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Repeated Inhalation Challenge with Diphenylmethane-4,4′-Diisocyanate in Brown Norway Rats Leads to a Time-Related Increase of Neutrophils in Bronchoalveolar Lavage After Topical Induction

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Diphenylmethane-4,4′-diisocyanate (MDI) is a low-molecular-weight chemical known to cause occupational asthma. The objective of this study was to evaluate the topical and inhalation routes of sensitization on the elicitation response of MDI in the Brown Norway (BN) rat model following repeated challenge exposures. BN rats were either induced topically (150 μl MDI on the flanks, booster administration to the skin of the dorsum of both ears using 75 μl/dorsum of each ear) or by inhalation (5×3 h/d, 28.3 ± 3.0 mg MDI/m^3 [$\pm$SD]). Inhalation challenge exposures with MDI (15.7 ± 1.4 mg/m^3, duration 30 min) were made on d 21, 35, 50, and 64. One day after each challenge, rats were rechallenged with methacholine (MCh) aerosol. Respiratory changes were monitored during challenges. One day after the MCh challenge, selected endpoints in bronchoalveolar lavage (BAL), the weights of lungs, and auricular and lung-associated lymph nodes were determined. After the first and last challenge, lymph nodes and lungs were examined by histopathology. Repeated challenge with MDI or MCh did not elicit marked changes in respiratory patterns at any time point. Mild but consistent time-related increased BAL neutrophils and slightly increased lung and lymph-node weights occurred in topically sensitized rats as compared to the remaining groups. In topically sensitized rats, in the lung histopathology revealed activated lymphatic tissue and an increased recruitment of airway eosinophils. Immunoglobulin (Ig) E determinations (serum and BAL) did not show any differences amongst the groups. Thus, high-dose topical induction with MDI was associated with a neutrophilic and eosinophilic inflammatory response in the lung after repeated inhalation challenge with MDI, with magnitude of effect dependent on the specific methodology used.

Investigation of the pathophysiological mechanisms of chronic asthma has been limited by the lack of satisfactory animal experimental models. Most of the currently applied bioassays utilize two phases: an induction phase, which includes single to multiple exposures to the test compound (sensitization) either via the respiratory tract or by dermal contact, followed by a challenge or elicitation phase. For any animal system to yield useful and valid insights into disease, it must exhibit an appropriate phenotype. It has became apparent that the more persistent phenotype of allergic lung inflammation and remodeling requires chronic repeated (high-level) exposures to antigens or haptens rather than an all-or-nothing, single sensitization excursion. Of considerable interest is the evidence that airway hyperreactivity does not appear to correlate with immunoglobulin (Ig) E titers or with the magnitude of the inflammatory response in the airway wall (Temelkowski et al., 1998). Among the issues that may contribute to some of the inconsistencies between animal studies is the fact that the employed routes,

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doses, and dosing regimens vary substantially from one animal model to another.

Most of the currently developed animal models of allergic airway inflammation have been restricted to acute inflammatory changes following relatively short periods of hapten or allergen exposure. 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 (Kumar & Foster, 2002; Leigh et al., 2002; Kumar et al., 2000). Repeated challenge exposure may exacerbate preceding events, allowing more conclusive and robust assessments when compared to short-term protocols (Johnson et al., 2004). Thus, studies investigating the sequence of inflammatory events after allergen challenge and the temporal association with specific endpoints might be critical for the definition of response, and to further our understanding of the factors involved in pathogenesis of respiratory hypersensitivity in this bioassay. Only a few studies have evaluated the sequence of inflammatory events taking place after repeated, chronic inhalation challenges. It was noticed that increases in maximal bronchoconstriction, associated increase in airway contractile tissue, and subepithelial fibrosis required a repeated chronic, not a single brief, allergen challenge protocol (Palmans et al., 2002a; Leigh et al., 2002; Kips et al., 2003).

The known human asthmagen diphenylmethane-4,4′-diisocyanate (MDI) has been evaluated in diverse animal models (Pauluhn et al., 1999). Opposite to studies with trimellitic anhydride (TMA), in most studies single challenge exposures with MDI aerosol, acute respiratory responses were equivocal. As shown for TMA, which is a benchmark respiratory sensitizer in all animal models currently in use (Arts et al., 1997, 1998, 2003; Cui et al., 1997; Elwood et al., 1991; Hilton et al., 1995; Pauluhn & Eben, 1991; Pauluhn et al., 1999, 2002; Warbrick et al., 2002), protocol determinants with regard to timing and frequency of challenge exposures can be instrumental in the outcome of the study (Pauluhn, 2003). The aim of the present study was to evaluate whether the sensitivity and robustness of this animal model could be increased by repeated challenge exposures.

METHODS

Test Material

Polymeric methylenediphenyl-4,4′-diisocyanate (MDI) was from Bayer AG, Leverkusen, Germany. The content of monomeric MDI (all isomers) was 42%. Methacholine chloride was from Sigma, Taufkirchen, Germany.

Animals and Maintenance

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 number 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 (±SD) were 230 g (±15 g). Animals were quarantined for at least 5 d prior to being placed on study. The light cycle was automatically controlled in the animal holding room to provide 12 h of fluorescent light and 12 h of darkness each 24 h. Temperature and relative humidity were continually monitored, with daily means in the range of 22°C and 40–60%, respectively. All experiments and procedures described took into account the European Union (EU) animal welfare regulations (Directive 86/609/EEC, 1986).

