Mast cell-nerve interactions in asthma

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Mestcel-zenuwinteracties in astma

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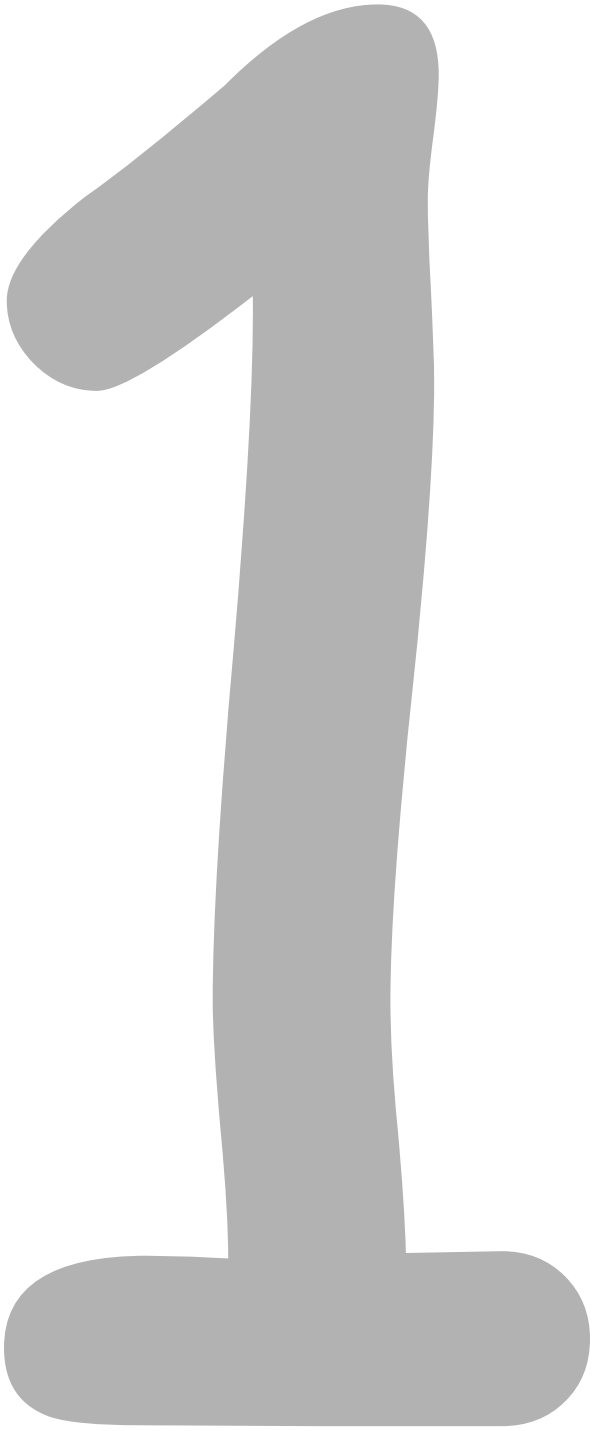
General Introduction



1. Asthma

Asthma is characterized by pulmonary inflammation and episodic reversible airway obstruction along with increased reactivity of airway smooth muscle to a wide variety of chemical, pharmacological or physical stimuli. It is currently accepted that asthma can be subdivided into two categories, although the two forms are not always strictly seperated. In the first category asthma is associated with atopy, the second category comprises the non-atopic asthmatics. The majority of patients suffer from atopic asthma, although the number of non-atopic patients is increasing. The most striking difference between atopic and non-atopic asthma is the serum IgE, which is increased in allergic asthma (1) whereas no elevated IgE levels can be detected in non-atopic asthma (2, 3). Amin and coworkers recently showed that besides the difference in serum IgE levels, atopic and non-atopic patients had different pathological changes present in their airways despite their similar clinical respiratory symptoms (4). Atopic patients have increased numbers of eosinophils in their airway tissue, whereas non-atopic asthma is dominated by the infiltration of neutrophils. A common feature observed in both groups is a significant involvement of inflammatory cells, either pulmonary resident, such as macrophages and mast cells, or infiltrated inflammatory cells, such as eosinophils, neutrophils and lymphocytes. In addition, tissue resident structural cells including endothelial cells, epithelial cells, fibroblasts and smooth muscle cells may also be an important source of inflammatory mediators in asthma (5).

2. Autonomic regulation of the airways

Inhibitory NANC nervous system

Inhibitory NANC nerves contain vasoactive intestinal peptide (VIP) and nitric oxide (NO). VIP is a potent relaxant of the airways. VIP and NO seem to counteract the bronchoconstriction. VIP also has been suggested to inhibit mediator release from mast cells by elevating the level of intracellular cyclic AMP (14). Undem and coworkers (15) reported that VIP inhibited antigen-induced histamine release. In cats, Miura and coworkers (16) demonstrated that NANC inhibitory electrical nerve stimulation inhibited antigen-induced bronchoconstriction and histamine secretion from histamine containing cells. Although dysfunction of inhibitory NANC nerves has also been proposed in asthma, no differences in inhibitory NANC responses have been found between asthmatics and healthy subjects (13, 17).

Excitatory NANC nervous system

In addition to inhibitory NANC efferent systems in the airways, there is a NANC afferent nervous system that protects the airways against inhaled irritants and chemical particles (6, 13, 17) . These excitatory NANC nerves play a regulatory role in airways. Their activation results in maintenance of pulmonary homeostasis via reflex pathways such as bronchoconstriction, mucus secretion, and cough. Excitatory NANC nerves innervate the airways of humans and other mammalian species (18).

Excitatory NANC nerves or so called sensory nerves are mainly localized between and beneath the airway epithelium. These sensory nerve fibers are also present in the vicinity of blood vessels and submucosal glands, and make direct contact with the smooth muscle layer and local tracheobronchial ganglion cells (19-21). The excitatory NANC nerves can be activated by different stimuli, that affect the chemosensitive C-fiber afferents in the airways and can lead to the local release of neuropeptides via axon reflex mechanisms (22). The tachykinins and calcitonin gene-related peptide (CGRP) are the predominant excitatory NANC-neuropeptides in the airways (23). These neuropeptides contract airway smooth muscle, dilate bronchial arteries, increase vascular permeability, increase mucus production and modulate ganglionic transmission. In addition, they are certain to influence the recruitment, proliferation and activation of inflammatory cells such as mast cells.

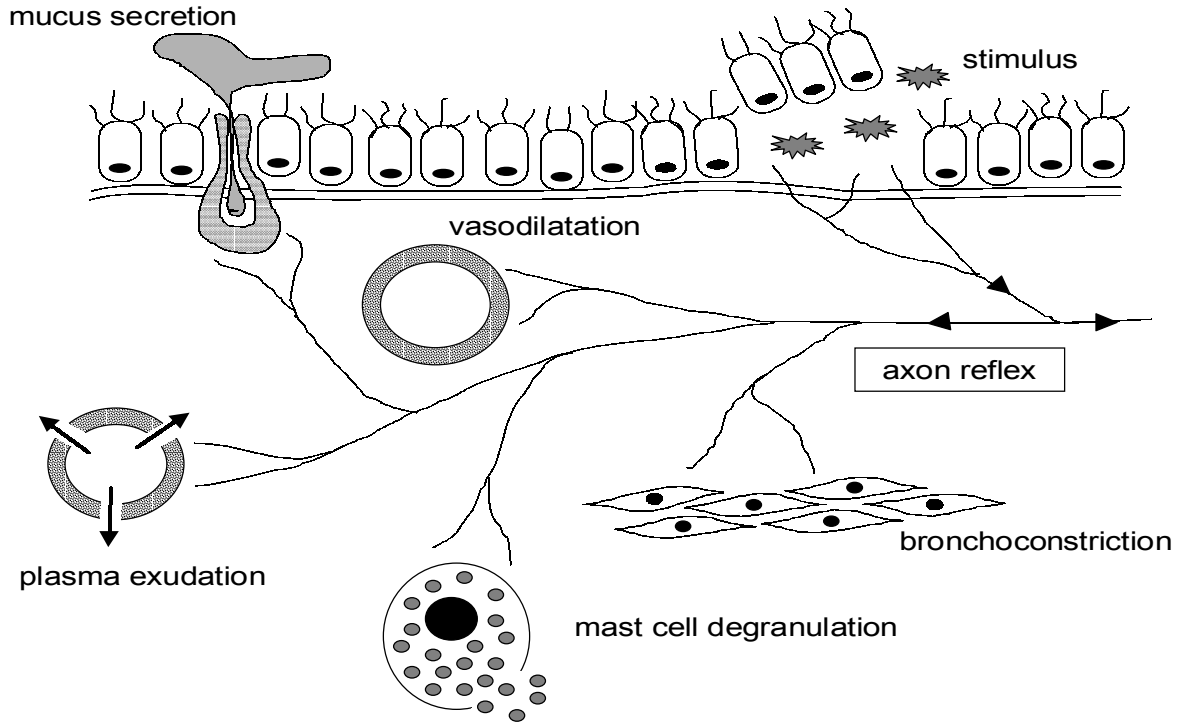
The effects of tachykinins result from signal transduction initiated by the interaction of tachykinins with specific receptors on effector cells. Three distinct subtypes of mammalian receptors have been identified and denoted as neurokinin 1 (NK-1), neurokinin 2 (NK-2) and neurokinin 3 (NK-3), which have the highest affinity for substance P, Neurokinin A en B, respectively (18, 23, 24). The tachykinin NK-3 receptors are predominantly present in the central and peripheral nervous system (25). Tachykinin NK-1 receptors are localized on smooth muscle cells, submucosal glands, blood vessels and inflammatory cells (24, 26). The NK-2 receptor is also found on smooth muscle. Animal studies have shown that the NK-2 receptors are involved in bronchoconstriction, whereas activation of tachykinin NK-1 receptors leads to neurogenic inflammation (27, 28).

3. Neurogenic inflammation

Neurogenic inflammation has been shown to occur in different tissues, including the skin, urinary tract, the digestive system and airways (19, 26, 29, 30). Neurogenic inflammation involves a change in function of sensory neurons due to inflammatory mediators, inducing an enhanced release of neuropeptides from the sensory nerve endings (31, 32). With the close proximity of mast cells and nerves to blood vessels in most tissues, these three components may be considered as an important functional unit in neurogenic inflammation (33).

Sensory neurons are characterized by their expression of a certain group of neuropeptides, the tachykinins such as substance P and neurokinin A. Tachykinins released from sensory nerves induce a wide range of inflammatory reactions including vasodilatation, increased vascular permeability, bronchoconstriction and adherence of inflammatory cells to the endothelium thereby facilitating cellular infiltration (18, 28). The effects produced by tachykinins (substance P, neurokinin A and neurokinin B) and CGRP released from peripheral endings of capsaicin-sensitive primary sensory neurons, are collectively referred to as neurogenic inflammation (34)(figure 1). Several studies have shown a role for neurogenic inflammation in the induction of airway hyperresponsiveness in animal models (35-37).

Figure 1. Schematic drawing of neurogenic inflammation in the airway via the release of neuropeptides from sensory nerve terminals.



4. Tachykinins and asthma

Substance P, neurokinin A and CGRP are present in airways of various mammals including man (19, 20). Both animal and human data strongly suggest a role for tachykinins in the pathogenesis of asthma. The role of excitatory NANC nerves, especially the tachykinins, has been described in animal models for atopic and non-atopic asthma (13, 17, 35, 38).

Exposure of guinea pigs to an aerosol of either capsaicin or substance P elicited airway hyperresponsiveness to bronchoconstrictor agents (37, 39-41). Recently, Ricciardolo and coworkers (42) have demonstrated that aerosolised neurokinin A caused a profound increase in total pulmonary resistance in guinea pigs, that was abolished by the combination of a NK-1- and a NK-2-receptor antagonist. Many more animal studies show that capsaicin-induced depletion of excitatory NANC neuropeptides or treatment with tachykinin receptor antagonists prevent airway responsiveness and pulmonary inflammation. For instance, airway hyperresponsiveness induced by ovalbumin (39), platelet-activating factor (43), toluene diisocyanate (44), dinitrofluorobenzene (35) or respiratory viral infection (39) can be attenuated by interruption or blockade of the excitatory NANC system. These data support the concept that excitatory NANC nerves are an important factor in the pathway leading to characteristics as hyperresponsiveness and inflammation as seen in asthma.

Immunohistochemical studies of neuronal tachykinins in airways of asthmatics have yielded conflicting results. While in some studies an increase in both the number and

length of tachykinin-immunoreactive nerve fibres was found in the airways (45, 46), others have detected significantly less substance P-like immunoreactivity in lung tissue from asthmatic compared to non-asthmatic patients (47, 48). However, this latter finding may reflect augmented release of substance P followed by degradation (48).

Studies on autopsy tissue (48), plasma levels (49), lung lavage fluid (46) and sputum (50) suggest that tachykinins are present in increased amounts in asthmatic airways. In addition, tachykinin levels can be elevated during exacerbations of asthma (51).

Substance P and neurokinin A elicit bronchoconstrictor effects of in asthmatic patients (22). Neurokinin A causes bronchoconstriction not only in asthmatics, but also in normal persons with the asthmatics being more sensitive than normal subjects (52, 53). Asthmatics were found to be hyperresponsive to substance P and neurokinin A (54). Effects of tachykinins on bronchial responsiveness in man are less well studied. Inhalation of substance P was found to enhance the maximal airway narrowing to metacholine in patients with asthma (55). This effect was observed 24 h after inhalation of substance P. An increase in mRNA transcripts for both tachykinin NK_1 and NK_2 receptors has been observed and possible changes in tachykinin receptor expression are found in asthma (56).

5. Mast cells

Mast cells are widely distributed throughout the body in connective tissues, particularly around blood vessels and nerves (57). They are abundant in the submucosa of the digestive tract (58), oral and nasal mucosa (59), respiratory mucosal surfaces (60) and skin (57, 61, 62).

Mast cells are involved in the regulation of their own overall tissue cell mass since mast cell degranulation leads to an overall increase in mast cells (63). Many factors are involved including generation and secretion by mast cells of factors such as GM-CSF, SCF and NGF which are all known to promote mast cell growth (64, 65).

Mast cells can be divided into various subpopulations with distinct phenotypes. There are two primary mast cell subtypes, the connective tissue mast cells (CTMC) and the mucosal mast cells (MMC), who differ markedly in their phenotypical and functional characteristics (66, 67). However, environmental factors such as cytokines, may induce differentiation in many more phenotypically different subsets (68). CTMC and MMC are considered to be derived from a common precursor in the bone marrow. Mast-cell progenitor cells originate from the bone marrow as undifferentiated cells that enter specific tissues and mature under microenvironmental influences. Their development and survival essentially depends on stem cell factor (SCF) and its receptor c-kit (69). Besides SCF, cytokines such as IL-3, IL-4 and IL-10 influence mast cell growth and differentiation (70), as does nerve growth factor (NGF) (71).

Mast cells are versatile cells capable of synthesis of a large number of pro- and anti-inflammatory mediators including cytokines, products of arachidonic acid metabolism, serotonin, histamine and growth factors including NGF and SCF. These mediators are pre-stored or can be newly synthesized upon stimulation. Pre-stored mediators, such as histamine, serine proteases, proteoglycans, sulfatases and TNF-α, are released within

minutes after degranulation of the cell (72). After this primary response a second wave of newly synthesized mediators are released and include PGD_2, LTC_4, LTD_4 and LTE_4. Subsequently, cytokines like IL-4, IL-5, IL-6, IL-8, IL-13 and TNF-α are induced and secreted (72).

Mast cells are best known for their involvement in type I hypersensitivity, allergic or anaphylactic reactions involving the interaction with IgE (73). However, mast cells also play a prominent role in non-IgE mediated hypersensitivity reactions (74-76). It was shown that upon skin sensitization, lymphocytes are induced to produce and release hapten-specific factors recently recognized as immunoglobulin light chain (IgLC)(77). IgLC will bind to mast cells and mediate an early and late phase of a pulmonary hypersensitivity reaction (78, 79).

The sensitivity of mast cells to activation by non-immunological stimuli as polycationic compounds, complement proteins, superoxide anions or neuropeptides is dependent on the population of the mast cells examined (80). A major distinction between the classical CTMC and MMC is their difference in responsiveness to non-immunological activators like neuropeptides. It has been reported that mast cells of mucosal origin including BMMC are not activated by neuropeptides (81), whereas CTMC were shown to respond significantly to stimulation by these compounds (81, 82).

Mast cell activation by neuropeptides do not need to involve one of the known NK receptor subtypes. Activation can be induced via direct interaction with pertussis toxin-sensitive G proteins through the N-terminal domain located in the inner surface of the plasma membrane (83, 84). Stimulation of G-proteins will activate a signal transduction pathway eventually leading to mast cell mediator production and release. However, it has been suggested that functional NK receptors are present on mast cells (85-87). Activation of the neurokinin receptors is dependent on the C-terminal domain of the tachykinins. In conclusion, activation of mast cells by tachykinins can either be receptor- or non-receptor-mediated. This process is highly influenced by their microenvironment, which is affected by pathological inflammatory conditions such as asthma.

6. Mast cell-nerve interaction

Histological studies reveal an intimate association between mast cells and neurons in both the peripheral and central nervous system (88). A close anatomical relationship between mast cells and substance P-containing sensory nerve endings has been reported in various tissues including skin (62, 89), intestine (90), dura mater (91, 92) and airway mucosa (61, 89). Other than an anatomical link, mast cells also form a functional link between the immune and nervous systems and mast cells appear to act as bi-directional carriers of information. Neuronal mechanisms are involved in mast cell activation and mast cells act as principle transducers of information between peripheral nerves and local inflammatory events (93). Peripheral nerves are highly populated with mast cells and manipulation of these peripheral nerves causes changes in mast cell densities (94).

Sensory neurons play a role in neurogenic inflammation (31, 95). It has becoming apparent that also the mast cell and its mediators are important in neurogenic inflammation

(33, 96). Association with the nervous system allows mast cells to act as sensory receptors for a variety of potentially noxious and newly encountered substances (97). They are therefore ideal to act in these ways to pass information on through afferent nerves to local tissues by axon reflexes. In various studies, tissue mast cells invariably show ultrastructural evidence of activation even in normal healthy conditions, suggesting that these cells are constantly providing information to the nervous system (91). The fact that they are located at sites under constant exposure to the external environment, such as the skin, respiratory and gastro-intestinal tract, emphasizes the significance of these associations.

The most evident neuronal response to mast cells is mast cell mediator-induced activation of sensory nerves causing the release of tachykinins such as substance P and neurokinin A (32, 98). Aside from the generation of action potentials, the C-fiber terminal is a secretory system, releasing tachykinins to cause neurogenic inflammation. Stimulation of these C-fibers by a range of chemical and physical factors results in afferent neuronal condition eliciting parasympathetic reflexes and antidromic impulses traveling along the peripheral nerve terminal. Such communication from one nerve to another, without passing through a cell body, is called the axon reflex and results in local release of tachykinins and CGRP from C-fiber terminals (figure 2) (23). Axon reflexes account for many of the local physiological responses to antigen for instance in sensitized lung (99, 100) and gut tissues (101), and have long been recognized to be involved in local vasodilatation in the skin (102). Antidromic stimulation of guinea pig vagal sensory fibers results in contractions of the isolated airway smooth muscle mediated by tachykinins (103). Further studies indicate that neuropeptide release can also be induced via direct depolarization of the terminal (104).

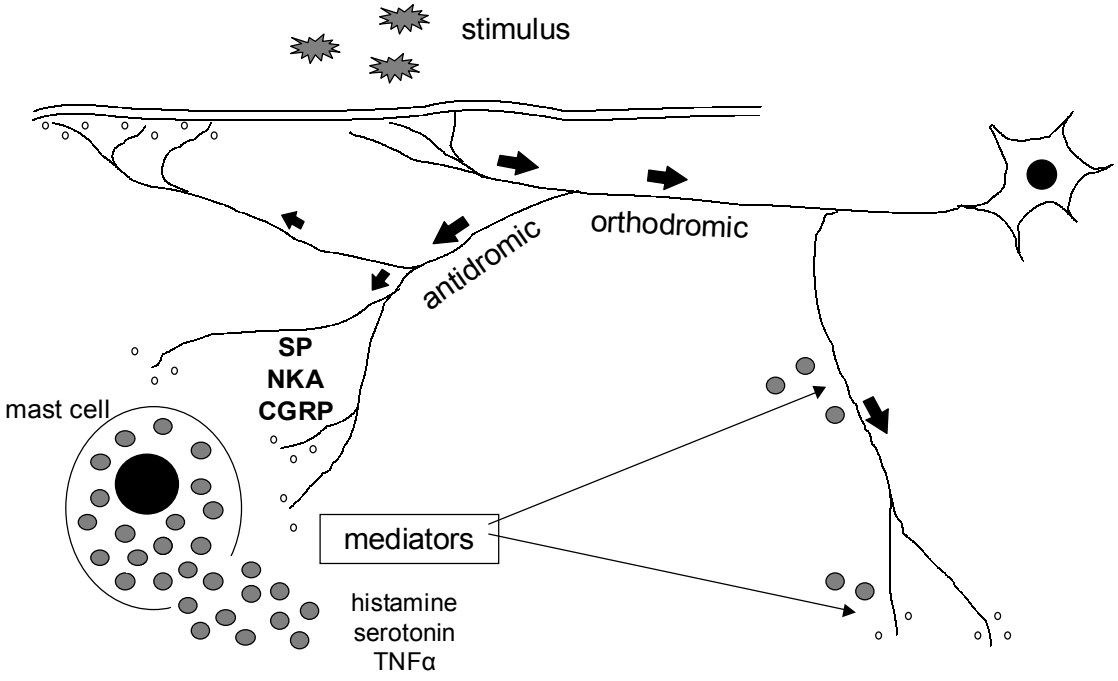


Figure 2. Representation of the involvement of the mast cell in the axon reflex. Stimuli activate sensory nerve fibers inducing the release of neuropeptides from the nerve terminals. This will induce mediator release from adjacent mast cells leading to further activation of other sensory nerve endings.

7. Mast cells and tachykinins

The relationship between mast cells and neurons seems to be bidirectional. Mediator release from mast cells can on one hand be induced by tachykinins and, on the other hand, cause the release of tachykinins from nerve endings (105).

Mediator release from mast cells can be induced by tachykinins like substance P and neurokinin A (92, 106). Stimulation of nerve fibers of humans and mammals induces mast cells to degranulate and release histamine and other mediators such as serotonin (107) and TNF-α (87). Janiszewski and coworkers (108) reported that mast cells respond electrophysiologically to very low concentrations of substance P without degranulation, but that degranulation occurred after repeated doses. These data indicate that substance P may act as a priming substance rather than a substance causing direct degranulation.

Many studies have shown that mast cell-derived mediators such as histamine, serotonin and cytokines modulate NANC neurotransmission (105, 109, 110). Furthermore, NANC nerve endings express receptors for histamine (H1 and H3) and serotonin (5-HT2a)(111-113), and histamine H1 receptor expression at least is upregulated on primary NANC nerves in inflammation (114). Mast cell mediators like TNF-α can sensitize afferent C fibers by lowering their treshold (109, 115) but can also cause direct release of substance P, neurokinin A and CGRP from unmyelinated fibers (105).

Priming may be a prominent aspect of nerve-mast cell interactions. Mast cells have been reported to be primed by different cytokine growth factors for subsequent different agonists (116). The concept of priming also applies to neurons. For instance, TNF-α may exert a priming, rather than a direct stimulatory effect on sensory activity (115, 117, 118).

8. Aim and scope of this thesis

From the previous paragraphs, it is clear that both mast cells and sensory neurons may play an important role in the development of atopic and non-atopic asthma. The goal of this thesis is to study mast cell-nerve interactions *in vitro* using primary cultured mast cells and neurons and *in vivo*, in a murine model for non-atopic asthma. Features of the murine model for non-atopic asthma are described in chapter 2. Chapter 3 focusses on the role of the tachykinin receptors in the development of non-atopic asthma using specific NK-1 or NK-2 receptor antagonists and genetically NK-1 receptor-deficient animals. We hypothesize a role for mast cells in the pathogenisis of non-atopic asthma. To explore this role, we studied hypersensitivity reactions associated with non-atopic asthma in genetically mast cell-deficient and congenic normal mice. TNF-α, a major mediator released from the mast cell, is studied in chapter 5. The role of TNF-α in our model was investigated by neutralizing anti-TNF-α antibodies. TNF-α receptor antibodies were used to identify which receptor subtype is involved in DNFB-induced tracheal hyperreactivity. Previously, we and other investigators have demonstrated that both TNF-α and substance P are involved in the inflammatory process associated with airway diseases. These two mediators also form a possible interaction between the neurologic and the immune system. Therefore, we

explored the effects of TNF-α and substance P on tracheal hyperreactivity and cellular accumulation in mice by intranasal application of TNF-α and substance P by itself or in combination. To further examine the mast cell-nerve interaction, we continued our studies *in vitro*. Primary mast cells were cultured (chapter 7) and sensitivity to substance P stimulation was studied under different culture conditions. We analyzed NK-1 receptor expression on these groups of primary mast cells and investigated the involvement of NK-1 receptors as a possible activation pathway. An *in vitro* coculture system of mast cells and neurites, to study direct interactions, is described in chapter 8. The main results obtained in this thesis are summarized and discussed in the final chapter (chapter 9: summarizing discussion).

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Murine model for non-atopic asthma: Dinitrofluorobenzene-induced hypersensitivity reactions in the airways

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Abstract

Asthma is characterized by episodic reversible airway obstruction along with bronchial hyperresponsiveness and airway inflammation. The majority of the patients suffer from atopic asthma, although the number of non-atopic asthmatics is increasing. The mechanisms involved in non-atopic asthma are poorly defined. In this chapter we describe a murine model for non-atopic asthma. Non-atopic asthma was induced in mice by skin sensitization with dinitrofluorobenzene (DNFB) followed by intranasal challenge with dinitrobenzene sulfonic acid (DNS). Features of the early phase pulmonary reaction included acute bronchoconstriction and mast cell activation shortly after challenge. Acute bronchoconstriction was observed in DNFB-sensitized mice immediately after DNS challenge and reached its maximum at 5 min after challenge. PenH values, a dimensionless value to monitor airway function, slowly returned to basal levels 15 min after the challenge. Mast cell activation, as measured by increase in serum mast cell protease-1 (mMCP-1), was observed in both early phase (0-3 h after challenge) and late phase responses (24-48 h after challenge). The most prominent rise in mMCP-1 was observed 30 min after intranasal hapten challenge. Tracheal hyperreactivity and mononuclear and neutrophilic cell infiltration were observed 24 and 48 h after the challenge. At both time points significant increases were seen in DNFB-sensitized mice compared to vehicle-sensitized animals. Although the infiltration of cells was comparable at both time points, the difference in reactivity between DNFB-sensitized animals and vehicle-sensitized mice was more profound at 48 h. Therefore, this time point was chosen in following studies to assess late phase features such as tracheal hyperreactivity and cellular accumulation.

Introduction

Delayed type hypersensitivity (DTH) or cell-mediated immunity plays a major role in the pathology and chronic aspects of many inflammatory disorders. Cell-mediated reactions are primarily investigated in the skin. It has become apparent that similar reactions can also occur in the airways (1) and the intestine (2) of sensitized animals after local antigen challenge. These reactions in the lung and the intestine are not mediated by IgE and will be referred to as non-atopic reactions in this thesis.

The cell-mediated reactions can be divided into three major subsets (Th1, Th2 and Th3) based on distinct cytokine profiles. Type I lymphocytes predominantly produce IL-2 and IFN-γ. Type II lymphocytes mainly produce IL-4/IL-5 whereas Type III lymphocytes are characterized by the production of IL-4 and TGF-β (3, 4). Previous studies have demonstrated that T helper-1 cells play a role in the induction of airway hyperresponsiveness associated with non-atopic asthma (5).

Asthma can roughly be divided into two categories. In the first category asthma is associated with atopy, the second category comprises the non-atopic asthmatics. The majority of patients have atopic asthma, characterized by an elevation of total and allergen-specific IgE in serum (6). The second category comprises the non-atopic asthmatics. Non-atopic asthmatics are skin test negative to common allergens and there is no evidence of allergen-specific serum IgE (7, 8). Amin and coworkers recently showed that besides the difference in serum IgE levels, atopic and non-atopic patients had different pathological changes present in their airways despite their similar clinical respiratory symptoms (9).

Atopic patients had increased numbers of eosinophils in their airway tissue, whereas non-atopic asthma is dominated by the infiltration of neutrophils. The number of mast cells showed a similar increase in both of the groups.

Non-atopic asthma is an increasing problem in the developed world. The mechanism involved in the induction and on-going respiratory impairments associated with non-atopic asthma are poorly investigated. Mast cells and lymphocytes showed to play an important role in non-atopic reactions in mouse skin, airway and intestine (2, 10, 11). It has been hypothesized that upon skin sensitization, lymphocytes are induced to produce and release hapten-specific factors recently recognized as immunoglobulin light chain (IgLC)(12). IgLC will bind to mast cells and mediate an early and late phase of a pulmonary hypersensitivity reaction (13, 14).

Low molecular weight substances (<5000 Da) are the most common agents causing non-atopic asthma (15). In the mouse, picrylchloride and toluene diisocyanate (TDI) have been shown to induce pulmonary hypersensitivity reactions. In the present study, a hypersensitivity reaction was elicited in the airways of mice with the low molecular weight compound dinitrofluorobenzene (DNFB) as the contact sensitizing hapten followed by intranasal challenge with dinitrobenzene sulphonic acid (DNS). We will describe this murine model for non-atopic asthma and its characteristics. In the following chapters 3, 4 and 5 this model will be used to further clarify the mechanisms responsible for DNFB-induced asthma.

Methods

Animals. Male BALB/c mice were obtained from the Central Animal Laboratory (GDL), Utrecht University, Utrecht, The Netherlands. All mice used were 6-8 weeks of age. The mice were housed in groups not exceeding 10 per cage and maintained under standard conditions. All experiments were conducted in accordance with the Animal Care Committee of the Utrecht University (Utrecht, The Netherlands).

Sensitization and experimental procedure. Mice were sensitized on day 0 with either DNFB (0.5% dissolved in acetone: olive oil [4:1] or vehicle control, both of which were applied epicutaneously to the shaved thorax (50 μl) and all four paws (50 μl). On day 1, DNFB or vehicle control (50 μl) was applied to the thorax alone. DNFB- and vehicle-sensitized mice were intranasally challenged with dinitrobenzene sulphonic acid (DNS, 50 μl 0.6 % in PBS, pH 7.2) on day 5. The sensitization and challenge were performed under light anesthesia (pentobarbitone, 40 mg/kg i.p.).

Mast cell activation in vivo. Mouse mast cell protease 1 (mMCP-1) is a protease specific for mouse mast cells, that appears in the blood after mast cell activation. To monitor mast cell activation in time, blood samples of DNFB and vehicle sensitized mice were taken 10, 30 min and 3, 6, 24 and 48 h after intranasal DNS challenge. Blood samples were collected and after centrifugation sera were stored at –70ºC until use. Levels of mMCP-1 were measured using a commercially available ELISA assay. 96 Wells flat bottomed Nunc-

immunoplates were coated with 50 μl sheep anti-mMCP-1 antibody (1 μg/ml in 0-1 M carbonate buffer, pH 9.6) in a humid chamber at 4ºC for 24 h. Plates were washed 5 times with PBS/Tween 20 (0.05% v/v) and were incubated for 30 min with 50 μl 1% BSA/PBS/ Tween 20 at 4ºC to block the residual binding sides. After 5 wash steps, plated were loaded with 50 μl mMCP-1 standard (0-20 ng/ml) or 1:5 diluted samples and the plates were incubated overnight at 4ºC. Subsequently, after washing the plates were incubated with rabbit anti-mMCP-1-horse radish peroxidase conjugate (1:300, 50 μl) for 2 h at 4ºC. Plates were washed again 5 times and were incubated for 30 min with 50 μl o-phenylenediamine at room temperature (2.2 mM in 0.1 M citric/phosphate buffer, pH 5.0). The reaction was stopped by adding 25 μl of 2.5 M H_2SO_4. The absorption was measured at 492 nm using a microplate reader. The mMCP-1 levels were obtained by comparison of absorbance of samples with these of the calibration curve.

Acute bronchoconstriction. Bronchoconstriction was measured as reported previously (16). In short, 5 min before intranasal DNS challenge, unrestrained conscious mice were placed in a whole body plethysmographic chamber to analyze the respiratory wave forms and obtain basal line. After 4 min and 30 sec, the mice were challenged under light anesthesia (inhalation of halothane 3%) and placed back in the chamber where resistance in each animal was measured over a 5 min period. Airway resistance is expressed as enhanced pause (PenH) PenH = pause x PEP/PIP
where PEP stands for peak expiratory pressure and PIP for peak inspiratory pressure. Pause is defined as (Te – Tr)/Tr, where T_e stands for time of expiration and T_r stand for the relaxation time -the time of pressure decay to 36% of the total expiratory pressure signal. During bronchoconstriction, the changes in the box pressure during expiration are more pronounced than during inspiration. Thus bronchoconstriction is reflected by an increase in PenH, a dimensionless value to empirically monitor airway function. After intranasal challenge, for each mouse maximal PenH readings were taken over 1 min time windows at the following time points : 2 min 30 sec, 5 min, 10 and 15 min.

Leukocyte accumulation in bronchoalveolar lavage fluid. Bronchoalveolar lavages (BAL) were performed in mice 24 and 48 h after the challenge. After sacrificing the animals, the trachea was canulated. Saline (37ºC) was slowly injected into the lung and withdrawn in 4 x 1 ml aliquots. The aliquots were pooled and maintained at 4° C. The lavage fluids were centrifuged (580 g, 5 min, 4ºC) to isolate the BAL cells. Total cells were counted using a haemocytometer and expressed as cells/lung. The BAL cell preparations were analyzed morphologically after centrifugation onto microscopic slides. Air dried preparations were fixed and stained with hematoxylin and eosin to ascertain the leukocyte populations. Results are expressed as leukocytes/lung for neutrophils and mononuclear cells in the airway lumen.

Tracheal reactivity in vitro. Mice were killed with an overdose of pentobarbitone at 24 and 48 h after intranasal DNS challenge. The trachea, which was resected *in toto*, was carefully cleaned of connective tissue using a binocular microscope as described before (1). A nine ring piece of trachea (taken from just below the larynx) was then transferred to a 10 ml

organ bath containing a modified oxygenated Krebs solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 25 mM $NaHCO_3$, 1 mM $NaHPO_4$ and 11.1 mM glucose). The trachea was directly slipped onto two supports, one coupled to the organ bath and the other coupled to an isometric transducer. The solution was aerated (95% O_2: 5% CO_2) and maintained at 37ºC. Isometric measurements were made using a force displacement transducer and a two channel recorder and measurements were expressed as changes in milligram (mg) force. An optimal preload, determined to be 1 g, was placed on the tissue at the beginning of the experiment. The trachea was allowed to equilibrate for at least 1 h before contractile effects were elicited. During this period, the bath fluid was exchanged every 15 min. At the end of the equilibrium phase, tracheal contractile reactivity was measured by recording cumulative concentration response-curves to carbachol (10^{-8} to 10^{-4} M).

