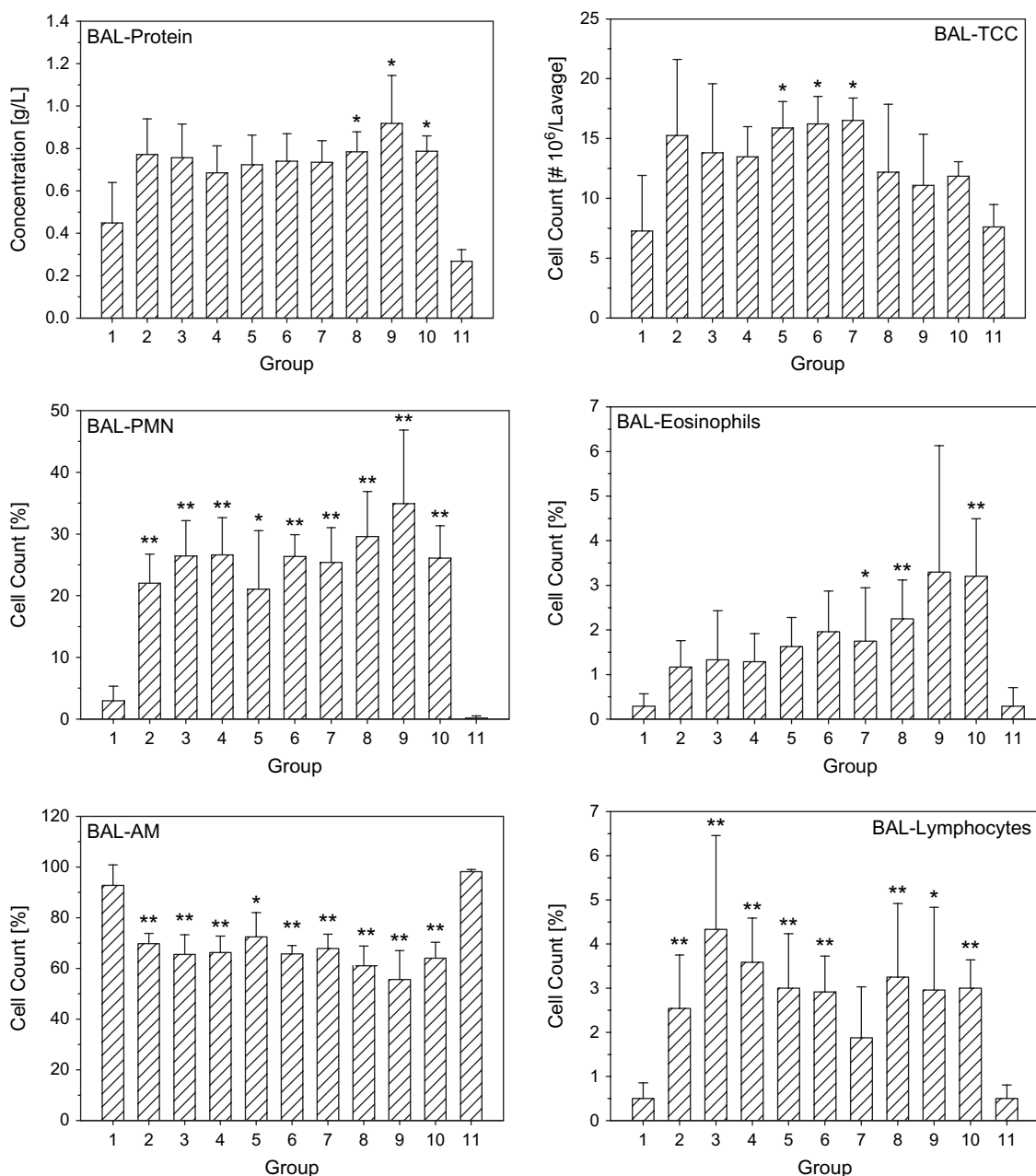


FIG. 3. Animals were topically sensitized with neat MDI and challenged with a mean concentration of 37.9 ± 3 mg MDI/m³ aerosol for a duration of 30 min (for group allocation and sensitizing regimen see Fig. 1/protocol 1 and Table 1). Animals were sacrificed for BAL one day after the fourth challenge. TCC: total cell count of lavaged cells, PMN: neutrophilic granulocytes. Cell differential counts were based on percentages. Bars represent means \pm SD ($n = 8$). Asterisks denote significant differences to the rechallenged naïve control (group 1). Differential counts were log-transformed prior to analysis. Significant differences are indicated by asterisks (* $p < 0.05$, ** $p < 0.01$).

respiratory responses showed a dependence on the elicitation dose (Figs. 5 and 6). The severity of respiratory responses is reflected best by the Penh-AUC_{20h}. However, the AUC value depends on both the magnitude of changes occurring approximately 2–4 h after the onset of data collection as well as the less pronounced but more sustained changes in breathing patterns over the remaining data collection period.

The results depicted in Figure 7 clearly illustrate that differences in BAL-endpoints between equally challenged naïve control rats and MDI-sensitized rats exist. However, with regard to elicitation-dependent dose-response analysis, with the exception of PMNs, most endpoints in BAL and lung or LALN weights did not demonstrate any remarkable dependence on the sensitization and challenge protocol applied. Consistent with

