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Hypersensitivity reactions in mouse airways after a single and a repeated hapten challenge

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Abstract. *Objective and design:* In this study, we examined the effect of a single and a repeated hapten-challenge on inflammatory processes in the airways of mice undergoing a hapten-induced non-IgE mediated hypersensitivity reaction.
Methods: BALB/c mice were skin-sensitized with the hapten dinitroflourobenzene (DNFB) and intra-airway challenged with dinitrobenzene sulphonic acid (DNS). Mucosal exudation, tracheal vascular permeability, cellular accumulation, and serum murine mast cell protease (MMCP) were investigated at different time points after the first DNS-challenge and 30 min after a repeated DNS-challenge.
Results: MMCP levels in serum were increased at all time points after single challenge and repeated challenge. Increased vascular permeability as determined by Monastral blue staining, was found in the trachea of DNFB-sensitized mice after single DNS-challenge. A second exposure to DNS profoundly enhanced the Monastral blue labeling of the tracheal blood vessels of DNFB-sensitized mice. Furthermore, increased mucosal exudation and polymorphonuclear cell (PMN) accumulation were present in DNFB-sensitized mice compared to vehicle-sensitized animals after the first DNS challenge.
Conclusions: Increased mucosal exudation, vascular permeability, and PMN accumulation are prominent inflammatory features of the DNFB-induced hypersensitivity reaction in the airways. Furthermore, mast cell activation is associated with this hapten-induced hypersensitivity reaction.

Key words: Hypersensitivity reaction – Lung – Inflammation – Mast cell – Dinitrofluorobenzene

Introduction

Hapten-induced hypersensitivity reactions are cell-mediated immune reactions, which take more then 12 h to develop and feature many types of inflammatory cells. The inflammatory cells can exert profound effects on the microvasculature. The local blood vessels become hyperpermeable resulting in extravasation of plasma with concomitant formation of edema.

Hapten-induced hypersensitivity responses have been extensively investigated in the skin but its has become apparent that a similar reaction can also occur in the airways. Skin-sensitization with the low-molecular weight compounds, such as toluene diisocyanate, dinitrofluorobenzene (DNFB) or picrylchloride induces hapten-specific hypersensitivity responses in mouse airways after intranasal challenge. These pulmonary hypersensitivity reactions are not associated with an elevated hapten-specific IgE levels in serum [1, 2]. Tracheal hyperreactivity, leukocyte accumulation and edema formation were prominent features 24 to 48 h after single antigen challenge [2–5].

Edema formation in the lung can be divided into two processes: vascular edema and mucosal edema. Vascular edema can be described as the passage of plasma from the microvasculature into the tissue. It is a consequence of an increase in permeability of the local blood vessels and will lead to thickening of the lamina propria. Often the extravasated plasma does not remain in the tissue but is reabsorbed into in the microvasculature or crosses the epithelial lineage thereby entering the airway lumen. The movement of plasma components across the epithelium into the airway lumen is called mucosal edema formation or mucosal exudation. Increased vascular permeability as well as enhanced mucosal exudation are both important in delivering inflammatory cells and inflammatory mediators from the vasculature into the airway lumen during a hapten-induced hypersensitivity reaction in the airways.

Mast cells and its mediators play an important role in hapten-induced hypersensitivity reactions in skin, lung and small intestine of mice [5–7]. It is hypothesized that upon skin-sensitization with low molecular weight compounds, hypersensitivity-initiating cells in the draining lymph nodes and spleen are induced to produce and release hapten-specific factors. These factors will bind to mast cells and mediate an early hypersensitivity phase [7–9]. Mast cells also participate in the late phase of a pulmonary DNFB-induced hyper-

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sensitivity reaction. Features like, tracheal hyperreactivity, increased mucosal exudation and leukocyte accumulation are not observed in DNFB-sensitized mast cell-deficient $WBB6F_1$-W/W^v mice [8, 9].