Histological examination. At 24 and 48 h after intranasal hapten challenge lungs were removed from mice after lethal anesthesia with pentobarbitone. **I.** Lungs were removed 24 h after challenge and filled intratracheally with acetic acid formalin fixing solution (0.8% formalin, 4% acetic acid). Lungs were fixed for at least 24 h in the fixative, dehydrated and embedded in paraplast. Four ìm sections were stained with hematoxylin and eosin. **II.** Due to optimized histological techniques, the lungs isolated 48 h after challenge were fixed in 4% formaldehyde in PBS and routinely embedded in GMA (glycol methacrylate)(17). Serial sections were cut at 3 μm and stained with hematoxylin in combination with eosin. The parameters were scored according to the method described by Enander and coworkers (18), as indicated in table 1.

Table 1. Histological score of cellular accumulation in the lung according to the method of Enander and coworkers (18).

Accumulation of mononuclear cells	0	1	2	3
Diffusely around Bronchioli	No Cells	< 10 cells layers thick	> 10 cell layers thick	Entirely surrounding bronchiolus > 10 cell layers thick
Around blood Vessels	No cells	≤ 3 cell layers thick	4-10 cell layers thick	≥ 10 cell layers thick

Materials. DNFB and olive oil were purchased from Sigma Chemical Co., St. Louis, USA. Hematoxylin and eosin were obtained from Sigma Aldrich, St.Louis, MO, USA. GMA came from Merck, Darmstadt, Germany. Sodium pentobarbitone was obtained from Sanofi, Maassluis, The Netherlands. Carbachol was purchased from Onderlinge Pharmaceutische Groothandel, Utrecht, The Netherlands. The mMCP-1 ELISA was from Moredun Scientific Ltd., Midlothian, UK. Maxisorp surface 96 well plates were purchased from Nunc Immuno plate, Roskilde, Denmark. The whole body plethysmographic chamber (Buxco) was obtained from Buxco Electronics, Inc. Shanon, CT. The force displacement transducer was purchased from Harvard Bioscience, Boston, MA, USA and the two channel recorder (Servogar type SE-120) from Plato BV, Diemen, the Netherlands.

Statistical analyses. Data are expressed as mean and standard error of the mean (SEM). EC50- and Emax-values for the carbachol-induced tracheal contractions were calculated by non-linear least-squares regression analysis of the measured contractions versus carbachol concentration using the sigmoid concentration-response relationship. The data were analyzed by performing a two-way analysis of variance (ANOVA). Data on the cellular accumulation in BAL fluid were processed by a distribution free Kruskal-Wallis ANOVA and the non-parametric Wilcoxon rank test was used to determine the significance of differences of histological scores. mMCP-1 data were analyzed by using a one-tailed unpaired t-test. Probability values of $P<0.05$ were considered significantly different. Analyses were performed by the usage of Graphpad prism (version 2.01, San Diego, U.S.A).

Results

Mast cell activation in vivo.

The appearance of mMCP-1 in the blood is indicative for the activation of mast cells (19). DNFB sensitization followed by intranasal DNS challenge resulted in a biphasic mast cell activation. An early rise was found 10, 30 min and 3 h after challenge in serum of DNFB-sensitized and DNS-challenged mice compared to vehicle-sensitized, DNS-challenged mice (table 2). The most prominent rise in mMCP-1 was observed 30 min after intranasal hapten challenge. No significant increase was found 6 h after challenge whereas in the late phase (24-48 h) mMCP-1 levels were again significantly enhanced in DNFB-sensitized mice compared to control animals.

Table 2. mMCP-1 levels in serum of vehicle- and DNFB-sensitized mice 10, 30 min and 2, 3, 24 and 48 h after intranasal DNS challenge.

	mMCP-1 (ng/ml serum)	
Time after challenge	Vehicle	DNFB
10 min	4.1 ± 0.5	9.1 ± 1.2*
30 min	5.4 ± 1.1	19.0 ± 3.8*
2 h	7.5 ± 2.1	15.7 ± 3.6*
3 h	4.1 ± 1.0	8.3 ± 1.4*
6 h	3.5 ± 0.7	5.3 ± 0.8
24 h	4.4 ± 0.4	10.7 ± 2.6*
48 h	3.9 ± 1.5	9.8 ± 1.8*

Results are expressed as mean (ng/ml serum) ± SEM, n=6-12 mice per group. Significant differences between the vehicle-sensitized and the DNFB-sensitized group are denoted by (*) for $p<0.05$.

Acute bronchoconstriction. Intranasal challenge was accompanied by an acute bronchoconstriction in DNFB-sensitized mice compared to vehicle-sensitized mice, as monitored by an increase in PenH values. Immediately after challenge Penh values increased, reaching a maximum 5 min after challenge. PenH values gradually returned to basal levels 15 min after the challenge (figure 2).

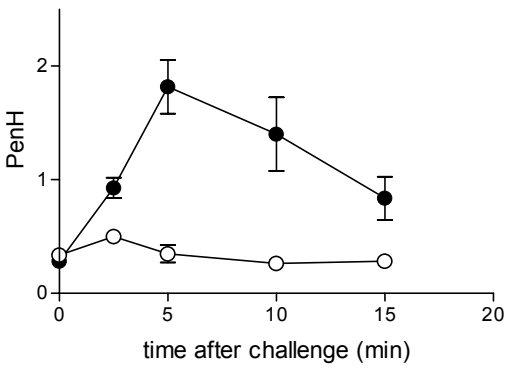

Figure 2. Acute bronchoconstriction is found in DNFB-sensitized BALB/c mice after intranasal DNS challenge. Bronchoconstriction is characterized by increases in Penh values, which were recorded in vehicle- and DNFB-sensitized mice 5 min before until 15 min after the challenge. For each mouse max PenH readings were taken over 1 min time windows at 0, 2 min 30 sec, 5, 10 and 15 min. * $p<0.05$, n=6 mice per group.

Tracheal reactivity.

The intranasal hapten application in DNFB-sensitized mice resulted in a tracheal hyperreactivity reaction to carbachol 24 h and 48 h after the challenge (Emax 24 h; Con: 2020 mg ± 180 mg, DNFB: 2841 mg ± 201 mg. n=6; $p<0.05$. Emax 48 h; Con: 2095 mg ± 175 mg, DNFB: 3203 mg ± 133 mg. n=6; $p<0.05$)(figure 3). Because the increase in reactivity was more profound at 48 h after challenge, this time point was chosen in following studies to measure tracheal hyperreactivity.

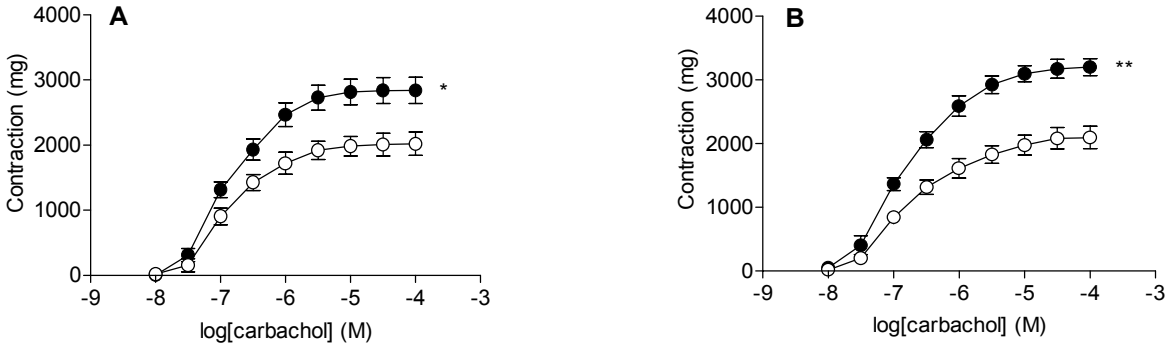

Figure 3. Mice were vehicle- or DNFB-sensitized and DNS challenged. Concentration-response curves were measured in DNFB- (closed circles) or vehicle- (open circles) sensitized mice 24 **(A)** and 48 h **(B)** after DNS challenge. Results are expressed as mean ± SEM (n=6). Significant differences between curves are denoted by (*) or (**) for $p<0.05$ and $p<0.01$, respectively.

DNFB-induced leukocyte accumulation in bronchial alveolar lavage fluid.

An increase in cellular accumulation was found in DNFB-sensitized, DNS-challenged mice 24 and 48 h after the challenge. The total cell counts from lung lavages from DNFB-sensitized mice differed significantly from vehicle-sensitized mice at both time points (table 3). Broncho-alveolar cells in these mice mainly consist of mononuclear cells and neutrophils. Both the numbers of mononuclear cells and neutrophils showed to be significantly increased in DNFB-sensitized animals compared to control mice at both 24 and 48 h after hapten challenge (table 3).

Table 3. Total, mononuclear and neutrophil cell numbers in BAL fluid. Mice were skin-sensitized with DNFB or vehicle and i.n. challenged with DNS. Cellular accumulation was studied studied 24 and 48 h after challenge.

Sensitization	Total cells (x1000 cells/lung)	mononuclear cells (x1000 cells/lung)	neutrophils (x1000 cells/lung)
24 h after challenge			
Vehicle	22.5 (21.0-31.5)	22.3 (20.1-29.4)	1.6 (0.9-2.0)
DNFB	40.5 (37.5-52.5)*	36.1 (33.1-47.3)*	5.0 (3.6-5.7)*
48 h after challenge			
Vehicle	23.3 (16.5-31.5)	22.6 (15.5-31.0)	0.7 (0.5-1.5)
DNFB	40.0 (33.0-46.5)*	35.1 (29.7-43.2)*	3.6 (2.8-5.4)*

Results are expressed as median (min-max) (n=6 animals/group). Significant differences between the vehicle-sensitized and the DNFB-sensitized group are denoted by (*) for $p<0.05$.

		Accumulation of inflammatory cells	
	Treatment	Around bronchioli	Around blood vessels
24 h	Vehicle	0.53 ± 0.08	0.17 ± 0.06
	DNFB	0.94 ± 0.04*	0.38 ± 0.02*
48 h	Vehicle	0.49 ± 0.09	0.17 ± 0.12
	DNFB	0.79 ± 0.10*	0.30 ± 0.17*

Table 4. Cellular infiltration in the lung. Mice were skin-sensitized with DNFB or vehicle and challenged intranasally with DNS. 24 and 48 h after challenge lungs were removed and studied for cellular infiltration (see Methods). Results are expressed as mean ± sem (4 mice/group). Significant differences between the vehicle-sensitized and the DNFB-sensitized group are denoted by (*) for ($p<0.05$).

Histological analysis. Within one day after intranasal DNS challenge, an accumulation of inflammatory cells around bronchioli and pulmonary blood vessels was induced in animals sensitized with DNFB. On the other hand, in vehicle-sensitized mice, DNS challenge did not result in cellular infiltration. Overall, a significant accumulation of inflammatory cells was seen in DNFB-sensitized mice around the bronchioli and around the blood vessels compared to vehicle-sensitized animals (table 4, figure 5 and 6). Differences between sensitized and control mice were shown to be significant at both 24 and 48 h after DNS challenge (table 4). No significant differences were observed between the two time points.

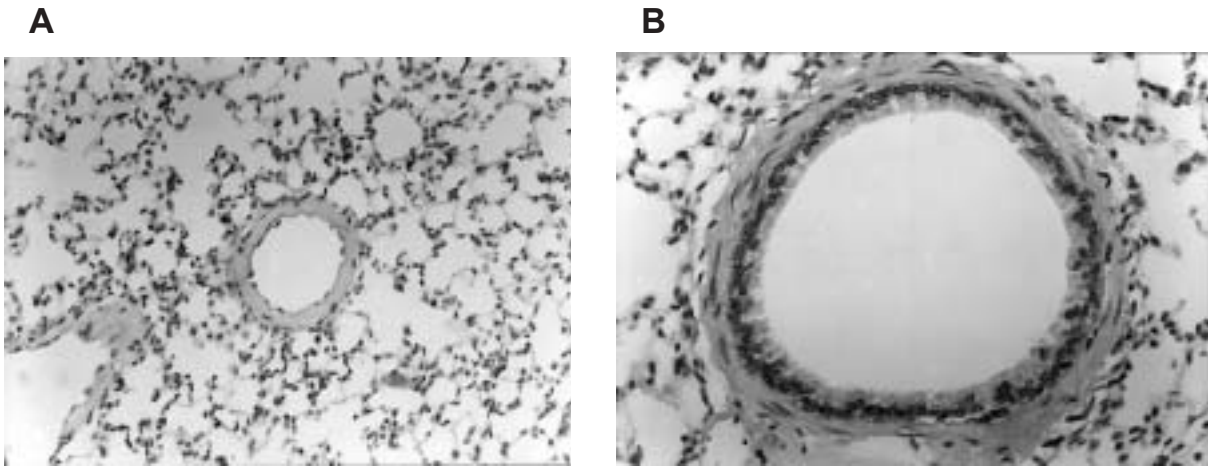

Figure 4. Lung of vehicle-sensitized mouse challenged with DNS. Specimen was taken 24 h after challenge. **A**. Blood vessel (x25), **B.** Bronchiolus (x40). No cellular infiltrates are found.

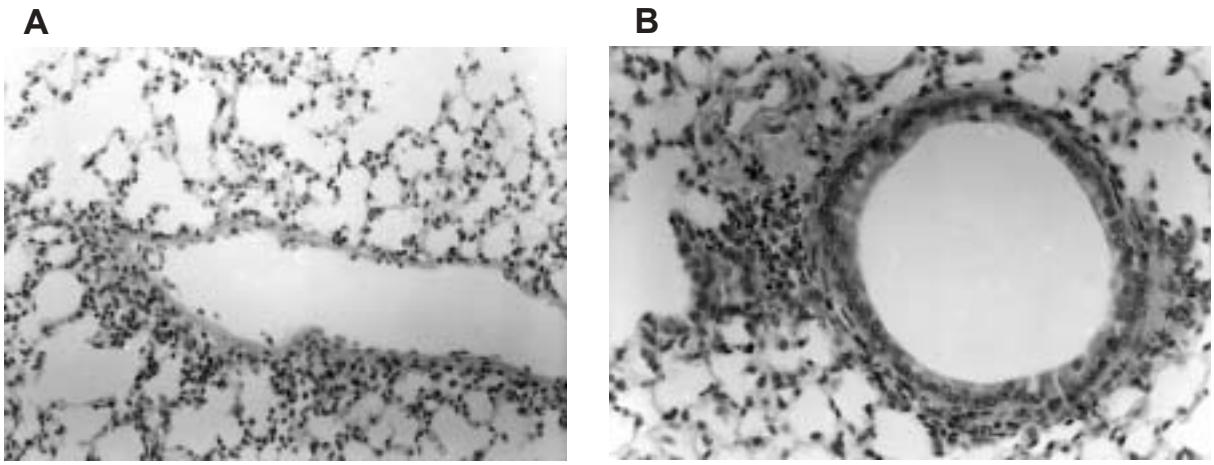

Figure 5. Inflammatory response to DNFB-sensitized in mouse challenged with DNS. Specimen was taken 24 h after challenge. **A**. Blood vessel (x25), **B.** Bronchiolus (x25). Cellular infiltrates were observed.

Discussion

In the present study, we describe a murine model for non-atopic asthma.
A pulmonary reaction was induced in contact sensitized and intranasally challenged mice. This pulmonary reaction was characterized by an early (0-3 h) and late phase hypersensitivity response (24-48 h after challenge). Features of the early pulmonary hypersensitivity reaction included acute bronchoconstriction and mast cell activation. The late phase was characterized by again mast cell activation, mononuclear and neutrophilic cell infiltration into the airway lumen and tracheal hyperreactivity. These results support data presented by previous studies performed in small intestine and airways in which edema formation, leukocyte accumulation and hyperreactivity were present 24 h after challenge (1, 20-22). Leukocyte accumulation was demonstrated 24 and 48 h after challenge in sensitized mice and was dominated by the accumulation of neutrophils. The role of the neutrophil in this model for non-atopic asthma will be further addressed elsewhere in this thesis.

The lymphocyte and the mast cell are two of the key inflammatory cells in the non-atopic response. Upon contact sensitization, lymphocytes are activated to produce hapten-specific immunoglobulin kappa light chains (IgLC) (12). The hapten-specific IgLC can arm mast cells (and possibly other cell types)(23). After a second encounter with the antigen or hapten, the challenge phase, the hapten will multivalent crosslink IgLC bound to the mast cell, causing activation and subsequent release of mediators. Vaso-active mediators such as histamine, serotonin and TNF-α increase the micro-vascular permeability and induce the infiltration of circulating hapten-specific effector cells, generated during the sensitization. These effector T-cells can recognize antigen in the context of MHC class II on antigen presenting cells after a second encounter with the hapten. The effector T-cells are now triggered to produce cytokines leading to the inflammatory response (see figure 1).

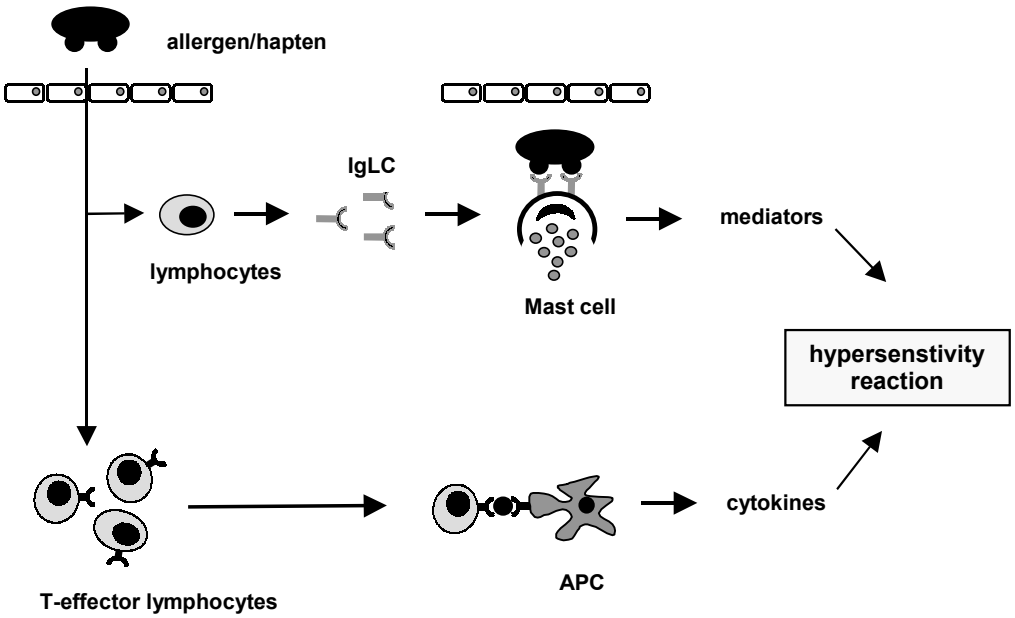

Figure 1. Schematic representation of the proposed mechanism leading to hypersensitivity reactions in the airways.

Both T lymphocytes and mast cells can be sensitive to sensory neuropeptides. Since pretreatment of human or guinea pig skin with capsaicin enhanced the cell-mediated reaction at the site of treatment, it was suggested that capsaicin-sensitive neurons modulate the reaction via release of neuropeptides (24). It is reasonable to speculate that neuropeptides, released from sensory nerve endings, play a role in the initiation and maintenance of inflammatory responses. This will be extensively addressed in the following chapters of this thesis.

In summary, our study shows that our murine model for non-atopic asthma is characterized by bronchoconstriction and mast cell activation in the early phase and mast cell activation, cellular accumulation and tracheal hyperreactivity in the late phase reaction. These characteristics bear marked similarity with those observed in non-atopic asthma in man. Therefore, this model can be used to further study the mechanisms responsible for the development of non-atopic asthma.

Acknowledgments

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Key role for mast cells in non-atopic asthma

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Abstract

The mechanisms involved in non-atopic asthma are poorly defined. In particular, the importance of mast cells in the development of non-atopic asthma is not clear. In the mouse, pulmonary hypersensitivity reactions induced by skin sensitization with the low molecular weight compound dinitrofluorobenzene (DNFB) followed by an intra-airway application of the hapten have been featured as a model for non-atopic asthma. In the present study, we employed this model to examine the role of mast cells in the pathogenesis of non-atopic asthma. Firstly, the effect of DNFB sensitization and intra-airway challenge with dinitrobenzene sulphonic acid (DNS) on mast cell activation was monitored during the early phase of the response in BALB/c mice. Secondly, mast cell-deficient *W/W^v*, *Sl/Sl^d* mice and their respective normal (+/+) littermate mice and mast cell-reconstituted *W/W^v* mice (BMMC→ *W/W^v*) were used. Early phase mast cell activation was found, which was maximal 30 min after DNS challenge in DNFB-sensitized BALB/c, +/+ mice, but not in mast cell-deficient mice. An acute bronchoconstriction and increase in vascular permeability accompanied the early phase mast cell activation. BALB/c, +/+ and BMMC→ *W/W^v* mice sensitized with DNFB and DNS-challenged exhibited tracheal hyperreactivity 24 and 48 h after the challenge when compared to vehicle-treated mice. Mucosal exudation and infiltration of neutrophils in bronchoalveolar lavage fluid associated the late phase response. Both mast cell-deficient strains failed to show any features of this hypersensitivity response. Our findings show that mast cells play a key role in the regulation of pulmonary hypersensitivity responses in this murine model for non-atopic asthma.

Introduction

Reversible airway obstruction, pulmonary inflammation and increased reactivity of airway smooth muscle to various stimuli are prominent features of asthma (1, 2). The majority of patients have atopic asthma, which starts during childhood and is characterized by an elevation of total and allergen-specific IgE in the serum (3). It is currently accepted that a subgroup of asthmatics is not demonstrably atopic (4). Non-atopic asthmatics are skin test negative to common allergens and there is no evidence of allergen-specific serum IgE (5, 6). Even total serum IgE levels are within the normal range. In addition, there is no clinical or family history of allergy (5, 6).

Atopic asthma has been extensively investigated. Considerable less information is available about the pathologic characteristics of non-atopic asthma (7). Very recently, Amin and coworkers have compared the cellular pattern and structural changes in the airways of atopic and non-atopic asthmatic patients (8). Both groups of asthmatics had respiratory symptoms, peak flow variability and bronchial hyperresponsiveness of similar severity. However, it was clearly demonstrated that in atopic asthmatics high numbers of eosinophils, mast cells and T lymphocytes characterized the airway inflammation, whereas non-atopic asthmatics mainly displayed high numbers of neutrophils and mast cells (8). These findings suggest that there are differences in the extent of the immunopathologic response of these two types of asthma.

The mechanisms involved in non-atopic asthma are poorly defined. In the mouse, several investigators have characterized hapten-induced pulmonary hypersensitivity (also referred to as delayed type hypersensitivity or type IV hypersensitivity) reactions induced

by skin sensitization followed by an intra-airway application of low molecular weight compounds such as picryl chloride, toluene diisocyanate and DNFB, as models for non-atopic asthma (9-13). These pulmonary hypersensitivity reactions were not associated with an elevated hapten-specific serum IgE (10, 13, 14). The features observed in these murine models resemble those found in non-atopic asthma and are hapten-induced acute bronchoconstriction, pulmonary edema, infiltration of neutrophils and mononuclear cells, *in vitro* tracheal hyperresponsiveness and *in vivo* airway hyperresponsiveness. Both the early (< 3 h) and the late (24 to 48 h) phases of the hapten-induced pulmonary reaction were found to depend on the presence of T lymphocytes, since in athymic mice airway hyperreactivity and cellular accumulation were suppressed (10, 12, 15).

Studies in mice contact-sensitized and locally challenged with low molecular weight haptens suggest that the pulmonary hypersensitivity response consists of a sequence of interactions between a variety of different cells rather than a direct T cell-mediated event. The mast cell is an important immunological and regulatory cell involved in the early mediation of tissue inflammation. Several human studies have suggested an important role for mast cells in non-atopic asthma. Furthermore, a role of the mast cell in hapten-induced pulmonary hypersensitivity reactions, the murine model of non-atopic asthma, has also been suggested (16-18). We hypothesized that mast cells may be critical in the development of non-atopic asthma.

Thus far no direct proof for the involvement of mast cells in hapten-induced pulmonary hypersensitivity reaction in the mouse has been presented. Therefore, the present study was undertaken to investigate the role of mast cells in DNFB-induced hypersensitivity reaction in the mouse lung. Firstly, the effect of DNFB sensitization and intra-airway challenge on mast cell activation was monitored during the early phase of the hypersensitivity reaction. Using genetically mast cell-deficient and congenic normal mice, the role of the mast cell was further established in this murine model for non-atopic asthma.

Methods

Animals. Male BALB/c mice were supplied by the central animal laboratory (GDL), Utrecht University, Utrecht, The Netherlands. Male mast cell deficient mice (WBB6F$_1$ *W/W^v*), their respective normal littermates (WBB6F$_1$ +/+), mast cell deficient mice (WCB6F1 *Sl/Sl^d*) and their respective normal littermates (WCB6F$_1$ +/+) were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were used at 6-8 weeks of age, with the exception of mast cell reconstituted *W/W^v* mice and age-matched +/+ and *W/W^v* mice that were used at 40 weeks of age. All the mice were housed in groups of not exceeding 10 per cage and maintained under standard conditions. The Animal Care Committee, Utrecht University, Utrecht, the Netherlands approved all experiments.

Mast cell reconstitution. Selective reconstitution of mast cells in mast cell-deficient W/W^v mice was carried out by the methods earlier described by Karimi and coworkers (19) and Williams and coworkers (20) with several modifications. Bone marrow-derived mast cells (BMMCs) were obtained from WBB6F$_1$ +/+ mice. Bone marrow was aseptically flushed

from femurs of +/+ mice and cultured for 4 to 5 weeks in complete RPMI (RPMI 1640 medium, which contained 10% FCS, 4 mM 1-glutamine, 0.5 μM β-mercaptoethanol, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin and 0.1 mM nonessential aminoacids). Pokeweed mitogen-stimulated spleen cell conditioned medium (20% v/v) was added to the culture medium as a source for IL-3. Medium was refreshed once a week. By flow cytometry analysis (c-Kit) > 90% purity of the BMMC population was determined. The culture contained a uniform cell population. Furthermore staining cells with toluidin blue indicated that nearly 99% of the viable cells were mast cells after 4-5 weeks culture. No stem-like cell was detected in the mast cell preparation. We suspect that the < 10% of the c-kit negative cells are unmature mast cells. Mast cell-deficient W/W^v mice were injected via the tail vein with 5×10^6 cultured BMMC cells and the recipients were studied 20 weeks later. Age-matched mast cell-deficient W/W^v mice and congenic normal mice were used when examining the BMMC-reconstituted W/W^v mice (BMMC→ W/W^v). BMMC→ W/W^v mice had 7.70 ± 1.33 mast cells/ mm trachea versus none or 8.04 ± 1.46 mast cells/ mm trachea for W/W^v or +/+ mice, respectively.

Preparation of tracheal tissue for histological examination. To establish the success of reconstitution of mast cells, trachea of +/+ and BMMC→ W/W^v mice were examined for the presence and distribution of mast cells. After animals were sacrificed, trachea were removed, fixed in 4% formaldehyde in PBS and routinely embedded in GMA (glycol methacrylate)(21). Serial sections were cut at 3 μm and stained with Heath's aluminium-Toluidin Blue (TB), or using chloro-acetate esterase (CAE) and peroxidase (PO) procedures, respectively (22).

Immunization and airway challenge with the hapten. Mice were sensitized on day 0 with either DNFB (0.5% dissolved in acetone: olive oil [4:1]) or vehicle control, both of which were applied epicutaneously to the shaved thorax (50 μl) and all four paws (50 μl). On day 1, DNFB or vehicle control (50 μl) was applied to the thorax alone. DNFB- and vehicle-sensitized mice were intranasally challenged with dinitrobenzene sulphonic acid (DNS, 50 μl 0.6 % in PBS, pH 7.2) on day 5. The sensitization and challenge were performed under light anesthesia (inhalation of halothane 3%). Acute bronchoconstriction was assessed in conscious mice directly after the challenge (as described below). Thereafter, the mice were sacrificed (sodium pentobarbitone, 0.3 ml, 60 mg/kg i.p.) at several time points after the challenge to determine *in vivo* mast cell activation, tracheal vascular permeability, mucosal exudation, *in vitro* tracheal reactivity and leukocyte accumulation in the bronchoalveolar lavage fluid.

Measurement of acute bronchoconstriction. Bronchoconstriction was measured as reported previously (23). In short, 5 min before intranasal DNS challenge, unrestrained conscious mice were placed in a whole body plethysmographic chamber to analyze the respiratory wave forms and obtain basal line. After 4 min and 30 sec, the mice were intranasally challenged and placed directly back in the chamber. Airway resistance in each animal was measured over a 25 min period. The airway resistance is expressed as enhanced pause (PenH). PenH = pause x PEP/PIP
where PEP stands for peak expiratory pressure and PIP for peak inspiratory pressure.

Pause is defined as $(T_e - T_r)/T_r$, where T_e stands for time of expiration and T_r stand for the relaxation time -the time of pressure decay to 36% of the total expiratory pressure signal. During bronchoconstriction, the changes in the box pressure during expiration are more pronounced than during inspiration. Thus bronchoconstriction is reflected by an increase in PenH, a dimensionless value to empirically monitor airway function. After intranasal challenge, for each mouse maximal PenH readings were taken over 1 min time windows at the following time points: 2 min 30 sec, 5 min, 7 min 30 sec, 10, 15 and 20 min.

Mast cell activation in vivo.

a. Histamine radioimmunoassay in plasma

To monitor mast cell activation, blood samples were taken from DNFB- and vehicle-sensitized mice 10 and 30 min after intranasal DNS challenge for measurement of histamine. Blood samples were collected into chilled tubes containing EDTA and placed on ice immediately. Plasma was obtained via centrifugation at 14000 rpm at 4ºC for 10 min. Supernatants were separated and frozen at -70ºC until assay. Histamine radioimmunoassay was performed according to the manufactures instructions (Immunotech International). Resulting counts per min from 0.5 to 150 nmol/L (assay standard enclosed with kit) and 0.1 to 50.0 ng/ml histamine standard solutions in plasma were used to construct standard curves. Histamine values for unknown samples were determined from the standard curve.

b. Mouse mast cell protease 1 ELISA in serum and tissue samples

Mouse mast cell protease 1 (mMCP-1) is a protease specific for mouse mast cells and will appear in the blood of mice after mast cell activation (24). To monitor mast cell activation blood samples were taken from DNFB- and vehicle-sensitized BALB/c mice 10, 30, 120 and 180 min after intranasal DNS challenge for measurement of mMCP-1. Blood samples were collected and after centrifugation sera were stored at -70^{o} C until assay. In addition, 30 min after the challenge and after perfusing the mice with 5 ml PBS (37ºC) via the right ventricle the lungs were isolated and homogenated in 1.5 M KCl at 4ºC. The lung homogenates were centrifuged for 10 min at 10,000 *g* and the supernatant were stored at -70ºC until assay. A commercially available mMCP-1 enzyme linked immunosorbent assay was used for the measurements of mMCP-1 in the sera and tissue homogenates. Serum and tissue homogenates supernatants were diluted 1:1 before assaying the samples using two anti-mMCP1 antibodies. 96 Wells flat bottomed micro ELISA plates were coated with sheep anti-mMCP-1 capture antibody (2 μg/ml) and kept for 24 h at 4ºC in a humid chamber before use. The coated plates were wash 6 times before loading standard mMCP-1 and samples for 24 h. After another wash step, plates were incubated with rabbit anti-mMCP-1-HPO conjugate for 1.5 h at room temperature. HPO activity was assessed by adding orthophenylenediamine/H_2O_2 (0.4 mg/ml). After stopping the reaction with 2.5 M H_2SO_4, OD was measured at a wavelenghth of 490 nm using a microplate reader. Results were expressed as ng mMCP-1 per ml serum or ng mMCP-1 per g tissue wet weight.

Determination of tracheal vascular permeability. Monastral blue pigment is a tracer to localize leakage at postcapillary venules in the trachea (25). Monastral blue was injected i.v. via the tail vein immediately before the challenge. 30 Min after the challenge mice were sacrificed with an overdose of sodium pentobarbitone and were perfused transcardially

for 5 min with PBS containing heparin (10 U/ml) followed by 1% paraformaldehyde for 10 min. Trachea were removed, opened longitudinally along the ventral midline and fixed in 4% paraformaldehyde overnight. Finally, they were hydrated in ethanol, cleared in xylene and prepared as whole amounts.

Leukocyte accumulation in bronchoalveolar lavage fluid. Bronchoalveolar lavages (BAL) were performed in separate groups of mice at 24 h after the challenge as previously described (11). After sacrificing the animals, the trachea was cannulated. Saline (37ºC) was slowly injected into the lung and withdrawn in 4 x 1 ml aliquots. After the collection of 1 + 3 ml BAL fluid samples per mouse, the samples were maintained at 4° C. The lavage fluids were centrifuged (1500 rpm, 580 *g*, 10 min, 4ºC) to isolate the BAL cells from the supernatant. After measuring the volume of the first ml, the supernatant was used to assess mucosal exudation. BAL cell pellets were pooled and resuspended in 150 μl for total and differential counts. The total BAL leukocyte count was determined for each mouse, using a hemocytometer. The BAL cell preparations were analyzed morphologically after centrifugation on microscopic slides. Air-dried preparations were fixed and stained with hematoxylin and eosin to ascertain the percentage of neutrophils, eosinophils and mononuclear cells (lymphocytes, macrophages and monocytes) in the airway lumen. 100 Cells per cytospin were examined to determine cell differential counts. The total number of the different leukocytes in the BAL fluid of each mouse was extrapolated from the data collected. Results are expressed as number of cell per lung.

Mucosal exudation. Mucosal exudation was assessed as previously described (26). At time of challenge or 22 h after the challenge, Evans blue dye (1.25%, 50 μl in sterile saline) was injected i.v. into anesthetized mice. One and 24 h after the challenge the mice were sacrificed and blood samples and BAL samples were taken as described above. The volumes of the first ml of lavage fluid recovered were recorded for each mouse for measurement of Evans blue content and for the calculation of mucosal exudation. No significant differences were found in the volumes of BAL fluids of vehicle sensitized and DNFB-sensitized mice. The extravasation of Evans blue dye-labeled macromolecules from the pulmonary microcirculation to the bronchoalveolar spaces was quantified by measuring the OD of the lavage and plasma samples at a wavelength of 595 nm with a microplate reader. The amount of mucosal leakage in the lavage fluid (μl/lung) was determined by dividing the Evans blue content in the total lavage fluid by the Evans blue content in 1 ml of plasma.