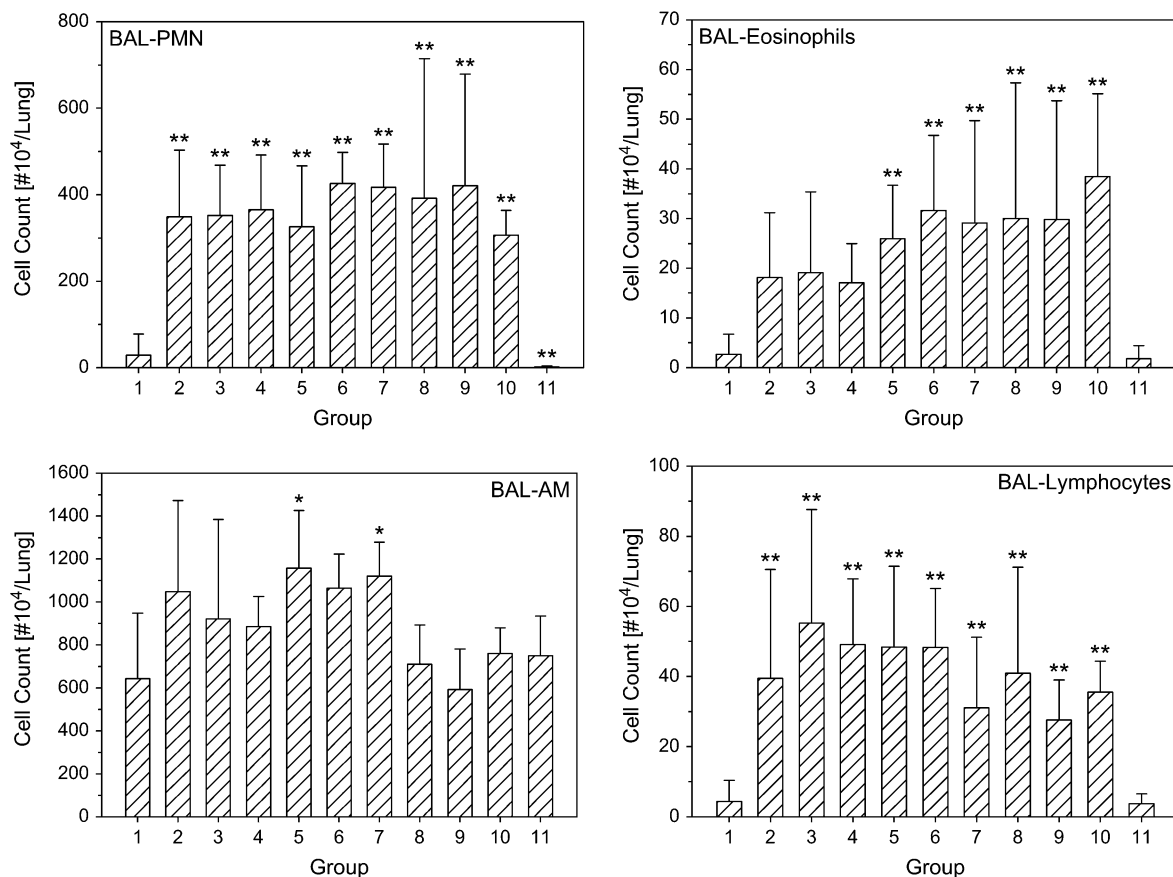


FIG. 4. Cell differential counts were adjusted on total cell counts. For further details see legend of Figure 3.

the findings of the dose metric study, PMNs in BAL appear to be most suitable to probe for elicitation-dependent differences in response. Following this paradigm, the elicitation threshold in “asthmatic,” that is, prechallenged rats clearly appears to be depending more on the sequence and dose of inhalation challenges than on the dermal sensitization dose (Fig. 7).

The Penh-AUC_{20h} of delayed respiratory responses correlated with the individual rats’ influx of PMNs in BAL (Fig. 8). The key inflammatory endpoint in BAL, PMNs, and the delayed respiratory response (Penh-AUC_{20h}) were dependent on the concentration used at the last challenge (Fig. 9). Based on the concentration-dependence of BAL-PMNs, 2.7 mg MDI/m³ (81 mg MDI/m³ × min) is considered to be the threshold concentration to elicit respiratory responses in “asthmatic” rats. Although these data represent entirely different types of responses their concentration-dependence paralleled.

DISCUSSION

With regard of hazard characterization of agents causing respiratory allergy, quantitative dose-response analyses are at

their infancy. Although there are many mechanistic similarities between induction and elicitation, in reality it is not normally possible to predict anything about elicitation from an appreciation of induction potency (van Loveren *et al.*, 2008). Ample published evidence support a concept that the threshold dose of elicitation is not an inherent property of an allergen, but may also be a consequence of the severity of the induction regime (Hostynek and Maibach, 2004).

Barometric plethysmography and the measurement of Penh appear to be an expedient tool to integrate several physiological endpoints (Kimmel *et al.*, 2002), most of them “breathing pattern” related, in a wholly noninvasive and nondisturbing manner so that nonspecific functional changes can readily be identified in studies where incremental rather than absolute changes over time are the primary focus. However, caution is advised to link these changes to any specific change in lung function or mechanics. It is generally conceived that measurements of Penh cannot directly be related to specific pathophysiological events, such as airway constriction. In this context it is also important to notice that nasal airway resistance is the largest component of the total airway resistance in obligate nasal breathing rodents which may superimpose all

