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ScienceDirect
Toxicology 246 (2008) 213–221
TOXICOLOGY
www.elsevier.com/locate/toxicol

The contact allergen dinitrochlorobenzene (DNCB) and respiratory allergy in the Th2-prone Brown Norway rat

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Received 30 October 2007; received in revised form 21 December 2007; accepted 21 January 2008
Available online 2 February 2008

Abstract

All LMW respiratory allergens known to date can also induce skin allergy in test animals. The question here was if in turn skin allergens can induce allergy in the respiratory tract. Respiratory allergy was tested in Th2-prone Brown Norway (BN) rats by dermal sensitization with the contact allergen dinitrochlorobenzene (DNCB; 1%, day 0; 0.5%, day 7) and a head/nose-only inhalation challenge of 27 mg/m^3 of DNCB (15 min, day 21), using a protocol that successfully identified chemical respiratory allergens. Skin allergy to DNCB was examined in BN rats and Th1-prone Wistar rats in a local lymph node assay followed by a topical patch challenge of 0.1% DNCB. Sensitization of BN rats via the skin induced DNCB-specific IgG in serum, but not in all animals, and an increased number of CD4$^+$ cells in the lung parenchyma. Subsequent inhalation challenge with DNCB did not provoke apneas or allergic inflammation (signs of respiratory allergy) in the BN rats. However, microarray analysis of mRNA isolated from the lung revealed upregulation of the genes for Ccl2 (MCP-1), Ccl4 (MIP-1beta), Ccl7 and Ccl17. Skin challenge induced considerably less skin irritation and allergic dermatitis in the BN rat than in the Wistar rat. In conclusion, the Th2-prone BN rat appeared less sensitive to DNCB than the Wistar rat; nevertheless, DNCB induced allergic inflammation in the skin of BN rats but even a relatively high challenge concentration did not induce allergy in the respiratory tract, although genes associated with allergy were upregulated in lung tissue.

Keywords: MIP1-beta; MCP-1; LLNA; Respiratory allergy; Skin allergy; Dinitrochlorobenzene

1. Introduction

Many low molecular weight (LMW) chemicals cause contact allergy in the skin, but only a limited number of chemicals are known to cause respiratory allergy. Interestingly, the respiratory allergens tested were all positive in the local lymph node assay (LLNA) and were able to induce skin allergy in test animals (Marignac et al., 1977; Basketter and Scholes, 1992; Kimber et al., 2007). The question here was if it is also the other way around: have skin allergens the potential to induce allergy in the respiratory tract?

Immune responses may be polarized toward either Thelper1 (Th1) or Thelper2 (Th2) production. Allergic contact dermatitis (mainly Th1) is the most common allergic disorder in the skin. Asthma and allergic rhinitis (mainly Th2) are most frequently encountered in the respiratory tract; asthma being so prominent that respiratory allergy has become almost synonymous with asthma. Thus, based on human evidence, the skin appears more prone to Th1 and the respiratory tract more prone to Th2 allergic disorders. This concept is in use to test chemicals for their potential to cause skin and/or respiratory allergy, although it is recognized as an oversimplification. Skin allergy also includes atopic dermatitis (mainly Th2) and respiratory allergy includes allergic alveolitis (hypersensitivity pneumonitis; mainly Th1; Belenky and Fuhrman, 2006), which is a serious and often insidious respiratory disease. Moreover, most LMW allergens examined today can activate both Th1- and Th2-cells, on the understanding that some of them preferentially induce either

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doi:10.1016/j.tox.2008.01.013

Th1 or Th2, whereas others do both almost equally well (Ulrich et al., 2001; Van Och et al., 2002; Dearman et al., 2003). Their action may depend on tissue factors, e.g. different manners of antigen presentation during sensitization. However, if Th1–Th2 divergence is tissue-dependent it is not so easy to understand why, in test animals, the skin can be such an effective route to sensitize the respiratory tract for Th2-mediated allergic reactions by LMW allergens (Botham et al., 1989; Warbrick et al., 2002a; Arts and Kuper, 2003; Johnson et al., 2004).

There is ample experimental and epidemiological evidence that respiratory allergens like isocyanates and acid anhydrides can induce Th1-type respiratory allergy and that some aspects of asthma are actually Th1-dependent (Bauer, 1995; Grammer, 1999; Merget et al., 2002; Arts et al., 2004; Matheson et al., 2005). Evidence that skin allergens can induce allergic reactions in the respiratory tract is only limited: Typical skin allergens like dinitrochlorobenzene (DNCB), dinitrofluorobenzene (DNFB) and trinitrochlorobenzene (TNCB) induced a slight mononuclear cell infiltrate in the larynx or lungs of sensitized Wistar rats and BALB/c mice but no changes in breathing parameters (Garssen et al., 1991; Zwart et al., 1994; Satoh et al., 1995; Arts et al., 1998). A challenge with 7.5 mg/m^3 DNCB did not induce respiratory allergy (apneus and/or allergic inflammation) in sensitized BN rats (Arts et al., 1998), nor were changes in breathing frequency, associated with allergy, observed in guinea pigs following a challenge of 10 mg/m^3 DNCB (Botham et al., 1989).