Tracheal reactivity in vitro. Determination of tracheal reactivity was assessed 48 h after the challenge as described previously (11). The trachea, which was resected *in toto*, was carefully cleaned of connective tissue under a binocular microscope. A nine-ring section of the trachea (taken from just below the larynx) was then transferred to a 10 ml organ bath containing a modified oxygenated Krebs' solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 25 mM $NaHCO_3$, 1 mM $NaHPO_4$ and 11.1 mM glucose). The tracheal section was slipped directly onto two supports of an organ bath, one of which was coupled to the organ bath and the other to an isometric transducer. The solution was aerated (95% O_2: 5% CO_2) at constant temperature (37ºC). Isometric measurements were made with a

force displacement transducer and a two channel recorder and were expressed as changes in milligram force. Optimal preload for the mouse trachea was determined to be 1 g. The trachea was allowed to equilibrate for 1 h in Krebs' solution. During equilibrium phase, the fluid in the bath was changed every 15 min. To assess reactivity, cumulative concentration-response curves for carbachol (concentration range: 10^{-8} to 10^{-4} M) were determined 24 or 48 h after the challenge of vehicle- or DNFB-sensitized mice.

Materials. DNFB, olive oil, carbachol, o-phenylenediamine dihydrochloride were purchased from Sigma Chemical Co., St. Louis, MO. DNS was purchased from Eastman Kodak, Rochester, NY. Tween 20 was purchased from Janssen Pharmaceutica, Beerse, Belgium. Sodium pentobarbitone was purchased from Sanofi, Maassluis, the Netherlands. RPMI 1640 medium was purchased from Life Technologies, Rockville, MD. Evans blue dye was obtained from Fluka Chemie AG, Munchen, Germany and heparin from Leo Pharmaceutical Products (Ballerup, Denmark). Monastral blue dye was a generous gift of Dr P. Baluk, Cardiovascular Research Institute (University of California, San Francisco, USA). Hematoxylin and eosin (Diff-Quik) were purchased from Merz & Dade A.G., Dubingen, Switzerland.
The histamine radioimmunoassay was purchased from Immunotech S.A., Marseilles, France. The mMCP-1 enzyme linked immunosorbent assay was from Moredun Scientific Ltd., Midlothian, UK. Maxisorp surface 96 well plates were purchased from Nunc Immuno plate, Roskilde, Denmark. The whole body plethysmographic chamber (Buxco) was obtained from Buxco Electronics, Inc. Shanon, CT. The force displacement transducer was purchased from Harvard Bioscience, Boston, MA, USA and the two channel recorder (Servogar type SE-120) from Plato BV, Diemen, the Netherlands.

Statistical analysis. All experiments were designed as completely randomized multifactorials with 4-14 mice per group. Emax values for the carbachol-induced tracheal contraction for each experimental animal were calculated separately by nonlinear least-square regression analysis (simplex minimalization) of the measured contractions versus carbachol concentration, using the sigmoid concentration-response relationship and including a theshold value. Histamine content in blood was analyzed using unpaired t-tests at the two diffentent time points of sampling. The following data obtained from individual animals were analyzed by two-way analysis of variance (ANOVA): mMCP-1 content in blood and lung homogenates; mucosal exudation values; EC50 and Emax values for the carbachol-induced tracheal contractility; followed by a post-hoc comparison between groups. In the figures group means ± SEM are given and a difference was considered significant when $P<0.05$. The cellular accumulation in BAL fluid was analyzed by using a distribution free Kruksal Wallis one way ANOVA. The cell data are expressed as medians (minimum-maximum). All data manipulation, non-linear fittings, unpaired t-test, ANOVA and post-hoc comparisons were carried out with a commercially available statistical package (SYSTAT, version 5.03; Wilkinson L. SYSTAT; The system for statistics. SYSTAT Inc. 1990, Evanston, IL).

Results

DNFB-induced pulmonary hypersensitivity reaction: early phase mast cell activation, acute bronchoconstriction and vascular permeability changes in the trachea.

The appearance of histamine and mMCP-1 in the blood is indicative for the activation of mast cells (24). An increase in histamine levels (± 150%) was found in the plasma of DNFB-sensitized BALB/c mice 10 and 30 min after intranasal DNS challenge when compared to vehicle-sensitized animals (10 min: vehicle/DNS: 158.4 ± 21.4 nM & DNFB/DNS: 240.1 ± 20.3 nM, $p<0.05$, n=4 mice per group ; 30 min: vehicle/DNS: 98.4 ± 29.9 & DNFB/DNS: 171.4 ± 12.8 nM, $p=0.07$, n=4 mice per group). The hapten-induced rise in serum histamine levels 30 min after the challenge was, however, not statistically significantly different, possibly due to the short half life of histamine in serum. Moreover, mast cells are not the only source for histamine; therefore we have examined a more specific marker for mast cell degranulation mMCP-1. mMCP-1 was markedly enhanced in serum of DNFB-sensitized and DNS-challenged BALB/c mice 10, 30, 120 and 180 min after the challenge (figure 1). The most prominent rise in serum mMCP-1 was observed 30 min after the intranasal challenge of DNFB-sensitized mice. At this time point, a concomitant reduction in lung tissue mMCP-1 levels was found in DNFB-sensitized and DNS-challenged BALB/c mice showing that the hapten-induced rise in serum mMCP-1 was of pulmonary origin. mMCP-1 levels in BAL fluid samples from vehicle- and DNFB-sensitized BALB/c mice were below the detection limit of the mMCP-1-ELISA.The early phase mast cell activation was associated with an acute bronchoconstriction in DNFB-sensitized and DNS-challenged BALB/c mice. As demonstrated by figure 2, intranasal DNS challenge in DNFB-sensitized BALB/c mice resulted in an increase in PenH values when compared to vehicle-sensitized mice (max PenH: saline/DNS=0.76 ± 0.07 and DNFB/DNS=2.92 ± 0.57, $p<0.05$, n=6). The DNFB/DNS-induced bronchoconstriction was evident within 15 min after the challenge. In addition, 30 min after the DNS challenge a significant increase in vascular permeability was evident in trachea of DNBF-sensitized BALB/c mice compared to controls (data not shown). Examination of Evans blue accumulation in BAL fluid 1 h after intranasal challenge, showed a significant mucosal exudation in the airways of DNFB-sensitized BALB/c mice when compared to vehicle-sensitized mice (table 1).

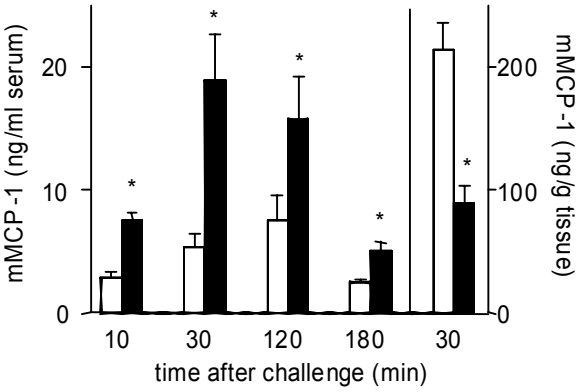
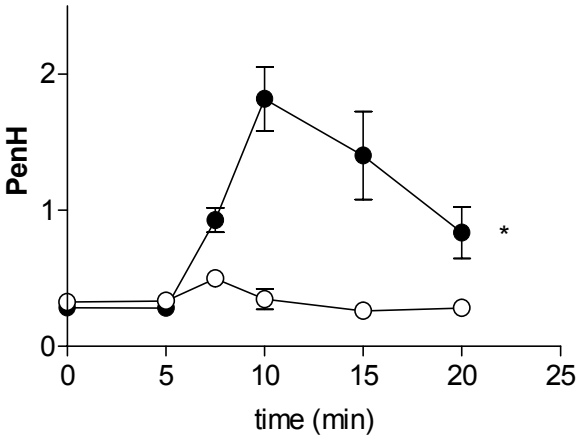


Figure 1. Mast cells are activated shortly after intranasal DNS challenge in DNFB-sensitized BALB/c mice. Mast cell proteases (mMCP-1) levels in serum and in lung tissue of vehicle- and DNFB-sensitized mice 10, 30, 120 and 180 min after intranasal DNS challenge. Results are expressed as mean (ng mMCP-1/ml serum or ng/g tissue wet weight) ± SEM, * $p<0.05$, n=6-12 mice per group.

Mouse	n	time[a] (hrs)	DNFB sensitization	mucosal exudation (µl plasma/lung)
BALB/c	6	1	-	4.20 ± 1.12
	6	1	+	8.51 ± 1.20*
+/+	6	1	-	3.85 ± 1.37
	6	1	+	8.65 ± 1.18*
W/W^v	6	1	-	4.00 ± 0.66
	6	1	+	3.45 ± 0.59
+/+	6	1	-	2.65 ± 0.18
	6	1	+	5.55 ± 0.61*
Sl/Sl^d	6	1	-	2.75 ± 0.36
	6	1	+	2.25 ± 0.29
BALB/c	6	24	-	3.17 ± 0.38
	6	24	+	6.30 ± 0.46*
[b]+/+	4	basal[c]		6.07 ± 0.81
	8	24	-	6.58 ± 0.85
	8	24	+	6.41 ± 0.33
W/W^v	7	24	-	5.51 ± 0.72
	8	24	+	4.61 ± 0.77
BMMC > W/W^v	5	24	-	5.84 ± 1.02
	6	24	+	4.95 ± 0.73

Table 1. Mucosal exudation in the airway lumen 1 and 24 h after DNS challenge of vehicle or DNFB-sensitized BALB/c, W/W^v, Sl/Sl^d and their respective normal littermate control(+/+) and reconstituted BMMC W/W^v mice[a]. (a) Mucosal exudation was determined in BAL fluid of vehicle (-) or DNFB-sensitized (+) mice 24 h after intranasal DNS challenge. Results are expressed as mean ml plasma/lung ± SEM. Significant differences between vehicle- and DNFB-sensitized groups are indicated (* $p<0.05$). (b) The mice examined in these experiments were age-matched (40 weeks). (c) basal mucosal exudation in airway of 40 weeks old non-treated +/+ mice.

Figure 2. Acute bronchoconstriction is found in DNFB-sensitized BALB/c mice after intranasal DNS challenge. Bronchoconstriction is characterized by increases in PenH values, which were recorded in vehicle- and DNFB-sensitized mice 5 min before until 20 min after the challenge. For each mouse max PenH readings were taken over 1 min time windows at -5 min, 0 min, 2 min 30 sec, 5, 10 and 15 min. * $p<0.05$, n=6 mice per group.



DNFB-induced pulmonary hypersensitivity reaction: late phase mucosal exudation, neutrophil infiltration in BAL fluid and tracheal hyperreactivity.

Bronchoalveolar lavage studies were performed in BALB/c mice to examine mucosal leakage and cellular infiltration 24 h after the challenge. Twenty-four hours after the intranasal DNS challenge a significant mucosal exudation was evident in the lungs of DNFB-sensitized BALB/c mice compared to controls (table 1). In addition, total cell numbers in BAL fluid of DNFB- or vehicle-sensitized BALB/c mice were determined. An increase in total BAL cell numbers recovered from DNFB-sensitized and DNS-challenged mice was found when compared to vehicle-sensitized mice (table 2). The increase in total BAL fluid cells was largely attributable to an increase in the number of neutrophils and mononuclear cells (table 2). At 24 and 48 h BALB/c mice exhibit a marked and significant tracheal

hyperreactivity to carbachol when DNFB-sensitized and DNS challenged (table 3). However,the observed tracheal hyperreactivity was more pronounced and reproducible at 48 h after the challenge. Therefore, this time point was chosen in mast cell reconstitution studies.

Table 2. Leukocyte accumulation and differentiation (neutrophils, eosinophils and mononuclear cells) in the lung air spaces 24 h after DNS challenge of vehicle or DNFB-sensitized BALB/c, W/W^v, Sl/Sl^d and their respective normal littermate control(+/+) and reconstituted BMMC→W/W^v mice[(a)]

Mouse	n	DNFB	leukocyte accumulation in BAL (cells/lung x 10^4)			
			total cells	neutrophils	eosinophils	mononuclear cells
BALB/c	6	-	2.25(1.05-2.78)	0.02(0.00-0.05)	0.00(0.00-0.11)	2.23(1.02-2.67)
	6	+	4.72(3.45-5.77)*	0.67(0.21-1.16)*	0.02(0.00-0.12)	3.81(3.12-4.50)*
+/+	8	-	2.10(1.35-2.78)	0.03(0.00-0.08)	0.00(0.00-0.01)	2.09(1.31-2.75)
	7	+	3.97(2.03-4.58)*	0.40(0.24-0.82)*	0.02(0.00-0.80)	3.25(1.66-3.71)*
W/W^v	8	-	2.10(1.20-2.70)	0.05(0.00-0.13)	0.00(0.00-0.03)	2.07(1.18-2.56)
	8	+	2.03(1.20-4.65)	0.05(0.02-0.06)	0.00(0.00-0.02)	1.97(1.16-4.19)
+/+	8	-	1.85(1.65-2.93)	0.03(0.00-0.20)	0.00(0.00-0.01)	1.81(0.23-2.93)
	8	+	2.93(1.98-5.40)	0.37(0.20-0.72)*	0.01(0.00-0.03)	2.54(1.30-5.02)*
Sl/Sl^d	8	-	2.02(0.83-4.28)	0.01(0.00-0.02)	0.00(0.00-0.01)	2.01(0.82-4.28)
	7	+	1.97(0.98-4.13)	0.01(0.00-0.06)	0.00(0.00-0.02)	1.97(0.96-4.13)
[(b)]+/+	4	B[(c)]	11.00(8.41-13.82)	0.03(0.00-0.14)	0.00(0.00-0.00)	10.99(8.41-13.75)
	8	-	12.66(3.90-20.10)	0.13(0.00-0.41)	0.00(0.00-0.00)	12.53(3.90-19.99)
	8	+	13.09(6.45-20.10)	0.89(0.29-1.58)*	0.04(0.00-0.13)	12.16(6.06-18.47)
W/W^v	7	-	12.41(5.10-18.90)	0.09(0.00-0.16)	0.03(0.00-0.15)	12.28(5.10-18.52)
	8	+	10.53(4.50-17.70)	0.12(0.00-0.49)	0.01(0.00-0.06)	10.39(4.28-17.61)
BMMC→W/W^v	5	-	11.88(7.95-13.80)	0.04(0.00-0.11)	0.00(0.00-0.00)	11.83(7.91-13.80)
	6	+	9.90(3.30-13.50)	0.58(0.27-0.90)*	0.06(0.00-0.27)	9.26(2.81-13.23)

(a) Leukocytes were determined in BAL fluid of vehicle (-) or DNFB-sensitized (+) mice 24 h after intranasal DNS challenge. Mononuclear cells being lymphocytes, macrophages and monocytes Results are expressed as median (minimum-maximum). Significant differences between vehicle- and DNFB-sensitized groups are indicated (* $p<0.05$). (b) The mice examined in these experiments were age-matched (40 weeks). (c) B: basal; leukocyte numbers of 40 weeks old non-treated +/+ mice.

Studies in two strains of mast cell deficient mice: the development of early and late phase reactions of DNFB-induced pulmonary hypersensitivity.

In separate experiments, several features from the DNFB-induced pulmonary hypersensitivity reaction were investigated in two strains of 8 weeks old mast cell-deficient mice (*W/W^v* and *Sl/Sl^d*) and their respective normal (+/+) littermates. Figure 3a shows that in both strains of normal (+/+) littermates DNFB/DNS-induced significant increases in mMCP-1 serum levels were found compared to vehicle sensitized animals. In both strains of mast cell-deficient mice, no changes in mMCP-1 serum levels were observed 30 min after the challenge of DNFB-sensitized mice compared to vehicle-sensitized animals (figure 3a).

Moreover, no mucosal exudation response was found 1 h after intranasal DNS challenge in DNFB-sensitized mast cell-deficient mice (table 1).To investigate the importance of the mast cell in the development of late phase responses 24 and 48 h after the DNS challenge in DNFB-sensitized mice, mucosal exudation, cellular infiltration into BAL fluid and tracheal reactivity were examined in the two strains of mast cell-deficient mice and their respective normal (+/+) littermates. In both strains of mast cell deficient mice no significant different mucosal exudation or total BAL cell numbers were found after DNFB-sensitization and DNS challenge when compared to vehicle-sensitized mice (tables 1 and 2). However, a significant mucosal exudation response and increase in BAL fluid neutrophils and to a lesser extent of mononuclear cells were demonstrated in the both normal (+/+) littermates 24 h after the challenge of DNFB-sensitized mice. Tracheal preparations taken from DNFB-sensitized congenic normal +/+ mice at 24 and 48 h after the DNS challenge exhibited a marked and significant hyperreactivity to carbachol when compared with responses of trachea of vehicle-sensitized mice (table 3). In both strains of mast cell deficient mice (*W/W^v* and *Sl/Sl^d*) no carbachol were found in DNFB-sensitized and DNS-challenged mice compared to control groups (table 3).

Table 3. Maximal tracheal reactivity (Emax) and pD2 (-log EC50) values derived from concentration response curves to carbachol (10^{-8} to 10^{-4} M) 24 and 48 h after DNS challenge in vehicle or DNFB-sensitized BALB/c, W/W^v, Sl/Sl^d and their respective normal littermate control(+/+) and reconstituted BMMC→W/W^v mice[(a)].

Mouse	n	time[(a)] (hrs)	DNFB sensitization	Emax (mg)	pD2
BALB/c	6	24	-	1928 ± 52	6.9 ± 0.1
	6	24	+	2402 ± 28*	6.8 ± 0.0
+/+	6	24	-	2277 ± 42	6.8 ± 0.1
	6	24	+	3012 ± 45*	6.7 ± 0.1
W/W^v	6	24	-	2324 ± 53	6.8 ± 0.1
	6	24	+	2488 ± 63	6.9 ± 0.1
+/+	6	24	-	1372 ± 25	6.7 ± 0.1
	6	24	+	2060 ± 41*	6.9 ± 0.1
Sl/Sl^d	6	24	-	1280 ± 33	6.7 ± 0.1
	6	24	+	1253 ± 14	6.6 ± 0.0
BALB/c	6	48	-	2177 ± 32	6.8 ± 0.1
	6	48	+	3116 ± 51*	6.8 ± 0.1
+/+	6	48	-	2266 ± 48	6.8 ± 0.1
	6	48	+	3091 ± 68*	6.7 ± 0.1
W/W^v	6	48	-	2042 ± 46	6.8 ± 0.1
	6	48	+	2115 ± 47	6.9 ± 0.1
[(b)]+/+	6	48	-	2347 ± 30	6.6 ± 0.0
	5	48	+	3332 ± 54*	6.6 ± 0.1
W/W^v	6	48	-	2421 ± 27	6.5 ± 0.0
	5	48	+	2616 ± 78	6.7 ± 0.1
BMMC→W/W^v	5	48	-	2480 ± 41	6.9 ± 0.1
	5	48	+	3326 ± 51*	6.7 ± 0.1

(a) Tracheal reactivity to carbachol in vitro of vehicle (-) or DNFB-sensitized (+) mice was assessed 24 and 48 h after intranasal DNS challenge. Results are expressed as means ± SEM. Significant differences between vehicle- and DNFB-sensitized groups are indicated (* $p<0.05$). (b) The mice examined in these experiments were age-matched (40 weeks). EC50: effective agonist concentration inducing 50% of the maximal response.

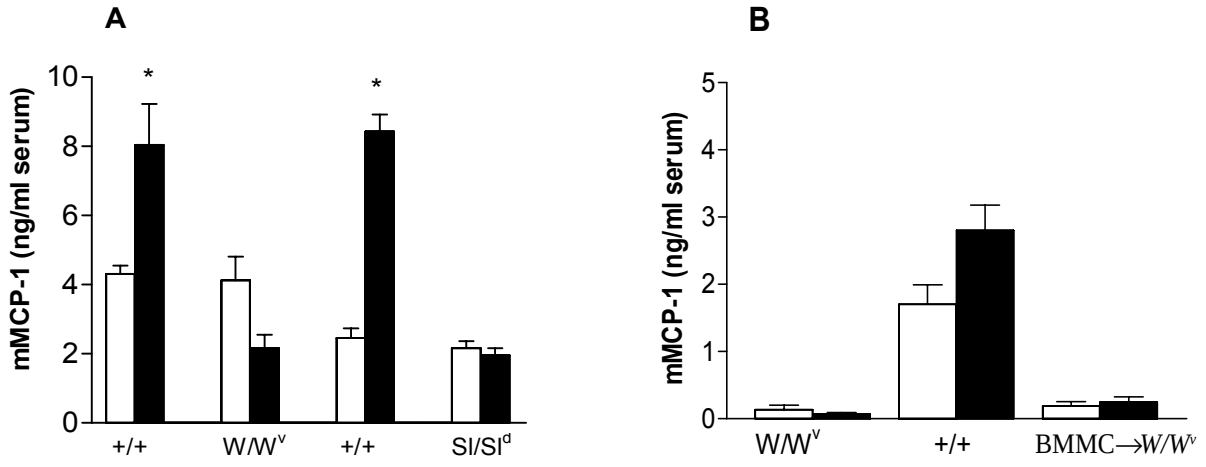


Figure 3. Mast cells are activated shortly after intranasal DNS challenge in DNFB-sensitized +/+ mice, but not in two strains mast cell-deficient mice. Mast cell protease (mMCP-1) levels in serum of vehicle- and DNFB-sensitized mast cell deficient *W/W^v*, mast cell deficient *Sl/Sl^d* and their respective normal littermate (+/+) and mast cell-reconstituted BMMC→*W/W^v* mice 30 min after intranasal DNS challenge. **a**) Results presented are obtained from 8 weeks old mice. **b**) Results presented are obtained from 40 weeks old mice. Results are expressed as mean (ng mMCP-1/ml serum) ± SEM, * $p<0.05$, n=6-12 mice per group.

Mast cells play a key role in the development of early and late phase reaction of DNFB-induced pulmonary hypersensitivity.

To confirm that the lack of mast cells was responsible for the failure of the development of the DNFB-induced pulmonary hypersensitivity reactions in the mast cell-deficient animals, we have determined the effect of reconstitution of *W/W^v* mice with mast cells cultured from bone marrow of +/+ mice (BMMC→*W/W^v*). An age-matched study was performed since the mast cell reconstitution took 20 weeks. To assess the establishment of mast cells in mast cell-reconstituted mice, trachea of +/+, *W/W^v* and BMMC→*W/W^v* mice were examined for the presence and distribution of mast cells. No mast cells were detected in tissues obtained from mast cell-deficient *W/W^v* mice (data not shown).

Staining with TB, revealed the presence of mast cells in tracheal sections of +/+ mice (figure 4a). In trachea of mast cell-reconstituted mice, TB did not stain any mast cells (figure 4d). Since CAE-staining is an accurate manner to identify mast cells in formalin fixed and GMA embedded tissue, CAE was also used to detect mast cells in the tracheal tissue. However, CAE staining not only detects mast cells but also some neutrophils. Therefore, serial sections were stained for PO-activity representative for neutrophils, but not for mast cells. Indeed, mast cells were observed in +/+ and BMMC→*W/W^v* mice using CAE staining (figure 4b,c,e,f). Serial sections revealed no overlap between PO and CAE positive staining, demonstrating that the CAE-positive cells were no neutrophils (data not shown). The number, morphology and anatomical distribution of mast cells in trachea of +/+ mice differ from mast cells in mast cell-reconstituted mice. It seems that latter mast cells are more mucosal-like: located in the mucosa, being smaller in size and having lower granular density (figure 4).

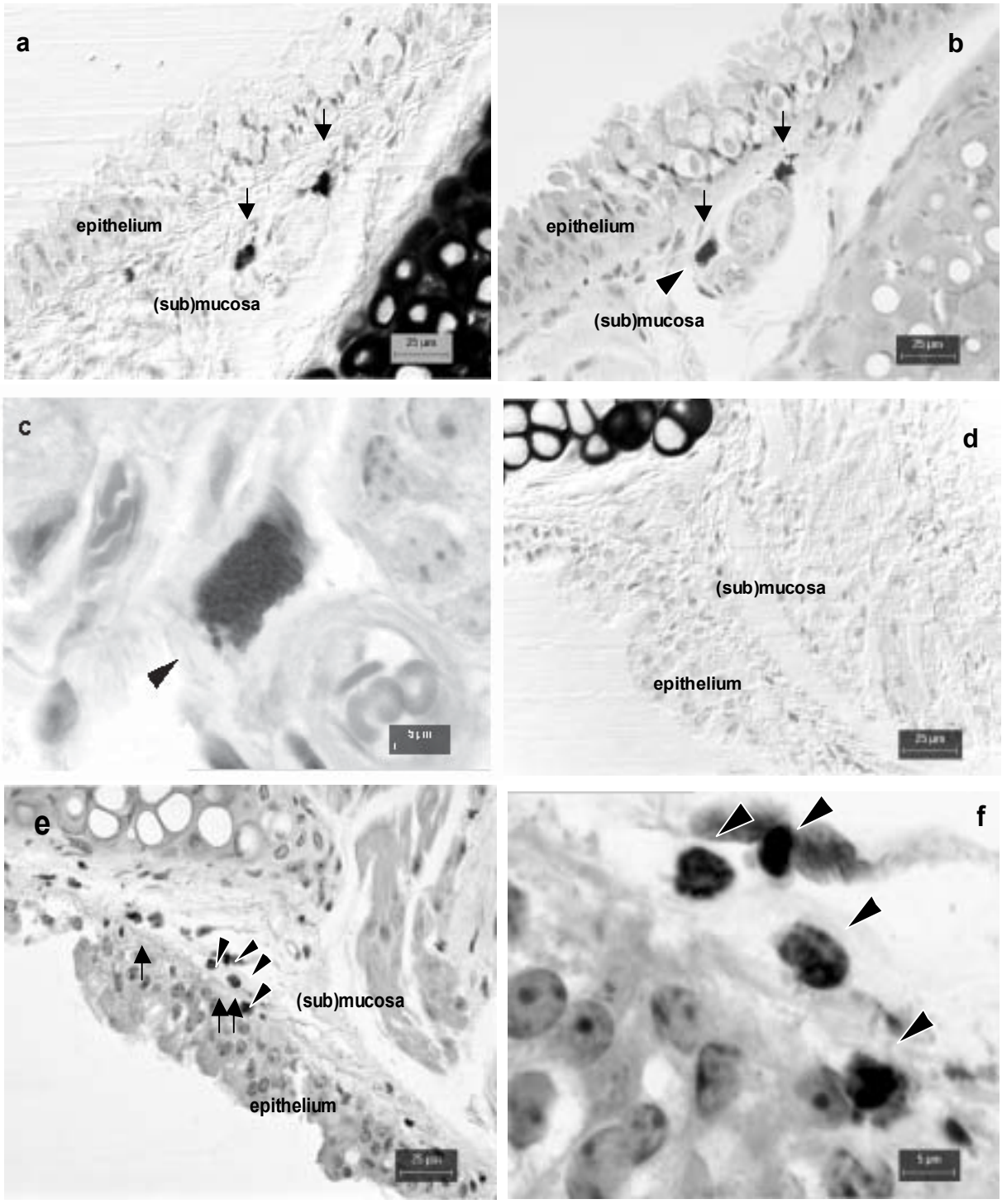


Figure 4. BMMC transplantation reconstitutes mast cells in trachea of mast cell-deficient *W/W^v* mice. BMMCs were injected intravenously into *W/W^v* mice. After reconstitution mice were housed for 20 weeks before being subjected to DNFB-sensitization and intranasal DNS challenge. At the time of experiment all mice were 40-weeks-old. Serial sections of GMA-embedded trachea from vehicle-sensitized +/+ (**a-c**) and BMMC→*W/W^v* mice (**d-f**) were stained with Heath's Aluminium Toluidin Blue (TB, **a** and **d**) and the chloro-acetate esterase reaction (CAE, **b,c,e** and **f**). The high magnification pictures (**c** and **f**) reveal a granular structure of CAE-positive mast cells. Arrows denote TB or/and CAE-positive mast cells. Arrowheads denote CAE-stained mast cells photographed at high magnification. Note the difference in size and granular density of CAE-stained mast cells in the wild-type and reconstituted animals (**c** and **f**).

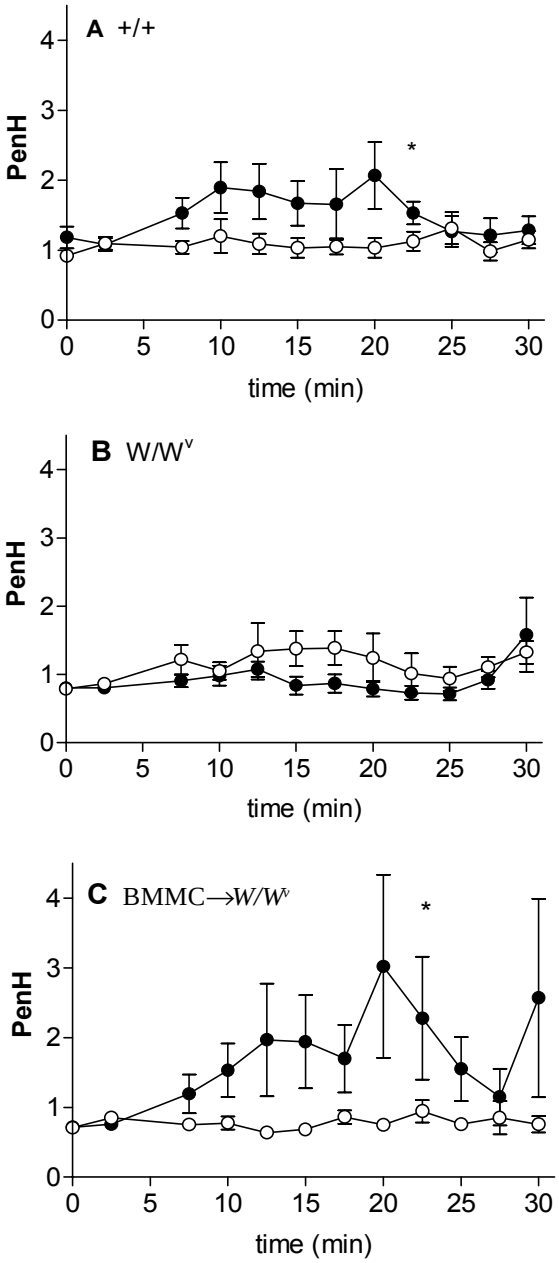


Figure 5. Acute bronchoconstriction is found in DNFB-sensitized +/+ and BMMC→ *W/W^v* mice, but not in mast cell deficient *W/W^v* mice. After reconstitution mice were housed for 20 weeks before being subjected to DNFB-sensitization and intranasal DNS challenge. At the time of experiment all mice were 40 weeks old. Bronchoconstriction is characterized by increases in PenH values, which were recorded in vehicle- and DNFB-sensitized mice 5 min before until 25 min after the challenge. For each mouse max PenH readings were taken over 1 min time windows at -5 min, 0 min, 2 min 30' sec, 5, 7 min 30 sec, 10, 12 min 30 sec, 15, 17 min 30 sec, 20, 22 min 30 sec and 25 min and 30 min. * $p<0.05$, n=5-6 mice per group.

As shown in Figure 5, DNFB-sensitization and DNS challenge resulted in a profound bronchoconstriction in 40 weeks old +/+ and BMMC→*W/W^v* mice, but mast cell deficient *W/W^v* mice failed to exhibit a bronchoconstrictive response directly after challenge. Although this response was associated in +/+ mice with a significant rise of mMCP-1 serum levels, no such changes were observed in *W/W^v* and BMMC→*W/W^v* mice (figure 3b). Nevertheless, an early phase-associated increase in tracheal vascular permeability observed in +/+ mice, that was absent in DNFB-sensitized *W/W^v* mice 30 min after challenge, could be completely restored after mast cell reconstitution (figure 6). Examination of BAL fluid of age-matched *W/W^v*, +/+, BMMC→ *W/W^v* mice, indicated that the presence of mast cells in the lung is important for the infiltration of neutrophils (table 2). Note that the total cell number of BAL cells is markedly risen in 40 weeks old mice compared to 8 weeks old mice (table 2). We were unable to assess mucosal exudation upon DNFB sensitization in 40 weeks old +/+ and BMMC→ *W/W^v* mice 24 h after intranasal challenge. The mucosal exudation values observed in +/+, *W/W^v* mice and BMMC→*W/W^v* mice after DNFB-sensitization and DNS challenge did not significantly differ from basal values in non-treated +/+ mice (table 1). Finally, reconstitution of mast cells in *W/W^v* mice restored the DNFB-induced tracheal hyperreactivity found 48 hours after the challenge (figure 7). The extent of tracheal hyperreactivity to carbachol in mast cell-reconstituted *W/W^v* mice was comparable to that observed in age-matched +/+ mice (table 3).