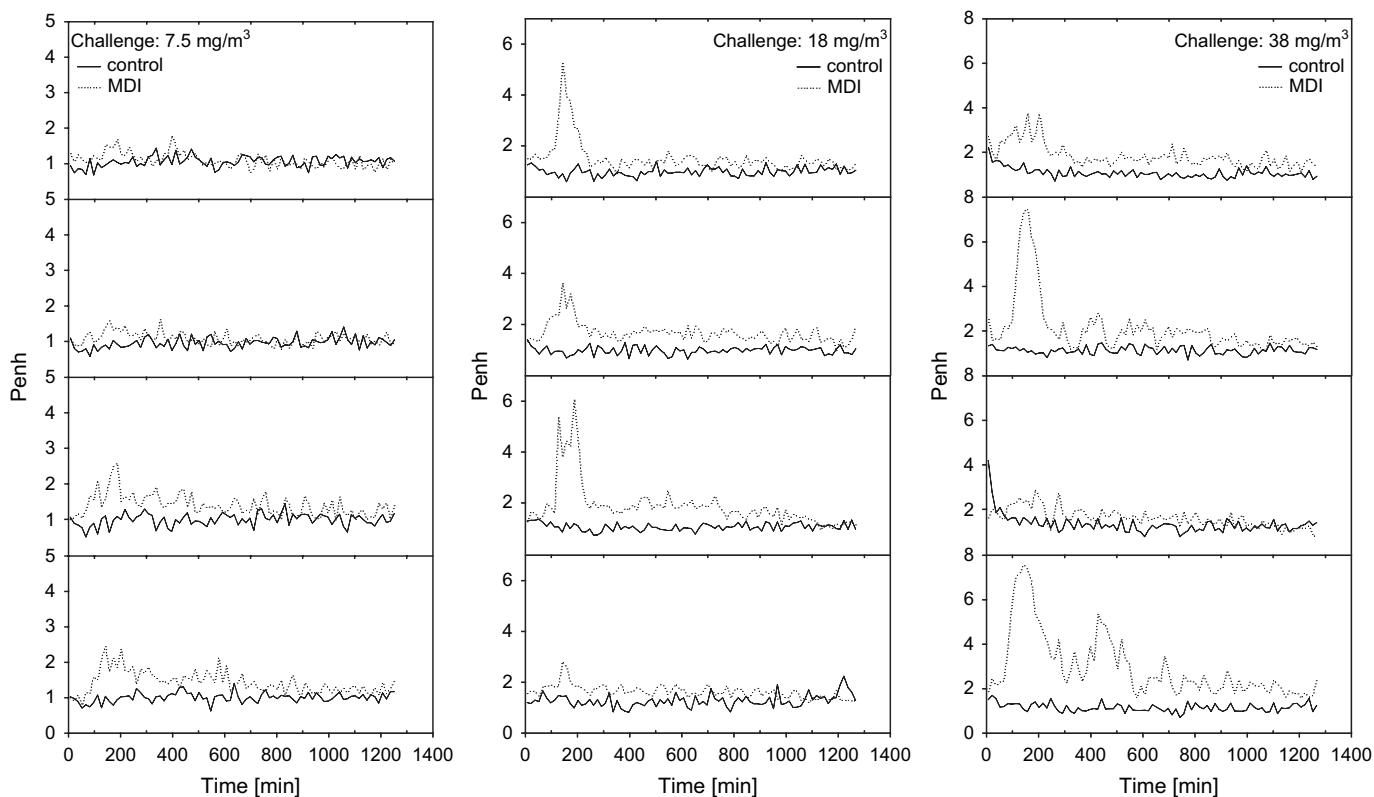


FIG. 5. Change of Penh in individual BN rats either naïve (control, groups 3–5, Table 2) or sensitized by dermal administration to and challenged with MDI (groups 7–9, Table 2). Three challenges were with 36.8 mg MDI/m³; the fourth challenge utilized a dose-escalation regimen. After the last challenge, measurements commenced within ½ h in whole-body barometric plethysmographs and continued during a data collection period of approximately 20 h (overnight). The four panels shown represent measurements from four individual rats per group.

other flow-restrictive effects occurring in the more distal airways. More detailed critiques regarding the interpretation of Penh have been published elsewhere (Adler *et al.*, 2004; Bates *et al.*, 2004; Hantos and Brusasco, 2002; Lundblad *et al.*, 2007; Mitzner *et al.*, 2003; Mitzner and Tankersley, 1998; Pauluhn, 2004). Accordingly, Penh might be viewed as a sensitive noninvasive but nonspecific functional endpoint suitable to probe for changes in respiration, secondary to specific response, in nonrestrained conscious rats.