Th2-prone animals like the guinea pig and the BN rat are generally used for investigating respiratory allergy, but they may not be very sensitive to Th1-type skin allergens. Therefore, a challenge concentration (27 mg/m^3) of the skin allergen DNCB was used in DNCB-sensitized BN rats, using a protocol that successfully identified chemical and protein respiratory allergens (Saloca et al., 1994; Pauluhn et al., 2002; Arts and Kuper, 2003; Zhang et al., 2004). The challenge concentration was chosen to ascertain that enough of the material reached the lungs (Arts et al., 1998). The concentration was relatively high when compared to the 10 mg/m^3 used by Botham et al. (1989) in guinea pigs, but it was not unphysiologically high because it induced only minimal pulmonary irritation. A whole genome analysis (microarrays) of lung tissue was included, to provide unbiased insight into potential allergy pathogenesis (Zimmerman et al., 2004). The relative sensitivity of BN rats to the allergic properties of DNCB was tested in a skin allergy test (a LLNA followed by a topical patch challenge) by comparing the skin response in BN rats with that of the Th1-prone Wistar rats.

2. Material and methods

2.1. Animals and maintenance

Female and male, 7–8-week-old, inbred Brown Norway (BN) rats and male Wistar WU (Crl:WI/WU, random-bred) were purchased from a colony maintained under SPF conditions at Charles River Deutschland GmbH (Sulzfeld, Germany). The animals were acclimatized for at least 5 days before the start of the study. They were kept under conventional laboratory conditions and received the Institute's grain-based open-formula diet and unfluoridated tap water ad libitum. All animal procedures were approved by the TNO Commission of Animal Welfare.

2.2. Study design

2.2.1. Respiratory allergy

The study was conducted with four groups of rats and according to the following scheme (Table 1): Blood was collected at 1 day before the start of the study. Female BN rats received 150 μl of 1% (w/v) DNCB (purity 97%; Sigma, St. Louis, MO) in a 4:1 (v/v) mixture of acetone (Merck; Darmstadt, Germany) and raffinated olive oil (AOO) (Sigma Diagnostics Inc. St. Louis, USA) as the vehicle on each flank (approximately 12 cm^2 each) which had been shaved with an electrical razor at least 2–3 days earlier. Seven days after the first sensitization, they received 75 μl of 0.5% (w/v) DNCB on the dorsum of each of both ears. Controls received vehicle AOO. On day 21, basal lung function (breathing frequency, tidal volume and breathing pattern) was assessed, followed by DNCB inhalation challenge and assessment of lung function during and post challenge. Animals were challenged by inhalation of a slightly (based on breathing frequency and pattern) to moderately irritating target concentration of 27 mg/m^3 of DNCB for 15 min (calculated total dose is 70 μg, based on 180 ml air per minute during 15 min × 26.7 mg/m^3). At day 22, lung function was assessed again, where after necropsy was performed (blood sampling, bronchoalveolar lavage (BAL); weighing and collection of organs and tissues).

Table 1
Treatment schedule of the respiratory and skin allergy tests against DNCB

Group designation: Skin allergy[a]	Sensitization Day 0: 300 μl on flanks Day 7: 150 μl on ears	Challenge Day 21: 15 min inhalation	Day 18: Necropsy
−/− unsensitized/unchallenged	–	–	- Lung function[b] - Serum Ig
+/− sensitized/not challenged	1% DNCB–0.5% DNCB	–	- Liver, kidneys, left lung weights
−/+ unsensitized/challenged	Vehicle	27 mg/m^3 DNCB	- Nasal passages, larynx, trachea fixed in formalin; left lung snap-frozen;
+/+ sensitized/challenged	1% DNCB–0.5% DNCB	27 mg/m^3 DNCB	- BAL of right lung lobes
Group designation: Skin allergy[c]	Sensitization days 0, 1 and 2: 75 μl on ears	Challenge day 21: 15 min inhalation	Day 18: Necropsy
−/+ unsensitized/challenged	Vehicle	0.1% DNCB	- Skin application site: macroscopic examination and fixation in formalin for microscopy
+/+ sensitized/challenged	1% DNCB	0.1% DNCB	

[a] Six female BN rats per group.
[b] Lung function parameters were determined before, during and after challenge.
[c] Five male BN and Wistar rats per control (−/+) group; ten male BN and Wistar rats per test (+/+) group. Groups E and G: BN rats; groups F and H: Wistar rats.