Study Design and Rationale for Dose Selection

This study consisted of naive controls (normal housing) and groups of BN rats that were sensitized epicutaneously or by repeated inhalation exposures. Two control groups were used: The control-1 group was repeatedly challenged with MDI on d 21, 35, 50, and 64, while control-2 was challenged once with MDI on d 64. For topical sensitization, the methodology used in previous studies with TMA (Pauluhn et al., 2002, 2003) and others (Arts et al., 1997, 1998) was used. For TMA, the sensitivity of the bioassay increased with increasing doses and number of challenges. Accordingly, starting on d 0, the rats received 150 μl MDI on the dorsal area of the trunk (treated area 5–8 cm^2), while on d 7, 75 μl MDI on the dorsum of each of both ears was administered. The skin areas were cleaned 1 d after administration. By the inhalation route, rats were sensitized using 3-h/d inhalation exposures on 5 consecutive days with 28.3 ± 3.0 mg MDI/m^3 (mean ± SD). The mass median aerodynamic diameter (MMAD) of aerosol was ~1.6 μm; the geometric standard deviation (GSD) was 1.6. On d 21, 35, 50, and 64 the rats were challenged to MDI aerosol (15.7 ± 1.4 mg/m^3, duration 30 min). Two days thereafter, eight rats per group and challenge time point were sacrificed, and lungs and lymph node weights (auricular, lung hilus) were determined. Lungs were lavaged for the analysis of endpoints suggestive of an inflammatory and immunological response. Serum total IgE was determined. The lung and lymph nodes (auricular, lung hilus) of rats sacrificed after d 21 and 64 challenges were examined by histopathology.

The selection of the concentration and regimen used for inhalation induction took into account previous studies in rats and guinea pigs and aimed at using a "high-dose" sensitization protocol. Karol and Thorne (1988) reported that mortality occurred on exposure day 3 in guinea pigs exposed to 72 mg MDI/m^3 for 3 h/d. Using rats, significantly increased protein and neutrophils in bronchoalveolar lavage (BAL) fluid and pneumonitis after a single 6-h exposure to 10 mg/m^3, which is equivalent to 20 mg/m^3 × 3 h, were observed (Kilgour et al., 2002). Significantly increased BAL-protein occurred in rats exposed to the 3-hr exposure equivalent of 6.2 mg MDI/m^3 (Pauluhn et al., 2002). At 39 mg/m^3, BAL protein was increased ~5 times above control (Pauluhn, 2004). With regard to changes in breathing patterns concentrations, ≥15 mg MDI/m^3 caused

slight, although distinct, changes in rats (Pauluhn, 2000) and guinea pigs (Pauluhn et al., 1999). This published evidence served as basis to use ~30 mg MDI/m^3 for 5 × 3 h/d for sensitization induction, a concentration likely to cause mild respiratory tract irritation. The challenge concentration of 15 mg MDI/m^3 for 30 min was believed to be high enough to cause minimal irritation without significant changes in breathing patterns in naïve rats.

Exposure Technique, Aerosol Generation and Characterization

Details addressing the exposure technology, methods, and lavage procedures used were published previously (Pauluhn, 2000). Briefly, at the end of the acclimatization period, rats were randomly assigned to the respective groups. Rats were exposed by *directed-flow* nose-only inhalation to analytically determined breathing zone concentrations. MDI was atomized using a digitally controlled Hamilton Microlab M pump (pump rate 10 μl MDI/min) and a modified Schlick-nozzle Type 970, form-S 3 (Schlick GmbH, Coburg, Germany) maintained at 40°C. The stability of the test atmospheres was monitored continuously using a RAS-2 real-time aerosol photometer (MIE, Bedford, MA). The exposure atmospheres were characterized using both the nitro reagent (*N*-4-nitrobenzene-*N*-*n*-propylammonium chloride) derivatization technique and filter analyses (glass-fiber filters, Sartorius, Göttingen, Germany). In essence, both methods revealed comparable results. The actual breathing concentrations shown in this article refer to filter analyses. Chamber air, sampled from the vicinity of the breathing zone of the rats, was sampled repeatedly during the study. For particle-size analyses, a low-pressure critical-orifice AERAS stainless steel cascade impactor (HAUKE, 4810 Gmunden, Austria) as used.

Bronchoalveolar Lavage

Details of the lavage technique have been published in detail elsewhere (Pauluhn, 2000). The supernatant was analyzed for the following factors: total protein, lactate dehydrogenase (LDH), nitrite, total IgE, and total cell count. For cytological evaluation of lavaged cells a Cytospin centrifuge (Centrifuge Cytospin 2, Shandon, Life Sciences International, Frankfurt, Germany) was used (2×10^5 per cytospot). The air-dried slides were fixed with a methanol:acetone mixture, stained according to Pappenheim, and mounted with Entellan (Merck). Cells were differentiated into macrophages, lymphocytes, granulocytes, and eosinophils by light microscopy (300 cells counted/cytospot). All granulocytes per cytospot were evaluated, and the number of eosinophils stained positively with the activation marker 3-nitrotyrosine (3-NT) was counted. Immunostaining utilized the following procedure: Cells were allowed to react with primary 3-NT antibody (Upstate Biotechnology, number 23446), followed by goat anti-rabbit IgG, $F(ab')_2$ fragment, alkaline phosphatase-conjugated (Molecular Probes number F-21456), and staining with fast red chromogen (DAKO EnVision System, number K4016) and counterstain with Mayers Hämalaun (Merck number 9249). NO (nitric oxide) was determined via nitrite that was measured using the Griess reaction. BAL supernatant was mixed with water and sulfanilic acid in phosphoric acid (5%). After addition of *N*-(1-naphthyl)-ethylenediamine, the corresponding azo dye was measured spectrophotometrically.

Total IgE in Serum and BALF

Total IgE was determined in the serum from blood collected by heart puncture at each necropsy and in the supernatant of BAL fluid (BALF). For determination, the following commercially available sandwich enzyme-linked immunosorbent assay (ELISA) was used: rat IgE ELISA quantitation kit, catalog number E110-117, Bethyl Laboratories, Inc., Montgomery, TX, including IgE monoclonal antibodies as standard (rat IgE myeloma; working range 50–0.78 ng/ml, 7 dilutions 1:2). All samples were tested in duplicates at 3 different dilutions which were for BALF 1:10, 1:30, and 1:100, and for serum 1:30, 1:100, and 1:300. Average values of duplicates were calculated from each standard and sample. Standard curves were calculated by KC4 software (Bio-Tek Instruments, Inc., Winooski, VT) using a logit/log fit curve (2-P). For evaluation an Elx808 automated microplate reader (Bio-Tek Instruments, Inc., Winooski, VT) was used. The "positive level" was set to a more than a factor of 2 increase over control values. Prior to storage the specimens were aliquoted and then kept frozen at −80°C to the day of evaluation. All specimens collected over time were assayed at once.