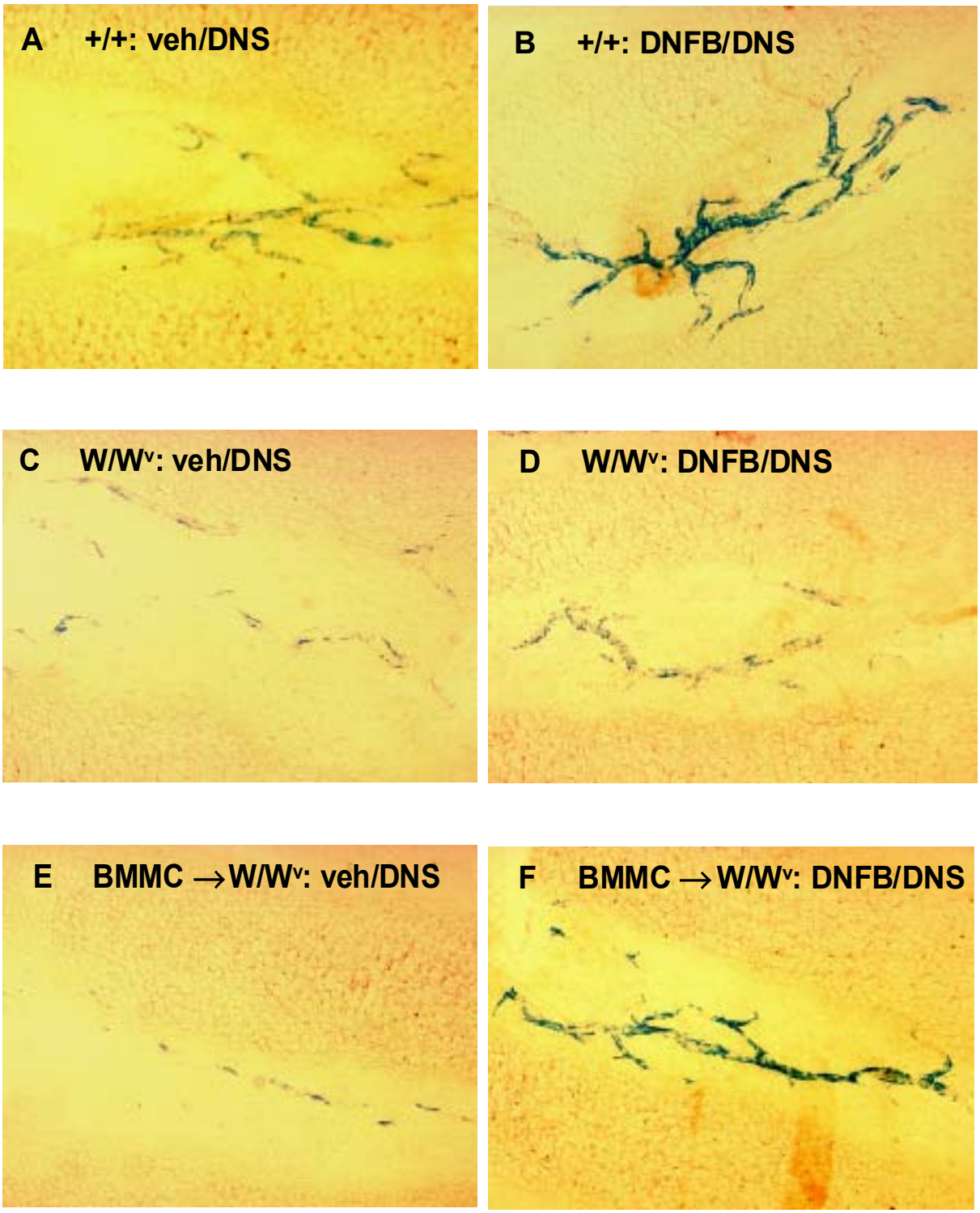


Figure 6. Tracheal vascular hyperpermeability is found in DNFB-sensitized +/+ (**a,b**) and BMMC→*W/W*^{v} mice (**e,f**), but not in mast cell deficient *W/W*^{v} mice (**c,d**). After reconstitution mice were housed for 20 weeks before being subjected to DNFB-sensitization and intranasal DNS challenge. At the time of experiment all mice were 40 weeks old. Typical individual samples of Monastral blue labeled blood vessels in trachea of DNFB- or vehicle-sensitized mice 30 min after DNS challenge.

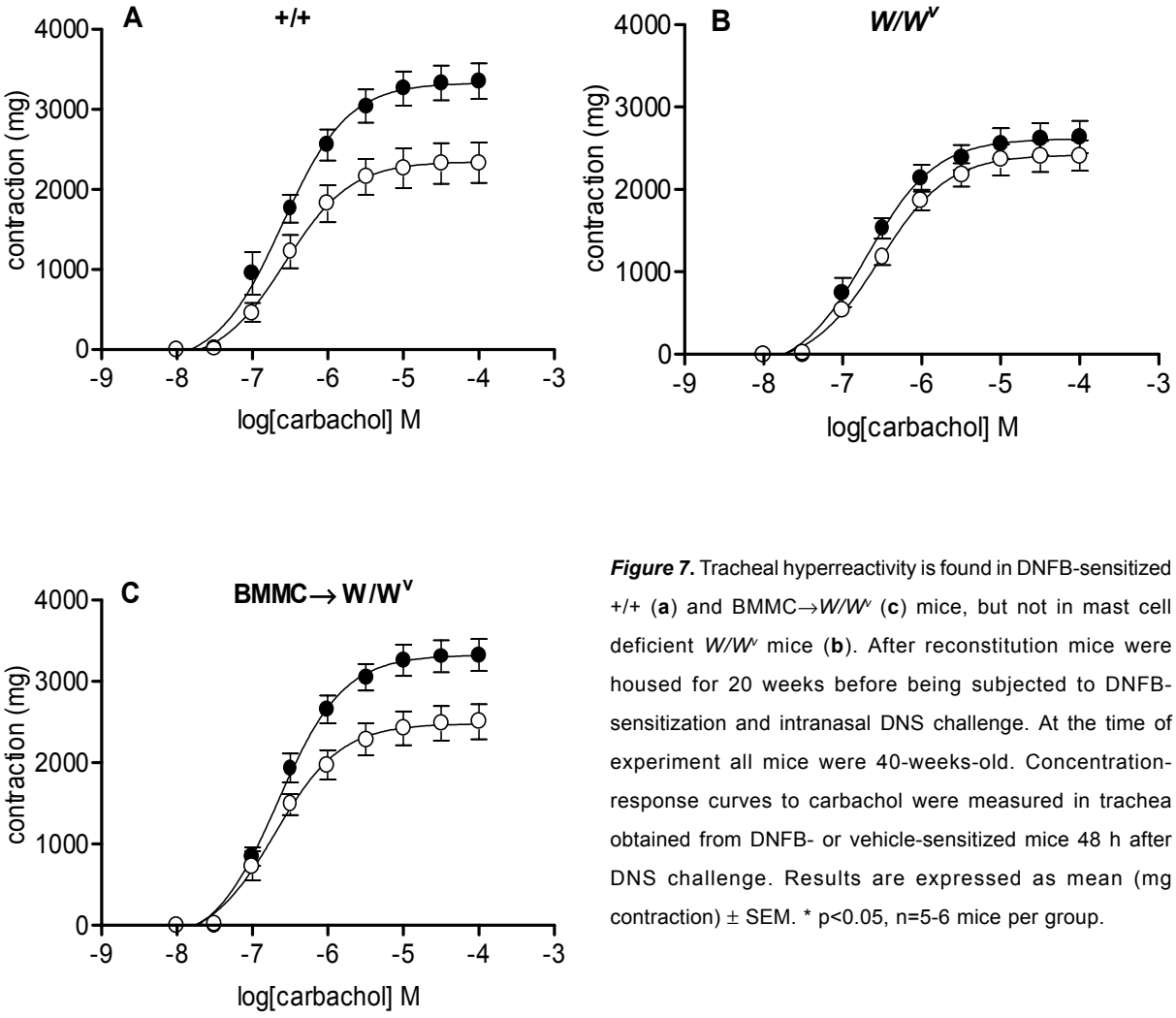


Figure 7. Tracheal hyperreactivity is found in DNFB-sensitized +/+ (**a**) and BMMC→*W/W*^{v} (**c**) mice, but not in mast cell deficient *W/W*^{v} mice (**b**). After reconstitution mice were housed for 20 weeks before being subjected to DNFB-sensitization and intranasal DNS challenge. At the time of experiment all mice were 40-weeks-old. Concentration-response curves to carbachol were measured in trachea obtained from DNFB- or vehicle-sensitized mice 48 h after DNS challenge. Results are expressed as mean (mg contraction) ± SEM. * $p<0.05$, n=5-6 mice per group.

Discussion

Mast cell mediators are known to contribute to the pathogenesis of asthma. Increased numbers of pulmonary mast cells have been demonstrated in atopic as well as in non-atopic asthmatic patients (8). The presence of increased mast cell numbers in non-atopic asthma focuses attention on the role of non-IgE mediated mast cell activation in asthma.

In the present experiments, we describe a murine model for non-atopic asthma and examine the putative role of mast cells. Cutaneous sensitization with the low molecular weight hapten DNFB, followed by an intranasal challenge with DNS, resulted in early and late phase hypersensitivity responses. An acute bronchoconstriction, tracheal vascular hyperpermeability and mucosal exudation characterized the early phase (within 1 h after hapten challenge). These responses were associated with mast cell activation shortly after the challenge. For each of these features, responses to intranasal DNS in DNFB-sensitized mast cell deficient mice were significantly lower or absent when compared to normal congenic +/+ mice. Similar results were found for the late phase responses of this murine model for non-atopic asthma, such as mucosal exudation, cellular infiltration and tracheal hyperreactivity. Moreover, DNFB-induced pulmonary hypersensitivity responses in mast cell reconstituted BMMC→*W/W*^{v} mice were indistinguishable from age-matched normal +/+ mice.

These data extend previous work performed by Garssen and coworkers and other investigators, which indicated that mast cells are involved in the elicitation of hapten-induced T cell-mediated responses in the airways of actively immunized mice (16, 18). There are several lines of evidence that favor a role for the mast cell in the early phase of hapten-induced hypersensitivity reactions. Firstly, these reactions are elicited preferentially at sites enriched with mast cells, such as lung, gastrointestinal tract, buccal mucosa and skin (12, 27, 28). Studies have reported the release of the mast cell mediator serotonin during contact sensitization in skin and lung early after the challenge (12, 29-31). This vaso-active amine can act locally by increasing vascular permeability and inducing vasodilatation, thereby facilitating cellular infiltration (29, 31). In addition, corticosteroid-induced mast cell depletion and treatment with mast cell stabilizers or serotonin receptor antagonists suppressed hapten-induced hypersensitivity reactions in lung, gastrointestinal tract and skin of mice and rat (12, 29, 31). Defective mucosal pulmonary hapten-induced hypersensitivity responses have been found in strains of mast cell deficient mice (12). This finding seems in contrast with hapten-induced contact hypersensitivity reactions at cutaneous sites, where skin responses were found in mast cell deficient mice (32). It was suggested that other cell types, such as the platelet, could be involved. However, very recently Biedermann and coworkers (33) have demonstrated, using mast cell-deficient mice and mast cell-reconstituted mice, that mast cells are necessary for the full development of a cutaneous hapten-induced delayed type hypersensitivity reaction.

No reports have described direct assessment of mast cell activation during the early phase of pulmonary hapten-induced hypersensitivity reactions in the mouse. In this study we have monitored *in vivo* mast cell activation up to 3 h after the challenge by means of measurement of histamine and mMCP-1. Our results demonstrate that mast cells are activated directly after the intra-airway DNS challenge of DNFB-sensitized mice. This early mast cell activation was associated with acute bronchoconstriction and mucosal exudation, which occurred within 1 h after the challenge indicating that an increase in vascular permeability had taken place. The latter feature was indeed demonstrated in trachea.

The role of the mast cell in DNFB-induced pulmonary hypersensitivity reaction was further evaluated using mast cell deficient W/W^v and Sl/Sl^d mice. No early phase bronchoconstriction, mast cell activation or increased vascular permeability, nor late phase infiltration of inflammatory cells into the airways nor tracheal hyperreactivity at 24 to 48 h were found in these mice. In +/+ littermate control mice normal hapten-induced early and late phase events were observed. In a previous study examining picryl chloride-induced pulmonary hypersensitivity reaction, no inflammatory infiltrates were found in the lungs of W/W^v mast cell deficient mice 48 h after the challenge (12). Tracheal reactivity during the late phase of this hypersensitivity reaction was not assessed. However, treatment with the mast cell stabilizer, nedocromil, resulted in an inhibition of the picryl chloride-induced tracheal hyperreactivity observed at 48 h after the challenge of BALB/c mice (12).

To provide more definitive evidence for the role of mast cells in this murine model for non-atopic asthma, we reconstituted mast cells in W/W^v mice by i.v. injection of in vitro cultured mast cells obtained from bone marrow of +/+ mice. Mast cell reconstitution restored the acute bronchoconstriction, tracheal vascular hyperpermeability, BAL neutrophilia and tracheal hyperreactivity observed after DNFB sensitization and intranasal DNS challenge.

However, the early phase mast cell activation as assessed by rises in serum mMCP-1 could be detected in +/+ but not in BMMC→*W/W^v* mice. One possible explanation could be that only a few pulmonary mast cells need to be activated in mice undergoing a DNFB-induced hypersensitivity reaction. Du and coworkers have described that in *W/W^v* mice that have been infused with bone marrow cell or BMMC, the density of pulmonary mast cells is 5 to 15-fold lower than in age-matched +/+ mice, suggesting minimal reconstitution (34). It has been demonstrated that only minimal reconstitution of a relatively small number of mast cells can restore IgE-mediated hypersensitivity reactions in the lung (35). It could be possible that the number of mast cell activated in reconstituted mice is too low to detect mMCP-1 levels in serum. In our study, however, tracheal mast cell counts did not indicate differences in the number of mast cells comparing +/+ and BMMC→*W/W^v* mice. A more plausible explanation could be that mMCP-1 may not be the right marker for mast cell activation in mast cell reconstituted *W/W^v*. Galli stressed that appropriate studies should be done to assess the number, phenotype and anatomical distribution of mast cells which develop in *W/W^v* recipients of BMMC (36). Indeed, histological examination of mast cells in trachea obtained from +/+ and BMMC→*W/W^v* mice showed differences in staining-sensitivity, morphology (granular density and size) and anatomical distribution. It was beyond the scope of this study to in-depth investigating this phenomenon.

The mechanism by which mast cells are activated in this murine model is currently under investigation. Mast cell activation can also be elicited via IgG1 in the mouse and low local levels of IgE, that are not detectable in serum, could result in mast cell sensitization. However, we have demonstrated that upon contact sensitization with low molecular weight haptens, hapten-specific proteins are produced. These hapten-specific proteins were devoid of IgG, IgE and IgM (unpublished observations). Purified hapten-specific proteins are able to passively sensitize naive mice. Intranasal challenge results in an acute bronchoconstriction, plasma leakage and mast cell activation. These features are similar as observed in the early phase of the hapten-induced asthma model described in this study. In addition, our studies indicate that hapten-specific proteins bind to mast cells and upon second contact with the hapten mast cells degranulate and thereby initiating a cascade resulting in hapten-induced hypersensitivity reaction in lung or skin.

In conclusion, our findings are confirmatory for the hypothesis of the initiating role of the mast cell in the cellular cascade leading to a hapten-induced hypersensitivity reaction as postulated earlier (12, 27, 30, 33, 37). Evidence from this study consistent with the hypothesis that mast cells significantly contribute to the initiation of non-IgE-mediated hypersensitivity responses in airways. Although the importance of mast cells in murine models for atopic asthma remains controversial, we are the first to demonstrate an essential role for mast cells in a murine model for non-atopic asthma.

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The neurokinin-1 receptor is crucial for the development of non-atopic asthma

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Abstract

Mast cell activation, bronchoconstriction, inflammation and airway hyperreactivity are prominent features of dinitrofluorobenzene (DNFB)-induced hypersensitivity reactions in the mouse airways, a murine model for non-atopic asthma. We studied the role of the tachykinin receptors in the development of non-atopic asthma using specific NK-1 (RP67580) or NK-2 (SR48968) receptor antagonists and genetically NK-1 receptor-deficient animals. NK-1 receptor blockade strongly reduced the DNFB-induced tracheal hyperreactivity and the accumulation of neutrophils and mononuclear cells in the bronchoalveolar lavage (BAL) fluid in DNFB-sensitized mice at 48 h after dinitrosulfonic acid (DNS) challenge. Treatment with the NK-2 receptor antagonist did not affect the DNFB-induced hypersensitivity reaction. The role for the NK-1 receptor was further confirmed in NK-1 receptor knockout mice. In previous studies, we have shown that mast cells played a crucial role in the development of non-atopic asthma. Although mast cell activation may be induced by stimulation of neurokinin receptors, we did not find an inhibitory effect of the tachykinin receptor antagonists or genetic absence of NK-1 receptors on the release of mast cell protease in serum of asthmatic animals. In conclusion, present study shows that, distal from mast cell activation, the NK-1 receptor is crucial for the development of tracheal hyperreactivity and pulmonary leukocyte accumulation in non-atopic asthma.

Introduction

Asthma and chronic obstructive pulmonary diseases are among the world's most prevalent airway diseases. Asthma patients can roughly be divided in two groups. In most cases, asthma is associated with atopy. Atopic asthma refers to the genetic predisposition of individuals expressing immunoglobulin E (IgE) specific for certain allergens. The second group of patients comprises patients in which asthma is not associated with atopy. In these patients no allergen-specific IgE can be detected in the blood. Non-atopic asthma, accounting for 5% to 15% of all new cases of asthma (1), is an increasing problem in the developed world. Low molecular weight substances (<5000 Da) are the most common agents causing occupational asthma without producing specific IgE (1). Sensitization and local challenge with the low molecular weight compound dinitrofluorobenzene (DNFB) has been shown to induce bronchoconstriction, airway hyperreactivity, cellular accumulation, mast cell activation and increased vascular permeability in the mouse airways. Sensitization is not associated with an increase in hapten-specific IgE. Therefore, this murine model is useful to study mechanisms of non-atopic asthma. Buckley and coworkers showed that DNFB-induced tracheal hyperreactivity and cellular infiltration were inhibited by capsaicin-induced depletion of excitatory non-adrenergic non-cholinergic (NANC) neuropeptides (2). From this study we hypothesize that sensory nerves play a role in the pathogenesis of non-atopic asthma.

Excitatory NANC pathway innervate the airways of human and other mammalian species (3). The excitatory NANC nerves can be activated by different stimuli, that affect the chemosensitive C-fiber afferents in the airways and lead to the local release of neuropeptides (4). Tachykinins and calcitonin gene-related peptide are the predominant excitatory NANC-neuropeptides in the airways (5). The tachykinins, substance P and neurokinin A, have various proinflammatory effects that could contribute to changes

observed in asthmatic airways such as smooth muscle contraction, vasodilatation, an increase in vascular permeability and infiltration and stimulation of inflammatory cells (3). Most of the effects have been observed in human and animal tissue and are mediated by specific tachykinin receptors. Two receptor types, neurokinin 1 (NK-1) and neurokinin 2 (NK-2), mediate the biological actions of tachykinins in the airways. The preferred ligand for these receptors are substance P and neurokinin A, respectively (6). Stimulation of the NK-1 receptor induces vasodilatation, plasma protein extravasation, mucus secretion, upregulation of adhesion molecules and stimulation of inflammatory cells such as mononuclear cells, neutrophils and mast cells (4). The NK-1 receptor is present on the surface of these effector cells and it has been shown to play a role in many inflammatory diseases including asthma, inflammatory bowel disease and experimental arthritis (7). Stimulation of the NK-2 receptor mediates a major part of the tachykinin-induced airway smooth muscle contraction (8, 9). Tachykinin effects on immune cells can also be non-receptor mediated. Substance P can cause degranulation of mast cells through direct activation of G proteins in the inner surface of the plasma membrane (10).

It is well appreciated that mast cells play a critical role in immediate hypersensitivity reactions, involving IgE. However, mast cells also play a prominent role in non-atopic hypersensitivity reactions (11,12,13). Firstly, mast cell degranulation is observed in DNFB-sensitized mice directly after DNS challenge (12,14,15). Furthermore, non-IgE hypersensitivity responses such as tracheal hyperreactivity and neutrophil infiltration are absent in WBB6F1-W/W^v and Sl/Sl^d mast cell deficient mice (14). As mast cells are in close proximity to excitatory NANC-nerves, the mast cell is thought to be an important mediator cell in neuroimmune interactions (16,17).

In the present study, we investigated the role of the neurokinin receptors in the non-atopic hypersensitivity reactions in the mouse airways leading to tracheal hyperreactivity and cellular accumulation in mice. Furthermore, mast cell activation was measured early after hapten challenge. The involvement of the tachykinin receptors in the development of airway hyperresponsiveness, pulmonary cellular influx and mast cell activation, was studied by using specific NK-1 and NK-2 receptor antagonists and NK-1 receptor knockout mice.

Methods

Animals. Male BALB/c mice were obtained from Charles River, Someren**,** the Netherlands. Neurokinin 1 receptor knockout mice (back-crossed to BALB/c) were developed and bred by Dr. N. Gerard, Harvard, Boston, USA (16). All mice used were 6-8 weeks of age. The experiments were conducted in accordance with the Animal Care Committee of the Utrecht University (Utrecht, The Netherlands).

Sensitization and experimental procedure. Mice were skin-sensitized on day 0 and 1 with dinitrofluorobenzene (DNFB, 50 μl 0.5 %) or vehicle (acetone:olive oil, 4:1). On day 5 the animals were intranasally challenged with dinitrobenzene sulfonic acid (DNS, 50 μl 0.6% in PBS). DNFB- or vehicle-sensitized mice were intravenously (i.v.) injected with the

neurokinin 1 (NK-1) receptor antagonist RP67580 (10^{-9} mol/mouse) or the neurokinin 2 (NK-2) receptor antagonist SR48968 (10^{-9} mol/mouse) at indicated times after the sensitization. As a control group, DNFB and vehicle sensitized mice were i.v. injected with RP65681, the inactive enantiomer of RP67580 (10^{-9} mole/mouse) or saline in the SR48968 study. The concentration SR48968 used in this study showed to be effective since this NK-2 receptor antagonist was capable of significantly inhibiting ear swelling in NK-1 receptor knockout mice caused by intradermal application of neurokinin A (100 pmol/site) (Saline/Saline: 94±22 μm; NKA/Saline: 218±22 μm, n=4; $p<0.05$. Saline/SR48968: 105±13 μm; NKA/SR48968: 134±16 μm, n=6; ns). Previous studies have shown that the mentioned dose RP67580 has been effective in inhibiting NK-1 receptor-mediated responses in the mouse (18,19)**.** The NK-1 receptor antagonist and its inactive enantiomer were administered i.v. using four treatment regimes:

I. 10 min before and 1 h after the challenge
II. 46 and 47 h after the challenge
III. 10 min before and 1 h, 24, 46 and 47 h after the challenge
IV. 10 min before challenge.

The NK-2 receptor antagonist, or saline as its control, was administered according to regime III and IV.

Tracheal reactivity in vitro. Mice were killed with an overdose of pentobarbitone 48 h after intranasal DNS challenge. The trachea, which was resected *in toto*, was carefully cleaned of connective tissue using a binocular microscope. A nine ring piece of trachea (taken from just below the larynx) was then transferred to a 10 ml organ bath containing a modified oxygenated Krebs solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 25 mM $NaHCO_3$, 1 mM $NaHPO_4$ and 11.1 mM glucose), aerated with 95% O_2: 5% CO_2, 37ºC. Measurements were expressed as changes in milligram (mg) force. An optimal preload, determined to be 1 g, was placed on the tissue at the beginning of the experiment. The trachea was allowed to equilibrate for at least 1 h before contractile effects were elicited. During this period, the bath fluid was exchanged every 15 min. At the end of the equilibrium phase, tracheal contractile reactivity was measured by recording cumulative concentration response-curves to carbachol (10^{-8} to 10^{-4} M).

Leukocyte accumulation in bronchoalveolar lavage fluid. Bronchoalveolar lavages (BAL) were taken from vehicle and DNFB-sensitized mice 48 h after the challenge. After sacrificing the animals, the trachea was carefully intubated and the catheter was secured with ligatures. The chest cavity was exposed for expansion. Saline (37ºC) was slowly injected via the catheter into the lung and withdrawn in 4 x 1 ml aliquots. The aliquots were pooled and maintained at 4ºC. The lavage fluids were centrifuged (1500 rpm, 10 min., 4ºC) to isolate the BAL cells. The cell pellet was resuspended in 150 μl PBS. Total cells were counted using a haemocytometer and expressed as cells/lung. The BAL cell preparations were analyzed morphologically after centrifugation on microscopic slides. Air dried preparations were fixed and stained with hematoxylin and eosin to ascertain the leukocyte populations. Results are expressed as leukocytes/lung for neutrophils and mononuclear cells in the airway lumen.

Mast cell activation in vivo. Blood samples of DNFB- and vehicle-sensitized mice were taken 30 min after intranasal DNS challenge. Blood samples were collected and after centrifugation sera were stored at −70° C until use. Levels of mouse mast cell protease 1 (mMCP-1), a selective marker for mast cell degranulation, were measured using a commercially available ELISA assay. Results were expressed as ng mMCP-1 per ml serum.

Materials. DNFB and olive oil were purchased from Sigma Chemical Co., St. Louis, USA. RP67580 and RP65681 were generous gifts from Rhône-Poulenc Rorer, Dr. C. Garrett in France. SR48968 was a generous gift from Dr. X. Emonds-Alt (Sanofi Research France). Carbachol was purchased from Onderlinge Farmaceutische Groothandel, Utrecht, The Netherlands. Sodium pentobarbitone was obtained from Sanofi, Maassluis, The Netherlands. The mMCP-1 ELISA was from Moredun Scientific Ltd., Midlothian, UK. Maxisorp surface 96 well plates were purchased from Nunc Immuno plate, Roskilde, Denmark.The force displacement transducer was purchased from Harvard Bioscience, Boston, MA, USA and the two channel recorder (Servogar type SE-120) from Plato BV, Diemen, the Netherlands.

Data analyses. Data are expressed as mean and standard error of the mean (SEM). EC50- and Emax-values for the carbachol-induced tracheal contractions were calculated by non-linear least-squares regression analysis of the measured contractions versus carbachol concentration using the sigmoid concentration-response relationship. The data were analyzed by performing a two way analysis of variance (ANOVA). Data on the cellular accumulation were studied by a distribution free Kruskal-Wallis ANOVA. mMCP-1 data were analyzed by using a one-tailed unpaired t-test. Probability values of $P<0.05$ were considered significantly different. Analyses were performed by the usage of Graphpad Prism (version 2.01, San Diego, U.S.A.).

Results

Effect of NK receptor antagonists on DNFB-induced tracheal hyperreactivity.
Intranasal hapten application in DNFB-sensitized mice resulted in the development of a tracheal hyperreactivity to carbachol at 48 h (Figures 1a and 2a). In previous studies, we have shown that neuropeptide depletion prevented the development of DNFB-induced tracheal hyperreactivity (8). We now further focussed on the role of NK receptors 1 and 2 in the development of this hyperreactivity. Mice were i.v. injected with the NK-1 receptor antagonist RP67580 or the NK-2 receptor antagonist SR48968 at indicated times after the sensitization. RP67580 had no effect on the DNFB-induced tracheal reactivity when administered 10 min before and 1 hr after the challenge (regimen I) or at 46 and 47 h after the challenge (regimen II) (table 1). Previously, it was demonstrated that it is important to have a sustained concentration of the antagonist present to inhibit the NK-1 receptor *in vivo* (20). After pretreatment with the NK-1 receptor antagonist RP67580 10 min before and 1 h and 24, 46 and 47 h after the challenge (regimen III) the development of tracheal hyperreactivity was abolished in DNFB-sensitized animals (Figure 1b, table 1). Pretreatment with the inactive enantiomer RP65681 according to all treatment regimens did not affect

the tracheal hyperreactivity observed in DNFB-sensitized mice 48 h after the DNS challenge (table 1). In vehicle-sensitized animals, treatment with RP67580 or RP65681 did not affect basal tracheal reactivity (figure 1b, table 1). Pretreatment with the NK-2 receptor antagonist SR48968, using regimen III, did not influence the tracheal hyperreactivity (figure 2b, table I) suggesting that only the NK-1 receptor played a role in the induction of non-atopic airway hyperreactivity.

Sensitization	Treatment	Regimes	Emax (mg)	pD_2
Vehicle	RP65681	I	1593 ± 98	6.9 ± 0.0
DNFB	RP65681	I	2334 ± 196**	6.8 ± 0.0*
Vehicle	RP67580	I	1384 ± 100	6.9 ± 0.0
DNFB	RP67580	I	2372 ± 167***	6.8 ± 0.1
Vehicle	RP65681	II	1736 ± 67	6.8 ± 0.1
DNFB	RP65681	II	2544 ± 368**	6.8 ± 0.1
Vehicle	RP67580	II	1533 ± 221	6.7 ± 0.1
DNFB	RP67580	II	2484 ± 173	6.8 ± 0.1
Vehicle	RP65681	III	1755 ± 185	6.6 ± 0.0
DNFB	RP65681	III	3368 ± 110***	6.9 ± 0.1
Vehicle	RP67580	III	1760 ± 247	6.7 ± 0.0
DNFB	RP67580	III	2070 ± 166	6.8 ± 0.1
Vehicle	Saline	III	1696 ± 157	6.9 ± 0.1
DNFB	Saline	III	3012 ± 202***	6.7 ± 0.0*
Vehicle	SR48968	III	1872 ± 359	6.7 ± 0.1
DNFB	SR48968	III	3292 ± 298**	6.8 ± 0.1

Table 1. Effect of NK-1 or NK-2 receptor inhibition on the development of tracheal hyperreactivity. Emax and EC50 values are derived from concentration-response curves to carbachol (10^{-8}-10^{-4} M). Mice were skin-sensitized with DNFB and challenged intranasally with DNS. Mice were i.v. injected with RP67580, the inactive enantiomer RP65681, SR48968 or saline following indicated treatment regimes (see Methods).Results are expressed as mean ± sem (n=6). Significant differences are denoted by (*) or (**) for $p<0.05$, $p<0.01$ between the vehicle-sensitized and the DNFB-sensitized group, respectively.

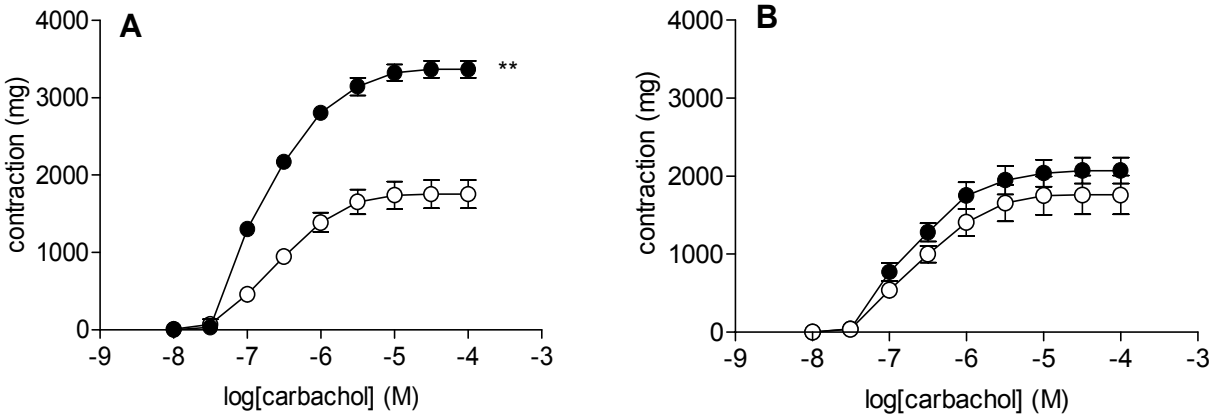

Figure 1. Effect of NK-1 receptor blockade on the development of tracheal hyperreactivity to carbachol 48 h after intranasal DNS challenge in DNFB-sensitized BALB/c mice. Concentration-response curves were measured in DNFB- (closed circles) or vehicle- (open circles) sensitized mice treated i.v. with 10^{-9} mol/mouse RP65681(control, **a**) or RP67580 (NK-1 receptor antagonist, **b**) at 10 min before, 1 h, 24, 46 and 47 h after the DNS challenge. Results are expressed as mean ± SEM (n=6). Significant differences ($p<0.01$) between curves are denoted by (**).

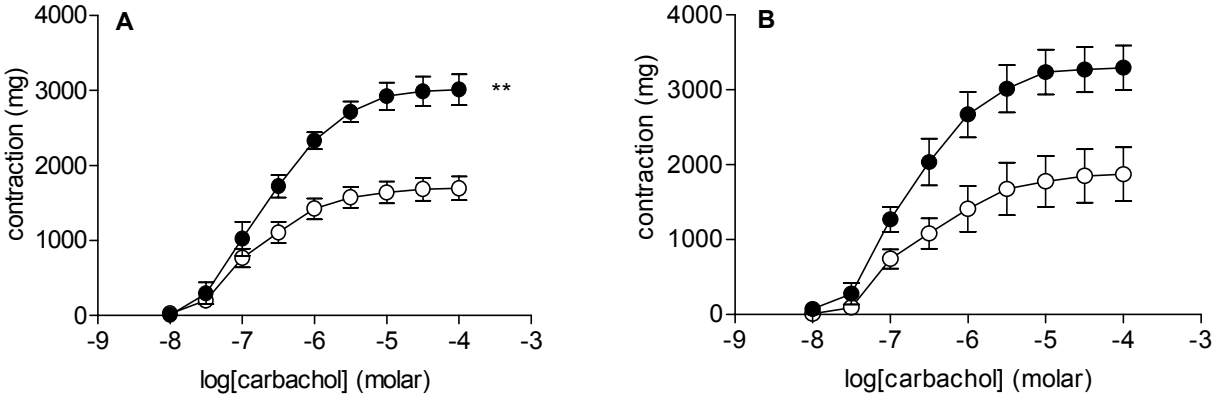

Figure 2. Effect of NK-2 receptor blockade on the development of tracheal hyperreactivity to carbachol 48 h after intranasal DNS challenge in DNFB-sensitized BALB/c mice. Concentration-response curves were measured in DNFB- (closed circles) or vehicle- (open circles) sensitized mice treated i.v. with saline (control, **a**) or with 10^{-9} mol/mouse SR48968 (NK-2 receptor antagonist, **b**) at 10 min before, 1 hr, 24, 46 and 47 h after the DNS challenge. Results are expressed as mean ± SEM (n=6). Significant differences between the vehicle-sensitized and the DNFB-sensitized group are denoted by (*) or (**) for $p<0.05$ or $p<0.01$, respectively.

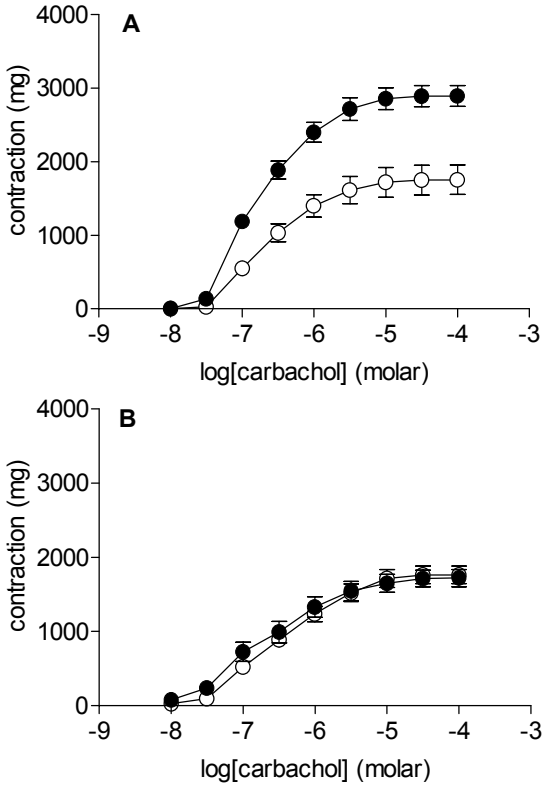

Figure 3. Effect of NK-1 receptor deficiency on tracheal hyperreactivity to carbachol 48 h after intranasal DNS challenge in DNFB-sensitized mice. Control Balb/c mice (**a**) and NK-1 receptor knock-out mice (**b**) were DNFB- (closed circles) or vehicle- (open circles) sensitized and DNS-challenged. Results are expressed as mean ± SEM (n=6). Significant differences ($p<0.05$) between curves are denoted by (**).