In this study, we investigated vascular permeability changes in the trachea of hapten-sensitized mice after single by usage of the two different dyes Monastral blue and Evans blue. Furthermore, mucosal exudation, and cellular accumulation were determined in the airways. Besides the effect of a single DNS-challenge, the effect of a second DNS-challenge within 24 h after the first DNS-challenge was examined. To study if mast cells are associated with this pulmonary hypersensitivity reaction, levels of mouse mast cell protease (MMCP) were quantified in the serum.

Materials and methods

Animals

Male BALB/c mice, aged 7 weeks upon delivery, were supplied by the Central Animal Laboratory (Utrecht, The Netherlands). They were housed in macrolon cages of six to ten animals per cage. Tape water and commercial chow-food (Hope Farms, Woerden, The Netherlands) were allowed ad libitum. All the experiments were approved by The Animal Care Committee of the Utrecht University (Utrecht, The Netherlands).

Induction of hapten-induced hypersensitivity responses

On day 0, mice received either DNFB (0.5%) or vehicle solution (acetone:olive oil = 4:1) epicutaneously applied onto the shaved abdomen (50 µl) and four paws (50 µl). On day 1, DNFB or vehicle was applied only onto the abdomen. During this sensitization procedure, animals were anesthetized with sodium pentobarbitone (50 mg/kg, intraperitoneally (i.p.)). On day 5, both DNFB-sensitized and vehicle-treated animals were challenged intranasally with DNS (0.6% in sterile phosphate-buffered saline (PBS, 50 µl) under light anesthesia sodium (pentobarbitone, 40 mg/kg, i.p.). In separate series of experiments, a second challenge was applied on day 6, 24 h after the first DNS-challenge.

Measurement of tracheal vascular permeability by Monastral blue

Monastral blue accumulation was measured in the tracheas as described by Baluk and coworkers [10]. Monastral blue is a colloidal dye that under normal conditions remain in the circulation. However, when the gaps between endothelial cells become large enough, e.g. an increase in permeability during inflammatory processes, the dye passes through the gaps where it is trapped in the basal lamina. Therefore, Monastral blue identifies vessels at the sites of extravasation. Monastral blue was injected i.v. via the tail vein immediately before the first or second DNS-challenge to vehicle- and DNFB-sensitized animal. In case of the 24-h time point, Monastral blue was adminstered 2 h before sacrificing the animals.

After killing the mice with an overdose of sodium pentobarbitone, they were perfused transcardially for 5 min with PBS containing heparin (10 U/ml) followed by 1% paraformaldehyde for 10 min. Tracheas were removed, opened longitudinally along the ventral midline and fixed in 4% paraformadehyde overnight. Finally, they were hydrated in ethanol, cleared in xylene, and prepared as whole amounts.

Measurement of mucosal exudation and vascular permeability by Evans blue

Evans blue dye (1.25% in sterile saline, 50 µl) was injected i.v. via the tail vein 2 h before the end of an experiment. Evans blue binds to plasma proteins and can be used to quantify permeability changes. After the animals were killed by an overdose of sodium pentobarbitone (60 mg/ml, 200 µl), blood samples were taken via cardiac puncture. Mucosal exudation was determined by measuring the Evans blue content in the first ml of the broncho-alveolar lavage (BAL) fluid samples as described by Buckley and Nijkamp [4]. Briefly, the trachea was carefully intubated and the catheter was secured with ligatures. Warm saline (37°C) was slowly injected into the lungs and withdrawn in 4 × 1 ml aliquots. The BAL fluid samples were kept on ice.

Plasma extravasation into tracheal tissue was quantified as described by Rogers et al [11]. After blood sampling and lavaging the lungs, the animals were perfused via the right ventricle with 10 ml warm saline. Blood and perfusion fluid were expelled through an incision in the vena cava. Tracheas were excised, dissected free of fat and connective tissue and were placed into formamide (250 µl). Evans blue dye was extracted from the tracheas overnight at 40°C. Tracheas were dried for 3 days at 40°C and tracheal dry weight was determined. The amount of Evans blue in plasma samples, BAL fluid samples, and formamide extracts was quantified by measuring the optical density at a wavelength of 595 nm with a Benchmark microplate reader (Biorad, California, USA). The amount of mucosal edema formation (µl/lung) was determined by dividing the Evans blue content in the first ml BAL fluid by the Evans blue content in 1 ml plasma. Changes in mucosal permeability were expressed as µl/lung. The amount of vascular edema formation in the trachea was calculated by dividing the Evans blue content in the formamide sample by the Evans blue content in 1 ml plasma. Changes in tracheal vascular permeability were expressed as µl leakage per mg tracheal dry weight.

