



Allergic inflammation in the upper respiratory tract of the rat upon repeated inhalation exposure to the contact allergen dinitrochlorobenzene (DNCB)

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ABSTRACT

Previously, the contact allergen dinitrochlorobenzene (DNCB) was identified as a sensitizer by inhalation in BALB/c mice; in addition, DNCB induced a lymphocytic infiltrate in the larynx of dermally sensitized Th1-prone Wistar rats upon a single inhalation challenge. In the present study, repeated inhalation exposures to DNCB were investigated using the same protocol as the single-challenge study: female Wistar rats were dermally sensitized with DNCB and subsequently challenged by inhalation exposure to 7 or 15 mg/m³ DNCB twice a week for 4 weeks. Allergy-related apnoeic breathing was not observed. DNCB-specific IgG antibodies were found in the serum and – predominantly lymphocytic – inflammations were found in the nasal tissues and larynx. Similar effects were observed in animals repeatedly exposed by inhalation without previous dermal contact, indicating sensitization by inhalation. The inflammation may be the upper respiratory tract analogue of hypersensitivity pneumonitis/allergic alveolitis. Possible progression of the airway inflammation upon long-term exposure should be investigated to support or dismiss discrimination between contact and respiratory allergens in relation to respiratory allergy.

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1. Introduction

Several low molecular weight (LMW) chemicals can cause contact allergy in the skin, but only a limited number of LMW allergens is known to cause respiratory allergy. Interestingly, all respiratory allergens tested in the murine local lymph node assay (LLNA) were positive upon skin application (Basketter and Scholes, 1992; Kimber et al., 2007), and thus are considered to sensitize the body via the dermal route. Based on human evidence, the skin appears more prone to Thelper1- and the respiratory tract more prone to Thelper2-mediated allergic disorders. However, in test animals the skin is considered a particularly effective route to sensitize the respiratory tract for Th2-mediated allergic reactions by LMW allergens (Arts and Kuper, 2003; Botham et al., 1989; Karol, 1986; Warbrick et al., 2002b), and there is evidence that this is true in humans as well (Redlich and Herrick, 2008). Various animal models for respiratory

allergy have therefore been developed which exploit this capacity, and depend on dermal sensitization followed by an inhalation challenge. The major advantage of separation of the routes of sensitization (a systemic process) and challenge (local reaction), is that effects of the challenge only can be investigated without interference by previous sensitization via the same route.

Data concerning the risk of inhalation exposure to Thelper1-mediated contact allergens are limited. Recently, the contact allergens DNCB and oxazolone (de Sousa and Parrott, 1969; Kimber and Weisenberger, 1989) were found positive in a respiratory LLNA, based on a stimulation index (SI) of 3 or more in the respiratory tract-draining lymph nodes (Arts et al., 2008). They could therefore be considered capable of sensitization by inhalation. In addition, both contact allergens induced a mixed Th1/Th2 cytokine profile in the respiratory tract-draining lymph nodes (de Jong et al., 2009), comparable to the cytokine profiles induced by skin application (Dearman et al., 2003; Ulrich et al., 2001; van Och et al., 2002). Guinea pigs, challenged by inhalation exposure to 10 mg/m³ DNCB, did not exhibit any changes in breathing frequency but demonstrated slightly elevated levels of DNCB-specific IgG1 (Botham et al., 1989). Furthermore, inhalation challenge with 7.5 or 27 mg/m³ DNCB did not provoke allergy-associated breathing changes or allergic inflammation in the Th2-prone Brown Norway (BN) rat, despite upregulation of several allergy-associated genes (Kuper et al., 2008). However, it did induce a minimal lymphocytic infiltrate in the larynx of dermally sensitized Th1-prone Wistar rats (Arts et al., 1998).

Abbreviations: BAL, bronchoalveolar lavage; BALT, bronchus-associated lymphoid tissue; BN, Brown Norway; DNCB, 2,4-dinitrochlorobenzene; LLNA, local lymph node assay; LMW, low molecular weight.

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The above-mentioned challenge studies demonstrated that a single inhalation challenge with the contact allergen DNCB in sensitized Wistar rats induced minimal allergic inflammation in the respiratory tract, but no distinct functional breathing changes. However, repeated inhalation exposure to DNCB might have more serious consequences. In the present study, we therefore used the low-IgE-responding, Th1-prone Wistar rat to investigate whether repeated inhalation exposures to DNCB could induce breathing changes indicative of respiratory allergy and a more vigorous airway inflammation than observed following a single inhalation challenge exposure. To that end, the study protocol that successfully identified chemical and protein respiratory allergens in the past using a single inhalation challenge (Arts et al., 1998; Arts and Kuper, 2003; Pauluhn et al., 2002; Saloga et al., 1994; Zhang et al., 2004), was extended with repeated inhalation exposures. The allergic and irritating responses were studied by analyzing breathing parameters in exposed animals; IgE and hapten-specific IgG levels in serum; histopathology of the airways; and cellular and biochemical changes in bronchoalveolar lavage (BAL) fluid.

2. Material and methods

2.1. Animals and maintenance

Female, 8-week-old Wistar WU rats (CrI:[W]WU BR, outbred) were purchased from a colony maintained under SPF conditions at Charles River Deutschland (Sulzfeld, Germany) and acclimatized for 13 days before the start of the study. They were kept under conventional laboratory conditions (3 animals per cage), and received a commercially available rodent diet (Rat & Mouse No. 3 Breeding Diet RM3; SDS Special Diets Services, Witham, England) and unfluoridated tap water ad libitum. Mean bodyweight (\pm standard deviation) was 189 (\pm 7) g at the start of the study. All animal procedures were approved by the TNO Committee on Animal Welfare.