No development of DNFB-induced tracheal hyperreactivity in NK-1 receptor knockout mice.

To further confirm the role for the NK-1 receptor, we studied the development of hyperreactivity in the airways of NK-1 receptor knockout mice. The animals were DNFB-sensitized and DNS-challenged and tracheal responses were measured 48 h after DNS challenge. In contrast to control BALB/c mice, in the NK-1 receptor knockout animals no hapten-induced development of tracheal hyperreactivity was observed in DNFB-sensitized mice compared to vehicle-sensitized animals (Emax: Con 1720 mg ± 113 mg, DNFB 1762 mg ± 118 mg. n=6; ns) (figure 3). Moreover, vehicle-sensitized and hapten-challenged NK-1 receptor knockout mice demonstrated a similar tracheal reactivity response upon stimulation with carbachol as vehicle-sensitized and DNS-challenged BALB/c mice (Emax: BALB/c mice 1752 mg ± 179 mg, NK-1 receptor knockout mice 1720 mg ± 113 mg. n=6; ns).

Tachykinin receptors in DNFB-induced leukocyte accumulation in bronchial alveolar lavage fluid.

An increase in mononuclear cells and neutrophils was found in DNFB-sensitized mice 48 h after DNS challenge (figure 4, table 2) compared to vehicle-sensitized mice. A separate set of mice were injected with the NK-1 or NK-2 receptor antagonist 10 min before and 1 h, 24, 46 and 47 h after DNS challenge (regimen III). The NK-1 receptor antagonist, RP67580, did significantly inhibit the accumulation of mononuclear cells and neutrophils in the airway lumen of DNFB-sensitized mice (figure 4a, table 2). In contrast, treatment with the NK-2 receptor antagonist SR48968 showed no significant decease in cellular accumulation associated with the pulmonary hypersensitivity response (figure 4b, table 2). In NK-1 receptor knockout mice, no significant differences in the accumulation of mononuclear cells and neutrophils were found comparing DNFB- and vehicle-sensitized animals 48 h after challenge (figure 4c, table 2).

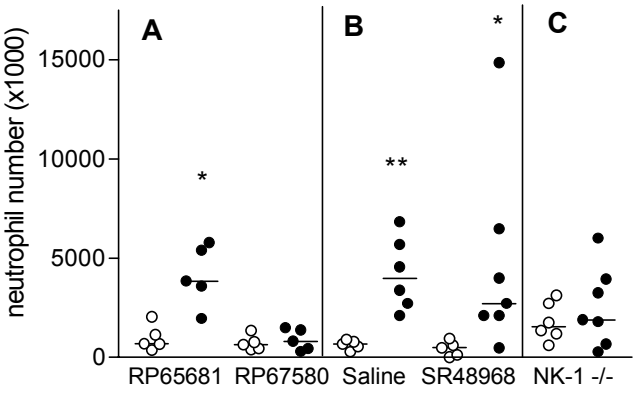

Figure 4. Effect of NK-1 or NK-2 receptor inhibition or deficiency of the NK-1 receptor on neutrophil accumulation in bronchoalveolar fluid. BALB/c mice were DNFB- or vehicle-sensitized, DNS-challenged and: (**a**) treated with RP67580 or the inactive enantiomer RP65681 at 10 min before and 1 h, 24, 46 and 47 h after challenge. (**b**) treated with SR48968 or the inactive control at 10 min before and 1 h, 24, 46 and 47 h after challenge. (**c**) NK-1 receptor knockout mice were DNFB- or vehicle-sensitized and DNS challenged. Open symbols represent vehicle-sensitized and closed symbols DNFB-sensitized mice. Results are expressed as mean number of neutrophils/ lung ± SEM (n=6). Significant differences between the vehicle-sensitized and the DNFB-sensitized group are denoted by (*) or (**) for $p<0.05$ or $p<0.01$, respectively.

Table 2. Effect of NK-1 or NK-2 receptor inhibition or deficiency of the NK-1 receptor on mononuclear cell number in bronchoalveolar fluid. Mice were skin sensitized with DNFB and challenged intranasally with DNS. Mice were i.v. injected with RP67580, the inactive enantiomer RP65681, SR48968 or saline following indicated treatment regimes (see Methods).

Sensitization	Treatment	Regimes	Median (min-max) of mononuclear cell (x1000)
Vehicle	RP65681	III	23.5 (15.8-36.4)
DNFB	RP65681	III	44.2 (26.4-48.6)*
Vehicle	RP67580	III	22.1 (19.1-25.7)
DNFB	RP67580	III	26.2 (14.7-33.1)
Vehicle	Saline	III	18.7 (10.0-26.7)
DNFB	Saline	III	29.2 (18.9-52.4)
Vehicle	SR48968	III	19.5 (11.9-29.4)
DNFB	SR48968	III	29.5 (18.9-39.9)
Vehicle	NK-1 receptor -/- mice		70.5 (29.4-85.3)
DNFB	NK-1 receptor -/- mice		52.1 (32.3-58.2)

Results are expressed as mean ± sem (n=6). Significant differences are denoted by (*) for $p<0.05$ between the vehicle-sensitized and the DNFB-sensitized group.

No involvement of tachykinin receptors in mast cell activation in vivo.
mMCP-1 levels in serum were measured to monitor mast cell activation. Previously, it was demonstrated that in mice the DNFB/DNS-induced hypersensitivity was associated with rapid mast cell activation as assessed by elevated serum levels of mMCP-1 at 30 min after challenge (15). Indeed, in our study, serum mMCP-1 levels were increased 30 min after-DNS challenge in DNFB-sensitized animals when compared to vehicle-sensitized mice (figure 5). The NK-1 receptor antagonist RP67580 nor the NK-2 receptor antagonist SR48968 were able to block early mast cell activation (Figure 5). Similar mast cell activation was found in DNFB-sensitized NK-1 receptor knockout mice compared to DNFB-sensitized BALB/c mice 30 min after hapten challenge (figure 5).

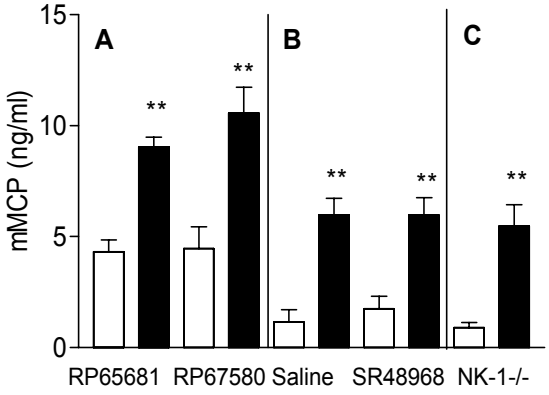

Figure 5. No effect of NK-1 or NK-2 receptor inhibition or deficiency of the NK-1 receptor on mast cell activation induced by intranasal hapten challenge of DNFB-sensitized mice. BALB/c mice were vehicle- or DNFB-sensitized, DNS challenged and: (**a**) treated with RP67580 or the inactive enantiomer RP65681 10 min before challenge. (**b**) treated with SR48968 or the inactive control 10 min before challenge. (**c**) NK-1 receptor knockout mice were DNFB- or vehicle-sensitized and DNS challenged. mMCP-1 levels in seum were measured 30 min after challenge. Open bars represent vehicle-sensitized and closed bars DNFB-sensitized mice. Results are expressed as mean ± SEM (n=6). Significant differences between the vehicle-sensitized and the DNFB-sensitized group are denoted by (**) for $p<0.01$, respectively.

Discussion

The present study provides evidence for the involvement of the NK-1 receptor in the pathogenesis of non-atopic asthma. To investigate the role of the NK-1 receptor, we induced non-atopic asthma in mice by DNFB skin-sensitization and intranasal DNS challenge. Blockade of the NK-1 receptor with a specific antagonist or the genetic absence of the NK-1 receptor prevented the development of tracheal hyperreactivity and cellular accumulation in the mouse airways. These results are in concord with our previous studies showing that neonatal neuropeptide depletion prevented the induction of tracheal hyperreactivity and cellular accumulation (21) and indicate that neuropeptide stimulation of the NK-1 receptor plays a crucial role in the development of this airway disease.

It has been demonstrated that, in the mouse adult lung, substance P-like immunoreactive nerve fibers are located in the smooth muscle of the airways, surrounding bronchi and bronchioli (22). Ek and coworkers (23) demonstrated that substance P and neurokinin A are released in response to epicutaneous application of the allergen oxazolone, a hapten known to cause hypersensitivity reactions in skin and airways. From our results, we hypothesize that intranasal DNS challenge causes tachykinin release from the sensory nerve endings in DNFB-sensitized airways.

The tachykinins substance P and neurokinin A, locally released from excitatory NANC nerves upon activation by different stimuli, have been shown to elicit inflammatory

airway responses, collectively referred to as neurogenic inflammation (24,25). These responses include bronchoconstriction, mucus secretion, vascular leakage, upregulation of adhesion molecules and recruitment and activation of inflammatory cells. These inflammatory responses are predominantly mediated by tachykinin receptors (26-30).

Despite the evidence for the involvement of excitatory NANC neuropeptides in non-atopic airway inflammation (12,21), it remained unclear which tachykinin receptors are implicated in the development of tracheal hyperreactivity and cellular accumulation. The present study shows that development of tracheal hyperreactivity and cellular accumulation, associated with DNFB-induced hypersensitivity reactions in the mouse, were inhibited by the selective NK-1 receptor antagonist RP67580. The NK-2 receptor antagonist SR48968 did not affect the non-atopic tracheal hyperreactivity and cellular accumulation. The role for the NK-1 receptor was further confirmed in NK-1 receptor knockout mice. Therefore, it can be concluded that the NK-1 receptor, but not the NK-2 receptor, plays an important role in the induction of tracheal hyperreactivity and cellular accumulation in the mouse lung.

Several studies have reported that exposure of guinea pigs to substance P elicited airway hyperresponsiveness to bronchoconstrictor agents. In the rat, a significant increase of airway responsiveness to acetylcholine was found after substance P pretreatment in the presence of a NEP inhibitor (31). Until now, no reports have described substance P-induced tracheal hyperreactivity in murine airways. Preliminary experiments in BALB/c mice have demonstrated that intranasal administration of substance P resulted in the induction of a profound tracheal hyperreactivity to carbachol (data not shown).

Excitatory NANC neuropeptides do not act only on bronchial smooth muscle, but it has recently become evident that neuropeptides are modulators of immune cells such as mast cells, eosinophils, neutrophils and lymphocytes. From these immune cells, mast cells, neutrophils and lymphocytes have been shown to be involved in this murine model for non-atopic asthma (14,32). In the skin and airways, it has been demonstrated that substance P is able to induce infiltration and activate lymphocytes and neutrophils to release their mediators such as cytokines, oxygen radicals and myeloperoxidase (MPO) (28). Furthermore, substance P can cause degranulation of mast cells in the respiratory system (11,12,16,17,28,33). Therefore, it is possible that excitatory NANC neuropeptides modulate smooth muscle activity indirectly via stimulation of infiltrating or resident immune cells.

Using a NK-1 receptor antagonist and NK-1 receptor knockout mice, we have shown that the antigen-induced infiltration of neutrophils is dependent on the presence of NK-1 receptors (Figure 4). These results correlate with the study of Bozic and coworkers (34), in which they have demonstrated that neutrophil accumulation was not found in NK-1 receptor knockout mice undergoing an immune-complex reaction. Moreover, studies have reported that substance P or specific NK-1 receptor agonists injected locally in skin or airways, was able to induce neutrophil infiltration (35-38). Potent inhibitory effects of NK-1 receptor antagonists on substance P induced neutrophil infiltration point out to an important role for the NK-1 receptor. The local microenvironment in the tissue seems te be important, because injection of substance P in normal rat and mouse skin did not induce neutrophil infiltration (39), but in inflamed skin the NK-1 receptor was involved in mediating neutrophil

accumulation (40). A possible mechanism of action of substance P in inducing infiltration of neutrophils, could be the upregulation of the expression of intracellular adhesion molecule (ICAM-1). Previously, we have demonstrated a prominent role for ICAM-1 in the development of tracheal hyperreactivity and cellular infiltration in DNFB-sensitized and DNS-challenged mice (41). Nakagawa and coworkers have shown that substance P is able to upregulate the expression of ICAM-1 on human endothelial cells (42). Besides the substance P-induced infiltration of neutrophils, it has been shown that substance P activates neutrophils, an effect that could be mediated by the NK-1 receptor. Stimulation of human PMN cells by substance P leads to superoxide anion production, IL-8 or MPO release (43). This mediator release in turn could lead to the development of tracheal hyperreactivity.

NK-1 receptor blockade or NK-1 receptor deficiency also prevented the DNFB/DNS-induced increased number of mononuclear cells in BAL fluid. An increase in lymphocytes mainly accounted for this rise in mononuclear cells in the airway. Tachykinin NK-1 receptors have been demonstrated on human and murine lymphocytes (44). Furthermore, substance P has been shown to stimulate chemotaxis (45), proliferation and activation of these cells (46). T-lymphocyte proliferation induced by substance P occurred both in the absence and presence of other stimuli and was mediated via the NK-1 receptor. Kaltreider and coworkers (47) have shown that mice undergoing a pulmonary immune reaction demonstrated a significant rise of BAL fluid lymphocytes expressing NK-1 receptor mRNA. Systemic treatment of mice with a selective NK-1 receptor antagonist reduced significantly the total number of lymphocytes, leukocytes and granulocytes retrieved in BAL fluid on day 5 of the pulmonary immune response (47). Taken together, we propose that DNFB/DNS-induced release of substance P and subsequent stimulation of the NK-1 receptor results in the infiltration and activation of neutrophils and lymphocytes. Besides the neutrophil and lymphocyte, the mast cell has been shown to be an essential immune cell in pulmonary hypersensitivity reactions (14,32). Mast cells and macrophages lining the mucosal layer of the respiratory tract, have been found in close vicinity of substance P- and CGRP-immunoreactive nerves (12,16,17). It has been shown that excitatory NANC nerves can modulate the activation state of mast cells. Stimulation of nerve fibers induces mast cells to degranulate and release histamine and other mediators such as serotonin and TNF-α in the respiratory tract of humans and animals (13). Mediator release from mast cells can be induced by neurotrophic factors such as NGF (48) and neuropeptides (49,50). Recently, Forsythe and coworkers (51) have demonstrated that substance P and neurokinin A induce histamine release from human airway mast cells. Activation of mast cells by substance P has been reported to be NK-1, NK-2 receptor mediated as well as non-receptor mediated, e.g. via direct activation of G-proteins (52,53). Whether NK receptors are involved or not in mediator release from mast cells, may be dependent on agonist concentration and mast cell origin. The interaction between mast cells and excitatory NANC nerves is bidirectional. Many studies have shown that mast cell derived mediators, such as histamine, serotonin, prostaglandins and leukotrienes, can modulate NANC neurotransmission. Moreover, NANC nerve endings express receptors for histamine (H_1 and H_3) and serotonin (5-HT_{2A}) (33,54,55). Under inflammatory-like conditions, it has been shown that primary NANC nerves show an upregulation of histamine H_1 receptor expression (56). Mast cell mediators can sensitize and cause release of substance P and CGRP from unmyelinated C-fibers (57,58). One of

the major cytokines released by mast cells under inflammatory conditions is tumor necrosis factor α (TNF-α). Very recently, we showed that mast cell-derived TNF-α has a priming effect on excitatory NANC nerves in (32). Thus, not only can tachykinins induce mast cell degranulation but also mediators released from mast cells cause the release of neuropeptides from sensory nerve endings. In our study, the NK-1 or NK-2 receptor blockade nor deficiency of the NK-1 receptor could inhibit mast cell activation associated with a pulmonary non-atopic response, which suggests that mast cells are activated by non-receptor mediated mechanisms or by other stimuli.

In conclusion, our study points to a role for excitatory NANC nerves in non-atopic asthma. The NK-1 receptor is crucial for the development of tracheal hyperreactivity and leukocyte accumulation, which occurs parallel to or distal from mast cell activation.

Acknowledgments

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TNF-α plays a central role in the development of airway hypersensitivity reactions associated with non-atopic asthma

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Abstract

Tumor necrosis factor alpha (TNF-α) is a multifunctional proinflammatory cytokine, rapidly released by mast cells, that elicits a large number of biological effects, including inflammatory and immuno-regulatory responses. The present study shows that TNF-α is an important mediator in the pathogenesis of non-atopic asthma. Non-atopic asthma was induced in mice by skin sensitization with dinitrofluorobenzene (DNFB) followed by intranasal challenge with dinitrobenzene sulfonic acid (DNS). Features of this pulmonary reaction include acute bronchoconstriction, tracheal hyperreactivity, cellular infiltration and an increase in mucosal exudation in the alveolar lumen, not characterized by an elevation of total and allergen-specific IgE in serum. Intranasal DNS challenge of DNFB-sensitized mice was accompanied with rapid mast cell activation as measured by increase in serum mast cell protease-1 (mMCP-1) compared to vehicle sensitized animals. Significantly increased levels of TNF-α were found in the bronchoalveolar lavage (BAL) fluid BAL fluid of DNFB-sensitized mice, 30 min to 3 h after DNS challenge (30 min DNFB/DNS: 147±27 pg/ml; vehicle/DNS 72±10 pg/ml: $p<0.05$. 3 h DNFB/DNS: 122±19 pg/ml; vehicle/DNS 77±11 pg/ml: $p<0.05$, n=3-6). Neutralizing anti-TNF-α antibodies did not affect mast cell activation. However, neutralizing anti-TNF-α antibodies strongly reduced the tracheal hyperreactivity and the accumulation of neutrophils and mononuclear cells in the BAL fluid in DNFB-sensitized mice at 48 h after DNS challenge. Pretreating animals with an antagonist for the TNFR1 receptor inhibited the development of tracheal hyperreactivity, whereas blockade of the TNFR2 receptor had no effect. These findings indicate that TNF-α and its TNFR1 contribute to the induction of airway hypersensitivity reactions in a murine model for non-atopic asthma.

Introduction

Tumor necrosis factor alpha (TNF-α) is a multifunctional proinflammatory cytokine that elicits a large number of biological effects such as hyperresponsiveness, vascular permeability, vasodilatation and the influx and accumulation of neutrophils (1-3).

A variety of cells such as alveolar macrophages, fibroblasts, monocytes, endothelial cells and neutrophils produce and release TNF-α upon stimulation (4-8). Moreover, the mast cell, a tissue resident inflammatory cell, contains immunologically inducible TNF-α and, in contrast to many other cell types, has a storage of preformed TNF-α (9). TNF-α is identified to be one of the main preformed mediators immediately released by the mast cell after cellular activation (10, 11). Furthermore, mast cells secrete newly synthetized TNF-α within 30 min following certain stimuli such as specific allergen and LPS (9).

Increased concentrations of TNF-α, in response to local injury, can play a role in cellular activation and the proliferation of the inflammatory response. Several investigators have shown increased amounts of TNF-α in BAL fluid of asthmatic patients (12, 13). In patients with toluene diisocyanate-induced asthma, a form of non-atopic asthma, TNF-α immunoreactivity was enhanced compared to control patients and the localization of the cytokine was predominantly to mast cells (14). These results indicate a possible role for mast cell-derived TNF-α in the pathogenesis of non-atopic asthma.

TNF-α has been demonstrated to upregulate the expression of adhesion molecules, effecting the neutrophil infiltration into the airways (15). Infiltration with neutrophils and

other inflammatory cells can subsequently lead to tissue destruction in the course of chronic inflammatory diseases such as asthma (16).

Biological activities of TNF-α are mediated by two distinct receptors, the p55 type 1 receptor (TNFR1) and p75 type 2 receptor (TNFR2). TNFR1 is associated with inflammation (1, 17) and neutrophilic infiltration (18), while TNFR2 seems to play a role in cell proliferation, the induction of tissue necrosis (1) and initiation of cutaneous immune responses (19, 20). The majority of cell types and tissues appear to express both receptors (1, 21).

In the present study, we investigated the role of TNF-alpha in pulmonary non-IgE-mediated hypersensitivity reactions in the mouse, a murine model for non-atopic asthma. It is currently accepted that asthma can roughly be divided into two categories. The majority of patients have atopic asthma, which is characterized by an elevation of total and allergen-specific IgE in serum (22). The second category comprises the non-atopic asthmatics. Non-atopic asthmatics are skin test negative to common allergens and there is no evidence of allergen-specific serum IgE (23, 24). Amin and coworkers recently showed that besides the difference in serum IgE levels, atopic and non-atopic patients had different pathological changes present in their airways despite their similar clinical respiratory symptoms (25). Atopic patients had increased numbers of eosinophils in their airway tissue, whereas non-atopic asthma is dominated by the infiltration of neutrophils. The number of mast cells showed a similar increase in both of the groups. Many studies have focused on the role of the mast cell in atopy showing that TNF-α released from the mast cell plays a role in the pathogenesis of atopic asthma (3, 26).

In our laboratory, a murine model for non-atopic asthma has been developed and characterized (27, 28). In this model, mice were skin sensitized with the low molecular weight compound dinitrofluorobenzene (DNFB) followed by an intra-airway challenge with dinitrobenzene sulphonic acid (DNS). Features of this pulmonary reaction included acute bronchoconstriction and mast cell activation shortly after challenge, and tracheal hyperreactivity, mononuclear and neutrophilic cell infiltration and an increase in mucosal exudation in the alveolar lumen 24 to 48 h after the challenge. This hypersensitivity reaction was not associated with an increase in hapten specific IgE (27, 28).

Previous studies in our laboratory have pointed to an important role for the mast cell in non-atopic pulmonary hypersensitivity reactions (29, 30). In this study, we report on the role of TNF-α in the airway hyperreactivity response and cellular infiltration observed in this murine model for non-atopic asthma.

Methods

Animals. Male BALB/c mice were obtained from the Central Animal Laboratory (GDL), Utrecht University, Utrecht, The Netherlands. All mice used were 6-8 weeks of age. Animal studies were approved by the Animal Care Committee of the Utrecht University (Utrecht, The Netherlands).

Sensitization and experimental procedure. Mice were sensitized on day 0 with either DNFB (0.5% dissolved in acetone: olive oil [4:1]) or vehicle control, both of which were

applied epicutaneously to the shaved thorax (50 μl) and all four paws (50 μl). On day 1, DNFB or vehicle control (50 μl) was applied to the thorax alone. DNFB- and vehicle-sensitized mice were intranasally challenged with dinitrobenzene sulphonic acid (DNS, 50 μl 0.6 % in PBS, pH 7.2) on day 5. The sensitization and challenge were performed under light anesthesia (pentobarbitone, 40 mg/kg i.p.). In stated experiments, neutralizing anti-TNF-α antibodies (XT22, 1 mg/mouse), anti-TNF R1 and R2 antibodies (50 μg/mouse) or control antibodies were injected i.v. 1 hr before and 24 h after DNS challenge.

Measurement of TNF-α in BAL fluid. Bronchoalveolar lavages (BAL) were taken from vehicle- or DNFB-sensitized animals at different time points after DNS challenge. After sacrificing the animals, the trachea was intubated and 1 ml saline (37° C) was slowly injected into the lung and withdrawn. The lavage fluids were centrifuged (1500 rpm, 10 min., 4ºC) to isolate the BAL cells. The concentration of TNF-α in BAL fluid was measured via ELISA. Plates were coated with anti-TNF-α antibody (4 μg/ml rat anti-mouse TNF-α) at 4ºC. The next day, plates were washed 5 times with 0.1% Tween 20 in PBS. Non-specific binding was prevented by incubating plates with 0.5% BSA in PBS for 2 h at room temperature. After washing, BAL fluid and standards were loaded on the plates together with biotinylated rabbit anti-mouse TNF-α antibody (1.25 μg/ml, 100 μl) and incubated for 2 h on an orbital shaker. Thereafter, plates were washed and streptavidin-poly-HRP (1:25000, 100 μl) was added and incubated for 1 h at room temperature on the shaker. Plates were washed and a solution of chromogen tetramethyl benzidine (TMB) 100 μl 1% TMB in DMSO, 10 ml 0.1 M sodium acetate buffer and 10 μl 3% H_2O_2) was added to each well. The plates were incubated for 15-30 min protected from light on an orbital shaker. The reaction was stopped with 100 μl 0.9 M H_2SO_4. The optical density was measured at a wavelength of 595 nm with a Benchmark microplate reader. Results are expressed as pg TNF-α per ml BAL fluid.

Mast cell activation in vivo. Blood samples of DNFB- and vehicle-sensitized mice were taken 30 min after intranasal DNS challenge. Blood samples were centrifuged and sera were stored at -70ºC until use. Levels of mouse mast cell protease-1 (mMCP-1), a selective marker for mast cell degranulation, were measured using a commercially available ELISA assay. Results were expressed as ng mMCP-1 per ml serum.

Leukocyte accumulation in bronchoalveolar lavage fluid. Bronchoalveolar lavages (BAL) were taken from vehicle and DNFB-sensitized mice 48 h after the challenge. After sacrificing the animals, the trachea was carefully intubated and the catheter was secured with ligatures. The chest cavity was exposed for expansion. Saline (37ºC) was slowly injected via the catheter into the lung and withdrawn in 4 x 1 ml aliquots. The aliquots were pooled and maintained at 4ºC. The lavage fluids were centrifuged (1500 rpm, 10 min, 4ºC) to isolate the BAL cells. The cell pellet was resuspended in 150 μl PBS. Total cells were counted using a haemocytometer and expressed as cells/lung. The BAL cell preparations were analyzed morphologically after centrifugation on microscopic slides. Air dried preparations were fixed and stained with hematoxylin and eosin to ascertain the leukocyte populations. Results are expressed as leukocytes/lung for neutrophils and mononuclear cells in the airway lumen.

Tracheal reactivity in vitro. Mice were killed with an overdose of pentobarbitone 48 h after intranasal DNS challenge. The trachea, which was resected *in toto*, was carefully cleaned of connective tissue using a binocular microscope as described before (27). A nine ring piece of trachea (taken from just below the larynx) was then transferred to a 10 ml organ bath containing a modified oxygenated Krebs solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 25 mM $NaHCO_3$, 1 mM $NaHPO_4$ and 11.1 mM glucose), aerated with 95% O_2: 5% CO_2, 37ºC. Measurements were expressed as changes in milligram (mg) force. An optimal preload, determined to be 1 g, was placed on the tissue at the beginning of the experiment. The trachea was allowed to equilibrate for at least 1 h before contractile effects were elicited. During this period, the bath fluid was exchanged every 15 min. At the end of the equilibrium phase, tracheal contractile reactivity was measured by recording cumulative concentration response-curves to carbachol (10^{-8} to 10^{-4} M).

Materials. DNFB and olive oil were purchased from Sigma Chemical Co., St. Louis, USA. DNS was obtained from Eastman Kodak Company (Rochester, New York, USA). Hamster anti-mouse TNFR1 and TNFR2 antibodies were purchased from Genzyme (R&D systems, Inc., Minneapolis, USA). Hamster serum, used as a control for the TNFR1 and R2 antibodies, was obtained from the Central Animal Laboratory (Utrecht, The Netherlands). Rat anti-mouse TNF-α antibodies and biotinylated rabbit anti-mouse TNF-α antibodies were obtained from Pharmingen (San Diego, USA). Sodium barbitone was purchased from Sanofi BN (Maassluis, The Netherlands). Carbachol was purchased from Onderlinge Pharmaceutische Groothandel, Utrecht, The Netherlands. Hematoxylin and eosin were obtained from Sigma Aldrich, St.Louis, MO, USA. The TNF-α ELISA kit was obtained from BioSource, Nivelles, Belgium and the mMCP-1 ELISA from Moredun Scientific Ltd., Midlothian, UK. Maxisorp surface 96 well plates were purchased from Nunc Immuno plate, Roskilde, Denmark. The Benchmark microplate reader came from Biorad, California, USA. The force displacement transducer was purchased from Harvard Bioscience, Boston, MA, USA and the two channel recorder (Servogar type SE-120) from Plato BV, Diemen, the Netherlands.

Statistical analysis. Data are expressed as mean and standard error of the mean (SEM). EC50- and Emax-values for the carbachol-induced tracheal contractions were calculated by non-linear least-squares regression analysis of the measured contractions versus carbachol concentration using the sigmoid concentration-response relationship. The data were analyzed by performing a two-way analysis of variance (ANOVA). Data on the cellular accumulation were studied by a distribution free Kruskal-Wallis ANOVA. mMCP-1 data were analyzed by using a one-way ANOVA followed by the Bonferroni's multiple comparison test. Probability values of $P<0.05$ were considered significantly different. Analyses were performed by the usage of Graphpad Prism (version 2.01, San Diego, U.S.A.).

Results

TNF-α levels in BAL fluid of DNFB-sensitized and DNS-challenged mice.

TNF-α was detectable in the BAL fluid of both vehicle- and DNFB-sensitized mice. At 30 min and 3 h after DNS challenge, the amount of TNF-α in the BAL fluid of DNFB-sensitized

animals was significantly increased compared to vehicle-sensitized animals. At later time points after challenge, the amount of TNF-α in BAL fluid of DNFB-sensitized animals declined to basal levels as found in vehicle-sensitized mice (figure 1).

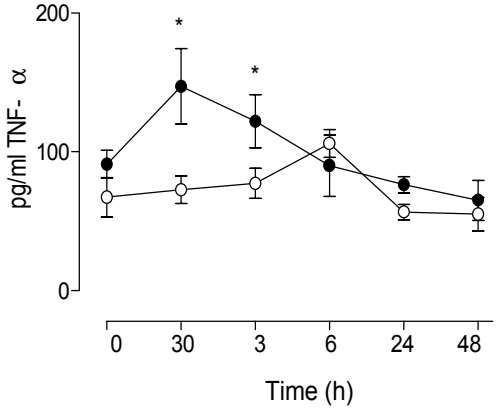


Figure 1. TNF-α levels (pg/ml) in BAL fluid of DNFB- and vehicle-sensitized mice at different time points after DNS challenge. Open bars represent vehicle-sensitized and closed bars DNFB-sensitized mice. Data are expressed as mean ± SEM, 3-6 animals per group, Significant differences are denoted by (*) for $p<0.05$ as compared to vehicle-sensitized animals at the same time point.

Effect of anti-TNF-α treatment on mast cell activation in vivo.

mMCP-1 levels in serum were measured to monitor mast cell activation. Previously, it was demonstrated that in mice the DNFB/DNS-induced hypersensitivity was associated with rapid mast cell activation as assessed by elevated serum levels of mMCP-1 at 30 min after challenge (31). Indeed, in our study, serum mMCP-1 levels were increased 30 min after-DNS challenge in DNFB-sensitized animals when compared to vehicle-sensitized mice (figure 2). No effects of anti-TNF-α antibodies on basal mMCP-1 levels were observed. Neutralizing anti-TNF-α did not affect the mMCP-1 release in DNFB-sensitized mice (figure 2).

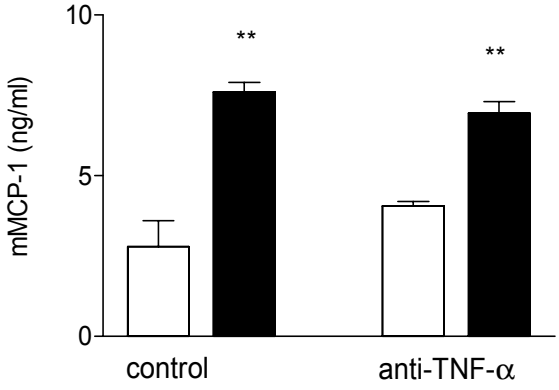


Figure 2. No effect of anti-TNF-α-antibodies on mast cell activation induced by intranasal hapten challenge of DNFB-sensitized mice. Mice were vehicle- or DNFB-sensitized, DNS challenged and treated with anti-TNF-α-antibodies (XT22, 1 mg/mouse) or rat IgG (control, 1 mg/mouse) 10 min before challenge. mMCP-1 levels in serum were measured 30 min after challenge. Open bars represent vehicle-sensitized and closed bars DNFB-sensitized mice. Results are expressed as mean ± SEM (n=6). Significant differences between curves are denoted by (**) for $p<0.01$.

Effect of anti-TNF-α antibody treatment in DNFB-induced leukocyte accumulation in bronchoalveolar lavage fluid.

An increase in cellular accumulation of mononuclear cells and neutrophils was found in DNFB-sensitized animals 48 h after DNS challenge (table 1). The neutralizing TNF-α-antibody significantly inhibited the accumulation of mononuclear cells and neutrophils in DNFB-sensitized mice (table 1).

Table 1. Effect of neutralizing anti-TNF-α antibodies on mononuclear and neutrophil cell numbers in bronchoalveolar fluid. Mice were skin sensitized with DNFB and challenged intranasally with DNS and studied 48 h after the challenge. Mice were i.v. injected with neutralizing TNF-α antibodies or IgG 1 h before and 24 h after challenge (see Methods).

Sensitization	Treatment	Leukocyte accumulation in BAL fluid (cells/lung x 10^3)		
		total cells	mononuclear cells	neutrophils
Vehicle	IgG	22.5 (12.0-42.0)	24.5 (12.0-32.7)	0.2 (0.0-1.0)
DNFB	IgG	40.5 (37.5-55.5)	38.5 (34.5-52.2)*	3.0 (2.0-3.3)*
Vehicle	anti-TNF-α	24.0 (19.5-30.0)	25.3 (19.3-33.0)	0.3 (0.0-0.5)
DNFB	anti-TNF-α	21.8 (19.5-22.5)	21.4 (18.3-22.3)‡	1.1 (0.2-1.7)‡

Results are expressed as median (min-max) (n=5-6 animals/group). Significant differences are denoted by (*) for $p<0.05$ between the vehicle-sensitized and the DNFB-sensitized group and by (‡) for $p<0.05$ between the control (IgG) and the anti-TNF-α-treated group.

Table 2. Effect of neutralizing anti-TNF-α antibodies and R1/R2 receptor inhibition on the development of tracheal hyperreactivity. Emax and pD_2 values are derived from concentration-response curves to carbachol (10^{-8}-10^{-4} M). Mice were skin-sensitized with DNFB and challenged intranasally with DNS. Mice were i.v. injected with anti-TNF-α, anti-R1, anti-R2 or hamster serum as a control (see Methods).