Analysis of Respiratory Response during MDI Challenge

All rats of the control-1, topical, and inhalation groups were challenged on d 21, 35, 50, and 64; the control-2 group was challenged only on d 64. Measurements were conducted in spontaneously breathing, conscious rats using a flow (volume displacement) mode nose-only plethysmograph. After acclimatization, baseline parameters were collected during a prechallenge air-exposure period (15 min), followed by the challenge period (30 min) and postchallenge measurements (30 min). Although other parameters describing the breathing cycle were measured, this analysis focused on the respiratory minute volume (MV) and expiratory time (ET). Further methodological details have been published elsewhere (Pauluhn, 1997, 2003).

Methacholine Challenge

Nonspecific airway responsiveness of rats to increasing concentrations of aerosolized methacholine hydrochloride (MCh) was measured in unrestrained, spontaneously breathing rats in calibrated barometric whole-body plethysmographs (Buxco Electronics, Troy, NY; modified; software used for data acquisition, BioSystem XA software version 2.1.8., Buxco Electronics, Troy, NY). Data analysis focused on "enhanced pause" (Penh). Measurements were made in the eight rats challenged with MDI the day before. After measurement of baseline respiratory patterns (exposure to air), either aerosolized isotonic

saline or an aqueous solution of MCh in increasing concentrations (spray solutions of 0.3%, 0.6%, 1.3%, 2.5%, and 5%, w/v) were nebulized sequentially through an inlet of the plethysmograph chamber for 1 min, followed by a data-collection period of 5 min for each step. More details are described elsewhere (Pauluhn, 2004).

Organ Weights Histopathology and Necropsy

Eight rats were used per interim necropsy performed 2 days after the MDI challenge on d 21, 35, 50, and 64 and 1 d after the MCh challenge. Animals were anesthetized with sodium pentobarbital (Narcoren; 120 mg/kg body weight, intraperitoneal injection). After blood sampling by heart puncture, complete exsanguination was achieved by severing the aorta abdominalis, which was examined grossly for abnormalities. After exsanguination, the lymph nodes, and the excised wet lungs of the animals were weighed. The lungs with trachea, the lung-associated lymph nodes, and the auricular lymph nodes were dissected and preserved in neutral, phosphate-buffered (10%, v/v) formaldehyde. The lung was intratracheally instilled with the fixative using a pressure of 20 cm H_2O. Next these organs were embedded in paraffin wax (Paraplast). The lungs were cut at five sagittal levels (one per lobe). Sections ($\sim$5 μm) were stained with hematoxylin and eosin (H&E) and examined by light microscopy. The grading utilized four scores: (1) minimal/very few/very small, (2) slight/few/small, (3) moderate/moderate number/moderate size, and (4) for severe changes (not applicable to this study). Histopathology was made on tissues from the first (d 23) and last sacrifice (d 64). The pathologist was aware of the exposure history but was unaware of any findings.

Statistical Evaluation of Data

Penh, organ and body weights, and IgE and BALF data were analyzed by one-way analysis of variance (ANOVA). Reported values are expressed as mean $\pm$ SD. The concentration of MCh causing a 100% increase in the individual animals' absolute baseline Penh following the exposure to saline (PC_{200}Penh) was calculated by interpolation of the dose-response curve using a polynomial function ($y = a + bx + cx^2$; $y =$ Penh, $x =$ percent of MCh aerosolized). The fitting of the parameters to this function to the experimental data used an iterative procedure (LSQR FORTRAN source code), and the PC_{200}Penh was calculated by interpolation starting with the saline exposure. The cumulative dose-response curves were compared through a one-way analysis of variance (ANOVA) followed by a Tukey–Kramer post hoc test. For all tests the criterion for statistical significance was set at $p < .05$ using a pairwise multiple-comparison procedure (Tukey-Kramer post hoc test). Quantal responses (histopathology) were compared with the concurrent control using the pairwise Fisher test with R $\times$ C chi-square test (Gad & Weil, 1982).

RESULTS

Clinical Observations, Body Weights, and IgE in Serum and BALF

About half the rats sensitized by repeated inhalation exposures with MDI aerosol displayed serous nasal discharge and labored breathing patterns. Some rats showed plugged/obstructed nostrils (polymerized MDI). One rat of this group succumbed on d 3 as a result of nasal plugs; upon mechanical removal of the nasal plugs the remaining animals recovered rapidly. Rats receiving a booster dose onto the dorsum of the ears showed skin lesions.

The determination of total IgE in serum did not reveal statistically significant differences between the groups (Figure 1). There was an apparent decrease in interanimal variability of total IgE over the duration of study (serum from all rats was determined at a single time point). Total IgE in BALF was in the range of the limit of detection ($\sim$3 ng/ml) without showing appreciable differences amongst the groups (data not shown).

Elicitation of Airway Hyperreactivity—MDI and MCh Challenge

Starting with d 21, all rats were challenged to an average concentration of 15.7 ± 1.4 mg MDI/m^3 on d 21, 35, 50, and 64, followed by an MCh challenge in 8 rats/group one day later. Control-1 rats were repeatedly challenged through d 64, whereas control-2 rats were challenged on d 64, only.