2.2. Materials

2,4-Dinitrochlorobenzene (DNCB; CAS No. 97-00-7; purity at least 98%) was obtained from Sigma (St. Louis, MO, USA); acetone from Biosolve (Valkenswaard, The Netherlands) and raffinated olive oil was supplied by Sigma (St. Louis, MO, USA).

2.3. Study design

The study design was based on a protocol which has successfully identified chemical and protein respiratory allergens in the past, using a single inhalation challenge. In short, 4 days before the first chemical application, rats were shaved on both flanks with an electrical razor and blood was sampled by orbital puncture for measurement of baseline levels of IgE and DNCB-specific IgG. Groups of rats (6 animals per group) received 150 μ l 1% (w/v) DNCB in a vehicle consisting of a 4:1 (v/v) mixture of acetone and olive oil (AOO) on each shaved flank (approximately 12 cm²) at day 0. Seven days later, they received 75 μ l 0.5% (w/v) DNCB on the dorsum of each of both ears. Another set of animals was exposed by inhalation only, without previous dermal contact. Starting on day 21, rats were challenged by inhalation exposure to 7 or 15 mg/m³ DNCB for 15 min, twice a week for 4 weeks (on days 21, 24, 27, 30, 34, 37, 41 and 43). Challenge concentrations were based on a previous experiment using a single inhalation exposure (Arts et al., 1998). Basal respiration parameters (breathing frequency, tidal volume and breathing pattern) were monitored of 2 animals per group on day 20 and subsequently during and after all successive challenges. Necropsy was performed on day 44, blood was sampled, bronchoalveolar lavage was performed, organs were weighed (liver, kidneys and left lung), and the ears and respiratory tract were collected for histopathological examination. An outline of the treatment schedule is depicted in Table 1.

2.4. Atmosphere generation and analysis

A compressed air driven atomizer (Schlick type 970/S, Coburg, Germany) was used to generate liquid aerosols from freshly prepared solutions of DNCB. Airflow to the nebulizer was monitored by a mass stream meter (Bronkhorst Hi Tec, Ruurlo, The Netherlands). DNCB was dissolved in acetone to a concentration of approximately 18 g/l and delivered to the nebulizer by a motor-driven syringe pump (WPI type SP220i, World Precision Instruments, Sarasota, FL, USA). The acetone concentration was kept below 1 g/m³, which is considered to be far below a level inducing sensory irritation (Alarie, 1973; de Ceaurriz et al., 1981; Schaper and Brost, 1991). Samples of the test atmosphere were collected prior to or after inhalation exposure and DNCB concentrations were determined gravimetrically by filter sampling at 5 l/min. Average actual concentrations (\pm standard deviation) of DNCB during inhalation exposure were 7.29 (\pm 0.59) and 15.47 (\pm 1.12) mg/m³ for the low and high

Table 1
Treatment schedule.

Group designation	Sensitization Day 0: 150 μ l per flank Day 7: 75 μ l per ear	Challenge 15 min inhalation Days 21–43 ^a
–/Low	Vehicle ^b	7 mg/m ³ DNCB
–/High	Vehicle ^b	15 mg/m ³ DNCB
+/Low	1% DNCB (day 0) 0.5% DNCB (day 7)	7 mg/m ³ DNCB
+/High	1% DNCB (day 0) 0.5% DNCB (day 7)	15 mg/m ³ DNCB

Respiration was monitored before the first, and during and after all successive challenges. At necropsy on day 44, animals were examined for gross pathological changes, blood was sampled (IgE and DNCB-specific IgG), BAL was performed, organs were weighed (liver, kidneys and left lung), and the nose, larynx, trachea, left lung and ears were collected for histopathological examination.

^a On days 21, 24, 27, 30, 34, 37, 41 and 43.

^b Acetone and olive oil in a 4:1 (v/v) mixture (AOO).

exposure groups, respectively. Particle size distributions of DNCB in the test atmospheres were determined using a 10-stage cascade impactor (Andersen, Atlanta, GA, USA) and the Mass Median Aerodynamic Diameter (MMAD) and geometric standard deviation (gsd) were calculated (Lee, 1972). MMAD and gsd were 2.7 μ m and 4.4 in the 7 mg/m³ DNCB atmosphere, and 2.4 μ m and 4.6 in the 15 mg/m³ DNCB atmosphere, respectively. Temperature and relative humidity (RH) were recorded during exposure; the average temperature was 21.7 \pm 0.3 °C for the low exposure and 22.3 \pm 0.3 °C for the high DNCB exposure groups. The average RH was 50.4 \pm 2.8% and 50.3 \pm 1.9% for low and high DNCB concentrations, respectively. Airflow through the exposure unit was 23 l/min.

2.5. Lung function measurements

Animals were exposed to the test atmosphere and lung function was measured as previously described (Arts et al., 1998). Groups of 12 rats were exposed simultaneously in a head/nose-only inhalation unit. The exposure unit permitted monitoring of the respiration of 4 of 12 animals (2 DNCB sensitized and 2 vehicle-treated controls), which were restrained in Battelle tubes and placed individually into one of four plethysmographs that were connected to the central exposure chamber. Thus, of each group of animals, the same two rats were used to analyze lung function during and after all inhalation exposures. Each plethysmograph was provided with a pressure transducer which sensed changes created by in- and expiration and transmitted amplified signals to a polygraph recorder, so respiratory frequency and pattern could be determined. Using this experimental setup, normal breathing pattern of the animals, exposed to fresh air, was assessed 1 day before the first inhalation exposure. Subsequently, respiration was monitored continuously during the 15 min inhalation challenge periods, 20 s/min for 15 min after each challenge, and 20 s/min for 6 min 1 day after each challenge. Mean values (breathing frequency, tidal volume and minute ventilation) were obtained from 6 exposure and 6 post-exposure values.