Sensitization	Treatment	Emax (mg)	pD_2
Vehicle	IgG	1756 ± 201	6.7 ± 0.1
DNFB	IgG	2896 ± 143**	6.9 ± 0.1
Vehicle	anti-TNF-α	1780 ± 240	6.7 ± 0.1
DNFB	anti-TNF-α	2213 ± 186	6.7 ± 0.0
Vehicle	hamster serum	1760 ± 186	6.7 ± 0.1
DNFB	hamster serum	2848 ± 216**	6.7 ± 0.1
Vehicle	anti-TNFR1	1731 ± 157	6.6 ± 0.1
DNFB	anti-TNFR1	1923 ± 218‡	6.7 ± 0.1
Vehicle	hamster serum	1885 ± 211	6.6 ± 0.0
DNFB	hamster serum	2973 ± 182**	6.7 ± 0.1
Vehicle	anti-TNFR2	1812 ± 83	6.6 ± 0.0
DNFB	anti-TNFR2	2515 ± 162*	6.7 ± 0.0

Results are expressed as mean ± sem (n=6). Significant differences are denoted by (*) or (**) for $p<0.05$, $p<0.01$ between the vehicle-sensitized and the DNFB-sensitized group, respectively and by (‡) for $p<0.05$ between the control (IgG) and the anti-TNF-α-treated group.

Effect of anti-TNF-α antibody treatment on DNFB-induced tracheal hyperreactivity. Intranasal DNS application in DNFB-sensitized animals resulted in the development of tracheal hyperreactivity to carbachol 48 h after challenge (figure 3a). To study the role of TNF-α in the development of tracheal hyperreactivity, neutralizing anti-TNF-α antibodies were i.v. injected 1 h before and 24 h after challenge. Figure 3 shows that anti-TNF-α treatment abolished the development of tracheal hyperreactivity in DNFB-sensitized mice. Anti-TNF-α itself did not affect basal reactivity as measured in vehicle-sensitized, hapten challenged mice (figure 3, table 2).

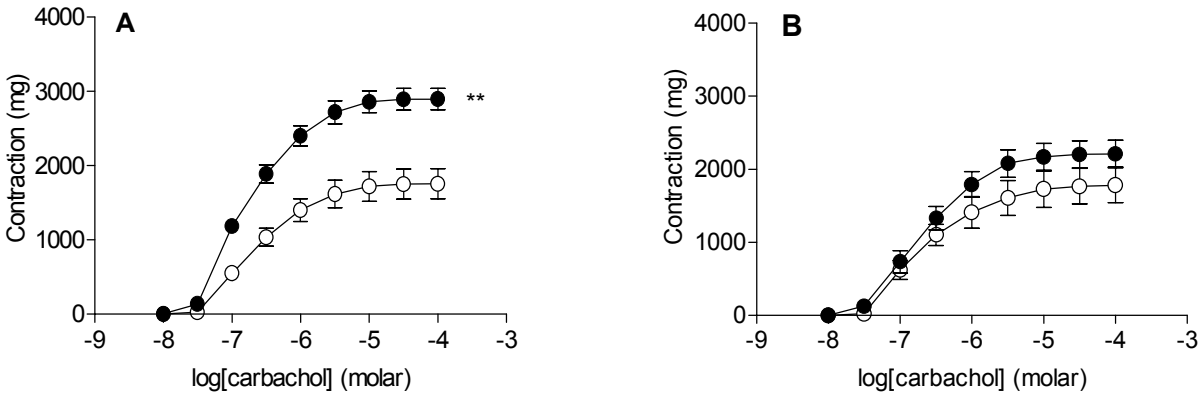
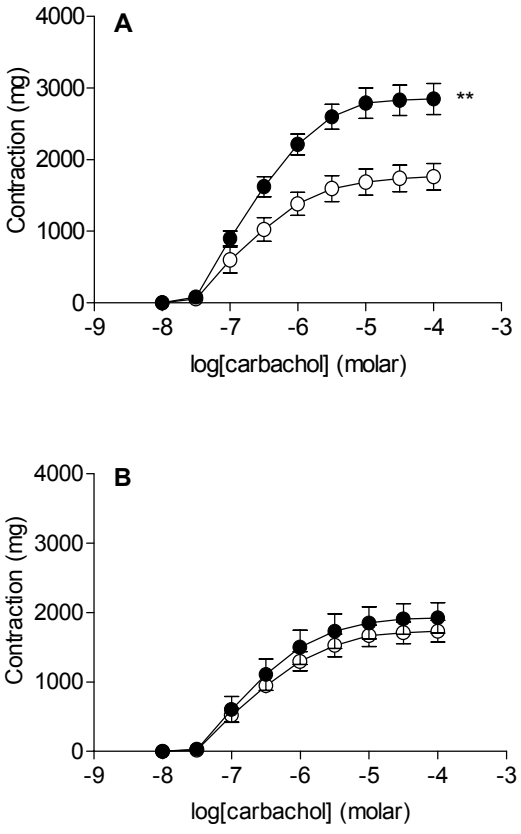


Figure 3. Effect of anti-TNF-α-antibodies on the development of tracheal hyperreactivity to carbachol 48 h after intranasal DNS challenge in DNFB-sensitized mice. Concentration-response curves were measured in DNFB- (closed circles) or vehicle- (open circles) sensitized mice treated i.v. with rat IgG (control, 1 mg/mouse, **a**) or anti-TNF-α-antibodies (XT22, 1 mg/mouse, **b**) at 10 min before and 1 h after the DNS challenge. Results are expressed as mean ± SEM (n=6). Significant differences ($p<0.01$) between curves are denoted by (**).



Effect of anti-*TNFR1 and anti-TNFR2 antibody treatment on DNFB-induced tracheal hyperreactivity.*

In a second set of experiments, we neutralized TNF-α by treatment with TNF-α receptor-specific antibodies. In previous studies, it has been shown that TNFR1 and R2-specific antibodies inhibited TNF-α induced activation of the receptors. Both receptor antibodies do not activate the receptor by itself (32). The role of TNFR1 and TNFR2 was studied by i.v. injection of antagonists against the R1 and R2 receptor 1 h before and 24 h after challenge. Figure 4 and 5 respectively, show that anti-TNFR1, but not anti-TNFR2, inhibited the development of tracheal hyperreactivity in DNFB-sensitized, DNS-challenged mice (table 2).

Figure 4. Effect of TNFR1 receptor blockade on the development of tracheal hyperreactivity to carbachol 48 h after intranasal DNS challenge in DNFB-sensitized BALB/c mice. Concentration-response curves were measured in DNFB- (closed circles) or vehicle- (open circles) sensitized mice treated i.v. with rat IgG (control, 1 mg/mouse) (**a**) or with 1 mg/mouse anti-TNFR1 (**b**) at 10 min before and 1 h after the DNS challenge. Results are expressed as mean ± SEM (n=6). Significant differences ($p<0.01$) between curves are denoted by (**).

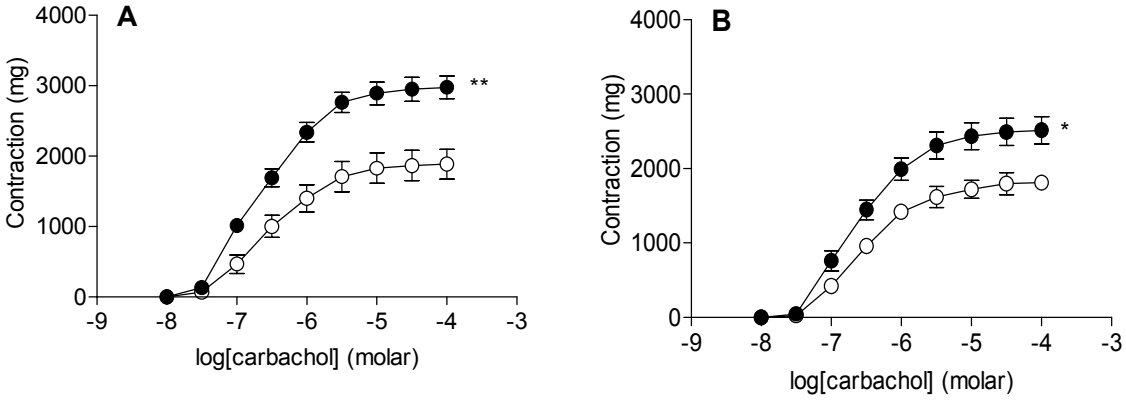


Figure 5. No effect of TNFR2 receptor blockade on the development of tracheal hyperreactivity to carbachol 48 h after intranasal DNS challenge in DNFB-sensitized mice. Concentration-response curves were measured in DNFB- (closed circles) or vehicle- (open circles) sensitized mice treated i.v. with rat IgG (control, 1 mg/mouse)(**a**) or with 1 mg/mouse α–TNFR2 (**b**) at 10 min before and 1 h after the DNS challenge. Results are expressed as mean ± SEM (n=6). Significant differences between curves are denoted by (*) or (**) for $p<0.05$ and $p<0.01$, respectively.

Discussion

The present study shows that TNF-α is an important mediator in the pathogenesis of non-atopic asthma. Neutralizing antibodies for TNF-α completely prevented the development of tracheal hyperreactivity and airway inflammation in mice.

TNF-α is increased in the sputa of patients with bronchial asthma (33) and several studies have shown that BAL fluid from atopic asthmatics contains increased amounts of TNF-α (34, 35). Workers with confirmed diiocyanate-induced occupational asthma, comprising of both atopic and non-atopic patients, showed increased levels of TNF-α in BAL fluid (36). To date no studies clearly report on changed TNF-α levels specifically in non-atopic asthma patients. In the present study, TNF-α levels in BAL fluid of non-atopic mice were significantly increased in sensitized animals shortly after challenge. This suggests that TNF-α is released by cells that contain prestored TNF-α leaving the mast cell as a potential source (11). Previous studies in our laboratory confirm this hypothesis. Van Houwelingen and coworkers (30) found that mast cell deficient mice failed to show an increase in TNF-α BAL levels 30 min after challenge in the murine model presented in this study. These data point to an important role of mast cell-derived TNF-α in mice undergoing a non-atopic asthmatic reaction.

TNF-α released from storage granules of mast cells appears to play a prominent role in neutrophil recruitment. For instance, neutrophil influx during IgE-dependent cutaneous inflammation is dependent on mast cells. TNF-α was identified as an important mediator involved in this response (37). In the present study, neutralizing TNF-α antibodies were capable of significantly diminishing the number of neutrophils and mononuclear cells in the BAL fluid of mice during a pulmonary hypersensitivity reaction. In accordance, neutralization of TNF-α *in vivo* has been shown to decrease the influx of neutrophils and subsequently suppress tissue injury in several models of inflammation. Furuta and coworkers

(38) found that the local administration of anti-TNF-α antibodies resulted in the inhibition of neutrophil accumulation in all layers of the stomach. In the airways, neutralization of TNF-α *in vivo* also affected the neutrophil infiltration, decreasing the percentage of cells by 50% (39). Kips and coworkers showed that anti-TNF-α antibodies partly diminished the influx of neutrophils into BAL fluid occurring after LPS exposure (2). In addition, they demonstrated that the increase in bronchial hyperresponsiveness after LPS exposure, was significantly diminished when pretreating rats with anti-TNF-α antibodies. This is in line with our study where anti-TNF-α antibodies diminished the development of tracheal hyperreactivity reaction in the murine model for non-atopic asthma.

The mechanisms of TNF-α involvement in the neutrophil response and tracheal reactivity reaction may center around the induction of adhesion molecules and leukocyte-specific chemokines, with the ability to participate in inflammatory responses (39). TNF-α has been shown to upregulate endothelial adhesion molecules, such as ELAM-1 and VCAM-1. The induction of these adhesion molecules has been shown to be essential for the migration of the neutrophil (40, 41). Moreover, monoclonal antibodies against adhesion molecules were able to block the occurrence of airway hyperresponsiveness in rat, sheep and guinea pig models (42-46). In the present model for non-atopic asthma, it was demonstrated that treatment with anti-LFA-1 or anti-ICAM-1 resulted in the abolishment of the accumulation of neutrophils into the airway lumen and a total suppression of the development of tracheal hyperreactivity (47, 48). These results suggest a relation between the migration of neutrophils into BAL fluid and the development of tracheal hyperreactivity.

Biological activities of TNF-α can be mediated by the TNFR1 and TNFR2 receptor. We demonstrated that the induction of tracheal hyperreactivity is abolished after treatment with α-TNFR1 but not after treatment with TNFR2 antibodies. Selective deficits in several host defense and inflammatory responses are observed in mice lacking the TNFR1 or both R1 and R2 receptor, but not in mice lacking only the R2 receptor. In the skin, Kondo and Sauder show that the inflammation induced by TNF-α in TNFR1 -/- mice is less than in TNFR2 -/- or control mice (1). This may be explained by the lack of induction of adhesion molecules as TNFR1-/- mice failed to show any upregulation of ICAM-1. In the presented study, the mechanism may involve the TNF-α R1 receptor-induced upregulation of adhesion molecules, facilitating the migration of neutrophils eventually leading to tracheal hyperreactivity. However, TNF-α may also directly modulate airway smooth muscle contractility by directly inducing calcium sensitization of intracellular contractile elements (49).

Taken together, we show that TNF-α is present in the BAL fluid shortly after challenge, probably released by mast cells. Additionally, we propose that TNF-α plays a central role in the development of airway hypersensitivity reactions in a murine model for non-atopic asthma.

Acknowlegments

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Substance P and TNF-α-induced neutrophil infiltration and tracheal hyperreactivity in mice

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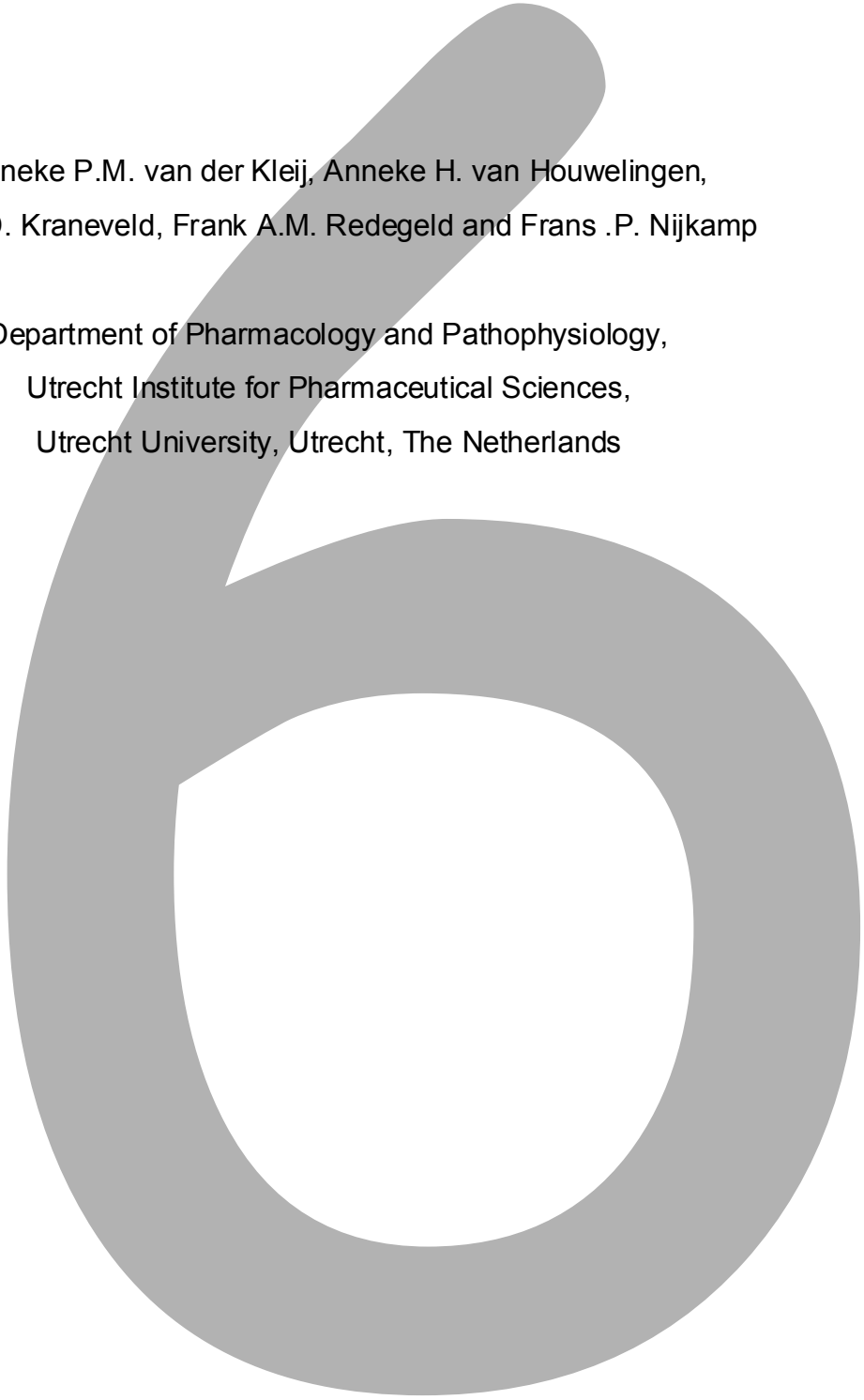


Abstract

Previously, we and other investigators have demonstrated that TNF-α and substance P are involved in the inflammatory process associated with airway diseases. In the present study we examined the effects of TNF-α and substance P on tracheal hyperreactivity and cellular accumulation and activation by intranasal application of TNF-α (250 pg/mouse), substance P (10^{-10}mol/mouse) or a combination of TNF-α and substance P to BALB/c mice on 2 consecutive days. Hyperreactivity occurred 24 h after the second treatment in all thee groups of mice compared to the PBS-treated group. Mice treated with the combination of TNF-α and substance P showed a more profound development of tracheal hyperreactivity than mice injected with TNF-α or substance P alone. TNF-α-treated animals, in contrast to the substance P-treated group, demonstrated a significant increase in the number of neutrophils in the bronchoalveolar lavage (BAL) fluid compared to the PBS-treated group. Neutrophil depletion caused a significant decrease in the TNF-α-induced hyperreactivity reaction, supporting the role of the neutrophil in the development of tracheal hyperreactivity by TNF-α. A significant increase in the number of neutrophils was also seen in the substance P/TNF-α-treated group compared to the PBS-treated group. Moreover, TNF-α- and TNF-α/ substance P-induced increase in BAL neutrophils was accomponied by a rise in myeloperoxidase levels in the BAL fluid, indicative for neutrophil activation. In summary, TNF-α and substance P both can induce tracheal hyperreactivity via possibly different pathways. Substance P amplifies the TNF-α-induced infiltration and subsequent activation of neutrophils.

Introduction

A close anatomical relationship between mast cells and nerves has been reported in virtually all tissues of the body including the airways (1-3). Close proximity to nerves, mostly of sensory origin, allows a bidirectional communication between mast cells and the nervous system (4, 5). Sensory neurons play an important role in neurogenic inflammation and it is becoming apparent that the mast cell may also play a role in this process (6, 7). Its inflammatory mediators can affect neuronal functioning by inducing an enhanced release of neuropeptides from the sensory nerve endings, a process that could be involved in the pathogenesis of asthma.

TNF-α, a major mast cell mediator, has been shown to be involved in the inflammatory process in asthma. Several investigators have shown increased amounts of TNF-α in BAL fluid of asthmatic patients (8, 9). In the airways, neutrophils, alveolar macrophages, airway epithelial cells, T lymphocytes and mast cells are all potential sources of TNF-α (10, 11). However, the mast cell is the only cell containing prestored TNF-α in its granules (12) which can be released by mast cells shortly after activation.

The airways are innervated with C-fiber terminals containing tachykinins, that are able to cause neurogenic inflammation (7, 13, 14). Substance P is a neuropeptide belonging to the family of tachykinins. It is stored in a subpopulation of afferent nerves characterized by their sensitivity to capsaicin. The release of tachykinins from sensory nerves can be evoked by a variety of stimuli such as capsaicin, electrical nerve stimulation, temperature, low pH, toluene diisocyanate, methacholine, leukotrienes, and prostaglandins (15). Subsequent to its release from nerve endings, substance P can increase microvascular

permeability, contract smooth muscle and stimulate immune cells such as the mast cell. Substance P showed to play an inflammatory role in several animal models for airway diseases (16, 17). In humans, BAL fluid and sputum samples taken from asthmatics after antigen challenge show an increase in substance P immunoreactivity (18). In addition, in asthmatic patients, an increase in both the number and the length of substance P-containing nerve fibers are found compared to subjects without asthma (19). These findings suggest a role for substance P in asthma.

Substance P and TNF-α are two important mediators which may link the neurologic and the immune system. Several studies have shown that mast cell mediators such as TNF-α can sensitize sensory neurons and cause the release of tachykinins such as substance P from unmyelinated C-fibers (20-22). Substance P is an important stimulus for cytokine expression in mast cells and can for instance selectively modulate mast cell TNF-α production (23). In the present study, the direct effects of TNF-α, substance P and the combination of TNF-α/substance P on tracheal hyperreactivity and neutrophil accumulation and activation, were studied by intranasal application of TNF-α and/or substance P to BALB/c mice.

Methods

Animals. Male BALB/c mice were obtained from the Central Animal Laboratory (GDL), Utrecht University, Utrecht, The Netherlands. All mice used were 6-8 weeks of age. Experiments were conducted in accordance with the Animal Care Committee of the Utrecht University (Utrecht, The Netherlands).

Intranasal treatment with TNF-α and substance P. Naïve mice were anesthetized with sodium pentobarbitone (50 mg/kg i.p.). The animals received TNF-α (250 pg/mouse), substance P ($5x10^{-10}$mol/mouse), a combination of TNF-α and substance P, or PBS as a control, intranasally, in a volume of 50 μl on day 0 and day 1. On day 2, mice were killed by an overdose of pentobarbitone and experiments were conducted.

Neutrophil depletion. In stated experiments, neutrophils were depleted from the circulation, diluted rabbit anti-PMN antiserum (1:10 in sterile saline, 12 ml/g body weight) or control serum (rabbit serum) was injected i.p. 1 day before starting TNF-α treatment and at day 0, together with intranasal TNF-α treatment. In a pilot study performed in naïve mice (n=3 mice/group), the effective neutropenia was approximately 98% with the total neutrophil count decreasing from 143.2 ± 12.1 x 10^4 cells/ml in the control serum treated mice to 4.7 ± 0.4 x 10^4 cells/ml in the anti-PMN antibody treated animals.

Tracheal reactivity in vitro. Mice were killed with an overdose of pentobarbitone at day 2 after starting the treatment of the mice. The trachea, which was resected *in toto*, was carefully cleaned of connective tissue using a binocular microscope as described before (24). A nine ring piece of trachea (taken from just below the larynx) was then transferred to a 10 ml organ bath containing a modified oxygenated Krebs solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 25 mM $NaHCO_3$, 1 mM $NaHPO_4$ and 11.1 mM

glucose, aerated (95% O_2: 5% CO_2, 37ºC)). An optimal preload, determined to be 1 g, was placed on the tissue at the beginning of the experiment. The trachea was allowed to equilibrate for at least 1 h before contractile effects were elicited. During this period, the bath fluid was exchanged every 15 min. At the end of the equilibrium phase, airway reactivity was measured by recording cumulative concentration response-curves to carbachol (10^{-8} to 10^{-4} M).

Leukocyte accumulation in bronchoalveolar lavage fluid. Bronchoalveolar lavages (BAL) were performed 24 h after the final treatment with either PBS, TNF, substance P or a combination of TNF-α and substance P. After sacrificing the animals, the trachea was carefully intubated and the catheter was secured with ligatures. The chest cavity was exposed for expansion. Saline (37ºC) was slowly injected via the catheter into the lung and withdrawn in 4 x 1 ml aliquots. The aliquots were pooled and maintained at 4ºC. The lavage fluids were centrifuged (1500 rpm, 10 min, 4ºC) to isolate the BAL cells. The cell pellet was resuspended in 150 μl PBS. Total cells were counted using a haemocytometer and expressed as cells/lung. The BAL cell preparations were analyzed morphologically after centrifugation on to microscopic slides. Air dried preparations were fixed and stained with hematoxylin and eosin to ascertain the leukocyte populations. Results are expressed as leukocytes/lung for neutrophils and mononuclear cells in the airway lumen. The remainder of the cell suspension (approximate 80 μl of the original 150 μl) was kept at –20ºC for myeloperoxidase activity of the BAL cells.

Myeloperoxidase in BAL fluid and BAL fluid cells. MPO activity, a marker for neutrophil activation, was measured in the BAL fluid and in the BAL fluid cells according to a standard spectrophotometrical technique (25). BAL cells samples were centrifugated and the supernatants were used to measure MPO activity. Both BAL fluid and BAL cell supernatant samples were diluted 1:1 in MPO dilution buffer (10 mM citratebuffer pH 5.0). Aliquots of 50 μl of each sample were pipetted into a 96-wells plate and 50 μl of substrate solution (0.46 mM tetramethylbenzidene, 0.88 mM H_20_2, and 120 μM resorcinol) was added to each well. The samples were incubated with substrate solution at protected from light at room temperature on a orbital shaker. The reaction was stopped after 15 min with a stop solution (4N H_2SO_4). The optical density (OD) at 450 nm was determined. As an additional control, 50 μl of citratebuffer + 50 μl of substrate solution with resorcinol was pipetted into 4 wells, followed by stop solution. Results were expressed as OD/ml BAL fluid or OD/ cells. Furthermore, the OD was corrected for the total amount of neutrophils in the BAL fluid samples and results were expressed as OD/neutrophil.

Materials. Substance P was purchased from Calbiochem-Novabiochem, Lufelfingen, Switzerland and TNF-α from PeproTech Inc. (Rocky Hills, USA). Sodium barbitone was purchased from Sanofi BN (Maassluis, The Netherlands). Carbachol was purchased from Onderlinge Pharmaceutische Groothandel, Utrecht, The Netherlands. Rabbit anti-PMN antiserum was obtained from Accurate Chemical & Scientific Corp., Westbury, New York, USA. Control rabbit serum was purchased from Central Animal Laboratory, Utrecht, The Netherlands. Hematoxylin and eosin (Diff-Quick) were purchased from Merz & Dade A.G.,

Dubingen, Switzerland. The force displacement transducer was purchased from Harvard Bioscience, Boston, MA, USA and the two channel recorder (Servogar type SE-120) from Plato BV, Diemen, the Netherlands.

Statistical analysis. Data are expressed as mean and standard error of the mean (SEM). EC50- and Emax-values for the carbachol-induced tracheal contractions were calculated by non-linear least-squares regression analysis of the measured contractions versus carbachol concentration using the sigmoid concentration-response relationship. The data were analyzed by performing a two-way analysis of variance (ANOVA). Data on the cellular accumulation were analyzed by a distribution free Kruskal-Wallis ANOVA followed by Dunn's multiple comparison test. MPO data were analyzed by using a one-way ANOVA followed by the Bonferroni's multiple comparison test. Probability values of $P<0.05$ were considered significantly different. Analyses were performed by the usage of Graphpad Prism (version 2.01, San Diego, U.S.A.).

Results

Effect of TNF-α and/or substance P on the development of tracheal hyperreactivity. Mice were intranasally treated with TNF-α, substance P, or a combination, on day 0 and day 1. On day 2, a significant tracheal hyperreactivity occurred in mice treated with TNF-α or with substance P compared to PBS-treated animals. Mice treated with a combination of TNF-α and substance P showed a more profound development of tracheal hyperreactivity than mice injected with TNF-α or substance P alone. This increase was significantly different from the reactivity in substance P treated mice, but not from the TNF-α treated group. When ΔEmax values of the different treatment groups were compared, it can be concluded that the effect of substance P and TNF-α were additive (figure 1 and table 1).

Table 1. Effect of TNF-α, substance P or TNF-α/substance P on the development of tracheal hyperreactivity. Mice were i.n. treated with TNF-α, substance P, TNF-α/substance P or PBS. Emax and pD_2 values are derived fromconcentration-response curves to carbachol (10^{-8}-10^{-4} M).

Treatment	Emax	Δ Emax	pD_2
PBS	1997 133	0	6.8 ± 0.1
TNF-α	2805 ± 122*	808 ± 179.7*	6.7 ± 0.1
Substance P	2553 ± 132*	556 ± 187.4*	6.9 ± 0.1
TNF-α/substance P	3436 ± 137*‡	1438 ± 190.0*‡	6.8 ± 0.1

Results are expressed as mean ± sem (n=6). Significant differences ($p<0.05$) denoted by (*) compared to the PBS and (‡) to the substance P group.

Effect of TNF-α and/or substance P on leukocyte accumulation in bronchalveolar lavage fluid. A significant increase in the number of neutrophils in the TNF-α treated group compared to control PBS-treated mice was observed (table 2). No significant differences in the number of neutrophils were seen in the substance P-treated mice compared to control

animals (table 2). Mice treated with the combination of TNF-α and substance P showed a significant increase in the number of neutrophils in the BAL Fluid (table 2). The neutrophil increase as seen in the TNF-α/substance P group is more profound although not significantly higher than in mice treated with TNF-α.

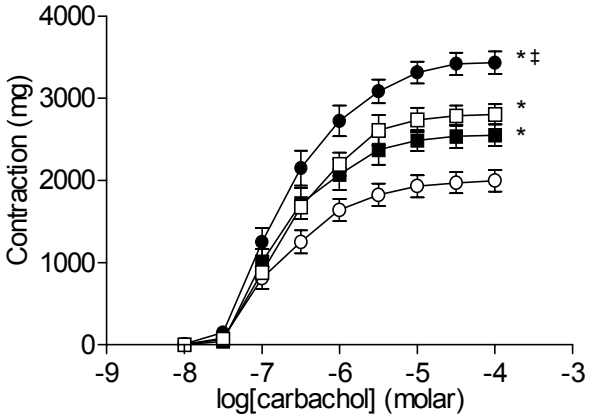


Figure 1. Effect of TNF-α, substance P or the combination of TNF-α and substance P on the development of tracheal hyperreactivity to carbachol. Concentration-response curves were measured in substance P- (closed squares), TNF-α (open squares), TNF-α/substance P- (closed circles) and PBS-treated mice (open circles). All animals were treated on 2 consecutive days. Reactivity was measured 24 h after the last treatment. Results are expressed as mean ± SEM (n=6). Significant differences ($p<0.05$) are denoted by (*) compared to the PBS group and by (‡) compared to the substance P group.

Table 2. Effect of TNF-α, substance P or TNF-α/substance P on leukocyte accumulation and differentiation. Mice were i.n. injected with TNF-α, substance P, TNF-α/substance P or PBS as a control.

Treatment	Leukocyte accumulation in BAL fluid (cells/lung x 10^3)		
	Total cells	Mononuclear cells	Neutrophils
PBS	28.5 (21.0-45.0)	25.9 (19.9-42.3)	1.8 (1.1-2.7)
TNF-α	48.0 (33.0-60.0)	31.7 (20.5-49.1)	15.4 (7.6-19.8)*
Substance P	27.8 (19.5-45.0)	24.4 (16.6-36.5)	3.5 (2.7-8.6)
TNF-α/Substance P	56.3 (37.5-76.5)	34.8 (23.6-40.0)	21.8 (12.2-38.2)*‡

Results are expressed as median (min-max). Significant differences ($p<0.05$) are denoted by (*) compared to the PBS group and (‡) to the substance P group.

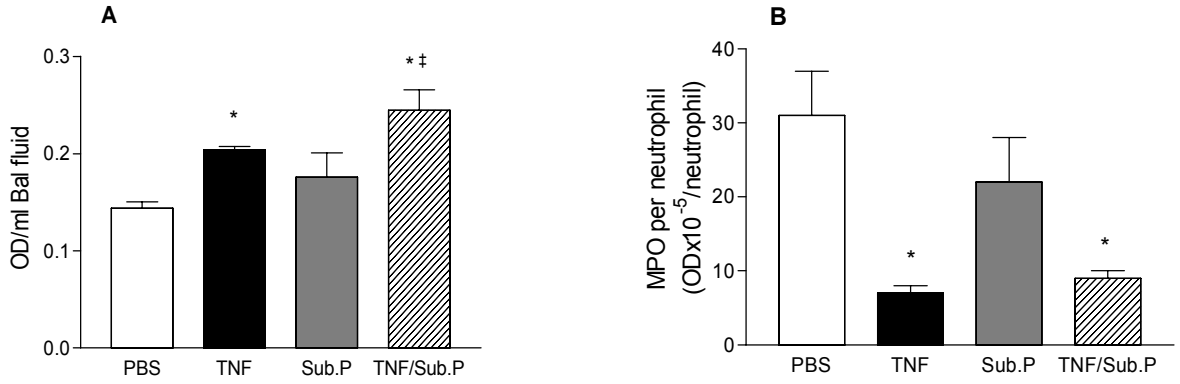


Figure 2. Effect of TNF-α, substance P or the combination of TNF-α and substance P on myeloperoxidase levels in: **A.** BAL fluid and **B.** neutrophils. All animals were treated on 2 consecutive days. Reactivity was measured 24 h after the last treatment. Results are expressed as mean ± SEM (n=6). Significant differences ($p<0.05$) denoted by (*) compared to the PBS group and (‡) to the substance P group.

Effect of TNF-α and substance P on myeloperoxidase levels in BAL fluid and neutrophils. Treatment with substance P did not change the MPO content in the BAL fluid when compared to PBS-treated mice (figure 2a). However, TNF-α treatment did significantly increase the MPO levels in the BAL fluid. A combination of TNF-α and substance P led to a profound increase in the MPO levels in the BAL fluid. This increase was significant compared to the PBS-treated group($p=0.002$) as well as to the substance P-treated animals ($p=0.04$). However, the increase was not significantly different from treatment with TNF-α alone ($p=0.08$). The amount of MPO per neutrophil was significantly decreased in the TNF-α and the TNF-α/substance P- treated groups suggesting that the neutrophils had released their MPO (figure 2b). This finding is consistent with the significant increase in MPO levels in the BAL fluid supernatant in both groups.