Cellular accumulation in the BAL fluid

After centrifuging the BAL fluid samples (1500 rpm, 4°C, 10 min) and removing the supernatant for measurement of mucosal exudation, the BAL cells were pooled and suspended in 150 µl PBS. Total number of cells was counted by using a Bürker-Türk chamber. For differential BAL cell counts, cytospin preparations were made and stained with Diff-Quick. One observer evaluated all cytospin preparations by oil immersion microscopy. Cells were identified into mononuclear cells (MNCs) and polymorphonuclear cells (PMNs) by standard morphology.

Measurement of mouse mast cell protease (MMCP)

Sera were collected as described above and stored at −80°C until the sera were measured. The MMCP levels in the sera were determined by a commercially available ELISA. The results were expressed as ng MMCP per ml serum.

Materials

DNFB and Evans blue were obtained from Sigma Chemical Company (St Louis, USA). DNS was purchased from Eastman Kodak Company (Rochester, New York, USA), pentobarbitone (Nembutal®) from Sanofi B.V. (Maasluis, The Netherlands), and heparin from Leo Pharmaceutical Products (Ballerup, Denmark). Diff-Quick staining kit was obtained from Merz&Dade A.G. (Düdingen). The MMCP elisa was purchased from Moredun Scientific Ltd. (Midlothian, Scotland).

Data analysis

The results are expressed as mean ± SEM or as medians (minimum-maximum). Differences between the groups were analyzed by one-way analysis of variance (ANOVA) and if applicable followed by a Tukey's post hoc test, which compared all pairs of columns (GraphPad Prism version 2.01, San Diego, USA). Data on the cellular accumulation were analyzed by a distribution free Kruskal-Wallis ANOVA. All P-values < 0.05 were considered to reflect a statistically significant difference.

Results

Vascular permeability changes

Changes in vascular permeability in tracheal tissue were determined in the DNFB-induced pulmonary hypersensitivity reaction by usage of Monastral blue dye and Evans blue dye. In tracheal whole-mount preparations from vehicle- and DNFB-sensitized animals almost none Monastral blue