2.6. Clinical signs, body and organ weights, serum collection, and necropsy

All animals were observed at least once daily and weighed weekly, shortly before the start of the experiment and prior to necropsy. Serum samples were prepared from blood withdrawn via the orbital plexus prior to sensitization, and via the abdominal aorta at necropsy. Serum samples were stored at –20 °C until analysis of total serum IgE and DNCB-specific IgG levels by means of ELISA. At necropsy, animals were anaesthetized with sodium pentobarbital (ip) followed by exsanguination from the abdominal aorta, and examined grossly for abnormalities. Liver, kidneys and the unlavaged left lung (see below) were weighed. The nasal tissues, larynx, trachea, left lung and ears (site of dermal application) were collected for histopathological evaluation.

2.7. Bronchoalveolar lavage

After binding of the left lobe at necropsy, which was used for histopathological evaluation, the right lung lobes were lavaged (Hooftman et al., 1988) two times with a volume of 23 ml saline per kg bw. The total amount of retracted lavage fluid was weighed and retained on ice. The bronchoalveolar cells were isolated from the supernatant by centrifugation (250 g) during 5 min at 4 °C and resuspended in 0.5 ml saline to assess total and differential cell numbers. Total cell numbers were counted using an automated haematology analyzer (K-800, Sysmex, Toa, Kobe, Japan). The percentage of viable cells was determined using an acridine orange/ethidium bromide staining method in combination with fluorescence microscopy. For differential cell counts, cytopspins were prepared and stained with May-Grunwald Giemsa. At least 200 cells were counted per animal to determine absolute numbers and percentages of macrophages/monocytes, lymphocytes, neutrophils and eosinophils. Supernatants were used for determination of total protein (Bradford, 1976), lactate

Table 2
Body and organ weights.

Group	Bodyweight (g)		Relative liver weight (g/kg bw)	Relative kidney weight (g/kg bw)	Relative lung weight ^a (g/kg bw)
	Day 0	Day 44			
-/Low	190.1 (7.2)	228.0 (9.3)	29.7 (1.5)	6.3 (0.4)	1.4 (0.1)
-/High	189.7 (5.9)	230.8 (10.0)	31.4 (0.9)	6.2 (0.3)*	1.5 (0.4)
+/Low	189.1 (8.9)	230.5 (12.4)	32.9 (2.2)*	6.6 (0.7)	1.4 (0.1)
+/High	186.9 (7.1)	226.3 (8.9)	32.2 (1.9)*	5.9 (0.1)*	1.5 (0.1)

Means (\pm SD) are expressed.

^a Relative weight of the left lung; the right lung lobes were used for bronchoalveolar lavage.

* Elevated relative liver weight in dermally sensitized animals relative to vehicle-treated groups, $p < 0.05$.

+ Decreased relative kidney weight in animals respiratory challenged with the high dose, relative to animals challenged with the low dose, $p < 0.05$.

dehydrogenase (LDH), γ -glutamyl-transferase (GGT), alkaline phosphatase (ALP) and N-acetyl glucosaminidase (NAG) using an automatic analyzer (Hitachi 911, Hitachi Instruments Division, Japan).

2.8. Total IgE and DNCB-specific IgG in serum

Serum IgE concentrations were measured by ELISA using a Rat IgE ELISA Quantitation Kit (Bethyl Laboratories Inc., Montgomery, TX, USA), performed according to the manufacturer's instructions. In brief, graded solutions of test samples (undiluted to 1:800) or rat IgE standard (0.78–50 ng/ml) were added in duplicate to 96 well plates, precoated with Sheep anti-Rat IgE capture antibody, and incubated for 1 h. Plates were washed and incubated with Sheep anti-Rat IgE-HRP conjugate for 1 h. Subsequently, enzyme substrate (TMB; 3,3',5,5'-tetramethylbenzidine; DAKO North

America, Inc., Carpinteria, CA, USA) was added and the reaction was terminated after 30 min by addition of 2 M sulphuric acid. Substrate conversion was measured as optical density at a wavelength of 450 nm using a BIO-RAD 3550 Microplate Reader (BIO-RAD, Richmond, CA, USA).

To determine DNCB-specific IgG levels, graded solutions of serum samples (1:50 to 1:3200) were added to 96 well plates, precoated with 50 μ g/ml dinitrophenyl-conjugated albumin (DNP-HSA; Sigma-Aldrich, St Louis, MO, USA) in 0.2 M carbonate-bicarbonate buffer (pH 9.5). After incubation (1 h at 37 °C) and washing, secondary antibody (1:1000 goat anti-rat IgG-HRP; Serotec, Düsseldorf, Germany) was added and plates were incubated for 1 h at 37 °C. Subsequently, TMB enzyme substrate was added and the reaction was terminated after 30 min by addition of 2 M sulphuric acid. Plates were analyzed as mentioned above. To compare levels of hapten-specific IgG in the various test samples, a standard curve was