The elicitation of respiratory allergy of potent respiratory tract irritants, such as diisocyanates, by single inhalation challenge is often biased to be (false) negative (Pauluhn *et al.*, 1999a). However, following repeated inhalation challenges this hazard potential became readily apparent with regard to increased counts of neutrophils in BAL and more rigorous respiratory responses delayed-onset (Pauluhn, 2005, 2008). The physiological effects (immediate and delayed-onset respiratory responses) of the IgE-dependent respiratory tract sensitizer trimellitic anhydride (TMA) could be satisfactorily modeled in this bioassay (Pauluhn, 2003; Pauluhn *et al.*, 2002), whereas for the known human asthmagen MDI a repeated inhalation challenge protocol was required to demonstrate the delayed-onset asthmatic response (Pauluhn, 2005). Karol *et al.* (1994) suggested that specific IgE might be associated with the

provocation of an early rather than a late asthmatic response in inhalation testing. Keskinen *et al.* (1988) found that subjects inflicted with diisocyanate asthma elaborating specific IgE had

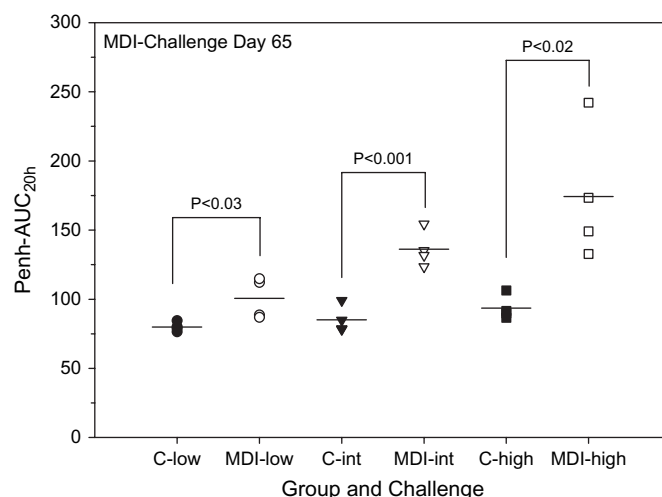


FIG. 6. Area under the curve (Penh-AUC_{20h}) of individual rats' based on changes of Penh after the fourth MDI-aerosol challenge (see Fig. 5). Naïve control rats (C) and MDI-sensitized rats (MDI) were challenged as indicated in Table 2. Suffixes represent challenge concentrations of MDI (mg/m³).