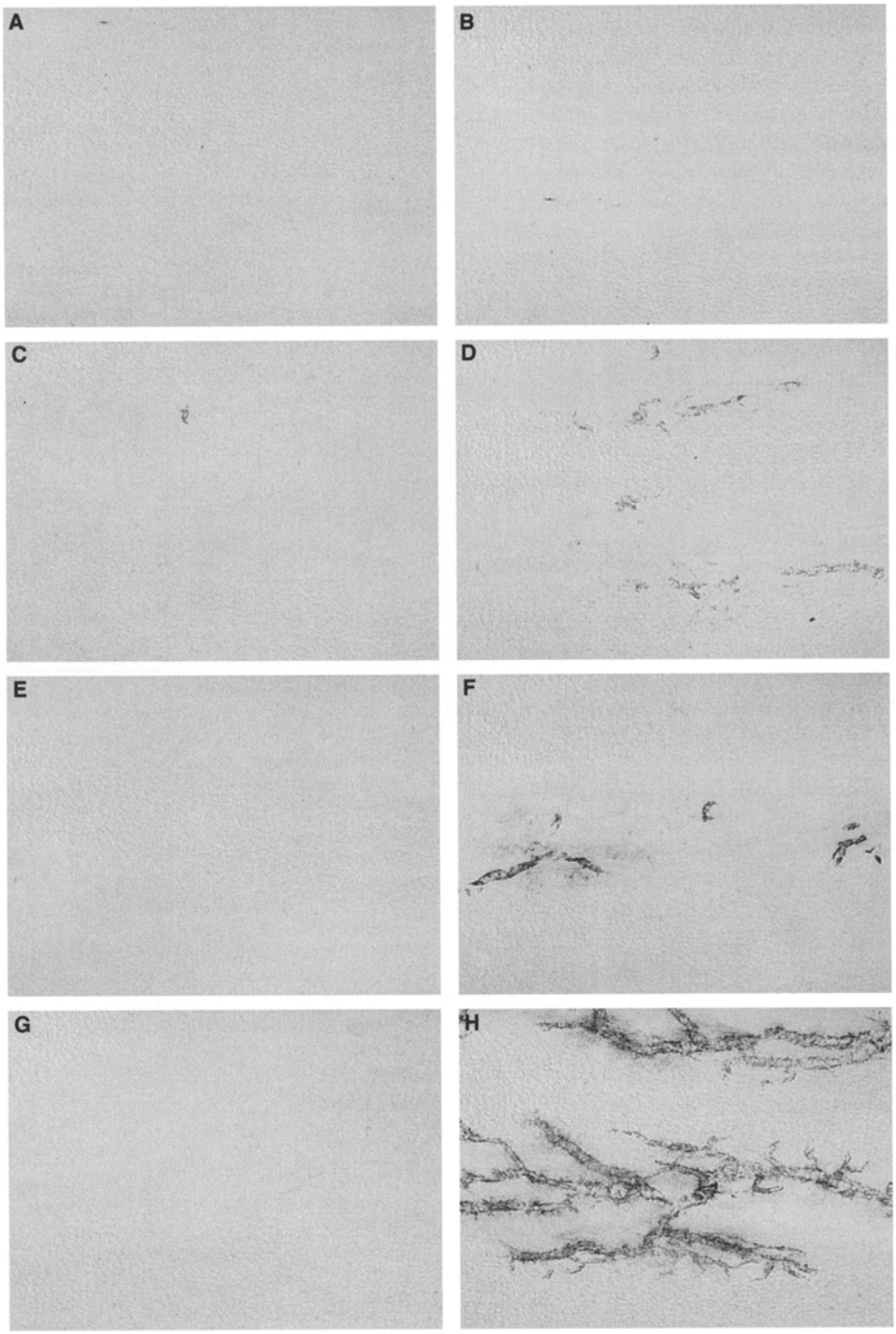

Fig. 1. Typical individual samples of Monastral blue-labeled blood vessels in the tracheas of vehicle-sensitized (**A**, **C**, **E**, **G**) or DNFB-sensitized (**B**, **D**, **F**, **H**) mice on day 5 before challenge (**A**, **B**), 30 min (**C**, **D**) and 24 h (**E**, **F**) after single DNS-challenge, and 30 min after a repeated challenge (**G**, **H**).

labeled vessels were present on day 5 before DNS-challenge. An increase in Monastral blue labeled blood vessels was present in tracheas of DNFB-sensitized mice 30 min and 24 h after single DNS-challenge compared to vehicle-sensitized mice (Fig. 1). Monastral blue dye accumulation was profoundly increased in DNFB-sensitized mice after a repeated DNS-challenge (Fig. 1). Monastral blue-labeled blood vessels were found mainly in mid and lower part of the tracheas after single DNS-challenge and throughout the whole trachea after repeated DNS-challenge.

No difference in basal Evans blue accumulation in tracheal tissue of DNFB-sensitized mice was observed before the challenge on day 5 compared to vehicle-sensitized animals (Fig. 2). In addition, no change in vascular permeability in tracheal tissue 30 min or 24 h after the first DNS-challenge was found compared to basal level on day 5. However, a profound increase in vascular permeability in DNFB-sensitized animals was found 30 min after a second exposure to DNS when compared to vehicle-treated mice (Fig. 2).