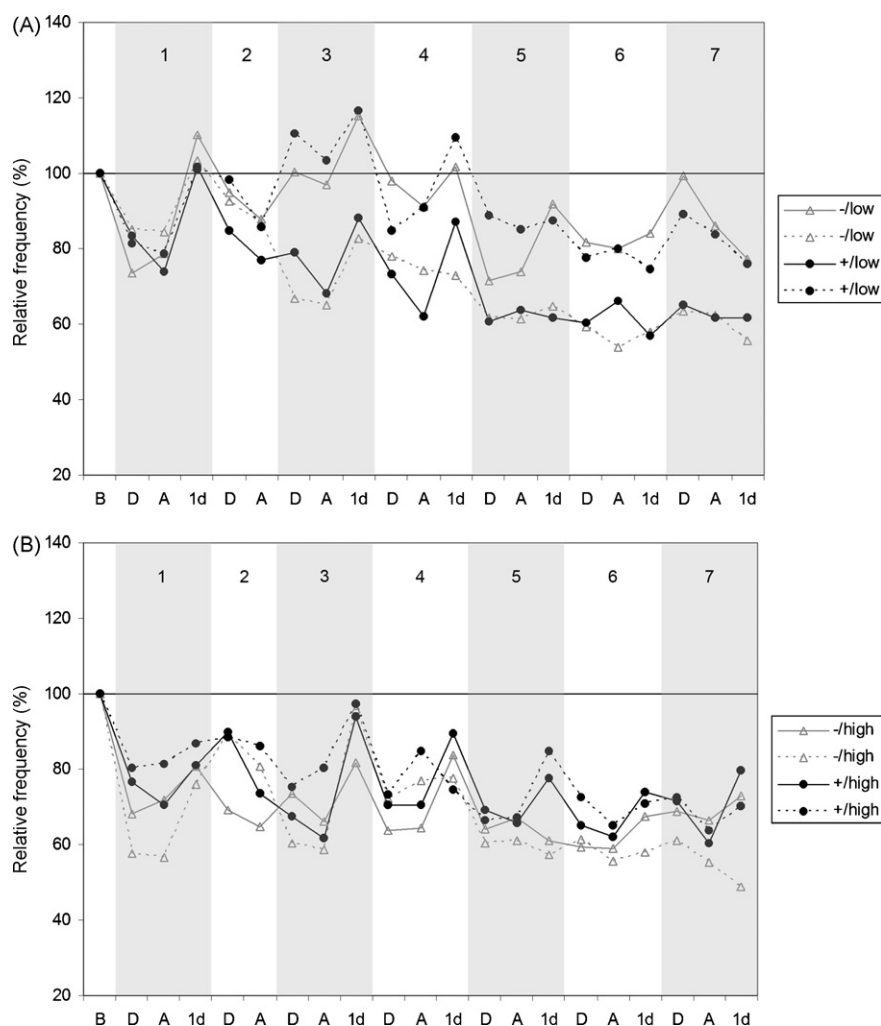


Fig. 1. Relative changes in breathing frequency upon challenge by inhalation. Animals were dermally sensitized with either vehicle or DNCB and repeatedly challenged by inhalation exposure to 7 mg/m³ (A) or 15 mg/m³ (B) DNCB, twice a week for 2 weeks (challenge numbers are indicated on top). Breathing frequency is expressed as percentage of prechallenge values. Respiration parameters were monitored of 2 animals per group (individual animals are represented), before challenge (marked as "B"), during challenge ("D"), immediately after ("A") and 1 day after challenge ("1d").

created using serial dilutions of positive serum (determined in a pilot ELISA) consisting of a pool of several sera obtained at necropsy. Thus, instead of determination of antibody titers, relative levels are expressed in arbitrary units (AU).

2.9. Histopathology

Neutral, phosphate-buffered 4% (v/v) formaldehyde was used to preserve the nasal tissues, larynx, trachea, the left lung and ears. The organs were embedded in paraffin wax and sectioned at 5 μ m. Nasal tissues were cut at six levels (Woutersen et al., 1994). The larynx was cut longitudinally through the epiglottis. The cranial part of the trachea was cut transversally, the caudal part longitudinally, together with the bifurcation and the two extrapulmonary bronchi. The left lung was cut at one sagittal level. The ears were cut at one median level, from the base to the tip. Sections were stained with haematoxylin and eosin and examined microscopically. In addition, microscopic slides (nose, larynx and lungs) of the single-challenge study with DNCB in Wistar rats (Arts et al., 1998) were examined retrospectively in a "blind" fashion.

2.10. Statistics

Body weights were analyzed by 2-way analysis of covariance (ANCOVA) using body weight on day 0 as a covariate. Organ weights, biochemical parameters and relative cell numbers in BALF were evaluated by 2-way ANOVA, and DNCB-specific IgG levels using a 3-way repeated ANOVA. Since the exposure equipment permitted measurement of respiratory parameters of only 2 animals per group, changes in respiration were only evaluated qualitatively and statistical analysis was not performed.

3. Results

3.1. Clinical signs, body and organ weights and bronchoalveolar lavage

Flank application of DNCB caused dermal erythema, scaliness or encrustations in all rats on day 2, persisting for 1–4 days. No visible local effects were observed after application of DNCB to the ears. Body weight gain was 40 (\pm 7) g between days 0 and 44 and did not differ significantly between groups. Dermal sensitized animals demonstrated slightly elevated relative liver weights at necropsy compared to vehicle-treated animals. Relative kidney weight was slightly decreased in rats exposed to high DNCB concentrations when compared to animals treated with the low concentration. No differences in relative lung weight were observed between the groups (Table 2).

Total and differential cell counts and levels of biochemical parameters (total protein, LDH, ALP, NAG and GGT) in BAL fluid were comparable to background data in all groups (untreated control animals of this strain and age (Arts et al., 2007)).