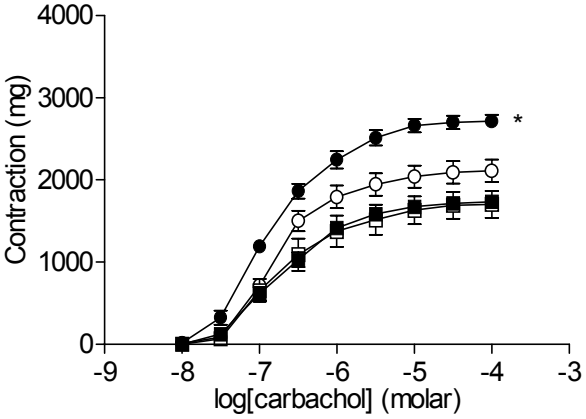


Figure 3. Effect of α-PMN antibody on the TNF-α-induced development of tracheal hyperreactivity to carbachol. Animals were treated with TNF-α on 2 consecutive days. a-neutrophil antibodies (300 ul/ mouse) or rabbit serum (control) were injected i.p. 1 day before TNF-α treatment and at day 0, together with intranasal TNF-α treatment. Reactivity was measured 24 h after the last TNF-α treatment. Concentration-response curves were measured in PBS/rabbit serum- (closed squares), PBS/α-PMN (open squares), TNF-α/rabbit serum (closed circles) and TNF-α/α-PMN-treated mice (open circles). Results are expressed as mean ± SEM (n=6). Significant differences ($p<0.05$) from the PBS group are denoted by (*).

Effect of anti-PMN antibody on the TNF-α-induced development of tracheal hyperreactivity. As is described above, TNF-α treatment caused a significant infiltration and activation of neutrophils, presumably followed by the development of tracheal hyperreactivity. To study this, an antiserum causing the depletion of neutrophils, was administered before and together with TNF-α treatment. Neutrophil depletion induced a significant inhibition of TNF-α-induced tracheal hyperreactivity. The reactivity of tracheas obtained from TNF-α/α-PMN-treated animals did not significantly differ from the reactivity of tracheas obtained from PBS/ control or PBS/α-PMN-treated mice (figure 3).

Discussion

As mast cells are in close proximity to excitatory NANC-nerves, the mast cell is thought to be a pivotal cell in neuroimmune interactions (2, 4, 5, 26). TNF-α and substance P are two important mediators in the link between the neurologic and the immune system. In this study we investigated the potential role of TNF-α and substance P in the development of tracheal hyperreactivity, cellular accumulation and neutrophil activation.

TNF-α showed to significantly induce the development of tracheal hyperreactivity. This is in accordance with earlier studies which show that application of TNF-α *in vivo*

causes bronchial hyperresponsiveness in rats (27, 28). In addition, inhaled TNF-α is able to increase the responsiveness of normal human subjects to methacholine (29). Indeed, neutralizing antibodies for TNF-α completely prevents the development of tracheal hyperreactivity in our murine model for non-atopic asthma (chapter 5 of this thesis).

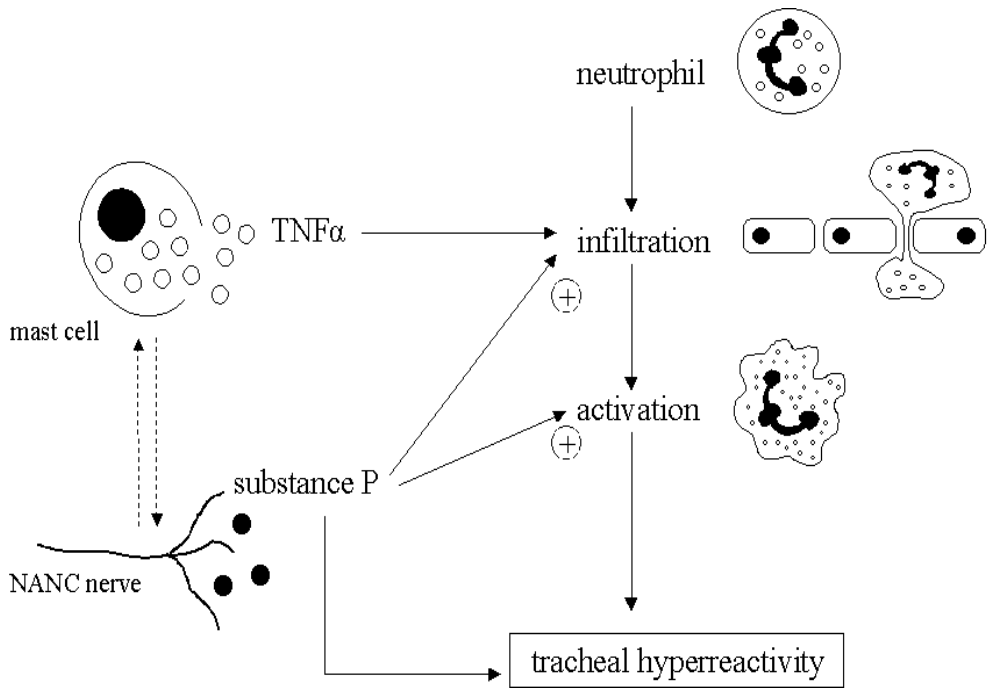
TNF-α injection in human skin has been shown to induce neutrophil accumulation within a few hours (30). We found that TNF-α was able to increase the number of neutrophils in the BAL fluid of mice. In several models of acute inflammation, TNF-α has been demonstrated to play a role in neutrophil infiltration (31-33). Neutralization of TNF-α *in vivo* has also been shown to decrease the influx of neutrophils and subsequently suppress tissue injury in several models of airway inflammation (27, 32). In chapter 5 of this thesis, it was also shown that neutralizing TNF-α antibodies prevented the accumulation of neutrophils in a murine model for non-atopic asthma. Summarized, TNF-α is a potent inducer of neutrophil infiltration. However, TNF-α per se is a weak chemoattractant. The chemotactic effect of TNF-α on tissue trafficking of neutrophils appears to be dependent on the induction of cell adhesion molecules and the release of chemokines in the vascular endothelium (32, 34-37). The murine CXC chemokines involved in neutrophil attraction comprises macrophage inflammatory protein-2 (MIP-2) and cytokine-induced neutrophil chemoattractant (KC). Selectins are another prerequisite for neutrophil adhesion and subsequent migration. Mercer-Jones and coworkers demonstrated that TNF-α derived from the mast cell is an important factor to induce endothelial selectins (38).

In the present study in mice, substance P is shown to cause tracheal hyperreactivity after repeated substance P treatment. Substance P is one of the important tachykinins released from the sensory nerve endings. It has been suggested that tachykinins, and substance P in particular, can contribute to the inflammatory response in asthma (1, 7, 39). Exposure of guinea pigs to substance P elicited airway hyperresponsiveness (16, 40). In the rat, a significant increase of airway responsiveness to acetylcholine was found after substance P pretreatment in presence of a neural endopeptidase inhibitor (41).

Substance P-induced development of tracheal hyperreactivity could have been mediated by an increased neutrophil influx. Serra and coworkers (42) indicated that substance P can stimulate the synthesis and direct release of the chemotactic cytokine IL-8 from polymorphonuclear cells. In this way, substance P may influence neutrophil adhesion and subsequent migration towards lung inflammatory sites as has been described in various studies (43-45). Moreover, substance P can increase the permeability of bloodvessels by the activation of NK-1 receptors, thereby facilitating the movement of neutrophils into the tissue compartment (46-48). A study by Tomoe and coworkers (49) demonstrated that substance P induces neutrophil infiltration in a concentration-dependent way in the skin, which was mediated though degranulation of mast cells. It can be speculated that substance P induces neutrophil infiltration via the release of TNF-α from mast cells in the present study. However, intranasal application of substance P showed only a minor, not significant effect on the infiltration of neutrophils. Possibly, the concentration of substance P used in this study, was not high enough to effect neutrophil infiltration or to evoke TNF-α release from mast cells. Yet, substance P did amplify TNF-α-induced neutrophil infiltration and activation suggesting that a treshold is passed by the effect of TNF-α.

The results obtained from this study suggest that TNF-α and substance P both affect airway hypersensitivity reactions. Application of the two compounds together leads to more profound effects. It is not clear what exact mechanisms are involved. The present report demonstrates that neutrophil migration is involved in the development of TNF-α-induced tracheal hyperreactivity as it could be significantly reduced by neutrophil depletion. Amrani and coworkers (50, 51) showed that TNF-α can modulate airway smooth muscle contractility by directly inducing calcium sensitization of intracellular contractile elements. This might not be the mechanism involved in the development of tracheal hyperreactivity by TNF-α in our study. Also, TNF-α can affect a subpopulation of C fibers resulting in the release of substance P (52). In turn, substance P could contribute to the induction of hypersensitivity reactions in the mouse and can induce TNF-α release from mast cells (53-56). Further research is needed to dissect the mechanimsms involved in substance P and TNF-α-induced airway hyperreactivity and neutrophil infiltration.

The results from this study show that TNF-α and substance P are both able to induce tracheal hyperreactivity via possible different pathways. Combination of both results in a profound tracheal hyperreactivity. We can conclude that the infiltration and activation of neutrophils is in turn, at least partly involved in the development of tracheal hyperreactivity (scheme 1).



Scheme 1. Proposed model for the effects of TNF-α and substance P on airway function. TNF-α and substance P both can induce tracheal hyperreactivity via different pathways. Substance P amplifies (as denoted by +) the TNF-α-induced infiltration and subsequent activation of neutrophils.

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Functional expression of neurokinin 1 receptors on mast cells induced by IL-4 and SCF

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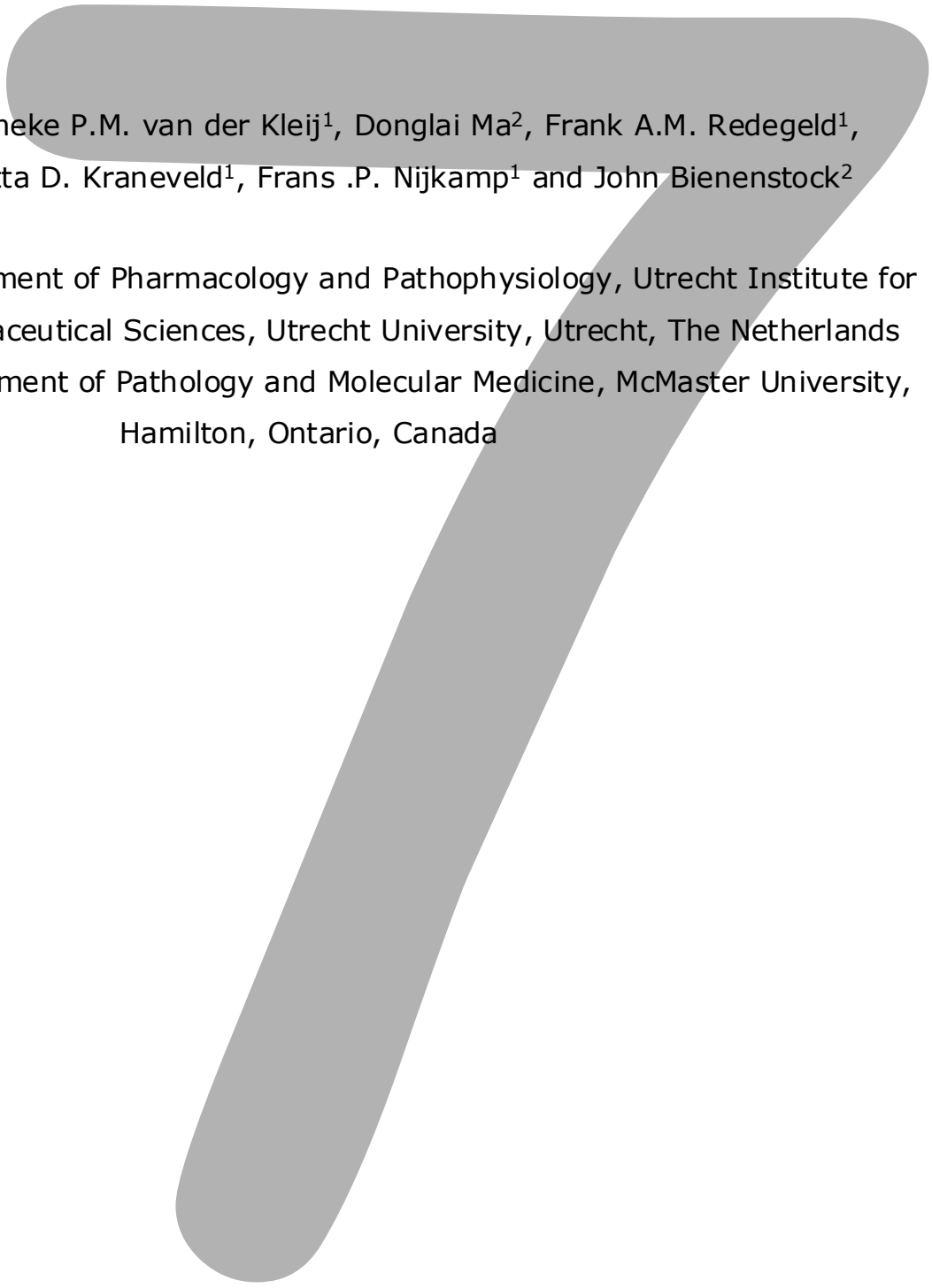

Abstract

It is widely accepted that NK-1 receptors are not generally expressed on mast cells but little is known about their expression in inflammation. The present study shows expression of NK-1 receptors on bone-marrow-derived mast cells (BMMC) under the influence of IL-4 or SCF. Highest expression was found when both cytokines are present. Six days of co-culture with the cytokines IL-4 and SCF showed significant expression of NK-1 receptors (NK-1 receptor$^+$/c-kit$^+$ BMMC; control: 8 %, IL-4/SCF: 16 %), while 12 days of cytokine-co-culture increased this expression to 38 % positive mast cells. A longer co-culture with IL-4 and SCF did not show an additional effect. Furthermore, we demonstrated the functional relevance of NK-1 receptor expression for the activation of BMMC resulting in an enhanced degranulation upon stimulation by substance P. This mast cell activation was significantly diminished by the NK-1 receptor antagonist RP67580 (10 μM) when stimulated with low concentrations of substance P. The inactive enantiomer RP65681 had no effect. In addition, BMMC cultured from bone marrow of NK-1 receptor knockout mice showed significantly decreased exocytosis to low concentrations of substance P. The present study clearly shows that NK-1 receptor-induced activation contributes significantly at low physiological substance P concentrations (<100 μM). At higher concentrations, activation of BMMC was largely due to receptor-independent mechanisms. In conclusion, BMMC were shown to express NK-1 receptors upon IL-4/SCF co-culture. This expression of NK-1 receptors has been demonstrated to be of functional relevance and leads to an increase in the sensitivity of BMMC to substance P.

Introduction

Mast cells are often found in the proximity of nerve endings, and may be activated by a number of neuropeptides, including tachykinins such as substance P. This close anatomical relationship in various tissues including skin (1), intestine (2), dura mater (3, 4) and airway mucosa (1) suggests the involvement of mast cells in the neurogenic component of inflammatory conditions. Furthermore, substance P can induce the release of histamine from certain types of mast cells such as peritoneal and mucosal mast cells (5, 6), human mast cells (7) and human pulmonary mast cells (8), further supporting the functional interaction between mast cells and substance P-containing nerves.

Mast cells can be divided into various subpopulations with distinct phenotypes. Two main subsets, connective tissue type mast cells (CTMC) and mucosal mast cells (MMC) are recognized as distinct mast cell populations with different phenotypical and functional characteristics (9, 10). However, environmental factors such as cytokines, may induce differentiation in various subsets (11). In spite of their differences, both CTMC and MMC are considered to be derived from a common precursor in the bone marrow. Mast-cell progenitor cells translocate from bone marrow to mucosal and connective tissues to locally undergo differentiation into mature forms. They possess a remarkable degree of plasticity, so that even apparently fully differentiated CTMC will transform their phenotype in MMC if transplanted into a mucosal environment (12). Their development and survival essentially depends on stem cell factor (SCF) and its receptor c-kit (13). Besides SCF, cytokines such as IL-3, IL-4 and IL-10 influence mast cell growth and differentiation (14), as does nerve growth factor (15, 16).

Classically, mast cells are associated with hypersensitivity reactions, involving the interaction with IgE (17). However, mast cells also play a prominent role in non-IgE mediated hypersensitivity reactions (18, 19). The sensitivity of mast cells to activation by non-immunological stimuli such as polycationic compounds, complement proteins, superoxide anions or neuropeptides is dependent on the subset of the mast cells examined (20).

Basic secretagogues trigger mast cell exocytosis though a mechanism called the peptidergic pathway. Instead of interacting with a membrane bound receptor, they appear to directly activate and bind to pertussis toxin-sensitive GTP-binding proteins (G-proteins) through the N-terminal domain located in the inner surface of the plasma membrane (21, 22). Stimulation of G-proteins will activate a signal transduction pathway eventually leading to mast cell mediator production and release.

It has been proposed that tachykinins like substance P can also induce mast cell activation via a receptor-dependent mechanism (23-25). Activation of the neurokinin receptors is dependent on the C-terminal domain of the tachykinins. C-terminal fragments of substance P cause histamine release from the mouse mast cell line MC/9 via an NK-2 receptor mediated pathway (26). Cooke and coworkers (25) demonstrated that RBL-2H3 cells, a rat mast cell line homologous to MMC, express the NK-1 receptor for substance P on their surface. However, it is widely accepted that mast cells do not express NK receptors under normal physiological conditions. The observation that mast cell lines express NK-1 receptors could represent an aberrance of these cell lines.

Most reports reveal that neuropeptides only cause degranulation at relatively high concentrations ($>10^{-5}$ M). However, some researchers have shown that physiological concentrations of substance P can have biological consequences under some conditions (27). Therefore, we wondered if NK-1 receptors might be expressed on mast cells cultured from bone marrow under different conditions involving co-culture with SCF and IL-4. We examined the expression of NK-1 receptors on mast cells by fluorescence activated cell sorting (FACS), and functionally by examining the release of ß-hexosaminidase in the presence or absence of NK-1 inhibitors and the NK-1 receptor itself.

Previously, we have shown that substance P causes dose-dependent degranulation in BMMC co-cultured with IL-4 and SCF for 6 days (28, 29). In this report, we have found that BMMC express functional NK-1 receptors under the influence of IL-4 or SCF but are expressed in highest numbers when IL-4 and SCF are both present. The IL-4/SCF-induced upregulation of the NK-1 receptor on BMMC was associated with an increased response to substance P.

Methods

Pokeweed mitogen-stimulated spleen cell conditioned medium (PWM-SCM).

Spleen cells from BALB/c mice (Charles River, Someren**,** the Netherlands or Harlan, Indianapolis, Indiana, USA) were cultured at density of $2x10^6$ cells/ml in RPMI 1640 medium containing 4 mmol/l L-glutamine , $5x10^{-5}$ mol/l 2-mercaptoethanol, 1 mmol/l sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin and 0.1 mmol/l nonessential amino acids (complete RPMI) containing lectin (8 μg/ml) and placed in 75-cm^2 tissue-culture flasks. The cells were

incubated at 37° C in a 5% CO_2 humidified atmosphere. After 5-7 days, medium was collected, centrifuged for 15 min. at 4000 RPM, filtered through a 0.22 μm millipore filter and used as PWM-SCM.

Mouse bone marrow cultures. BALB/c mice, in stated experiments NK-1 receptor knockout mice (a generous gift from Dr. N. Gerard, Harvard, Boston, USA), were killed by cervical dyslocation and bone marrow was aseptically flushed from femurs into complete RPMI 1640. The cell suspension was washed twice in complete RPMI by centrifugation at 400 g for 10 min and finally resuspended in complete RPMI containing 10% vol/vol FCS and placed in 75-cm^2 tissue culture flasks at density of 1-2x10^5 cells/ml. PWM-SCM, 20% vol/vol was added to the culture medium. Flasks were incubated at 37°C in a 5% CO_2 humidified atmosphere. Cells were centrifuged and resuspended in fresh media every week to achieve a final concentration of 1-2x10^5 cells/ml. After 3-4 weeks, the BMMC were resuspended in complete RPMI and cultured with IL-4 (300 U/ml), SCF (50 ng/ml) or IL-4 plus SCF. Cytokine-treated BMMC were centrifuged and resuspended in fresh medium every three days to achieve a final concentration of 2x10^5 cells/ml.

FACS analysis for NK-1 receptor expression. Immunofluorescence analysis was performed to determine the presence of NK-1 receptors on BMMC positive for c-kit. Therefore BMMC were monitored by double staining with the antibodies against the NK-1 receptor and c-kit. RBL-2H3 cells were used as a positive control as these cells are known to express NK-1 receptors. Cell staining analysis was performed on BMMC cultured under different culture conditions : BMMC, IL-4 co-cultured BMMC at 6 and 12 days of co-culture, SCF co-cultured BMMC at 6 and 12 days of co-culture, IL-4/SCF co-cultured BMMC at 6, 12 and 18 days of co-culture. Cells were harvested and resuspended at a concentration of 1x10^6 cells/ml. 10^5 cells were aliquoted per tube and cells were washed using ice-cold staining buffer (PBS supplemented with 1% BSA, 0.02% EDTA and 0.02% NaN_3). Cells were centrifuged at 1000 rpm for 5 min at room temperature and resuspended in staining buffer with the primary rabbit antibody against either the NK-1 receptor (1:100) or rabbit IgG (isotype control) was added. Biotin conjugated c-kit or isotype control (rat IgG) was added to the designated tubes at a final concentration of 1 μg/ml and incubated for 30 min at room temperature. Next, cells were centrifuged, washed twice and resuspended in blocking buffer (staining buffer containing 5% goat serum). FITC-conjugated goat anti-rabbit IgG was used for the detection of the NK-1 receptor, PE-conjugated streptavidin was used for detection of c-kit, incubated for 30 min. Again, cells were centrifuged, washed twice and resuspended in 500 μl FACS buffer per tube. Cells were analyzed using FACS WinList (version 5.0).

Activation of cells. Bone marrow cells from BALB/c mice or NK-1 receptor knockout mice were cultured for 3 to 4 weeks to develop into BMMC. At 3 to 4 weeks, these cells were cultured with SCF and IL-4 as described above. Cells were washed twice with Tyrode's buffer supplemented with 0.1% BSA and resuspended in Tyrode's buffer at density of 0.6x10^6 cells/ml. 2-3x10^4 cells were aliquoted in 96 well plates. In stated experiments cells were pretreated with RP67580, a specific NK-1 receptor antagonist or its inactive

enantiomer RP65681 (10^{-6} M) for 10 min. Cells were activated with different concentrations of substance P (0-200 μM) for 30 min. Total release was established by adding NP40 to get complete lysis of cells. After one hour incubation of the supernatant with assay solution containing 4-methylumbelliferyl glucosaminide in 0.1 M citrate buffer (pH=4.5), the reaction was stopped by adding 0.2 M glycine buffer, pH 10.7. Fluorescence was measured using a multi-well plate reader at an emission wavelength y= 360 nm and excitation wavelength y= 450 nm. The percentage of degranulation was calculated as: [(a-b)/(t-b)]x100, where a is the amount of ß-hexosaminidase released from stimulated cells, b is that released from unstimulated cells and t is total cellular content.

Reagents. RPMI 1640, fetal calf serum and nonessential amino acids were purchased from GibCo BRL Life Technologies, Paisley Scotland. Penicillin, streptomycin, L-glutamine, sodium pyruvate, 2-mercaptoethanol were obtained from Sigma, St. Louis, MO, USA and lectin from Phytocalla Americana. Murine recombinant SCF came from PeproTech, London, England. Murine recombinant IL-4 was kindly provided by W.E. Paul NIH, USA. Substance P was purchased from Novabiochem, Laufelfingen, Switzerland. Rabbit anti-NK_1 was obtained from Chemicon International, Temecula, Canada and rabbit IgG from DAKO Diagnostics, Missisauga, Ontario, Canada. Goat anti-rabbit-FITC was purchased from Vector Lab, Burlingame, Canada. Streptavidin-PE conjugate was from Becton Dickinson, USA. Biotin anti-mouse CD117 (c-kit) was from PharmaMingen, San Diego, CA, USA. RP67580 and RP65681 were generous gifts from Rhône-Poulenc Rorer, Dr. C. Garrett in France.

Results

NK-1 receptor expression on BMMC

Previously we have shown that co-culture of BMMC in presence of IL-4 and SCF increased the sensitivity to stimulation by substance P (29).

To investigate if co-culture was paralleled by an increase in expression of the NK-1 receptor, we analyzed binding of an NK-1 receptor specific antibody using flow cytometry. Cells were double stained for NK-1 and c-kit to show the population of mature mast cells positive for the NK-1 receptor. As a positive control, we first studied RBL-2H3 cells, which were known to express NK-1 receptors on their surface (25). These cells indeed showed NK-1 receptor expression (NK-1 receptor$^+$: 18.5%, table 1). To further confirm specificity, bone marrow from NK-1 receptor knockout mice were cultured and studied for NK-1 receptor expression. These NK-1 receptor cells were negative for the expression of the NK-1 receptor (figure 1a). Additionally, NK-1 receptor knockout BMMC cells co-cultured with IL-4 and SCF were also negative for the expression of the NK-1 receptor (figure 1b). We next analyzed NK-1 receptor expression on BMMC cultured in PWM-SCM. On these cells, only 8.1 % of the cells showed expression of the NK-1 receptor (figure 2a). BMMC were now studied after 6, 12 and 18 days of treatment with IL-4 and/or SCF. These levels of NK-1 receptor positive mast cells represent significant increases compared to untreated cells. After 6 days of cytokine treatment, the percentage of cells expressing c-kit and the NK-1 receptor, increased significantly compared to non-treated BMMC. IL-4 by itself (figure 2b)

showed no effect on the expression of the NK-1 receptor compared to non-treated cells BMMC at this time of culture, whereas treatment with SCF displayed a slight increase

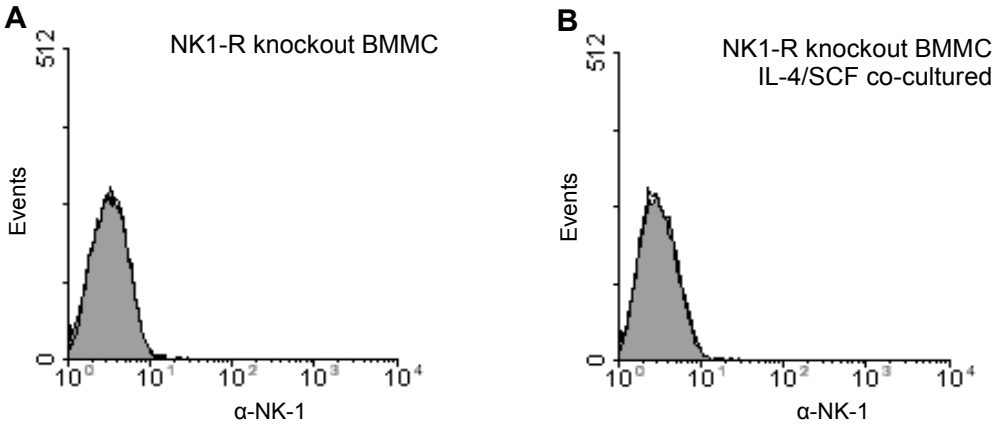

Figure 1. FACS analysis of expression of NK-1 receptor on NK-1 receptor knockout BMMC in different culture conditions. Mouse bone marrow cells were cultured for 28 days in the presence of PWM-SCM (**A**) or for 22 days with PWM-SCM plus 6 days of IL-4/SCF co-culture (**B**) as described in methods. Cells were stained with anti-NK-1 receptor antibody and analyzed by flow cytometry. Isotype controls are represented by the shaded histograms. The clear histograms represent NK-1 receptor$^+$ cells. Both untreated and IL-4/SCF treated cells showed negative for NK-1 receptor expression.

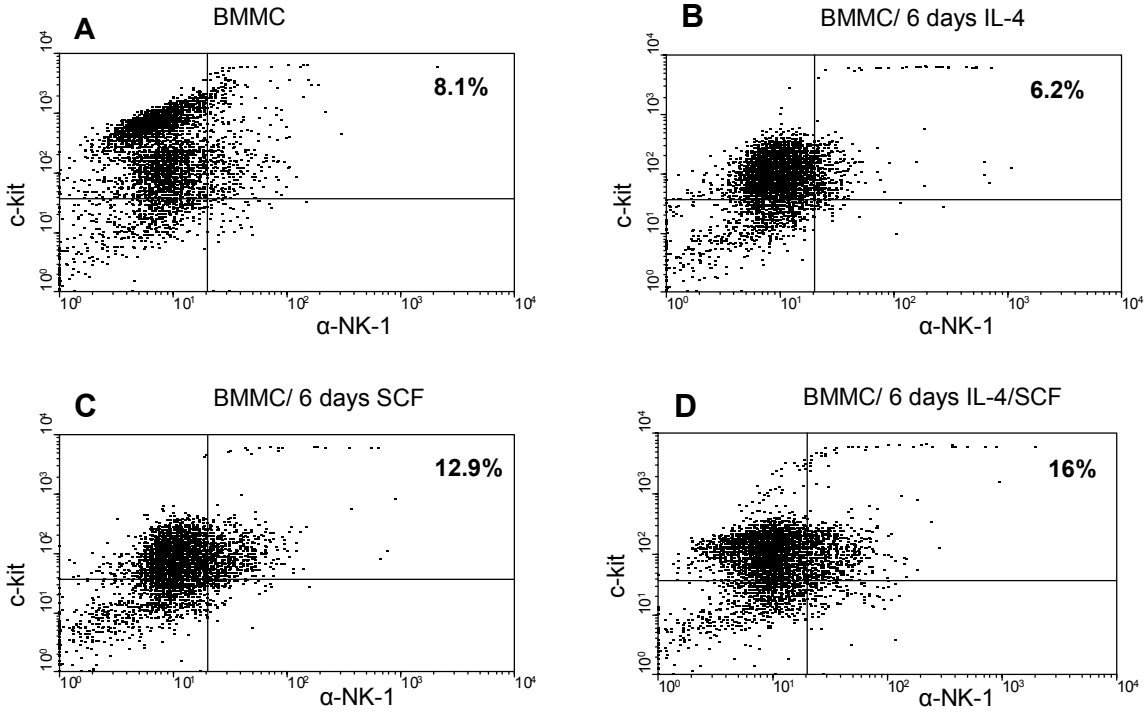

Figure 2. FACS analysis of NK-1 receptor$^+$-c-kit$^+$ cells in different culture conditions. Mouse bone marrow cells were cultured for 28 days in the presence of PWM-SCM (**A**) or for 22 days with PWM-SCM plus 6 days with IL-4 alone (300 U/ml)(**B**), SCF alone (50 ng/ml)(**C**) or a combination of IL-4 and SCF (**D**). Different cell populations were double stained with FITC-conjugated anti-NK-1 receptor (1:100) and streptavidin PE-conjugated c-kit specific biotinylated Ab (1 ìg/ml) and analyzed by flow cytometry.

compared to non-treated mast cells (figure 2c). Combining IL-4 and SCF demonstrated an additional increase in NK-1 receptor expression (figure 2d). After 12 days of co-culture, the percentage NK-1 receptor positive cells further increased (figure 3). No difference in fluorescence background at 12 days was observed (figure 3). Treatment for 12 days with IL-4 resulted in an increased number of positive mast cells compared to 6 days treatment (figure 3b). The percentage of positive mast cells after single SCF treatment was also increased at day 12 (figure 3c). IL-4/SCF co-cultured BMMC showed a percentage of 37.9 % positive cells for the expression of the NK-1 receptor (figure 3d).

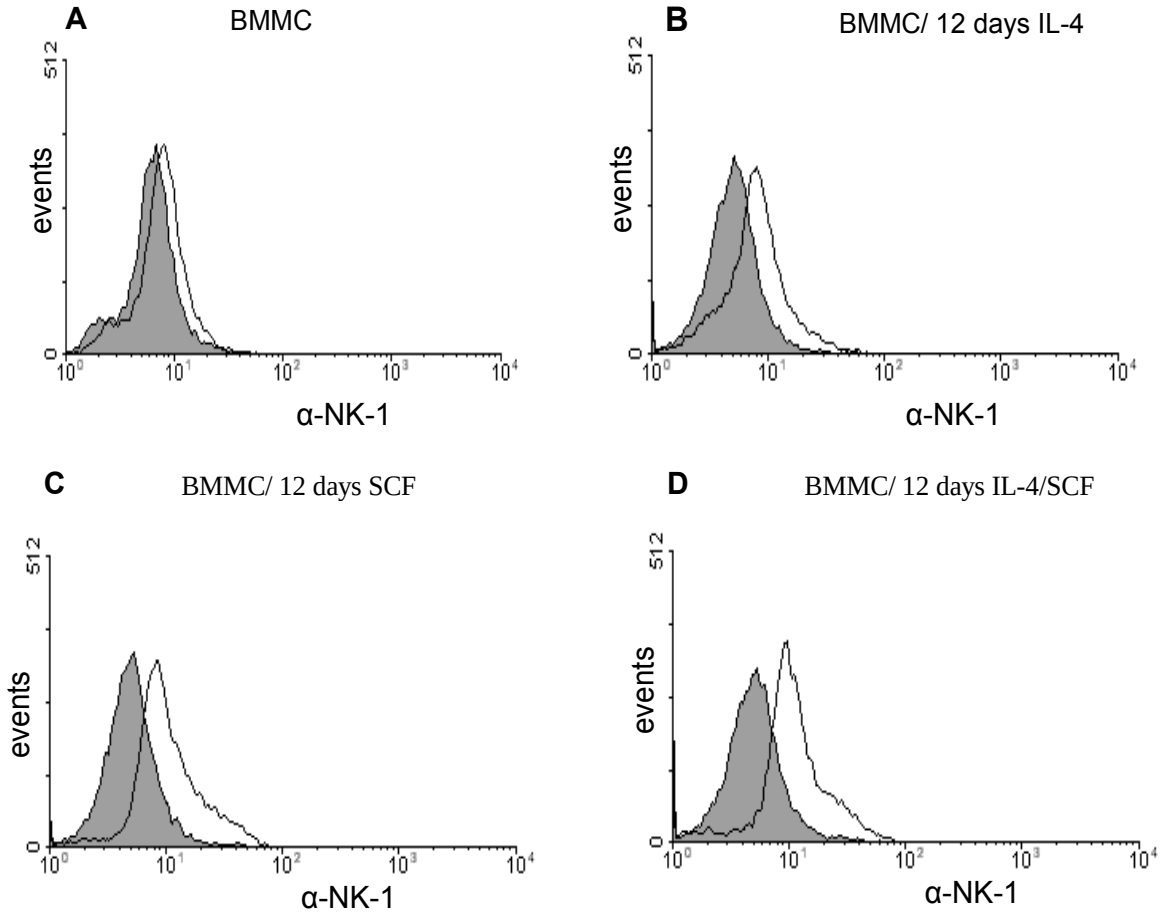

Figure 3. FACS analysis of NK-1 receptor$^+$-c-kit$^+$ cells in different culture conditions. Mouse bone marrow cells were cultured for 28 days in the presence of PWM-SCM (**A**) or for 16 days with PWM-SCM plus 12 days with IL-4 alone (300 U/ml)(**B**), SCF alone (50 ng/ml)(**C**) or a combination of IL-4 and SCF (**D**). Different cell populations were double stained with FITC-conjugated anti-NK-1 receptor (1:100) and streptavidin PE-conjugated c-kit specific biotinylated Ab (1 ìg/ml) and analyzed by flow cytometry. Isotype controls are represented by the shaded histograms. The clear histograms represent NK-1 receptor$^+$-c-kit$^+$

18 Days of IL-4 and SCF treatment did not further increase the percentage of positive mast cells (NK-1 receptor$^+$/c-kit$^+$ BMMC; 12 days IL-4/SCF: 37.9 % vs 18 days IL-4/SCF: 38.6 %, data not shown), indicating a plateau level. Taken together, these data suggest that IL-4 and SCF individually promote the expression of the NK-1 receptor on BMMC, but together their effects are synergistic. An overview of all the experiments, with their respective numbers of NK-1 receptor positive and NK-1 receptor/c-kit double positive cells is summarized in table 1.