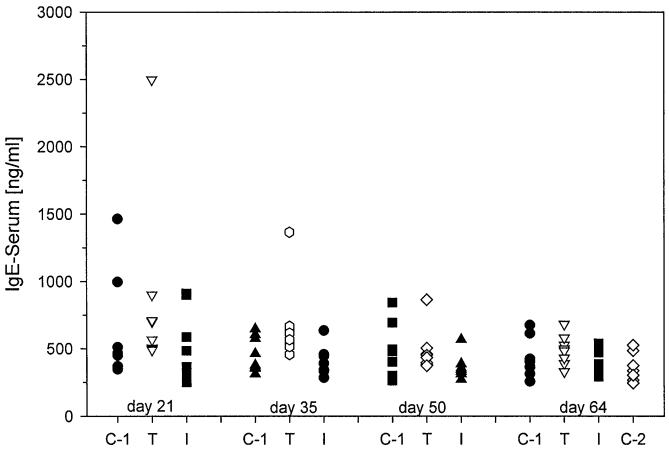

FIG. 1. Total serum IgE levels of individual Brown Norway rats that were sensitized either by (T) topical administration of undiluted MDI (150 μl on the shaved flanks on d 0 and 75 μl on the dorsum of each ear on d 7) or by (I) 5 $\times$ 3-h/d inhalation exposures to 28 mg MDI/m^3 on d 0–4. All rats were challenged with $\sim$15 mg MDI/m^3 for 30 min on d 21, 35, 50, and 64. Naive rats of the control-1 (C-1) group were repeatedly challenged, whereas those of the control-2 (C-2) group were challenged on d 64 only. Blood was sampled 2 d after MDI challenge at each interim sacrifice (8 rats/group) by heart puncture.

TABLE 1
Respiratory response upon inhalation challenge with MDI aerosol

Parameter	Day	Induction			
		Control-1	Topical	Inhalation	Control-2
MV_{1500}	21	0/8	0/8	4/8	—
	35	0/8	1/8	0/8	—
	50	0/8	0/8	0/8	—
	64	0/8	1/8	0/8	0/8
ET_{2000}	21	0/8	1/8	4/8	—
	35	0/8	1/8	1/8	—
	50	0/8	0/8	0/8	—
	64	0/8	0/8	0/8	1/8

Note. Indexes represent the intensity of response as area under the curve exceeding $\pm 3 \times$ SD of the individual prechallenge period of Brown Norway rats that were either sensitized by topical administration of MDI or by 5 × 3-h/d inhalation exposures to 28 mg MDI/m^3. All rats were challenged with ~15 mg MDI/m^3. Control-1 was repeatedly challenged; control-2 was challenged on d 64 only. MV, respiratory minute volume; ET, expiratory time. Indexes represent the arbitrarily selected threshold to define positive response. —, No challenge performed. #/#, Number of positive responders per group/number of rats per group evaluated.

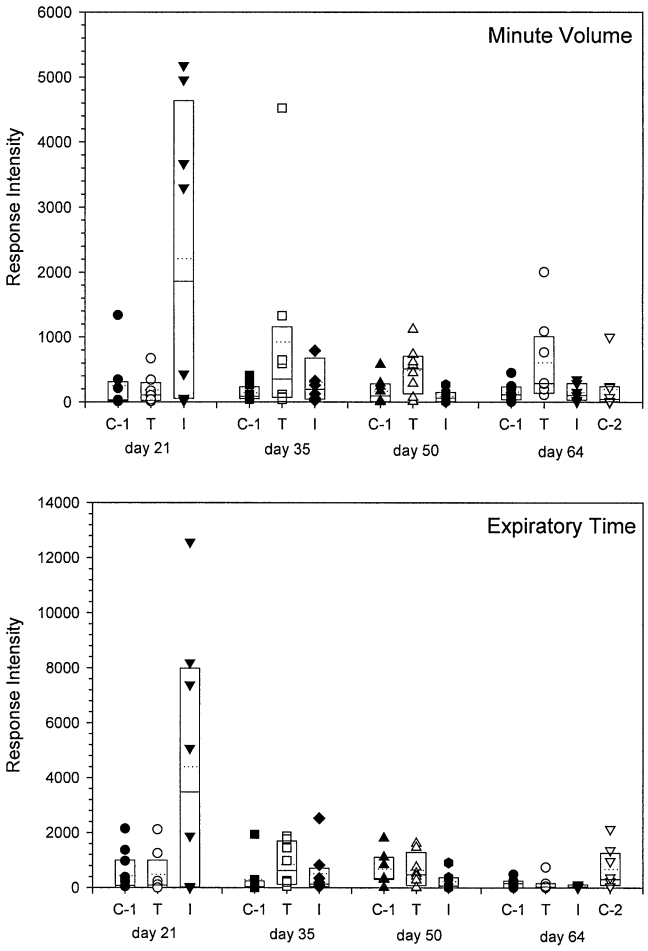

FIG. 2. Analysis of the intensity of respiratory response of Brown Norway rats that were sensitized either by (T) topical administration of undiluted MDI (150 μl on the shaved flanks on d 0 and 75 μl on the dorsum of each ear on d 7) or by (I) 5 × 3-h/d inhalation exposures to 28 mg MDI/m^3 on d 0–4. All rats were challenged with ~15 mg MDI/m^3 for 30 min on d 21, 35, 50, and 64. Naive rats of the control-1 (C-1) group were repeatedly challenged, whereas those of the control-2 (C-2) group were challenged on d 64 only. Data represent the area under the curve of individual rats' measurements of the respiratory minute volume (upper panel) and expiratory time (lower panel) and Tukey box plots (dotted line, mean; solid line, median).

During the 30-min MDI challenge and ~30-min postchallenge periods, data for breathing parameters were recorded. Changes in respiratory minute volume and expiratory time were more pronounced in the inhalation induction group on d 21, while subsequent rechallenges to MDI did not elicit any specific changes in breathing patterns (Table 1 and Figure 2). With regard to the decreased minute volume and increased expiratory time, the response occurring in the inhalation induction group differed qualitatively from the remaining groups (data not shown). The respiratory response during MDI challenge in the control groups receiving either a single or repeated challenge was essentially identical (Figure 2).

Penh measurements using a stepped MCh challenge protocol did not illustrate any shift in baseline levels (exposure to saline aerosol prior to MCh challenge) or provide evidence of increased airway responsiveness to MCh aerosol (Figure 3). Accordingly, also PC200Penh did not show any consistent time-related or route-of-induction-dependent differences among the groups, which could also be attributed to the high interanimal variability (data not shown).