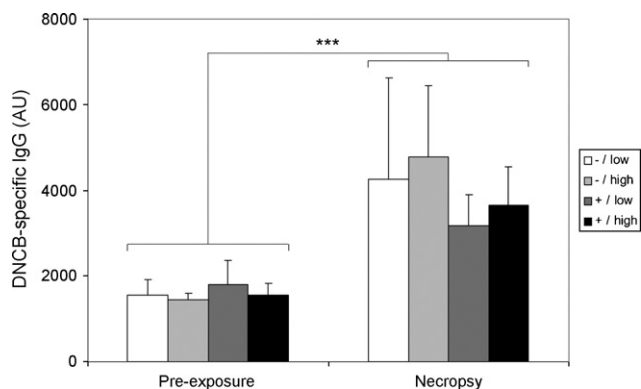


Fig. 2. DNCB-specific IgG levels in serum. Groups of animals ($n=6$) were dermally sensitized with DNCB or vehicle on days 0 and 7, and repeatedly challenged by inhalation exposure to 7 mg/m³ (low) or 15 mg/m³ (high) DNCB on days 21–43 twice a week. Serum obtained on day –4 (orbital puncture) and at necropsy on day 44 was analyzed for hapten-specific IgG by ELISA. Mean IgG levels (\pm SD) are expressed as arbitrary units (AU); statistics: 3-way repeated ANOVA; (***) $p < 0.0001$.

Table 3

Histopathological changes in Wistar rats treated dermally with vehicle or DNCB, followed by repeated inhalation exposure to DNCB.

Changes	Incidence of changes ^a			
	–/Low	–/High	+/Low	+/High
Nasal cavity, level I	(6)	(6)	(6)	(5)
Lymphocytic cell infiltrate				
Very slight	3	1	4	3
Slight	1	3	1	1
Moderate	2	2	1	1
Nasal cavity, level II	(6)	(6)	(6)	(5)
Lymphocytic cell infiltrate				
Very slight	2	1	2	3
Slight	2	4	1	0
Slight focal squamous hyperplasia	0	0	0	1
Nasal cavity, level III	(6)	(6)	(6)	(5)
Focal lymphocytic cell infiltrate				
Very slight	3	1	2	2
Slight	0	3	0	1
Nasal cavity, levels IV, V, VI	(6)	(6)	(6)	(5)
No abnormalities detected	6	6	6	5
Larynx	(6)	(5)	(6)	(5)
Lymphocytic cell infiltrate				
Very slight	1	3	4	3
Slight	5	2	2	2
Squamous metaplasia				
Very slight	4	1	5	2
Slight	1	3	0	2
Trachea	(6)	(6)	(6)	(5)
No abnormalities detected	6	6	6	5
Lungs	(6)	(6)	(6)	(5)
No abnormalities detected	6	6	6	5
Skin/subcutis (ears)	(6)	(6)	(6)	(5)
No abnormalities detected	6	6	6	5

^a Number of animals = 6; one animal of the +/high group died on day 9, the cause of death was unrelated to the treatment.

3.2. Respiratory changes

Breathing frequencies of all rats decreased during and directly after the first DNCB respiratory challenge (Fig. 1A and B). One day later, breathing rates of the rats challenged by inhalation with the low concentration had returned to normal levels, whereas animals exposed to the high concentration still exhibited a slightly reduced frequency. No apparent differences were observed between dermally sensitized and vehicle-treated animals. Recovery of the breathing frequency diminished after subsequent inhalation exposures (Fig. 1A and B). Tidal volumes tended to increase upon respiratory challenge and generally increased over time in all groups, which in part reflected an age-related effect (data not shown). Minute ventilation was not affected by repeated inhalation exposure to DNCB (data not shown).

Breathing patterns of all animals appeared normal and apnoeic pauses in respiration – considered to be indicative of respiratory allergy (Arts et al., 1998; Arts et al., 2003) – were not observed.

3.3. Serum immune globulin levels

Total serum IgE levels were very low or undetectable before sensitization (4 ng/ml average) and were not significantly altered after exposure to DNCB (data not shown). DNCB-specific IgG levels in serum obtained at necropsy were elevated in all groups when compared to pre-exposure levels ($p < 0.0001$; Fig. 2). Specific IgG levels tended to be even higher in dermally unsensitized animals at necropsy than in rats which received the topical DNCB treatment, although this was not supported by a significant interaction with the dermal sensitization ($0.05 < p < 0.10$).

3.4. Airway histopathology

Microscopic examination of the respiratory tract and pulmonary parenchyma revealed an inflammatory response in the nasal passages and in the larynx. The severity, nature and localization of the inflammation were not clearly dependent on the dermal sensitization or the respiratory challenge concentration (Table 3). The inflammation in the nasal tissues was a predominantly lymphocytic cell infiltrate, together with some monocytes around the vascular bed in the lamina propria, which extended into the epithelium. It was more or less diffuse at

level I (Fig. 3); localized at the tips of the naso- and maxilloturbinate, in the middle and ventral meatus, including the lateral walls, at level II; and localized in the ventral meatus at level III. The nasal epithelium was not hyperplastic, but mitoses were occasionally observed. The infiltrate in the larynx was at the ventral side of the epiglottis and contained, in addition to the lymphocytes, some monocytes and medium-sized macrophages (Fig. 4). It was accompanied by squamous metaplasia. The non-keratinized squamous epithelium was slightly hyperplastic and showed epithelial cell desquamation with occasional epithelial cell vacuolization.