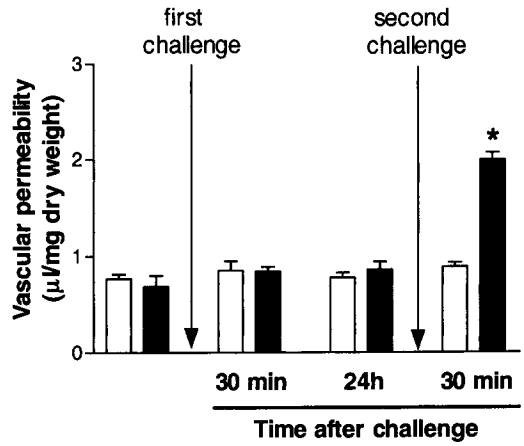

Fig. 2. Tracheal vascular permeability changes (µl/mg tracheal dry weight) 30 min and 24 h after first DNS-challenge and 30 min after second DNS-challenge in vehicle-sensitized (open bars) and DNFB-sensitized (closed bars) mice. Data are expressed as mean ± SEM for n=6 animals per group. * $p<0.001$ compared to all groups.

Mucosal permeability changes

Increased mucosal exudation is a prominent feature of the DNFB-induced hypersensitivity reaction in the mouse airways as can be seen in Fig. 3. No difference in basal mucosal permeability was observed between vehicle- and DNFB-sensitized animals before the DNS-challenge on day 5. Also 30 min after the challenge, no difference was found in mucosal exudation. However, a significant increase in mucosal exudation, as measured by an increased Evans blue dye accumulation in the BAL fluid, was found in DNFB-sensitized mice 24 h after the first challenge compared to vehicle-sensitized and DNS-challenge animals (Fig. 3). A second exposure to did not further enhance the mucosal permeability in DNFB-sensitized mice (Fig. 3).

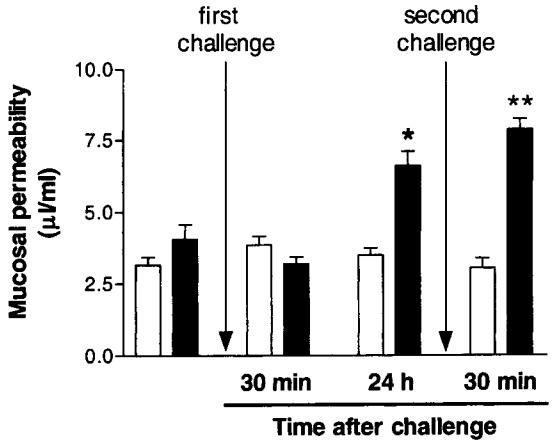

Fig. 3. Mucosal permeability changes (µl/lung) 30 min and 24 h after first DNS-challenge and 30 min after second DNS-challenge in vehicle-sensitized (open bars) and DNFB-sensitized (closed bars) mice. Data are expressed as mean ± SEM for n=6 animals per group. * $p<0.001$ compared to all groups, except to DNFB-sensitized animals 30 min after the second challenge. ** $p<0.001$ compared to all groups with the exception of DNFB-treated animals 24 h after the first challenge.

Cellular accumulation

The total cell numbers and differential cell count in the BAL fluid from DNFB-sensitized mice and vehicle-treated mice are presented in Table 1. No difference in total cell numbers

Table 1. Total cell numbers and leukocyte differentiation in the BAL fluid of vehicle- and DNFB-sensitized mice at different time points after challenge.

Sensitization		Cells/lung (×1000)		
		Total cells	MNC	PMN
No challenge	Vehicle	31 (20–39)	30 (19–39)	0.4 (0.3–0.7)
	DNFB	29 (15–35)	28 (15–34)	0.2 (0.2–0.5)
Time after first DNS challenge				
30 min	Vehicle	25 (12–46)	24 (12–46)	0.3 (0.1–0.7)
	DNFB	34 (21–51)	33 (21–50)	0.5 (0.1–1.3)
24 h	Vehicle	25 (17–41)	24 (16–38)	1.1 (0.3–3.7)
	DNFB	38 (30–42)	30 (20–39)	4.2 (2.7–16.0)*
Time after second DNS challenge				
30 min	Vehicle	23 (20–29)	18 (15–22)	2.8 (2.5–4.3)
	DNFB	34 (24–39)	30 (21–35)	7.4 (5.3–10.3)**

Data are expressed as median (min-max). * $p < 0.01$ compared to vehicle-sensitized and single DNS-challenged mice. ** $p < 0.01$ compared to vehicle-sensitized and repeated DNS-challenged mice.