Bronchoalveolar Lavage

The results from analyses in BAL are summarized in Table 2. BALF nitrite and BALF LDH of topically sensitized rats were somewhat different from the remaining groups at isolated time points; however, conclusive route-of-induction-related or time-related changes were not observed. BAL protein, neutrophils, and lymphocytes were significantly increased with increasing number of challenges in topically sensitized rats when compared to the control or inhalation induction groups. The total cell counts and eosinophil counts in BAL were similar in all groups on d 21 and 35, while at later challenges there was an apparent time-dependent decrease in total cell count in all groups except the topical induction group. From the analysis of the time course of the percentage of eosinophilic granulocytes in BAL, it emerges that the already spontaneously increased presence of these cells in this strain

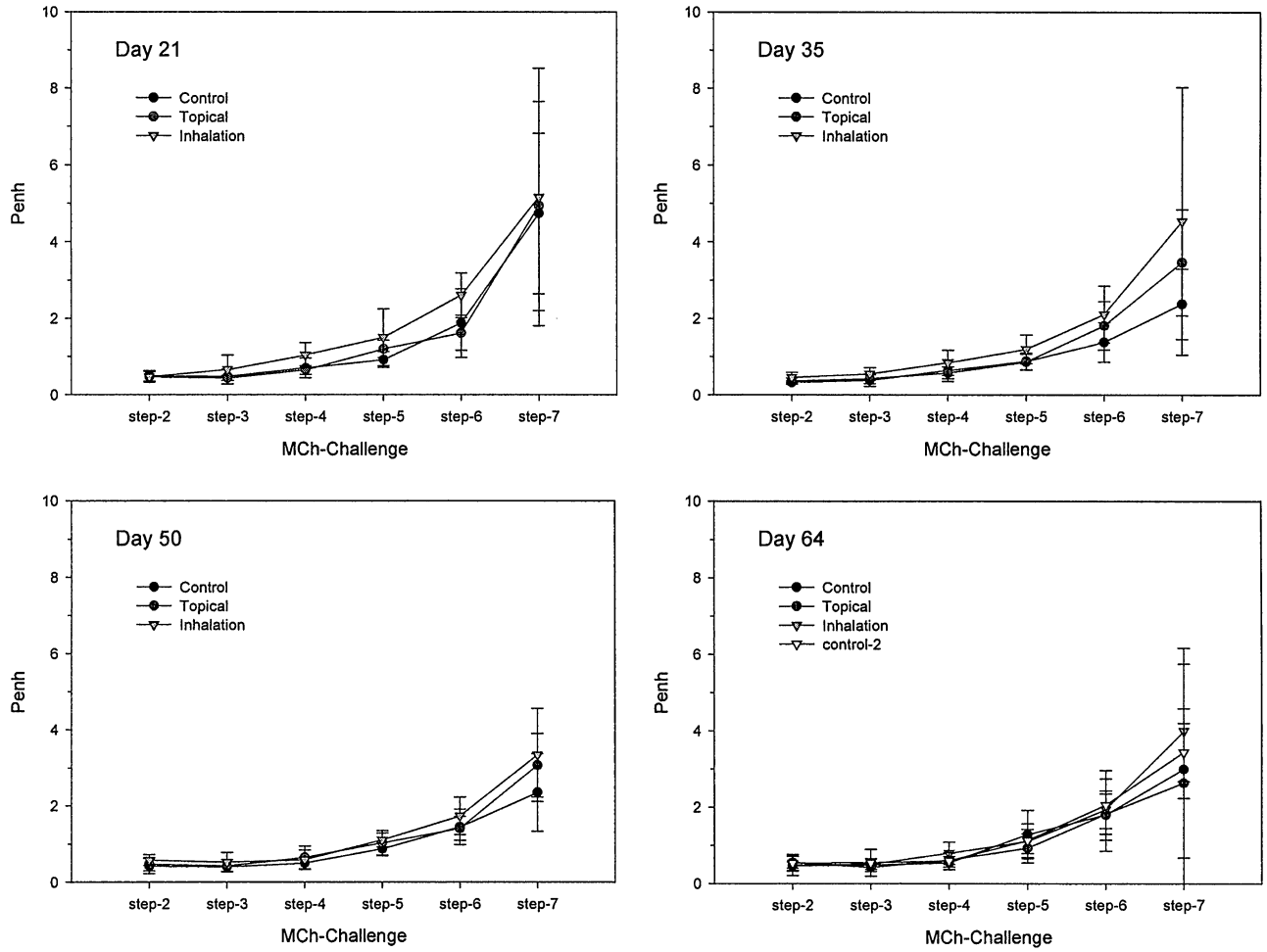

FIG. 3. Change of Penh (enhanced pause) during a stepped challenge with methacholine hydrochloride (MCh) aerosol in concentrations of 0% (saline), 0.3%, 0.6%, 1.3%, 2.5%, and 5% (w/v saline) of Brown Norway rats that were sensitized either by topical administration of undiluted MDI (150 μl on the shaved flanks on d 0 and 75 μl on the dorsum of each ear on d 7) or by 5×34-h/d inhalation exposures to 28 mg MDI/m^3 on d 0–4. All rats were challenged with ~15 mg MDI/m^3 for 30 min on d 21, 35, 50, and 64. One day later, they were challenged with MCh. Naive rats of the control-1 group were repeatedly challenged, whereas those of the control-2 group were challenged on d 64 only. Data points represent the means ± SD of eight rats per group.

of rats decreases with increased holding period. Despite this trend, the percentage eosinophils had a tendency to be somewhat higher in the topical induction group when compared to the remaining groups at each time point and especially at the end of the study. The remaining endpoints were indistinguishable from the controls. Anti-3-nitrotyrosine-stained eosinophilic granulocytes, which served as a marker of activation of these cells, did not show consistent effects at any time point.