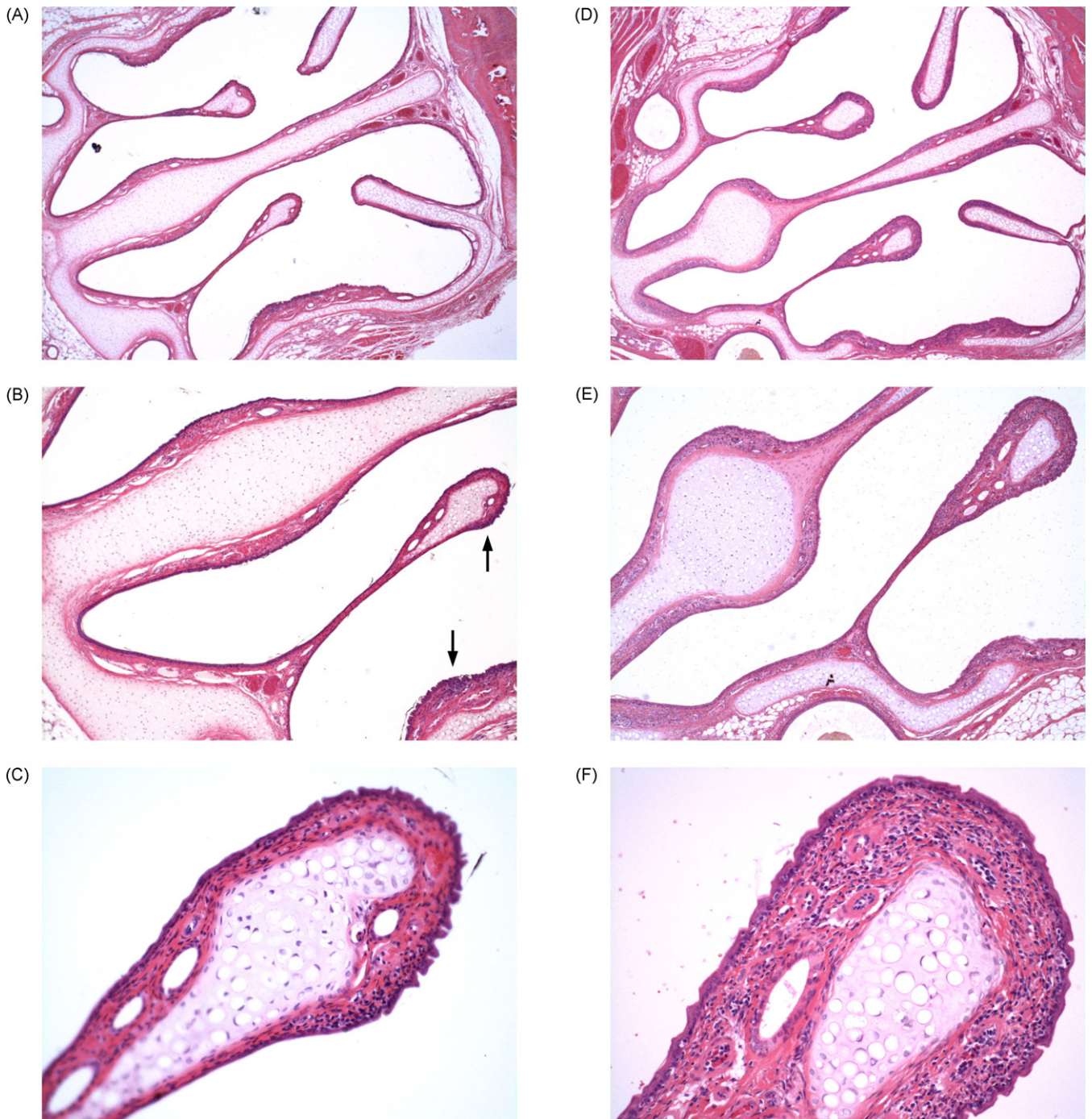


Fig. 3. Cross sections through the nose at level I (Woutersen et al., 1994), which demonstrate the inflammatory response in the nasal tissues of dermally sensitized Wistar rats upon a single inhalation challenge exposure to 7.5 mg/m³ DNCB ((A–C) study performed by Arts et al., 1998) or repeated inhalation exposure to 7 mg/m³ DNCB ((D–F) present study). Arrows point to minimal, localized infiltrates in the singly challenged animal, whereas the inflammation in the repeatedly challenged animal was largely diffuse. H&E-stained sections at 25×, 50× and 200× magnification, respectively.