2.2.2. Skin allergy

Skin allergy was tested in male BN and Wistar rats by using the protocol for a local lymph node assay (LLNA) for sensitization, followed by a topical challenge as in the guinea pig maximization test (Table 1). The doses for sensitization were based on previous on previous work (Arts et al., 1996). DNCB (75 μl of 1% in AOO) was applied to the dorsal side of the ears, on days 0, 1 and 2. The challenge dose was tested in a pilot study with a topical patch with 0.01, 0.05 and 0.1 % DNCB in 30 μl vaseline, in DNCB-sensitized Wistar rats (data not shown). A topical patch with 0.1% DNCB was selected and applied for 24 h to the flank, on day 16 (calculated total dose is 30 μg). The flank was examined macroscopically directly after removal of the patch and 24 h thereafter (skin reaction scores according to Magnusson and Kligman, 1969). Thereafter, the animals were killed and the skin at the flank was removed and preserved in 4% buffered formalin. After fixation, the skin was processed for microscopic examination by paraffin embedding and staining the 4 μm sections with hematoxylin and eosin (H&E).

2.3. Atmosphere generation and analysis

To generate the test atmosphere, the DNCB was nebulized after dissolving in acetone using a motor-driven syringe pump (WPI type SP22i, World Precision Instruments Sarasota, FL, USA) and an air-driven all glass nebulizer (Institue's design) at a preferred particle size distribution with a MMAD between 1 and 4 μm. The acetone concentration was kept at approximately 300 ppm (~0.7 g/m^3), which level is considered to be far below the level inducing sensory irritation (Alarie, 1973; De Ceaurriz et al., 1981; Schaper and Brost, 1991) and which indeed did not induce changes in breathing pattern in either sensitized or unsensitized rats (Arts et al., 1998). Air flow through the unit was 27 l/min; temperature and relative humidity were kept at 22 ± 2 °C and between 40 and 70%, respectively. Atmospheric concentrations of DNCB were determined gravimetrically by filter sampling and those of acetone by calculations based on the nominal concentration and its complete evaporation. The particle size distribution of DNCB in the test atmosphere was determined using a 10-stage cascade impactor (Andersen, Atlanta, GA). Gravimetry and cascade impactor samples were not collected during the challenge exposure but immediately prior to or after challenge, due to the small sampling air flow rate and the large total volume required for analytical and particle size determinations. The mean DNCB concentration was 26.7 ± 2.2 mg/m^3. The mass median aerodynamic diameter (MMAD) of the aerosolized DNCB particles was 1.5 μm with a gsd of 1.9.

2.4. Lung function measurements

For exposure to DNCB and for measurement of changes in respiration before, during and after exposure, rats were individually restrained in Battelle tubes and each tube was placed into one of four whole body plethysmographs connected to the central exposure unit. Using this experimental set up, two DNCB-sensitized (+/+) and two vehicle-treated (−/+) rats at a time, were first exposed to fresh air for at least 25 min (pre-challenge period) and then to the DNCB atmosphere for exactly 15 min (challenge period), followed by a recovery (post challenge) period of 30 min. Breathing parameters (breathing frequency, breathing pattern and tidal volume) were monitored by means of recording the pressure signal before, during and post challenge. Before challenge, breathing parameters were monitored approximately 20 s each minute, starting 6 min prior to challenge. During challenge, breathing parameters were monitored continuously, whereas after challenge, they were monitored approximately 20 s during each minute for the first 10 min followed by 20 s each 3 min for 9 min, and followed by 20 s each 5 min for 10 min. Furthermore, breathing parameters were monitored approximately 20 s each minute for 6 min 24 h after challenge. Mean values were therefore obtained based on 6 pre-exposure, 15 exposure, 15 post-exposure (immediately after), and 6 post-exposure (24-h after) values.

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

The animals were observed daily and weighed shortly before the DNCB application, at weekly intervals thereafter, and just prior to necropsy. Individual serum samples were prepared from blood withdrawn via the orbital plexus prior to sensitization, and via the abdominal aorta at necropsy. The serum samples were stored at −20 °C until analysis of total serum IgE and DNCB-specific IgG levels by means of an ELISA. At necropsy, animals were anaesthetized with Nembutal®, killed by exsanguination from the abdominal aorta and examined grossly for abnormalities. Liver, kidneys and the unlavaged left lung were weighed (left lung after bronchoalveolar lavage had been performed on the right lung lobes; see below). The left lung was inflated with 50% Tissue Tek in saline, quick-frozen on dry ice and kept at −70 °C for microarray analysis or immunohistochemistry. The nasal tissues, trachea and larynx were collected and fixated in neutral, phosphate-buffered 4% (v/v) formaldehyde for histopathological evaluation.

2.6. Bronchoalveolar lavage

At necropsy, the right lung lobes were lavaged two times with a volume of 23 ml saline per kilogram bw after ligating the bronchus of the left lung. 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 cell 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 microscopic evaluation. For differential cell counts, cytospins 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 dehydrogenase (LDH), alkaline phosphatase (ALP), and gamma-glutamyltransferase (GGT), using an automatic analyzer (Hitachi 911, Hitachi Instruments Division, Japan).