Lung and Lymph Node Weights

Sacrifice of rats 1 d after the MCh challenge revealed mildly although significantly increased lung weights in the topical induction group after challenge d 50 and 64 (Table 3). In this group, the weights of the lung-associated and auricular lymph nodes were increased throughout the study. Relative to the control, in the topical group the increased weight of the auricular lymph nodes decreased over time, while that of the lung-associated lymph node remained elevated (Table 3). The interanimal variability prevented these differences to gain statistical significance.

Histopathology

Some mild findings were observed in the lung of rats from all groups after the challenge on d 21, which decreased during the course of study. After the last challenge (d 64), in the topical induction group an increased incidence of bronchial epithelial thickening, recruitment of peribronchial/perivascular eosinophils, and activation of the bronchus-associated lymphoid tissue (BALT) was observed when compared to the

TABLE 2

Analysis of bronchoalveolar lavage fluid of Brown Norway rats that were sensitized either by topical administration of undiluted MDI (150 μl on the shaved flanks on d 0 and 75 μl on the dorsum of each ear on d 7) or by 5 × 3 h/d inhalation exposures to 28 mg MDI/m^3 on d 0–4.

Group	Total cell count ($\times 10^6$/lung)	LDH (U/L)	Protein (g/L)	Nitrite (μmol/L)	Alveolar macrophages (%)	Neutrophils (%)	Lymphocytes (%)	Eosinophils (%)	3-Nitrotyrosine-positive cells (per 100 eosinophils)
					Day 21				
Control-1	15.0 ± 4.2	72.1 ± 14.1	0.32 ± 0.05	6.24 ± 0.32	90.5 ± 3.4	0.76 ± 0.31	3.12 ± 1.16	5.54 ± 3.30	0.08 ± 0.18
Topical	16.2 ± 6.1	111.7 ± 28.3[a]	0.38 ± 0.06	6.74 ± 0.59	89.2 ± 3.9	1.18 ± 0.69	3.69 ± 1.46	6.40 ± 3.10	0.68 ± 0.53
Inhalation	16.0 ± 3.7	102.5 ± 29.7	0.37 ± 0.04	6.60 ± 0.89	95.5 ± 2.3[a]	0.33 ± 0.29[a]	1.83 ± 1.15	2.19 ± 1.88	1.36 ± 2.09
					Day 35				
Control-1	15.1 ± 3.5	108.4 ± 30.8	0.32 ± 0.04	6.34 ± 1.05	94.6 ± 1.3	0.49 ± 0.24	2.13 ± 0.57	2.66 ± 1.17	4.20 ± 4.67
Topical	15.6 ± 1.5	96.7 ± 18.1	0.45 ± 0.08[b]	8.46 ± 2.29	87.5 ± 4.4[b]	1.49 ± 0.94[a]	4.18 ± 1.84[a]	6.38 ± 3.14[a]	0.39 ± 0.90
Inhalation	13.7 ± 1.5	71.2 ± 4.0[a]	0.34 ± 0.03	6.99 ± 0.55	94.6 ± 2.1	0.45 ± 0.20	2.54 ± 1.55	2.24 ± 1.48	0.31 ± 0.88
					Day 50				
Control-1	9.8 ± 1.0	61.3 ± 14.5	0.26 ± 0.06	6.34 ± 0.79	96.3 ± 1.2	0.53 ± 0.47	1.88 ± 0.61	1.18 ± 0.70	3.20 ± 5.23
Topical	16.5 ± 4.7[a]	95.9 ± 14.5[b]	0.41 ± 0.04[b]	8.46 ± 1.09[b]	90.6 ± 1.6[b]	2.79 ± 1.03[b]	4.98 ± 1.01[b]	1.56 ± 0.49	2.90 ± 3.42
Inhalation	11.8 ± 5.3	75.3 ± 21.2	0.34 ± 0.09	7.57 ± 0.65[a]	94.7 ± 2.7	1.06 ± 0.74	2.53 ± 0.68	1.56 ± 2.52	1.38 ± 2.23
					Day 64				
Control-1	7.96 ± 1.6	84.6 ± 24.3	0.29 ± 0.04	9.17 ± 3.17	97.6 ± 1.1	0.63 ± 0.70	1.27 ± 0.61	0.37 ± 0.45	14.5 ± 10.2
Topical	16.5 ± 4.3[b]	128.3 ± 30.5[a]	0.61 ± 0.08[b]	6.51 ± 2.29	90.0 ± 2.6[b]	3.59 ± 1.92[a]	4.51 ± 1.02[b]	1.80 ± 0.80[b]	2.95 ± 4.43
Inhalation	9.08 ± 1.6	88.2 ± 22.2	0.33 ± 0.05	8.57 ± 1.50	96.0 ± 1.7	0.64 ± 0.52	2.94 ± 1.34	0.28 ± 0.20	0.56 ± 1.34
Control-2	7.72 ± 0.9	77.6 ± 26.4	0.30 ± 0.03	6.58 ± 1.47	97.9 ± 0.9	0.39 ± 0.27	1.05 ± 0.60	0.50 ± 0.54	0.09 ± 0.23

Note. All rats were challenged with ~15 mg MDI/m^3 for 30 min on d 21, 35, 50, and 64. One day later they were challenged with methacholine. Naive rats of the control-1 group were repeatedly challenged, whereas those of the control-2 group were challenged on d 64 only. Sacrifice was 1 d after the methacholine challenge. Data represent group means and SD of eight rats/group and sacrifice.

[a]Significant difference from control-1, $p < .05$.

[b]Significant difference from control-1, ($p < .01$).