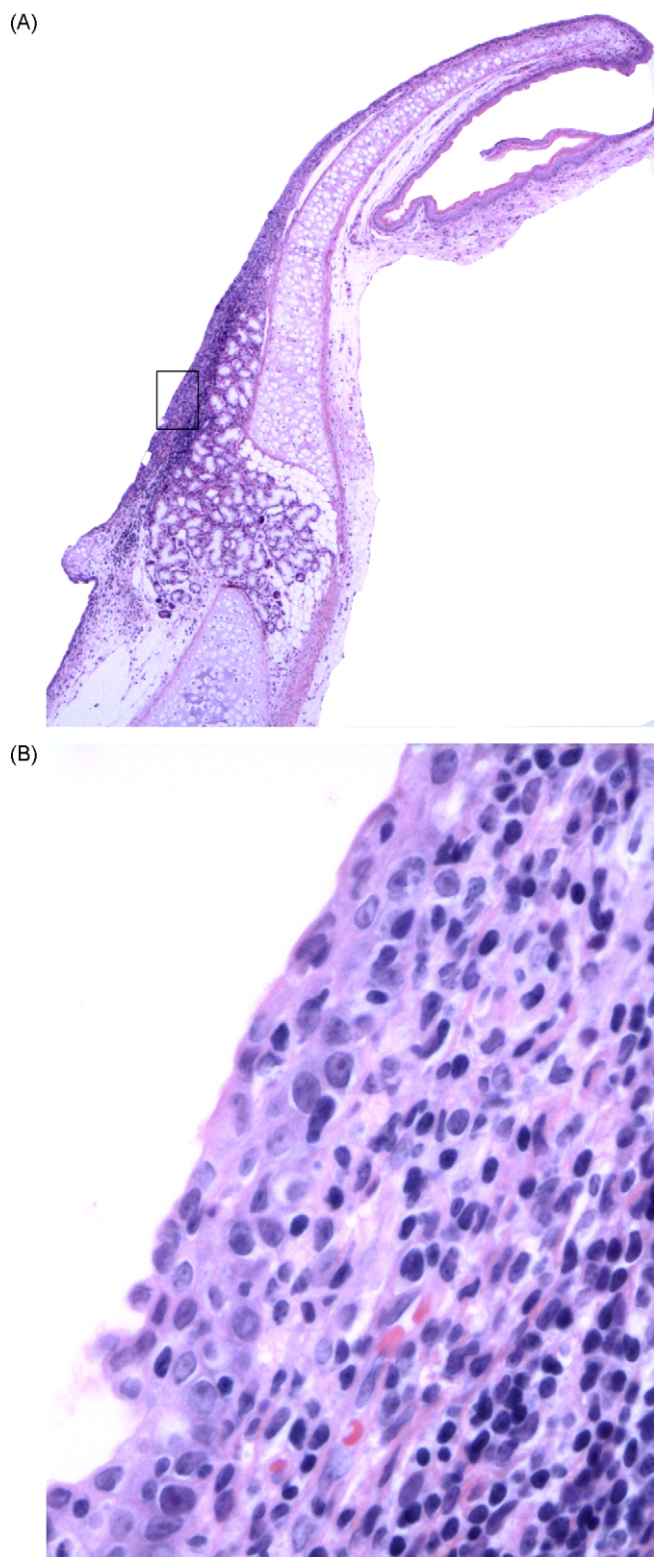


Fig. 4. The inflammatory response at the ventral side of the epiglottis of the larynx after dermal sensitization and repeated inhalation challenge exposure to 15 mg/m³ DNCB. The transitional epithelium was replaced by squamous epithelium, which was slightly hyperplastic. In addition, desquamation and vacuolization of epithelial cells are shown. H&E-stained sections at 25× and 400× magnification, respectively.

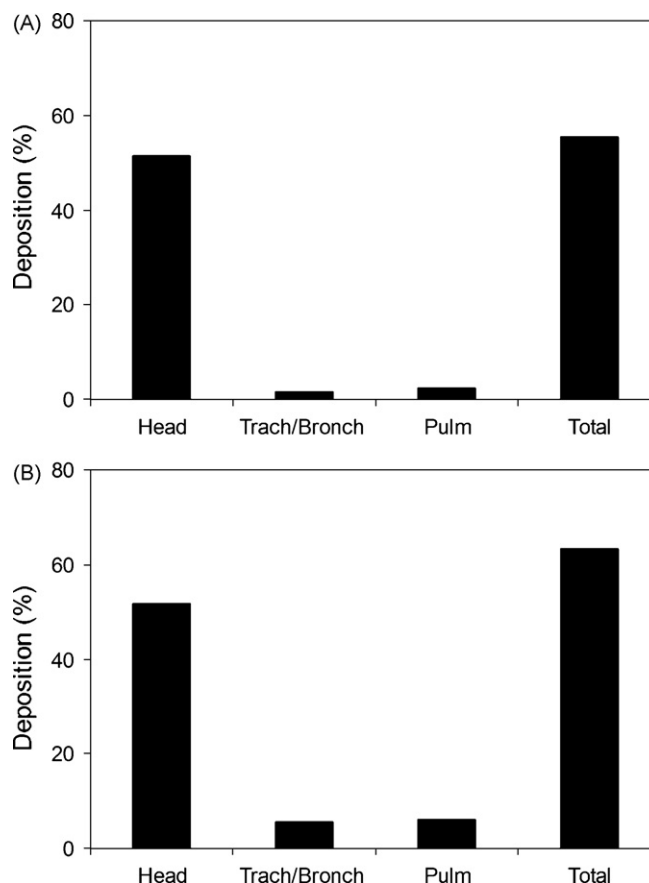


Fig. 5. Estimated aerosol deposition in the airways of the rat and human. The percentage of the inhaled DNCB aerosol concentration deposited in the head, tracheobronchial (Trach/Bronch), and pulmonary (Pulm) region, and total deposition in the airways was calculated, based on aerosol characteristics (see Section 2) and breathing parameters, using the MPPD model v2.0 (Copyright©CIIT and RIVM 2001). Data are presented for 7 mg/m³ DNCB; 15 mg/m³ DNCB yielded similar deposition patterns (data not shown). (A) Rat, breathing frequency: 150.7/min; tidal volume: 1.69 ml (determined during challenge); (B) human, breathing frequency: 12/min; tidal volume: 625 ml (default values in the MPPD model).

Retrospective microscopic examination (“blind”) of the single-challenge study with DNCB in Wistar rats (Arts et al., 1998) revealed that very slight focal lymphocyte infiltrates were present in 4 out of 6 DNCB-sensitized rats, but not in any of the unsensitized rats upon a single DNCB challenge. The infiltrates were localized on the naso- and maxilloturbinates at levels I and II and/or the lateral wall at level I. Microscopic examination of the ears did not reveal inflammatory cell infiltrates or epithelial alterations.