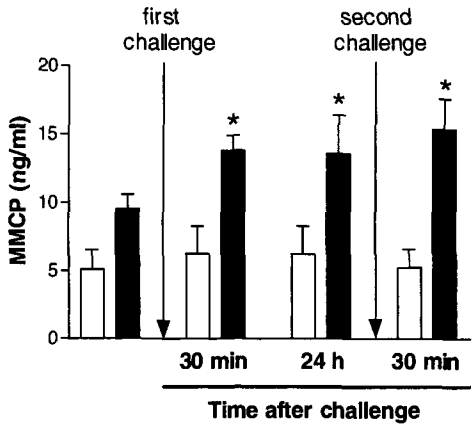

Fig. 4. MMCP levels in serum of vehicle-sensitized (open bars) and DNFB-sensitized (closed bars) mice on different time points after first and repeated challenge. Data are expressed as mean ± SEM for n=6 animals per group. * $p<0.001$ compared to all control groups.

was found in the BAL fluid on day 5 before challenge and at 30 min after the first DNS-challenge. There was an increase, although not significant, in total numbers of BAL fluid cells recovered from DNFB-sensitized mice 24 h after the first challenge compared to vehicle-sensitized mice. In additionally, total cell numbers recovered from the BAL fluids of DNFB-treated mice were slightly increased 30 min after the second challenge. When examining the BAL fluid cells differentially, a significant increase in the number of PMN was found 24 h after a single DNS-challenge. A repeated DNS-challenge to DNFB-sensitized animals further enhanced the number of PMN in the BAL fluid (Table 1).

Serum MMCP levels

Mast cells release MMCP into the serum when they are activated and serum MMCP levels can be used as a marker for mast cell activation. Serum MMCP levels were increased at all time points after the first challenge in DNFB-sensitized animals when compared to vehicle treated mice (Fig. 4). A second challenge did not further enhance the MMCP levels, but increased MMCP levels were still evident in mice that were DNFB-sensitized and DNS-challenged.

Discussion

In this study, we have shown that an early vascular permeability response, as measured by Monastral blue accumulation, was found in tracheas of DNFB-sensitized mice 30 min after the first challenge. 24 h after single DNS-challenge mucosal exudation and PMN accumulation were prominent features associated with a pulmonary DTH reaction in mice. In addition, a repeated challenge, 24 h after the first challenge, induced a vascular hyperpermeability response in tracheas of mice sensitized with DNFB as measured by both Monastral blue and Evans blue accumulation. Moreover, mast cell activation measured by MMCP levels in serum was evident in DNFB-sensitized mice after single and repeated DNS-challenge. The appearance of MMCP in serum is a selective indicator of mast cell degranulation. Therefore, it can be concluded that the DNFB-induced hypersensiti-vity reaction in the airways is associated with mast cell activation.

Low molecular weight compounds, like DNFB, picrylchloride and toluene diisocyanate, have shown to be potent contact sensitizers and induce hypersensitivity responses in skin, intestine, and airways. It is hypothesized that during sensitization, antigen specific lymphocyte factors are produced and released systemically by hypersensitivity-initiating lymphocytes in lymph nodes and spleen. These factors can bind to mast cells and other cells throughout the body. Redegeld and coworkers demonstrated in vitro that the picrylchloride-specific factor was able to bind to mast cells [12]. Very recently, we have characterized the biological compound of picrylchloride-specific lymphocyte-derived factor as a 27 kD protein, which has shown identical to immunoglobulin kappa light chain [13].