2.7. Total IgE and DNCB-specific IgG levels

Total IgE was measured in serum by ELISA as described earlier (Arts et al., 1997). The concentration of IgE in the samples was calculated using a standard curve obtained with known quantities of monoclonal rat IgE and expressed as μg/ml serum.

DNCB-specific IgG was measured by ELISA. The DNCB–BSA (bovine serum albumin) conjugate was prepared by dissolving 10 mg BSA in 1 ml carbonate buffer (pH 9.4). 1.5 g DNCB was added and the solution was stirred for 2 h. The conjugate solution was then dialyzed against phosphate-buffered saline for 48 h. ELISA plates (NUNC A/S, Roskilde, Denmark) were coated with 50 μg conjugate or BSA control (10 mg/ml diluted 200× in carbonate buffer, pH 9.4). Rat serum samples were diluted serially, starting with 1/50 dilution. OPD (phenylenediamine dihydrochloride, Sigma, and hydrogen peroxide) was added and the reaction was stopped by the addition of 4 M H_2SO_4. The absorbance was measured by 492 nm using a Microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

2.8. Histopathology and immunohistochemistry

The formalin-fixed nasal tissues, larynx and trachea (respiratory allergy test) and the flank skin (skin allergy test) were embedded in paraffin wax and sectioned at 5 μm. Nasal tissues were cut at six levels according to 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 skin was sectioned through the center of the application site. The sections were stained with hematoxylin and eosin.

Cryostat sections were made from the deep-frozen left lung of three animals per group (the other three were used for microarray analysis (see below). The 7 μm sections were stained for epsilon chain of rat IgE (MARE; Oxford Biotechnology Ltd., Oxford, England; 1:500), CD4 (W3/25; Serotec Ltd., Oxford, England; 1:800), CD8 (OX8; Serotec; 1:800) or CD161 (10/78; Serotec Ltd.; 1:800), using a 2-step indirect immunolabeling. After drying for one night at room temperature the sections were fixed in cold acetone for 7 min at 4 °C. The sections were washed two times for 5 min with PBS. Unspecific binding was blocked with 25% normal goat serum in PBS for 15 min. The sections were incubated for 60 min with the monoclonal antibodies listed above. Control sections were processed in the same way, except for the specific antibodies. For the second step the sections were incubated with powervision poly HRP-anti-mouse IgG mixed with normal rat serum (100:5, first pre-incubation for 60 min) for 30 min. The sections were then treated with the chromogen NovaRed for 8 min and were additionally contrasted with heamaluin for 20 s. All incubations were carried out at room temperature in a humid chamber and were followed by a three times washing step with PBS.

2.9. Microarray analysis

Total RNA was isolated from the frozen left lung of three rats per group, using TriZol reagent. RNA was purified using the RNeasy purification columns (Qiagen Mini kit, Westburg) and the quantity and quality (purity and stability) was determined using an Agilent BioAnalyzer. The RNA was labeled and hybridized to Affymetrix rat 230-2.0 GeneChip, containing probesets for 31000 rat genes at ServiceXS (Leiden, The Netherlands). Data were loaded into GCOS software (Affymetrix, USA). GCOS data were transferred to GeneSpring (Agilent/Silicon Genetics, USA) for GC-RMA normalization. Log transformed, normalized data were analyzed using Rosetta Resolver (RosettaBio, USA), Spotfire and Excel (Microsoft, USA). Data from GCOS were annotated with Gene Ontology information via the SOURCE website (Stanford University, USA). Automated annotation updates were also performed with BRB ArrayTools. A subset of 'expressed genes' was selected by filtering on expression level >15 in at least three samples (18624 probesets). To explore the variability in gene expression groups, the explorative multivariate methods principal component analysis (PCA) and hierarchical clustering were performed with Spotfire, Rosetta and GeneSpring (Demo version). Selection of differentially expressed genes was done by *t*-tests or by filtering on more than 25% up- or down-regulation in all three rats in a group.

A multiple testing correction (estimation of False Discovery Rate or FDR) was incorporated into the statistical analysis of the microarray data. An ANOVA was performed to test for significant differences in expression between the four treatment groups. In addition, *t*-test was performed to test for differences between the groups.

The microarray dataset was also analyzed on the pathway or gene group level. To infer gene groups that are significantly co-expressed we used T-profiler (Boorsma et al., 2005). T-profiler uses the unpaired *t*-test to score changes in average activity of pre-defined groups of genes. The gene groups are defined on the basis of Gene Ontology and KEGG categorization and have to contain at least six gene members (GO; Ashburner et al., 2000; Boyle et al., 2004). T-profiler uses the log ratios of genes between the treatment and reference as input. Therefore, log 2 ratios were calculated by taking the ratios of the mean-centered treatment groups and the mean-centered reference group (concurrent untreated BN rats plus three untreated BN rats from another experiment performed at the same time). In case of individual experiment comparison, the ratios were taken of the individual treatment experiments and the mean-centered reference group. After applying the unpaired *t*-test for each gene group, a *P*-value was calculated from t using the *t*-distribution with $N-2$ degrees of freedom where N is the total amount of genes tested. The *P*-value was corrected for multiple testing by multiplying it by the number of gene groups that are being tested in parallel (Bonferoni correction). The *P*-value now becomes an *E*-value. All groups with a corrected *E*-value of ≤ 0.05 are considered to be significantly regulated. In order to make the procedure more robust against outliers the highest and lowest value of each gene group was discarded. This is similar to the jack-knife procedure. A major advantage of T-profiler is that no parameters have to be tuned; the complete transcriptome is used for the calculation. The analysis is supported by http://www.t-profiler.org.