4. Discussion

Inhalation of the contact allergen DNCB can lead to sensitization of BALB/c mice (Arts et al., 2008), and a single respiratory challenge induced slight inflammation of the upper airways after dermal sensitization, but failed to provoke any functional changes in Wistar rats (Arts et al., 1998), guinea pigs (Botham et al., 1989) or BALB/c mice (Satoh et al., 1995). Here, we investigated whether repeated inhalation exposure to DNCB could induce functional breathing changes and a more vigorous airway inflammation, indicative of allergy, in the dermally sensitized Th1-polarized Wistar rat.

As expected, exposure to DNCB did not affect total serum IgE levels in the low-IgE-responding Wistar rats. Accordingly, other investigators did not observe IgE induction after dermal DNCB exposure in less Th1-skewed, more IgE-responding animals like the BALB/c mouse (Dearman and Kimber, 1991; Fukuyama et al.,

2008), BN rat (Arts et al., 1997; Warbrick et al., 2002a), nor induction of cytophilic antibodies in guinea pig (Botham et al., 1989). The absence of histopathological abnormalities in the skin of the ears in the present study indicated that repeated challenges with DNCB via inhalation (head/nose-only exposure) did not induce allergic reactions in the skin—not even in animals which had received DNCB on the ear prior to the inhalation exposure. Most probably, the deposited amount per unit area was too low to induce dermal reactions (Upadhye and Maibach, 1992; White et al., 1986). Instead, inhalation exposure to DNCB induced slight respiratory irritation in all animals (decreased breathing frequency). The observed changes slightly aggravated and recovery was slower after repeated inhalation challenges, which might indicate an increasing sensitivity towards respiratory irritation.

The absence of differences in breathing parameters between dermally unsensitized and dermally sensitized animals together with the absence of apnoeic breathing during respiratory challenge (hallmark of asthmatic effects) suggests that DNCB did not induce respiratory allergy upon repeated inhalation challenges. However, all animals showed DNCB-specific IgG antibodies to a similar extent in the present study. DNCB-specific IgG has also been found in BALB/c mice exposed by inhalation to 15 mg/m³ DNCB on 3 consecutive days (Dearman et al., 1991). The most plausible explanation of these findings is that the inhalation exposures itself induced immunization/sensitization, and thus that dermal applications were not a prerequisite in this protocol and with this allergen. This is in accordance with the recently demonstrated capability of DNCB to sensitize BALB/c mice by inhalation (Arts et al., 2008). Sensitization by inhalation of DNCB can explain the presence of a lymphocytic inflammation in the upper respiratory tract (nasal tissues and larynx) of dermally sensitized, as well as dermally unsensitized animals. The mononuclear (predominantly lymphocyte) cell inflammation was similar to, but more prominent than observed upon a single challenge in dermally sensitized Wistar rats (Arts et al., 1998; see Section 3). This inflammatory response in the upper respiratory tract resembles the delayed-type allergic inflammation observed in the skin of guinea pigs sensitized and challenged dermally with DNCB (Medenica and Rostenberg, 1971). Furthermore, this type of inflammation is also observed in the lungs of humans in Th1-dominated allergic reactions, as described for hypersensitivity pneumonitis (or allergic alveolitis), often accompanied by induction of antigen-specific IgG (Girard et al., 2009). The absence of an allergic inflammation in the Th2-skewed BN rat after an inhalation challenge with 7.5 (Arts et al., 1998) or 27 mg/m³ (Kuper et al., 2008) DNCB underlines the importance of genetic factors in predictive models for allergy.

Repeated inhalation exposure to DNCB did not induce inflammation in the lungs of the Wistar rats (histopathology and BAL). The DNCB aerosol concentration may have been too low, exposure duration too short, and/or particle size too large (although the latter was within the 1–4 and the 1–3 μm ranges described in the OECD guidelines on inhalation toxicology testing) to have penetrated sufficiently into the lower airways. Indeed, estimation of the aerosol fractions deposited in different parts of the airways using the Multiple Path Particle Deposition (MPPD) model (Cassee et al., 1999; Subramaniam et al., 1999) shows that impaction of particles was greatest in the nasal tissues of the rats (Fig. 5A). Extrapolation to humans using the same aerosol characteristics shows a similar deposition pattern (Fig. 5B). This seems to be in accordance with the observation in humans that allergic rhinitis almost always precedes allergic asthma (Leynaert et al., 2000). Intranasal application of the allergen, dissolved or suspended in an appropriate solution (followed by holding the animal in an upright position to increase the amount of allergen reaching the lungs), may lead to a higher pulmonary dose than following inhalation exposure: indeed, intranasally applied nitrochlorobenzenes induced

interstitial pneumonia characterized by lymphocytic infiltration, in sensitized BALB/c mice and Wistar rats (upper respiratory tract not examined) (Garssen et al., 1989; Satoh et al., 1995; Zwart et al., 1994). The contact allergen dinitrofluorobenzene (DNFB) induced immediate pulmonary hypersensitivity and acute bronchoconstriction after intranasal challenge in a murine model for nonatopic asthma (Kraneveld et al., 2002; van Houwelingen et al., 2002).

In summary, repeated inhalation exposure to DNCB induced mild respiratory irritation but no functional respiratory allergic reactions, as judged by breathing parameters. Allergic inflammation in the upper respiratory tract was found, together with DNCB-specific IgG antibodies in serum. The genetic make-up of the host was considered to have played a pivotal role in the response. Possible progression of the allergic inflammation – in severity as well as in extension to the lower respiratory tract – upon long-term inhalation exposure should be investigated. Furthermore, the present data confirm the potential of DNCB to sensitize by inhalation. More research is needed to support or dismiss discrimination between contact and respiratory allergens in relation to respiratory allergy.

Conflicts of interest

All authors declare that there is no conflict of interest.

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