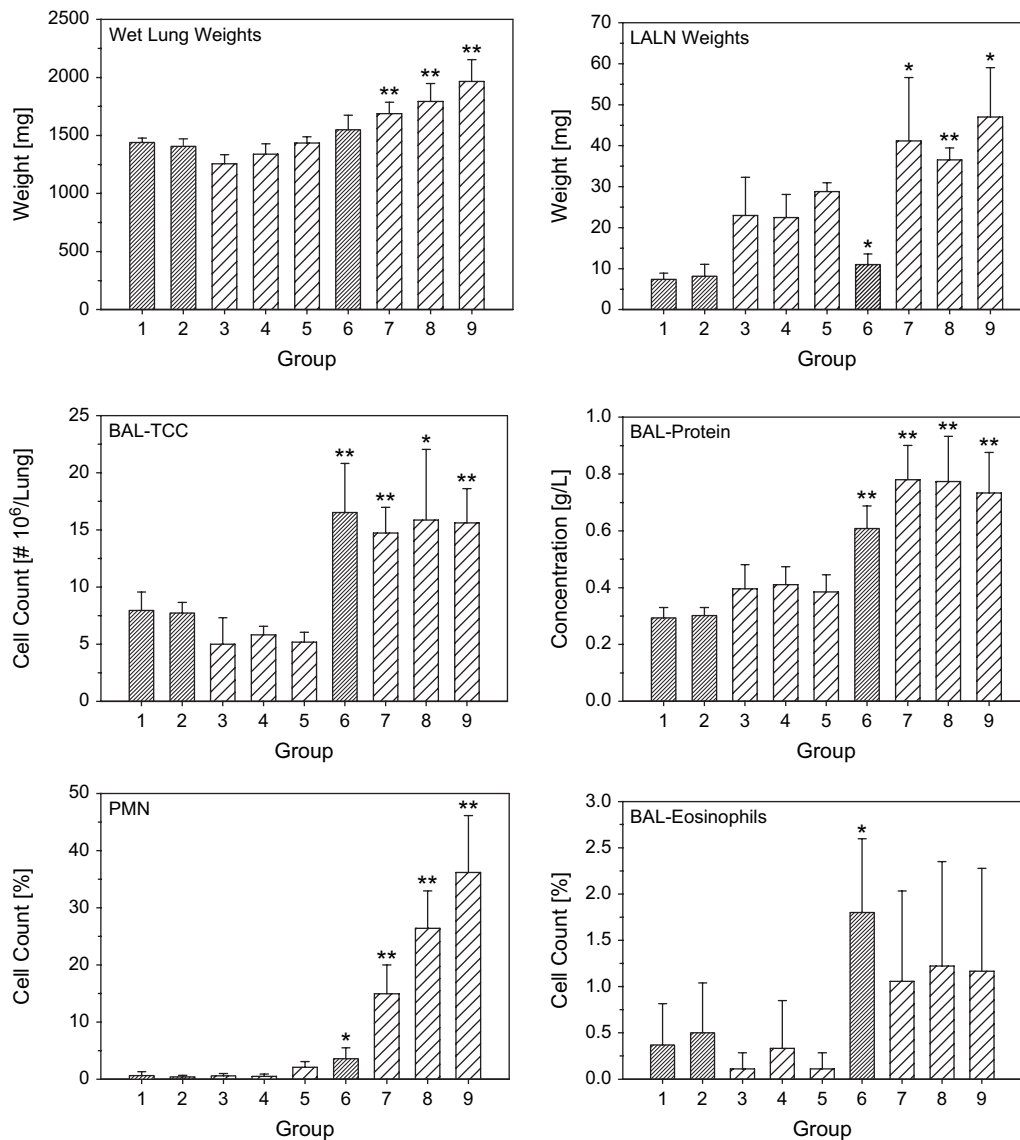


FIG. 7. Wet lung weights, LALN weights, total cell counts of lavaged cells (TCC), neutrophilic granulocytes (PMN), protein, and LDH in BAL fluid of rats challenged with MDI aerosol. Groups 1–5 represent naïve controls challenged with MDI aerosol, groups 6–9 were sensitized to MDI (for details see Table 2). Hashed bars (fine): four challenges with 15.7 mg MDI/m³, hashed bars (coarse): three challenges with 36.8 mg MDI/m³, fourth challenge utilized a dose-escalation regimen. Bars represent means \pm SD ($n = 6$). Asterisks denote significant differences to the re-challenged naïve control (group 1). Significant differences are indicated by asterisks (* $p < 0.05$, ** $p < 0.01$).

an immediate asthmatic response, and none of those with late responses had specific IgE. Hence, in humans, this type of asthma appears to be associated with inflammatory changes and significant functional changes, often delayed or dual in onset (Aul *et al.*, 1999; Leroyer *et al.*, 1998; Vogelmeier *et al.*, 1991). In humans, a mixed eosinophilic and neutrophilic inflammation has been shown, but neutrophil inflammation seems to be more common (Lemière *et al.*, 2002).

Similar conclusions can be drawn from the BN rat MDI-asthma model as the BN rat model appears to mirror common findings observed in human diisocyanate asthma. Although of ultimate importance for hazard characterization, the $C \times t$

exposure relationships required to induce (sustained) airway inflammation and asthmatic reactions is often ill-appreciated in animal models, in spite of robust published evidence from controlled human challenge studies demonstrating such a relationship (Lemière *et al.*, 2002). It is important to note that the $C \times t$ relationship has also been defined as a key feature that determines the asthmatic reaction to isocyanates in humans (Malo *et al.*, 1999). It also serves as unifying concept for the comparison of different exposure regimens.

The applied challenge regime utilized 2-week spacing between each 30-min challenge period in order to minimize carry-over effects of irritation from one challenge to another.