2.10. Statistics/data analysis

Body weights were analyzed by one-way analysis of co-variance, followed by the two-sided Dunnett's multiple comparison test. Organ weights, immunoglobulin levels, BAL biochemical parameters and absolute cell numbers were determined by Anova–Dunnett's test. Analyses were performed by the usage of Graphpad Prism (Version 3.0, San Diego, CA, USA).

Statistical analysis of the microarray data are described under 'Microarray analysis'.

3. Results

3.1. Breathing parameters/lung function

Breathing frequencies shortly before challenge with 27 mg/m^3 of DNCB (estimated total dose 70 μg) were comparable between the unsensitized/challenged (−/+) and sensitized/challenged (+/+) groups (Fig. 1a). During challenge, the breathing frequency in both groups decreased by approximately 13%, indicating that the concentration was only minimally irritating to the lungs (Alarie, 1973), and it was still decreased 24 h after challenge. Tidal volume was unaltered during and shortly after challenge, but was increased in the +/+ group 24 after challenge (Fig. 1b). Minute ventilation, a function of breathing frequency and tidal volume, decreased slightly during and shortly after challenge and returned to normal values at 24 h

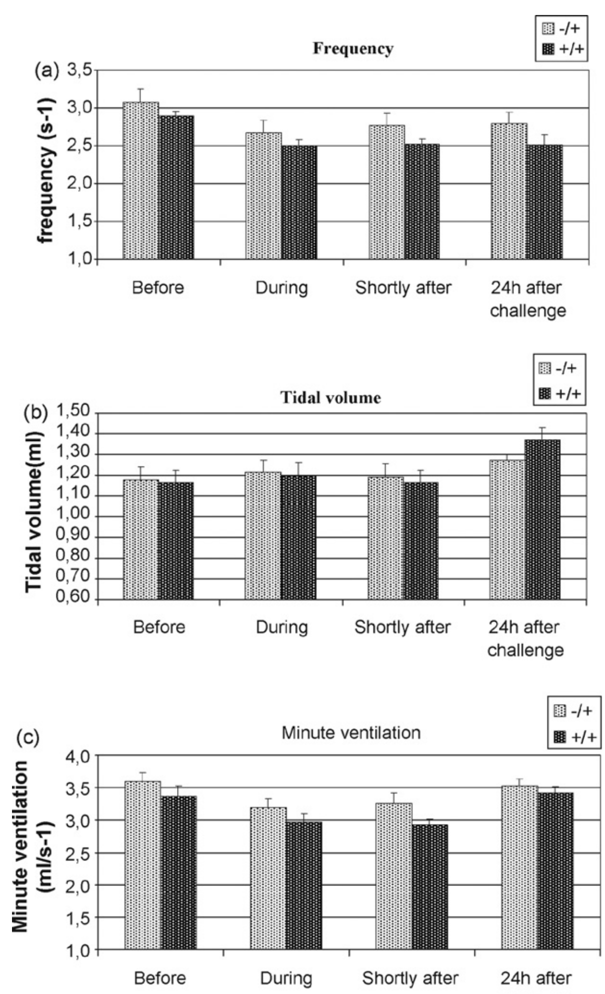

Fig. 1. Mean relative changes in respiratory frequency, tidal volume and minute ventilation in groups of six BN rats after dermal sensitization and inhalation challenge with DNCB. Treatment: unsensitized (vehicle-treated)/challenged (−/+; lightly dotted columns) and sensitized/challenged (+/+; heavily dotted columns).

Table 2
Number of neutrophils in BAL[a] and immunohistochemically stained cells[b] in lung parenchyma in the respiratory allergy study with DNCB

Group[c]	Neutrophils in BAL $\times 10^5$	$CD161^+$ cells	$CD4^+$ cells	$CD8^+$ cells	IgE+ cells
−/−	0.1 ± 0.0	6.3 ± 4.2	8.5 ± 5.5	2.8 ± 1.4	2.0 ± 1.8
+/−	0.0 ± 0.0	5.4 ± 2.8	12.7 ± 4.4*	4.5 ± 2.5	3.2 ± 1.7
−/+	0.0 ± 0.0	4.1 ± 2.2	7.3 ± 3.3	2.5 ± 2.0	2.1 ± 2.2
+/+	0.2 ± 0.0#	4.5 ± 2.9	14.5 ± 6.1*	4.1 ± 3.2	2.8 ± 2.1

\# $P<0.05$ Anova/Dunnett's test (−/− group as control group).
* $P<0.05$, sensitization effect; sensitized (+/− and +/+) versus unsensitized (−/− and −/+) groups.
[a] Mean cell numbers ± S.E.M. per milliter.
[b] Mean number of positive cells per square field of 0.4 mm^2 lung parenchyma ± S.E.M. of each animal, 5 square fields were counted per left lung at 250× magnification.
[c] $N = 6$ BN rats/group for BAL counts and three BN rats/group for immunohistochemistry.

after challenge, indicating that the +/+ group compensated the slightly decreased breathing frequency with an increased tidal volume (Fig. 1c).