TABLE 3

Analysis of body weights, lung weights, lung-associated lymph node weights, and auricular lymph node weights of Brown Norway rats that were sensitized either by topical administration of undiluted MDI (150 μl on the shaved flanks on d 0 and 75 μl on the dorsum of each ear on d 7) or by 5 × 3-h/d inhalation exposures to 28 mg MDI/m^3 on d 0–4

Group	Body weights (g)	Lung weight (mg)	Lung-associated lymph-node weight (mg)	Auricular lymph-node weight (mg)
			Day 21	
Control-1	281.5 ± 16.5	1476 ± 67	11.0 ± 3.9	14.9 ± 3.8
Topical	256.4 ± 8.9[a]	1348 ± 104[a]	16.5 ± 6.6	30.6 ± 18.9
Inhalation	277.8 ± 15.7	1426 ± 58	11.9 ± 4.2	17.3 ± 6.6
			Day 35	
Control-1	276.9 ± 10.8	1443 ± 67	11.8 ± 3.4	16.1 ± 5.4
Topical	289.6 ± 20.5	1529 ± 119	15.1 ± 6.1	37.3 ± 22.7
Inhalation	281.9 ± 11.6	1395 ± 82	11.6 ± 3.8	20.1 ± 5.3
			Day 50	
Control-1	296.6 ± 16.9	1394 ± 59	7.5 ± 2.3	19.3 ± 6.0
Topical	300.4 ± 24.8	1534 ± 56[b]	13.4 ± 6.5	22.6 ± 11.5
Inhalation	301.3 ± 10.4	1385 ± 79	8.6 ± 2.7	18.6 ± 4.3
			Day 64	
Control-1	303.6 ± 8.0	1439 ± 40	7.4 ± 1.5	14.0 ± 4.8
Topical	301.1 ± 13.7	1548 ± 127	11.0 ± 2.6[a]	17.1 ± 4.9
Inhalation	317.7 ± 20.6	1430 ± 99	9.3 ± 2.1	12.0 ± 2.9
Control-2	301.1 ± 18.7	1405 ± 66	8.1 ± 2.9	18.5 ± 7.4

Note. All rats were challenged with ~15 mg MDI/m^3 for 30 min on d 21, 35, 50, and 64. One day later they were challenged with methacholine. Naive rats of the control-1 group were repeatedly challenged, whereas those of the control-2 group were challenged on d 64 only. Sacrifice was 1 d after the methacholine challenge. Data represent group means and SD of eight rats per group and sacrifice.

[a] Significant difference from control, $p < .05$.

[b] Significant difference from control, $p < .01$.

concurrent controls. In this group the lung-associated lymph nodes and auricular lymph nodes showed an increased activation, including lymphocytolysis and plasmocytosis, especially after challenge d 21 (Table 4).

DISCUSSION

A repeated-challenge "chronic" bioassay in Brown Norway (BN) rats was utilized for the evaluation of respiratory allergy using the known human asthmagen diphenylmethane-4,4′-diisocyanate (MDI). In previous single-challenge protocols in guinea pigs, MDI caused an increase in airway eosinophils but did not elicit remarkable changes in immediate-inset respiratory patterns as compared to TMA (Pauluhn et al., 1997, 2000). In terms of the dose used for induction for each route, the "maximum feasible dose" was selected because previous studies with the reference compound TMA have demonstrated that most conclusive changes in animal models are obtained by using high-dose rather than low-dose protocols (Pauluhn, 2003). Particular emphasis was directed toward the simulation of more realistic exposure patterns (except dose) by omitting any vehicle, use of artificial routes of elicitation (e.g., intranasal challenge), or synthesis of ill-defined conjugates of the hapten. Therefore, a rechallenge protocol was devised in BN rats, a strain susceptible to induce IgE, to test the hypothesis that a buildup of acute-on-chronic changes is more apt to identify MDI as respiratory sensitizer. Recent published evidence supports the view that rechallenge protocols are experimentally more robust and lead to phenotypical changes in the airways that typify asthma (Haczku et al., 1994; Palmans et al., 2000, 2002a, 2002b; Kips et al., 2003). In this study a challenge regimen was used to minimize carryover effects from one challenge to another. This was based on the time course of neutrophils and eosinophils in BALF shown to occur in BN rats after challenge with ovalbumin (Schneider et al., 1997).

The induction of IgE is dependent on the induction dose (Warbrick et al., 2002). Despite the use of high-dose protocols, total IgE in serum or BALF sampled at necropsy 2 d after MDI challenge did not show a route of induction-specific effects at any time point. The total IgE levels in serum were similar to those reported by Arts et al. (2004) for naive BN rats. This finding supports the views generally expressed, namely, that

TABLE 4
Incidence of exposure-related histopathologic lesions in Brown Norway rats either sensitized by topical administration of MDI or by 5 × 3-h/d inhalation exposures to 28 mg MDI/m^3

	Challenge						
	Day 21			Day 64			
Tissue and lesion[a]	C-1	T	I	C-1	T	I	C-2
Number of tissues examined	(8)	(8)	(8)	(8)	(8)	(7)	(8)
Lung							
Bronchial epithelial thickening							
Grade 1	1	2	3	0	3	0	0
Grade 2	1	3	0	0	3	0	0
(Per)Bronchial eosinophils							
Grade 1	5	5	5	1	6[b]	0	3
Grade 2	0	3	0	0	0	0	0
(Per)Vascular eosinophils							
Grade 1	6	5	7	2	7[b]	3	0
Grade 2	2	3	0	0	0	0	0
BALT activation							
Grade 1	0	0	1	0	2	0	0
Grade 2	0	4	1	0	2	0	0
LALN							
Activation							
Grade 1	4	0	3	5	3	3	3
Grade 2	2	6	2	1	2	1	0
Grade 3	0	1	0	0	0	0	0
Lymphocytolysis							
Grade 1	3	5	5	4	7	4	3
Grade 2	1	1	0	0	0	0	0
Plasmocytosis							
Grade 1	0	0	1	0	0	0	0
Grade 2	0	3[c]	0	0	1	0	0
Auricular LN							
Activation							
Grade 1	2	0	0	3	0	1	5
Grade 2	0	5[c]	2	1	2	1	0
Grade 3	0	2	0	0	0	0	0
Lymphocytolysis							
Grade 1	1	7[c]	4	0	3[b]	1	2
Plasmocytosis							
Grade 1	0	1	0	0	1	0	0
Grade 2	0	1	0	0	0	0	0

Note. All rats were challenged with ~15 mg MDI/m^3. Animals were sacrificed 2 d after the first (d 21) or last (d 64) MDI challenge. C-1, control group 1 (repeatedly challenged); C-2, control group 2 (challenged on d 64 only); T, topical induction; I, inhalation induction. Grade1/2/3: minimal/slight/moderate. LALN, lung-associated lymph nodes. LN, lymph nodes.
[a]Sacrifice 2 d after MDI challenge.
[b]Significant at $p < .05$, Fisher's exact test (unilateral comparison).
[c]Significant at $p < .01$, Fisher's exact test (unilateral comparison).

classical IgE-mediated mechanisms do not appear to be crucial in isocyanate-induced asthma, and that such antibodies bear a poor relationship to disease occurrence or severity (Matheson et al., 2001).