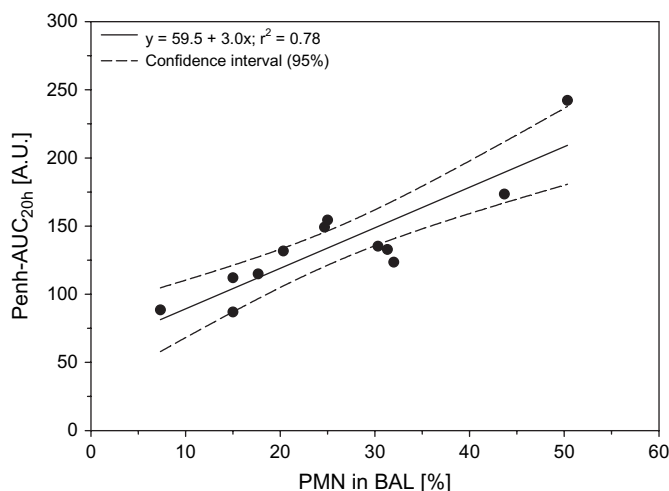


FIG. 8. Correlation of area under the curve (Penh-AUC_{20h}) of individual rats' after the fourth MDI-aerosol challenge of group 7–9 (see Figs. 4, 5 and Table 2) and neutrophilic granulocytes (PMN) in BAL.

This time period has been demonstrated to be sufficient by time-course analyses of neutrophils and eosinophils in BAL in BN rats after challenge with ovalbumin (Schneider *et al.*, 1997). Thus, the protocol was designed to focus on “allergic memory” rather than cumulative irritant responses. In a previous time-course study it was shown that with each inhalation challenge with 15.7 mg MDI/m³ a time-related increase of PMNs in BAL occurred without concomitant elevations of irritation-related endpoints (Pauluhn *et al.*, 2005).

Overall the findings from this study support the notion that skin exposure may be priming a graded “immunological memory” eventually leading to increased susceptibility to future inhalation challenges. Under the conditions of study no specific dependence on the surface area dose or dose-to-body weight could be demonstrated. In contrast, the magnitude of the lung response appears to be more depended on repeated inhalation challenge doses rather than the past initiating topical induction dose. This observation complements the findings from other authors demonstrating that if the concentration of hapten applied to the skin is kept constant, changing the area and hence the total dose applied, made very little difference to sensitivity (Friedmann *et al.*, 1990; Rees *et al.*, 1990; White *et al.*, 1986). From previous studies it was not apparent that small modifications in sensitization protocols caused specific biases of effects following repeated challenge exposures (Pauluhn, 2008). Therefore, the slightly differing sensitization protocols of groups detailed in Table 2 are not considered to be relevant for the outcome of study.

With regard to acute pulmonary irritation in naïve rats a challenge concentrations of ≈ 40 mg MDI/m³ \times 30 min, a $C \times t$ dose of ≈ 1200 mg/m³ \times min, is considered to be minimally irritant, whereas ≈ 180 mg MDI/m³ \times min represents the acute irritant threshold $C \times t$. In contrast, the dose-dependence of BAL-PMNs in repeatedly challenged “asth-

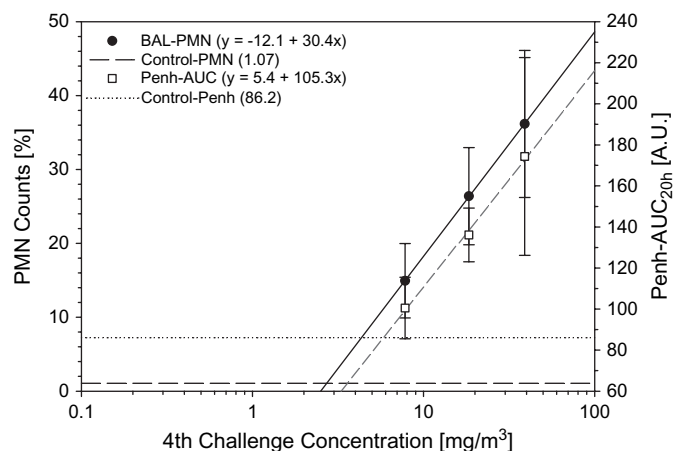


FIG. 9. Concentration-response analysis by linear regression of neutrophilic granulocytes (PMN) in BAL and area under the curve (Penh-AUC_{20h}) after the fourth MDI-aerosol challenge of groups 7–9 (Table 2). The means from the control (all challenge groups combined) for PMN and Penh-AUC_{20h} are indicated by dashed and dotted lines, respectively. Bars represent means \pm SD ($n = 6$).