3.2. Clinical signs, body and organ weights and bronchoalveolar lavage (BAL)

All animals gained approximately 15 g between days 0 and 22. The weights of kidneys, liver and left lung were unaffected. The total cell numbers in BAL were not affected in the challenged groups. There was a two-fold increase in the number of neutrophils in the sensitized and challenged (+/+) animals when compared to the untreated (−/−) controls, but their number still remained low (Table 2).

3.3. IgE and IgG levels

Serum levels of total IgE were low (mean level ± S.E.M.: 0.50 ± 0.04 μg/ml) in all groups before treatment and stayed low during the entire study, regardless the treatment. After the second immunization, on day 22, relatively high DNCB-specific IgG levels were observed in 3/6 sensitized and challenged rats, compared to unsensitized/challenged rats (Fig. 2).

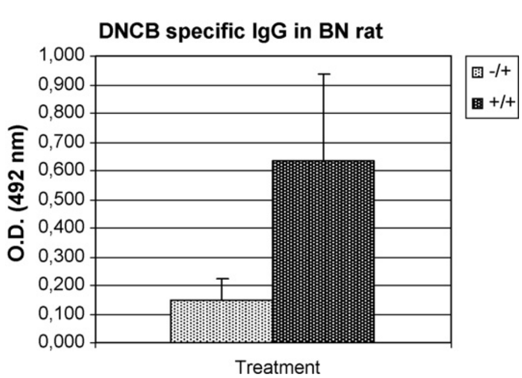

Fig. 2. Mean ± S.D. of DNCB-specific IgG in the unsensitized/DNCB-challenged (−/+; lightly dotted columns) group and the DNCB-sensitized/DNCB-challenged group (+/+; heavily dotted columns). Six BN rats per group were measured.

3.4. Nasal passages, larynx and trachea histopathology, and lung immunohistochemistry

The nasal passages and trachea did not exhibit histopathological changes. In the larynx of the challenged animals (−/+ and +/+), signs of irritation were observed at the ventral base of the epiglottis, i.e. erosion and thinning of the epithelium with microhaemorrhages and mixed inflammatory cell infiltrate. There was no difference in severity and character of the inflammation between the two challenged groups. Sensitization and/or challenge did not alter significantly the number of $CD8^+$, $CD161^+$ and IgE+ cells in the interstitium of the left lung. Sensitization significantly increased the number of $CD4^+$ cells (+/− and +/+ groups compared to the −/− and −/+ groups) (Table 2).

3.5. Microarray analysis of lung tissue

3.5.1. Data exploration and differentially expressed genes/single gene comparison

PCA and hierarchical clustering did not show a clear separation of the treatment groups. This was caused by variation between samples within a group as well as by a limited effect of the treatment. Single gene comparison between the groups (ANOVA and *t*-test analyses with multiple testing correction by estimation of FDR and setting the FDR threshold on 10%) resulted in low numbers of differentially expressed genes, below the number of differentially expressed genes by chance.

3.5.2. Gene group analysis (T-profiler)

A few groups of genes were significantly differentially expressed upon comparison of the treatment groups (Table 3). The treatment group that differed most from the other three treatment groups was the unsensitized/challenged (−/+) group. This −/+ group differed significantly from the other three groups for the gene groups 'Chemokine activity', 'Inflammatory response' and 'Chemotaxis'. The sensitized/challenged (+/+) group did not differ significantly from the sensitized/unchallenged (+/−) group; from the untreated control (−/−) and the unsensitized/challenged (−/+) groups it differed for the gene group

Table 3
T-values of differentially expressed groups of genes (GO Ontology) in left lung tissue of BN rats after inhalation challenge with DNCB

Group comparison	Chemokine activity	Extracellular space	Inflammatory response	Extracellular region	Chemotaxis	Immune response
+/+ versus −/−	3.14	4.68*	2.94	1.86	0.77	0.73
+/+ versus +/−	1.99	2.39	2.10	−0.35	1.09	1.04
+/+ versus −/+	16.64***	10.90***	10.04***	8.53***	7.31***	5.40**
+/− versus −/−	1.85	3.14	1.80	1.80	−0.05	−0.14
+/− versus −/+	14.22***	7.90***	8.49***	8.17***	6.49***	4.84*
−/+ versus −/−	−8.02***	−2.90	−4.04*	−3.82	−4.86*	−3.34

* *E*-value < 0.05.
** *E*-value <0.001.
*** *E*-value <0.0001.