When challenged with the MDI aerosol, BN rats sensitized to MDI by inhalation elaborated distinct, although mild, changes in respiratory patterns at the first challenge (d 21). These were characterized by a decreased respiratory minute

volume and increased expiratory time. Thus, functional evidence suggests that previous inhalation exposures (sensitization) rendered the pulmonary region functionally slightly more susceptible to an irritant stimulus 2 wk after cessation of the inhalation induction period. At this time point, histopathology did not reveal morphological evidence of induction-related carryover of pulmonary inflammation. Upon rechallenge on d 35, 50, and 64, all groups appeared to be indistinguishable. In contrast, in TMA-sensitized/rechallenged BN rats a time-related increased responsiveness was observed (Pauluhn, 2003). Although speculative, this finding appears to be more suggestive of a reflexively induced rather than immunologically mediated etiopathology. With regard to the methacholine aerosol challenge 1 d after the MDI challenge, conclusive route-of-induction-related differences among the groups were not observed at any time point.

In BN rats sensitized topically to and challenged with MDI, a time-related exacerbation of pulmonary inflammation occurred as indicated by mildly, although statistically significantly, increased protein and neutrophil counts in BAL as compared to either control or the inhalation induction group. The toxicological significance of eosinophil counts in BAL is difficult to judge, due to the age-dependent decrease in eosinophil and total cell counts observed in the BALF. In control BN rats of the study performed by Palmans et al. (2002), the percentage of eosinophils in BALF decreased from 14.2% to 1.5% over a time period of approximately 2 mo (the total cell number in BALF was not different in young and adult rats). Despite the time-related trends occurring to a comparable extent in the control-1 and inhalation groups, the time-course-related changes in topically sensitized rats were different.

Conventional histopathology of the lungs of topically induced BN rats revealed an increased incidence of eosinophilic bronchiolitis and BALT activation. Due to the time-related decrease in airway eosinophils in the control, this finding appeared to be more distinct after the d-64 challenge when compared to the d-21 challenge. It appears that focal changes in the airway wall, which are not necessarily accessible to the lavage fluid, can be better appreciated by histopathology than by bronchoalveolar lavage, which is a sensitive although non-site-specific technique. Therefore, the changes observed in bronchoalveolar lavage are believed to mirror best those that occur at the alveolar rather than airway level. Thus, the findings presented corroborate the notion that each method chosen to probe for a generalized or a focal response within the heterogeneous lung has its particular advantages and disadvantages. Thus, the two approaches are complementary and can be used to best advantage in concert.

Increased nitric oxide (NO) levels in the exhaled air in workers with suspected sensitization with MDI have been reported (Allmers et al., 2000). It was demonstrated that NO plays a pivotal role in the eosinophil migration and infiltration in non-allergic inflammation (Ferreira et al., 1998). Thus, published evidence supports the hypothesis that tyrosine nitration might result in the formation of inactive "footprints" of reactive nitrogen intermediates, but might also be functionally related to the pathobiology of inflammatory diseases (van der Vliet et al., 1999; Iijima et al., 2001). Hence, the 3-nitrotyrosine (3-NT) determinations in BAL cells (especially eosinophils) served as an indicator of oxidation reactions mediated by NO-derived oxidants. Although there is evidence of neutrophil and eosinophil involvement, anti-3-NT determinations in BAL cells and the nitrite determination in BALF did not provide any conclusive evidence that eosinophils and/or neutrophils in BAL were specifically activated to produce nitric oxide in any group. Interleukin (IL)-17 in serum and lavage, a factor that can modulate the neutrophil recruitment (Lindén & Adachi, 2002), was below the limit of detection in all groups (data not shown).

The changes and time course of local lymph-node weights appear to be consistent with the time and location of induction and are not at variance with the lymph-node activation described to occur following topical application of skin sensitizers (Kimber & Dearman, 1997; Dearman & Kimber, 1999). In terms of relative sensitivities of endpoints to analyze route- and time-dependent effects, the measurements in BALF (cytology) appear to be most straightforward and sensitive but not necessarily specific enough to probe for mucousal changes at the airway level. Indeed, the resolution of histopathology can be improved by using morphometric methods, which, however, were beyond the scope of this study.

In summary, high-dose topical induction with MDI induced a neutrophilic and eosinophilic inflammatory response in the lung after inhalation challenge to MDI. Differences in the extent of neutrophilic versus eosinophilic inflammatory response appear to be related to the methodology-dependent analysis of specific lung compartments. The cumulative dose used for the topical route was 1696 mg/kg body weight (based on an average body weight of 230 g and a specific density of MDI of 1.3 g/ml) and for the inhalation route 25 mg/kg body weight (based on a respiratory minute volume of 1 L/(min × kg-rat; Mauderly, 1986). Thus, it remains unresolved whether the effects observed in rats sensitized topically is a true route-dependent phenomenon or simply a high-dose phenomenon. From comparison with previous single-inhalation-challenge studies in guinea pigs, it appears that the results obtained in BN rats and guinea pigs are not at variance and that variables in protocol design appear to play a greater role than species selection. The results of this study supplement the conclusion from other authors, namely, that repeated-challenge protocols increase significantly the sensitivity and robustness of this bioassay.

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