matic” rats revealed 81 mg MDI/m³ \times min to be the threshold dose to elicit respiratory responses. Hence, the trigger dose in prechallenged rats for the acute asthmatic exacerbation is about half the trigger dose to elicit acute irritation in naïve rats. No doubt, asthmatic rats may experience an allergic response; however, the close relationship to the “irritant dose” makes it difficult to conclude unequivocally the relative contribution of immunological effects to irritant-related neural stimuli.

The level at which no health risk exists is usually operationalized by the NOAEL/UF (no-observed-effect level/uncertainty factor) approach. In such a pragmatic approach the respiratory tract irritant NOAEL should be identified in both naïve and “asthmatic” animals. Suffice it to say, the deposited dose at bronchial target sites in sensitized humans as well as the associated (patho)physiological airway responses differ in humans and rodents. To adjust for this quality of uncertainty an overall assessment factor of 30 (one for irritation at the site of initial deposition of aerosol, three for interspecies differences in dosimetry, and 10 for interspecies differences in relative abundance and susceptibility of bronchial musculature) appears to be reasonable. Accounting for these uncertainties, the estimated human 8-h working day threshold concentration to elicit respiratory responses in asthmatic subjects should be close to ≈ 6 μ g MDI/m³-8h (81 mg MDI/m³ \times min \times 1/30 \times 1/480) which is equivalent to ≈ 0.2 ppb total NCO (Bello *et al.*, 2007). This estimated value appears to be coherent with the respective empirically derived threshold dose from human inhalation challenge studies with MDI. When adjusted to an 8-h working day the respective concentration is ≈ 9 μ g MDI/m³-8h (0.15 mg/m³ \times 30 min = 4.5 mg MDI/m³ \times min; adjusted to 1/480 = 9.4×10^{-3} mg/m³-8h) (Leroyer *et al.*, 1998). This comparison shows a reasonable agreement of predicted and observed threshold concentrations.

Applying this paradigm to nonsensitized subjects, this means the adjustment focuses solely on differences in dosimetry of acute irritation-related effects. This would then lead to $\approx 0.13 \text{ mg/m}^3\text{-8h}$ time-weighted average (TWA) ($\approx 180 \text{ mg MDI/m}^3 \times \text{min} \times 1/3 \times 1/480$). Taking into account the current occupational exposure level of MDI (0.05 mg/m^3), the conclusions derived from this bioassay of respiratory irritation and allergy are not at variance with existing human evidence and current occupational workplace standards (Bello *et al.*, 2007; DFG-MAK, 2007; Lemièrre *et al.*, 2002; Malo *et al.*, 1999).

In summary, this dermal induction repeated inhalation challenge BN rat bioassay supports the conclusion that the topical concentration *per se* rather than the total dose per surface area skin or the dose per body mass appears to be the most important limiting factor for quantitative dose-response analyses and risk assessment. Hence, skin exposures may prime subjects to develop an increased susceptibility toward repeated inhalation challenges with MDI. However, in order to attain this state of increased susceptibility, it is conceived that acute irritant threshold $C \times t$ relationships must be repetitively exceeded. This threshold may be lowered by repeated high-level exposures. However, the difference between normal and hypersensitive rats was unexpectedly small. Overall, the comparison of various induction and elicitation protocols demonstrates that this animal model is amenable for dose-response analyses and risk characterization. The derived elicitation threshold appears to be plausible relative to human evidence. More research is needed to investigate whether the conclusions drawn for MDI are also valid to other diisocyanates.

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