Table 4
Chemokines, which contributed most to the GO ontology gene groups 'Chemokine activity', 'Inflammatory response', 'Chemotaxis' and 'Extracellular space' and their up- or downregulation[a]

Gene symbol, accesion no.	Gene description	Mean fold change (log 2 ratio) −/+ versus −/−	Mean fold change (log 2 ratio) +/+ versus −/+
Ccl2, NM_03153	Chemokine (C-C motif) ligand 2; monocyte chemotactic protein 1 or MCP-1; small inducible cytokine A2; small inducible gene JE	−2.07	3.49
Ccl4, U06434	Chemokine (C-C motif) ligand 4; macrophage inflammatory protein-1 beta or MIP1beta; small inducible cytokine A4	−1.86	2.35
Ccl7/RGD1359152, BF419899	Chemokine (C-C motif) ligand 7; chemotactic protein-3	−2.00	3.96
Ccl17, NM_05715	Chemokine (C-C motif) ligand 17; small inducible cytokine subfamily A (Cys-Cys), member 17	−2.60	6.61

[a] Differential expression as mean fold changes of the unsensitized/challenged (−/+) group versus unsensitized/unchallenged (−/−) group, and the sensitized/challenged (+/+) group versus the unsensitized/challenged (−/+) group. *P* value of *t*-test was >0.01, for all genes.

Table 5
Macroscopy and microscopy of the skin response against DNCB, in the LLNA with challenge[a,b]

	Control BN mean ± S.E.M.	Control Wistar mean ± S.E.M.	Ratio BN:Wistar	Sensitized BN mean ± S.E.M.	Sensitized Wistar mean ± S.E.M.	Ratio BN:Wistar
Number of animals	(5)	(5)		(10)	(10)	
Macroscopy score 24 h	0.8 ± 0.8	0.8 ± 0.8	1	1.7 ± 0.7	1.8 ± 0.6	0.9
Macroscopy score 48 h	0.6 ± 0.9	2.2 ± 0.8	0.3	2.6 ± 0.5	3.8 ± 0.4	0.7
Microscopy: mononuclear inflammation score	Not present	Not present	Not present	1.2 ± 0.4	2.2 ± 0.4	0.6

[a] Macroscopy score is presented as the mean of scores, according to Magnusson and Kligman (1969).
[b] Microscopy: mononuclear inflammation was scored on a scale of 1 (very slight) to 3 (moderate) and presented as mean score.

'Extracellular space'. The chemokines Ccl2, Ccl4, Ccl7, and Ccl17 contributed most to these gene groups (Table 4).

3.6. Skin allergy scores and microscopy

DNCB, 0.1% (estimated total dose 30 μg), was slightly irritating to the skin of the BN rat and the irritation diminished during the 24 h observation period, whereas it was slightly irritating at first but became considerably irritating to the skin of the Wistar rat at the end of the 24 h observation period (control animals; Table 5). Sensitized BN rats also reacted less than the sensitized Wistar rats to the challenge with DNCB. This was especially true for the microscopic parameter 'inflammatory cell infiltrate'. The macroscopically observed allergic reaction in sensitized Wistar rats was somewhat obscured by the irritation (low relative increase in sensitized compared to unsensitized Wistar rats).

4. Discussion

The potential of the contact allergen DNCB to induce respiratory allergy (allergic inflammation and/or changes in breathing pattern) was examined in the highly Th2-polarized BN rat (Table 6). The sensitivity of the BN rat for the allergic properties of DNCB was tested in a skin allergy test and compared with that of the highly Th1-polarized Wistar rat.

DNCB induced a specific IgG antibody response in the BN rat upon dermal sensitization, but the response could not be demonstrated in all animals. This is in accordance with the finding that BN rats did not have a vigorous and consistent IgG antibody response against DNCB in contrast to the response against the LMW respiratory allergen trimellitic anhydride (TMA), when the allergens were tested at concentrations with comparable overall immunogenicity with regard to lymph node activation and the induction of lymph node cell proliferation (Warbrick

Table 6
Summary of the respiratory allergy test with DNCB in BN rats

Group	Effects
+/+ (sensitized/unchallenged)	- Increased number of lung parenchymal $CD4^+$ cells
−/+ (unsensitized/ challenged)	- Differentially expressed gene groups: chemokine activity, inflammatory response; Chemotaxis - Down-regulation of mRNA for the chemokines Ccl2, Ccl7 and Ccl17
+/+ (sensitized/ challenged)	- Increased number of lung parenchymal $CD4^+$ cells - Increased number of neutrophils in BAL - Differentially expressed gene group: Extracellular space - Upregulation of mRNA for the chemokines Ccl2, Ccl4, Ccl7 and Ccl17