After a local challenge, the hapten will bind to the lymphocyte factor on the mast cell and induce the release of vasoactive amines like histamine, serotonin and cytokines as tumor necrosis factor-α and vascular endothelial growth factor [5, 9]. These vasoactive amines could be responsible for the local edema formation associated with the early hypersensitivity response. Monastral blue labeling showed leaky blood vessels 30 min after single DNS-challenge. This indicates that there is an early hypersensitivity response present in the tracheas of DNFB-sensitized and single DNS-challenged mice. Furthermore, mast cell activation, measured by serum MMCP levels, was present directly after the first challenge. This suggests that mast cells can be responsible for the early hypersensitivity reaction. In addition, no increase in Evans blue accumulation was found directly after single DNS-challenge. A possible explanation could be the detection limit of the Evans blue accumulation into the tracheal tissue; this method could be too insensitive to detect a very small, but significant, change in permeability. Another possibility is that the DNFB-induced Evans blue accumulation could be increased at a later time point after challenge. In an early hapten-induced hypersensitivity response in the skin, a maximal increase in edema formation was found 2 h after local ear challenge [9].

Besides the early hypersensitivity response, a late phase hypersensitivity reaction was profoundly present in the airways of DNFB-sensitized mice compared to vehicle treated animals. Enhanced mucosal exudation, vascular permeability and PMN accumulation in the BAL fluid 24 h characterized this late hypersensitivity phase after challenge. These results support data presented in previous studies performed in small intestine and airways in which edema formation, tracheal hyperreactivity, and leukocyte accumulation were present 24 h after challenge [3, 4, 14]. In addition, MMCP levels were increased 24 h after the challenge suggesting that mast cell activation is an ongoing process in a hapten-induced hypersensitivity reaction.

Neutrophilic infiltration has been reported to be a prominent feature in cutaneous hapten-induced hypersensitivity responses in rodents [15–17]. Buchanan and colleagues showed that neutrophils were the first cells that enter a Cryptococcus neoformans-induced inflammatory DTH reaction

site [17]. Furthermore, Kudo and colleagues observed that depletion of neutrophils by usage of a selective monoclonal antibody resulted in inhibition of the DTH reaction in rats induced by sheep red blood cells [15].

A repeated challenge induced a vascular hyperpermeability response in tracheas of mice sensitized with DNFB as measured by Evans blue and Monastral blue accumulation in the tracheas. The vascular hyperpermeability response is not due to increased mast cell activation since there was no further enhancement of the MMCP values after a repeated DNS-challenge. Previous studies showed that this increased susceptibility is not primarily restricted to DNS and that the susceptibility to neurogenic inflammation is altered in DNFB-sensitized mice [18]. Furthermore, the tracheal hyperpermeability response to DNS is mediated via the neurokinine-1 receptor, which suggests that DNS has the ability to release substance P. In addition, capsaicin, a sensory nerve stimulant, and exogenous substance P could mimic this hyperpermeability response in the trachea. This increased tracheal vascular permeability response to DNS could be a result of several factors. Firstly, it could be a result from increased sensitivity of the sensory nerves for various stimuli since capsaicin could mimic the hyperpermeability response [18]. Secondly, angiogenic process could be present in the trachea of DNFB-sensitized and single DNS-challenged mice, which explains the effect of substance P in these mice. Newly formed blood vessels are extremely sensitive to neurogenic inflammation by upregulation of neurokinine-1 receptors [19]. The present data do not permit a conclusion in respect to the occurrence of angiogenesis. Furthermore, decreased degradation or increased release of neurokinins are other explanations for the increased tracheal hyperpermeability.

A repeated challenge did not further enhance the mucosal exudation and mast cell activation. This suggests that both mast cell activation and mucosal exudation are already maximal after the first DNS-challenge. Only the PMN numbers in the BAL fluid tended were increased after the second challenge.

In conclusion, our study shows that besides a late phase reaction in the airways, an early phase reaction is detectable in mice undergoing a pulmonary DNFB-induced hypersensitivity reaction. Furthermore, mucosal exudation and PMN accumulation are characteristic features of the late phase hypersensitivity reaction after a single challenge. In addition, the susceptibility of tracheas to DNS is increased in DNFB-sensitized and single DNS-challenged mice. Evidence from this study indicates that mast cell may play a role in the pulmonary hypersensitivity reaction induced by DNFB.

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