et al., 2002b). Immunization by DNCB in BN rats was also apparent in previous positive LLNA tests, although the Wistar rat had a much higher stimulation index than the BN rat (Arts et al., 1996). Interestingly, both dermally sensitized groups of BN rats (+/− and +/+) in the present respiratory allergy test showed an increased number of $CD4^+$ cells in the lung parenchyma (Table 2). Immunization/sensitization by DNCB was indirectly shown by the allergic skin responses in all BN rats upon dermal challenge. Again, BN rats had less vigorous allergic reactions compared to the Wistar rat, especially with regard to the microscopically observed mononuclear cell infiltrate, a parameter that solely represented allergy. Genetic factors are considered to play a role in occupational asthma (Dermchuk et al., 2007) and allergic alveolitis (Belenky and Fuhrman, 2006). It may, therefore, be worthwhile to take genetic factors of the test animal into account in predictive tests of allergens .

In the present study, the calculated total dermal dose used at skin challenge was 30 μg and the calculated total dose at inhalation challenge was twice as high, i.e. 70 μg DNCB. The inhalation challenge concentration was considered relatively high with respect to the larynx changes observed in the challenged rats (−/+ and +/+ groups), although it did not induce histopathological lesions in the bronchi and lung parenchyma. Nevertheless, the inhalation challenge did not induce apneas or allergic inflammation (or histopathological lesions indicative of toxicity), as found with the respiratory allergen trimellitic anhydride in BN rats using the same protocol (Arts et al., 1998). The absence of respiratory allergy against DNCB upon a single inhalation challenge could not be ascribed to a lack of allergen reaching the lower airways: DNCB inhalation induced increased number of neutrophils in BAL of sensitized and challenged (+/+) rats, and differential expression of the gene groups ‘Chemokine activity’, ‘Inflammatory response’, ‘Chemotaxis’ and ‘Extracellular space’ in lung tissue (microarray analysis) (Table 3). Interestingly, gene groups were more profoundly expressed in the unsensitized/challenged (−/+) group, which can be considered as a group in the very early phase of sensitization by inhalation.

In the gene groups, mRNA for the chemokines Ccl2 (MCP-1), Ccl4 (MIP-1beta), Ccl7 and Ccl17 was upregulated in the allergic (sensitized/challenged or +/+) group. Upregulation of Ccl2/MCP-1 was also found in the skin of rats affected by DNCB-induced contact dermatitis (allergy phase; Hartmann et al., 2006). Ccl2/MCP-1 (to a lesser extent Ccl7 and Ccl17; reviewed by Bloemen et al., 2007) is associated with respiratory allergy: early and prolonged upregulation of Ccl2/MCP-1 (and Ccl7) was observed after respiratory allergen (ovalbumin) challenge in murine lung (allergy phase; Fulkerson et al., 2004; Zimmerman et al., 2004); it was found in diisocyanate asthma in mice (Johnson et al., 2004); in man, Ccl2/MCP-1 stimulation by diisocyanates in human blood mononuclear cells was considered to have a greater sensitivity and specificity than specific IgE in serum (Bernstein et al., 2002); finally, the gene for Ccl2/MCP-1 was included in the top-100 list of asthma genes (Ober and Hoffjan, 2006). In contrast, mRNA for the chemokines Ccl2/MCP-1, Ccl7 and Ccl17 were down-regulated in the unsensitized/challenged (−/+) group, as stated above a group possibly in the very early phase of sensitization by inhalation. Down-regulation of these genes was also observed in human dendritic cells, upon stimulation by DNCB (sensitization phase; Ryan et al., 2004; Schoeters et al., 2005). These data suggest that DNCB by inhalation sets into motion a chemokine reaction in the lung that is comparable to that involved in sensitization via the skin. Chemokines attract inflammatory cells, but an inflammatory cell infiltrate into the lungs was restricted to a minimal increase in the number of BAL neutrophils in sensitized/challenged (+/+) rats. The significance of this neutrophil infiltrate for respiratory allergy is unknown. Using the same protocol, the respiratory allergen TMA induced also a slight increase in neutrophils and additionally in eosinophils (Arts et al., 2003).

The variable DNCB-specific IgG response and the slight to moderate dermal response in BN rats to a high dermal dose challenge of DNCB demonstrated that the Th2-prone BN rat was less sensitive to DNCB than the Th1-prone Wistar rat. It is concluded that the contact allergen DNCB did not induce respiratory allergy in the BN rat, despite the fact that the immune system of the rat recognized DNCB and that upregulation of some allergy-associated genes in lung tissue suggested the induction of an allergic response.

Acknowledgements

The authors gratefully acknowledge CEFIC-LRI Brussels, Belgium, and the Dutch Ministry of Social Affairs and Employment for financial support. They also thank E. Duistermaat, F. Hendriksma, L. van Oostrum and G. Roverts for expert technical assistance; Dr. H. Muijser for the statistical analyses and Dr. M. van Erk for her help in the microarray analysis.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tox.2008.01.013.

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