	Day 6		Day 12	
BMMC	**NK_1-R^+/c kit^+**	**NK_1-R^+**	**NK_1-R^+/c-kit^+**	**NK_1-R^+**
untreated	8.1	9.1	11.4	13.6
IL-4	6.2	8.0	19.2	22.4
SCF	12.9	15.2	28.3	34.2
IL-4+SCF	16.0	18.9	37.8	45.4
NK1-R KO				
untreated	c-kit^+ 74.9	0		
IL-4+SCF	c-kit^+ 77.3	0		
RBL-2H3		18.5		

Table 1. Percentage of cells positive for the NK-1 receptor and cells double positive for the NK-1 and c-kit receptor. Different mast cell populations were stained with FITC-conjugated anti-NK-1 receptor (1:100) or double stained with FITC-conjugated anti-NK-1 receptor and streptavidin PE-conjugated c-kit specific Ab (1 μg/ml). Cells were analyzed by flow cytometry.

Substance P-induced mast cell activation.

In the next set of experiments, we studied whether the increased NK-1 receptor expression on BMMC was accompanied by enhanced sensitivity to stimulation by substance P.

IL-4/SCF co-culture increased substance P-induced mast cell activation.

BMMC showed only minor degranulation upon stimulation with substance P (figure 4). In comparison, BMMC co-cultured for 6 days with SCF and IL-4 showed significant dose-dependent degranulation upon substance P stimulation (figure 4). A significant increase in responsiveness was seen at all concentrations of substance P used to stimulate mast cells (10-200 μM). Moreover, continued co-culture with IL-4/SCF for 12 days increased the response to substance P significantly (figure 4). The increased responsiveness was seen at all substance P concentrations but was more marked in the lower concentration range.

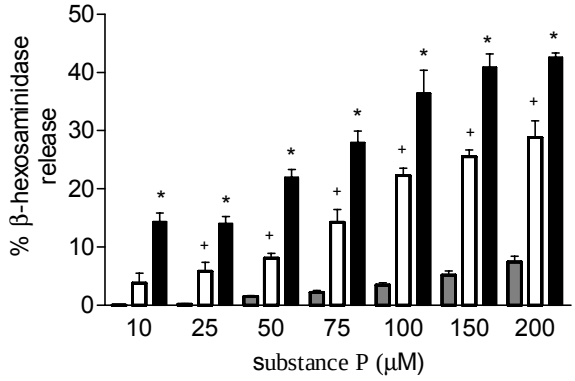

Figure 4. BMMC activation. Short term co-culture with IL-4 and SCF increases the responsiveness of BMMC to substance P. BMMC were cultured for 6 and 12 days in presence of IL-4/SCF. Grey bars represent non-treated BMMC, white bars represent IL-4/SCF co-cultured BMMC for 6 days and black bars represent BMMC co-cultured for 12 days. Results are expressed as mean ± SEM of quadruplicate samples. Significant differences ($p<0.05$) between the 6 days co-culture group and the non-treated group are denoted by (+) and by (*) between the 12 days co-culture group and both other groups.

NK-1 receptor antagonist RP67580 affects substance P-induced mast cell activation.

We addressed whether stimulation of BMMC was NK-1 receptor-dependent. Therefore, IL-4/SCF BMMC co-cultured for 12 days were incubated with RP67580, a specific NK-1 receptor antagonist, before activation with substance P. Blockade of the NK-1 receptor resulted in a significant decrease of substance P-induced β-hexosaminidase release at low concentration range (10 -75 μM, figure 5). Stimulation at higher concentrations of substance P (100-200 μM) was not significantly affected by RP67580 (figure 5). The inactive enantiomer RP65681, used as a control for specificity, did not affect the dose-dependent release of β-hexosaminidase (figure 5).

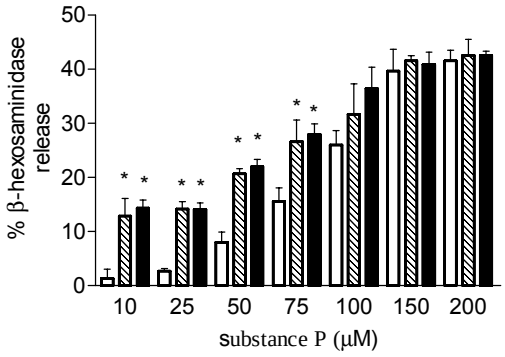

Figure 5. Effect of NK-1 receptor blockade on BMMC activation by substance P. All cells were co-cultured with IL-4 and SCF. Cells were pretreated with the NK-1 receptor antagonist RP67580 (10^{-3} M) or its inactive enantiomer RP65681(10^{-3} M) 10 min before activation with substance P (10-200 μM) for 30 min. White bars represent BMMC pretreated with RP67580, hatched bars represent BMMC pretreated with RP65681 and black bars BMMC not pretreated with an antagonist. Results are expressed as mean ± SEM of quadruplicate samples. Significant differences ($p<0.05$) compared to BMMC pretreated with RP67580 are denoted by (*).

NK-1 receptor knockout BMMC show a decreased response to low substance P concentrations.

In addition, BMMC were grown from NK-1 receptor knockout mice and were co-cultured with IL-4/SCF. At high concentrations (100-200 μM), the release of β-hexosaminidase by substance P was comparable in BMMC from BALB/c mice and BMMC grown from NK-1 receptor knockout mice (figure 6). However, NK-1 receptor knockout BMMC released significantly less β-hexosaminidase in the lower concentration range (0-75 μM) than did normal BMMC (figure 6). These results suggest that at lower concentrations of substance P, both a receptor-dependent and independent mechanism are involved in the induction of mast cell degranulation. At higher concentrations, the contribution of the receptor-dependent pathway seems to be insignificant.

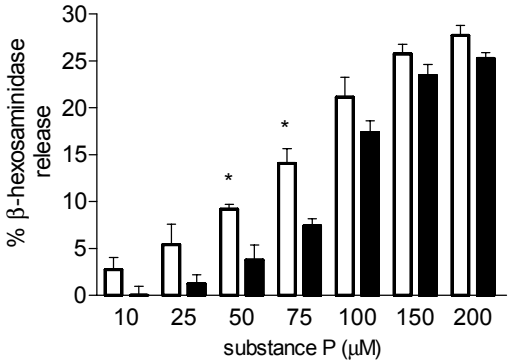

Figure 6. BMMC activation. BMMC were grown from NK-1 receptor knockout mice and were co-cultured with IL-4/SCF. Cells were incubated with different concentrations of substance P (10-200 mM) for 30 min and degranulation was assessed by the release of b-hexosaminidase. White bars represent BMMC grown from BALB/c mice and black bars represent BMMC grown from NK-1 receptor knockout mice. Results are expressed as mean ± SEM of quadruplicate samples. Significant differences ($p<0.05$) between groups are denoted by (*).

Discussion

The present study investigated functional expression of the NK-1 receptor on BMMC. Our study clearly shows increased time dependent NK-1 receptor expression on BMMC after co-culture with the cytokines IL-4 and SCF. In addition, we show that the expression of the NK-1 receptor is of functional relevance for the activation of mast cells.

In previous work, we have shown that BMMC gained sensitivity to substance P in the presence of IL-4 and SCF (28, 29). Tsai and coworkers (30) showed that SCF induces proliferation of mouse mast cells *in vitro* and *in vivo*. However, optimal murine mast cell proliferation and differentiation in response to SCF may require cofactors such

as IL-4 (14). IL-4 synergistically enhanced the proliferation of various mast cell lines in the presence of IL-3 or SCF (31-34). In the present study, IL-4 functioned as an efficient cofactor for SCF to induce the expression of NK-1 receptors on BMMC. A combination of IL-4 and SCF is sufficient to induce stable expression of the NK-1 receptor on primary cultured mast cells. However, in time IL-4 and SCF alone also induced a significant increase in expression of NK-1 receptors

Mast cells may exhibit enhanced expression of NK receptors in certain pathophysiological conditions. In line with our results, it has been shown that in murine peritoneal macrophages the level of expression of the NK-1 receptor can be increased following exposure to IL-4 (35). This suggests an increased expression of NK-1 receptors in inflammatory conditions. *In vivo*, several investigators have discussed the increased expression of the NK-1 receptor during inflammation. Mantyh and coworkers (36, 37) have shown that NK-1 receptors were significantly upregulated in inflamed tissues, on epithelium, blood vessels and in lymphoid accumulations. Pothoulakis and coworkers (38) provide evidence for increased NK-1 receptor expression in the intestinal epithelium shortly after exposure to Clostridium Toxin A. Furthermore, increased levels of BAL lymphocyte mRNA encoding NK-1 receptors were found in a murine model of immune inflammation in the lung (39). Upregulation of NK-1 receptors on cultured macrophages following exposure to lipopolysaccharide in rats and salmonella in mice has been reported (40). However, *in vivo* no studies have been reported on the increased expression of the NK-1 receptor on the mast cell.

SCF and IL-4 have been implicated to play an important role in the pathogenesis of inflammatory diseases including atopic dermatitis (41, 42) and asthma (43). We hypothesize that elevated levels of IL-4 and SCF in tissues undergoing inflammation could result in the enhanced expression of NK-1 receptors on inflammatory tissue resident cells, such as mast cells.

Substance P can induce histamine release from several types of mast cells such as rat peritoneal mast cells, dural mast cells (44), human skin mast cells (45) and mucosal mast cells (6). Since high concentrations of substance P (micromolar) are required to induce mast cell activation, and dependent on the positively charged N-terminal amino acid residues of the substance P molecule (46, 47), the mechanism of degranulation by substance P is believed to be the result of a direct G-protein activation rather than a specific receptor-mediated process (48). However, several studies support the hypothesis that neurokinins can activate mast cells via a specific receptor-dependent pathway. Ogawa and coworkers (5) showed on rat peritoneal mast cells that substance P stimulated NK-1 receptors to release histamine. This was significantly blocked by the NK-1 receptor antagonist CP96345. Okada and coworkers (49) demonstrated that peritoneal mast cells of rats express functional NK-1 receptors but this is, so far, the only report of NK-1 receptor expression on mast cells *in vitro*.

In this study, we show for the first time inducible expression on primary cultured mast cells. Co-culture of BMMC with SCF and IL-4 results in a time-dependent increased expression of the NK-1 receptor and leads to an increased sensitivity to degranulation by substance P. Several lines of evidence indicate that substance P can stimulate BMMC by a receptor-dependent route. First, an NK-1 receptor antagonist was able to partially block

mast cell activation by substance P. Secondly, BMMC cultured from NK-1 receptor knockout mice show significant exocytosis but only upon exposure to high concentrations of substance P. Therefore, the data presented in this study clearly show a receptor-dependent mast cell activation. However, since mast cell activation is not completely blocked, other pathways must also be involved. Furthermore, decreased exocytosis induced by inhibition or absence of the NK-1 receptor was only found at lower concentrations of substance P. Thus, at low (physiological) concentrations substance P induced degranulation in a receptor-dependent manner. However, at high concentrations substance P effected mast cell degranulation in a receptor-independent way since substance P-induced degranulation was not altered in the higher concentration range. Several mechanisms for substance P action on mast cells have been suggested. Previously in our laboratory, we showed that the IL-4/SCF-induced response to substance P was partially blocked by the G-protein inhibitors pertussis toxin and benzalkonium chloride (unpublished observations). Furthermore, substance P-induced activation of BMMC is blocked by specific tyrosine kinase and protein kinase C inhibitors (unpublished observations). These results delineate a pathway for substance P-induced secretion via G-protein(s), phospholipase C, calcium, and protein kinase C and are in accordance with data in the literature. For instance, Mousli and coworkers demonstrated the involvement of pertussis toxin-sensitive G proteins and phospholipase C as targets for substance P in rat peritoneal mast cells (50) and human skin mast cells (51), without requiring substance P specific membrane receptors.

In conclusion, the present study provides clear evidence for the inducible expression of functional NK-1 receptors on BMMC in response to co-culture with IL-4 and SCF. Increased expression of the NK-1 receptor is accompanied by enhanced sensitivity of the mast cells to stimulation by substance P. Our data suggest that under specific conditions *in vivo* such as those accompanying inflammation, mast cells could gain increased responsiveness to substance P, which may propagate and enhance neurogenic inflammatory responses. In turn, we suggest that NK-1 receptor antagonists may be partially effective in minor inflammatory conditions, but are less likely to be effective when inflammation is more severe.

Acknowledgements

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Direct neurite-mast cell communication in an *in vitro* co-culture model

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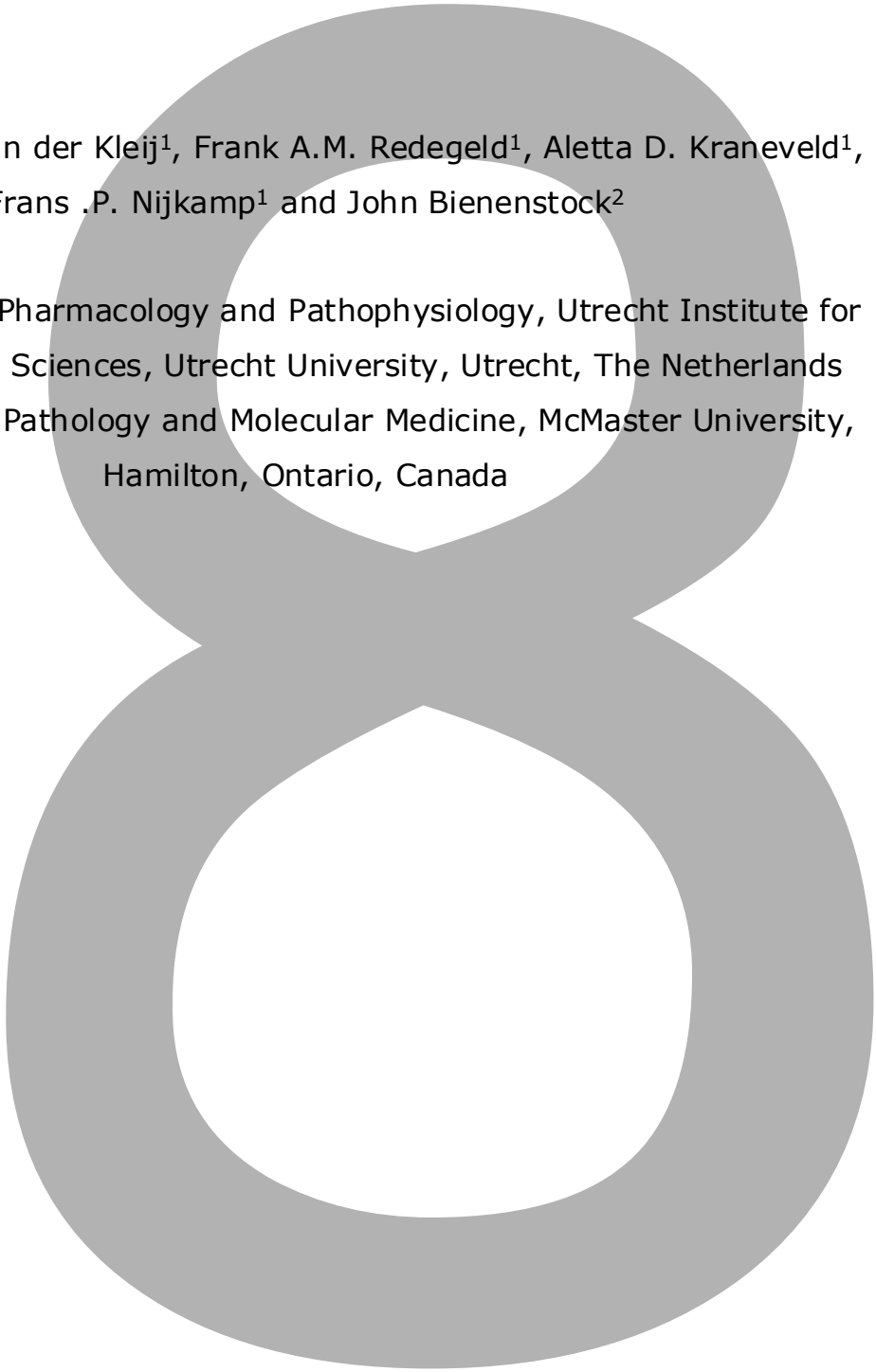

Abstract

Mast cells may play a role in neurogenic inflammation. Close proximity to nerves, mostly of sensory origin, allows direct communication between mast cells and the nervous system. By electron microscopy we showed that substance P *in vivo* is capable of inducing mast cell degranulation. However, it is not clear if this is a direct response. To date, very few studies report on co-cultures of mast cells and neurons. To study functional interactions between mast cells and peripheral nerves, a tissue culture model of murine neurons and mast cells, was developed. Bone marrow-derived mast cells (BMMC) and superior cervical ganglia (SCG) were co-cultured to study bidirectional interaction using a fluorescent calcium indicator as an index of cellular activation. Scorpion venom, a stimulus for SCG, did not have an effect on BMMC by itself. Scorpion venom caused an increase in neurite fluorescence within 10 sec after addition of the venom. Neurite activation is subsequently followed by activation of the mast cell approximately 5 sec after the signal appeared in the neurite, presumably by substance P release from the neurite. We proceeded to assess the role of substance P, the preferred ligand for the neurokinin 1 (NK-1) receptor, as the mediator responsible for the direct neurite-mast cell communication. Therefore, BMMC cultured from bone marrow of NK-1 receptor knockout mice, were studied for their response to neurite stimulation in the co-culture system. Preliminary results show that mast cells cultured from the NK-1 receptor knockout mice did not respond to neurite activation as elicited by scorpion venom. These data suggest an NK-1 receptor dependent process. We demonstrate that mast cell activation can occur as a direct response to the release of presumably substance P from neurites.

Introduction

Mast cells are often found in the proximity of nerve endings, and may be activated by a number of neuropeptides including tachykinins such as substance P and neurokinin A. It has been postulated that mast cells may play a role in neurogenic inflammation for example in asthmatic disease (1). Neurogenic inflammation involves changes in function of the sensory neurons due to inflammatory mediators which results in an enhanced release of tachykinins from the nerves (2). Indeed, mast cell mediators such as serotonin, histamine and nerve growth factor have been suggested to influence neuronal function (3, 4). Moreover, mediators released by nerves, such as acetylcholine and substance P can induce the activation of mast cells resulting in mediator release (4-7).

Close proximity to nerves, mostly of sensory origin, allows a bidirectional communication between mast cells and the nervous system (8, 9). A close anatomical relationship between mast cells and substance P-containing sensory nerve endings has been reported in various tissues including skin (10), intestine (11), dura mater (9, 12) and airway mucosa (10). Morphometric analysis has shown mast cells associated with nerves along their axial path, for example in the intestine (13, 14). Burnstock and coworkers have shown that cells within 200 nm of a nerve can be affected by an action potential moving down a nerve fiber. This lends more meaning to the likelihood of two-way communication.

Mast cell-nerve interaction has been shown in infected/inflamed tissue as well as in healthy tissue. Increased numbers of mast cells are seen in inflammatory or allergic conditions. Additionally, enhanced mast cell-nerve contacts appear in the infected/inflamed

areas. For instance, rats infected with nematodes have been shown to have more frequent mast cell –nerve associations in the intestinal mucosa than normal counterparts (11).

To date, very few studies report on co-cultures of mast cells and neurons. *In vivo* studies only revealed possible interactions between both cell types (15). Most *in vitro* studies demonstrate the effect of single mediators, released by either neurons or mast cells, on the other cell type (5, 16). To study functional interactions between mast cells and peripheral nerves, a tissue culture model of murine neurons and rat basophilic leukemia cells (RBL-2H3), was developed (17). Results from this study demonstrate direct communication between mast cells and superior cervical neurites in co-culture. In the present study, we examined the direct communication of co-cultured primary murine bone marrow-derived mast cells (BMMC) and neurite-sprouting murine superior cervical ganglia (SCG). BMMC and SCG were co-cultured to study direct mast cell-to-neuron communication measuring calcium-fluorophores as an index of cellular activation.

Methods

Animals. For the culture of BMMC, 6-8 weeks old male BALB/c mice were used. To grow SCG, 0-48 h old BALB/c mice were used. Mice were obtained from Harlan, Indianapolis, Indiana, USA. Neurokinin 1 receptor knockout mice (back-crossed to BALB/c) were a generous gift from Dr. N. Gerard, Harvard, Boston, USA

Electron microscopy. Mice were intranasally treated with substance P ($3x10^{-9}$mol) for 10 min. Ultrastructural changes in mast cells as a sign for mast cell activation was studied by performing electron microscopy of murine tracheal mast cells after exposure to substance P. Mice were killed with an overdose of pentobarbitone, the trachea was isolated and fixed in Karnovsky. Samples were cut and washed with osmium tetroxide, dehydrated and embedded on epoxy resin. Ultrathin sections were contrasted with aqueous uranyl acetate and Reynolds lead citrate and examined in an electron microscope.

Mouse bone marrow cultures. Bone marrow cells were isolated according to a standard protocol, as described in chapter 7 of this thesis. Cells were centrifuged and resuspended in fresh media every week to achieve a final concentration of $1\text{-}2x10^5$ cells/ml. After culturing the cells for 3-4 weeks, the BMMC were co-cultured for 6 days with SCF (50 ng/ml) plus IL-4 (300 U/ml). Cytokine-treated BMMC were centrifuged and resuspended in fresh medium plus IL-4 and SCF every three days to achieve a final concentration of $2x10^5$ cells/ml.

Nerve-mast cell co-culture. SCG were dissected from newborn (0-48 h old) BALB/c mice and rinsed in HBBS containing 10 mM HEPES (pH 7.4). Each ganglion was divided in 2 pieces and incubated for 60 minutes in at 37° C in 2 ml of HEPES containing 0.125% trypsin. The resultant cell suspension was plated at a density of $0.5\text{-}1x10^4$ nerve cells onto matrigel-coated 35-mm diameter glass dishes. The neurons were grown in RPMI medium enriched with 0.2 mM L-glutamine, 1% Pen/strep, 10% FBS,10 ng/ml nerve growth factor, and cytosine-β-d-arabinofuranoside (Ara-C, 10^{-6} M) to kill nonganglionic cells in the culture.

For co-culture, BMMC ($2x10^4$ cells/dish) were added to 48 h old cultures of SCG-neurites and incubated for another 72 h in the presence of IL-4 (300 U/ml) and SCF (50 ng/ml).

Cellular activation. After 72 h of co-culture, cells were loaded with Fluo-3-AM (2.5 μM, 30 min, 37ºC), and washed 3 times with HEPES buffer. Cells were examined using confocal microscopy to study calcium mobilization by activation of fluorophores as index of cellular activation (18, 19). A special computer program was developed capable of measuring the fluorescence of an indicated area during time. Neurite activation was evoked by scorpion venom.

Reagents. RPMI 1640, fetal calf serum and nonessential amino acids were purchased from GibCo BRL Life Technologies, Paisley Scotland. Penicillin, streptomycin, L-glutamine, sodium pyruvate, 2-mercaptoethanol, pokeweed, trypsin, scorpion venom were obtained from Sigma, St. Louis, MO, USA and lectin from Phytocalla Americana. Murine recombinant SCF came from PeproTech, London, England. Murine recombinant IL-4 was kindly provided by W.E. Paul NIH, USA. Matrigel was purchased at Becton Dickinson, Bedford, MA.

Results

1. Electron microscopy: the effect of substance P on mast cell *in vivo*

Substance P affects mast cells in vivo. Figure 1a shows an intact control mast cell from the trachea of a mouse. Figure 1b depicts a tracheal mast cell of a mouse after intranasal treatment with substance P. Substance P induced marked morphological changes to mast cells located in the mouse trachea. The plasma membrane is intact however, granular swelling and loss in electron density were observed as indicated with arrows.

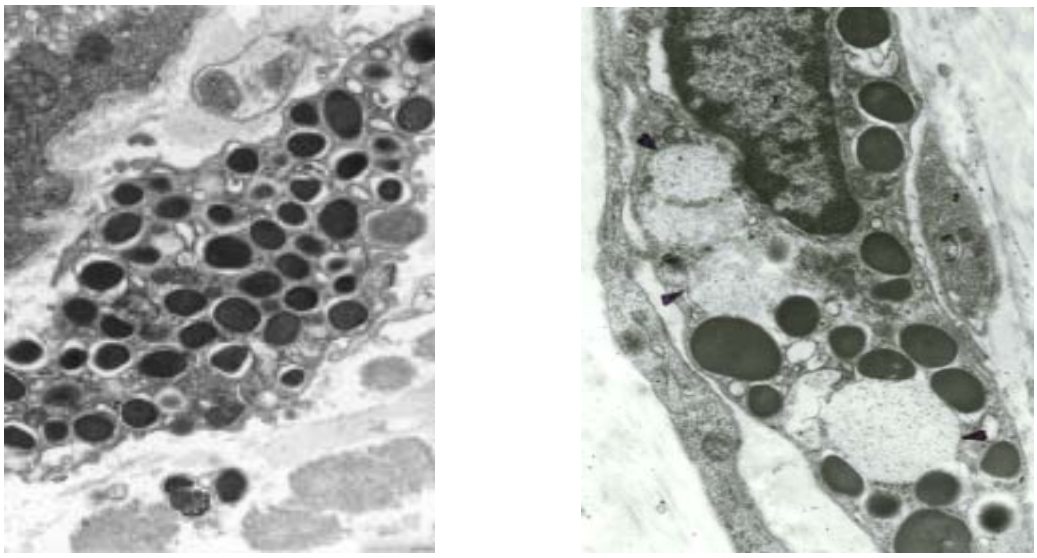

Figure 1. Substance P induces marked morphological changes in mast cells located in the mouse trachea. **A.** Control mast cell (x12740). Note the close proximity of the unmyelinated nerve to the mast cell membrane (arrow). **B.** Intranasal application of substance P ($3x10^{-9}$mol; 10 min)(x16100; trachea preparation). Plasma membrane is intact however, a high proportion of granules are less electron dense and/or oedematous.

2. Confocal laser scanning microscopy: BMMC-SCG co-culture

Previously, a tissue culture model of murine neurons and rat basophilic leukemia cells (RBL-2H3), was developed. This tissue culture system was used to study functional interaction between BMMC and sympathetic neurons. Unlike RBL-2H3 cells, BMMC do not adhere to the laminin coated wells. These primary mast cells normally grow in suspension. From the BMMC-neurite co-culture we can conclude that BMMC specifically adhere to neurites. Adhered cells, in contrast to non-adhered cells, were not removed from the culture after washing the dishes before conducting the experiments.

Scorpion venom selectively induces an increase in calcium mobilization in the neurite. Activation of the neurite was measured by calcium mobilization by activation of fluorophores using confocal fluorescence microscopy. Scorpion venom showed an almost instantaneous increase in neurite fluorescence, which was maximal within 10 sec after addition of the venom (figure 2a). The neurite activation peaked at 15 sec and returned to basal levels again at 30 sec after addition of the venom. In a BMMC culture scorpion venom did not have any effect meaning that scorpion venom exclusively affects the neurite *in vitro* (data not shown).

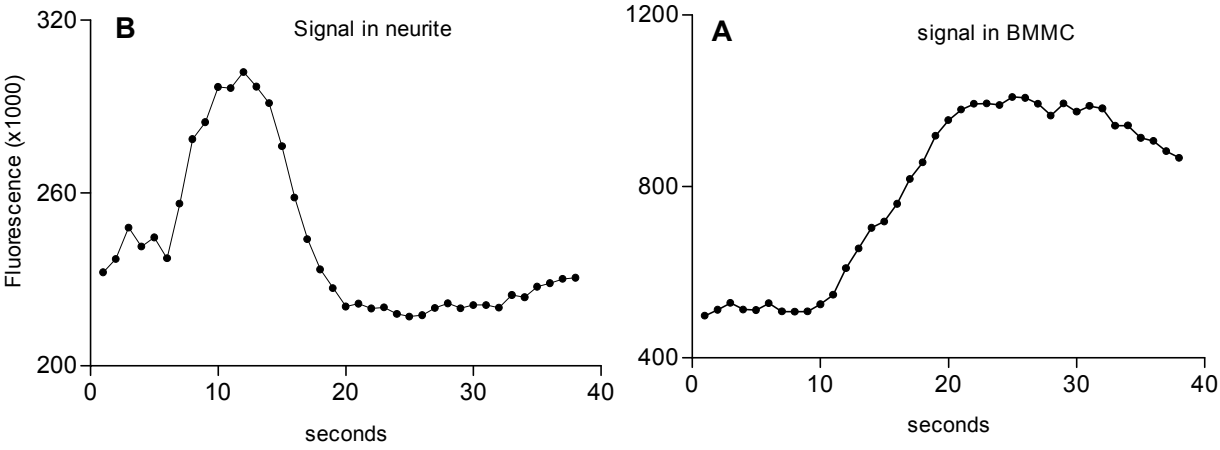

Figure 2. A representative trace illustrating the sequential increase in fluorescence evoked by scorpion venom (100 pg/ml) in BMMC-SCG co-cultures. **A**. Increase in fluorescence in neurite after addition of scorpion venom to the co-culture. **B.** Increase in fluorescence in the BMMC in response to the neurite activation.

Effect of neurite stimulation on BMMC calcium mobilization.

BMMC were co-cultured with SCG. Scorpion venom caused an increase in neurite fluorescence, maximal within 10 sec after addition of the venom (figure 2a and 3). The increase in neurite activation was followed by a rise in BMMC calcium mobilization (figure 2b and 3). Calcium levels in the BMMC slowly increased 10 sec after addition of scorpion venom and reached its maximum at 25 sec after addition of the venom to the co-culture. After 25 sec, calcium levels slowly decreased but remained elevated till the end of the experiment (40 sec)(figure 2b and 3). In all co-culture experiments examined, scorpion venom-evoked neurite activation preceded BMMC activation.

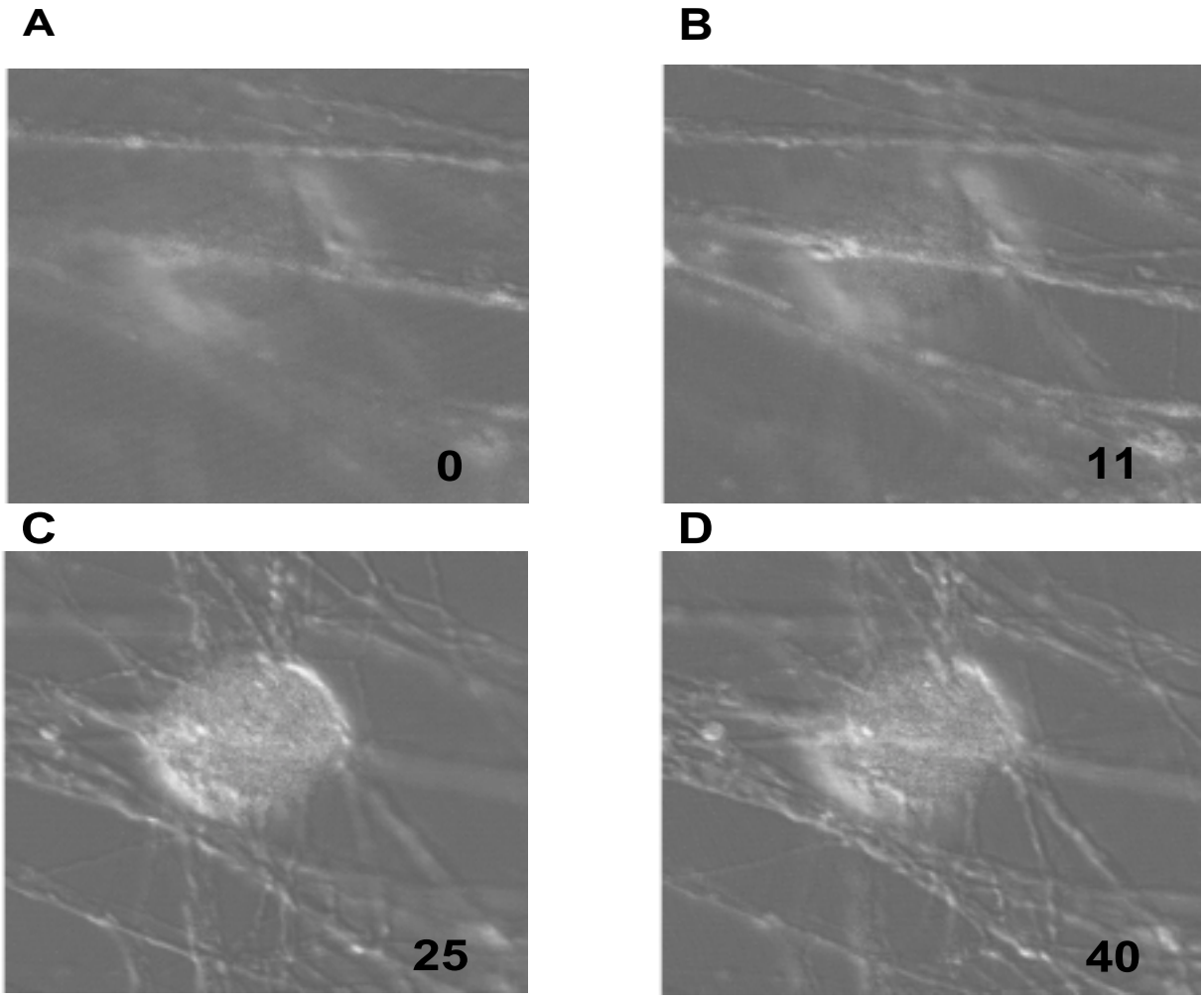

Figure 3. Fluorescence images of a BMMC-SCG co-culture in time. **A.** Basal fluorescence image of a co-culture. **B.** Fluorescence image showing the increase in the neurite (11 sec) evoked by scorpion venom (100 pg/ml). **C.** Image showing the sequential effect of neurite activation on the BMMC (25 sec after scorpion venom addition). **D.** 40 sec; end of the time course. Fluorescence is still elevated in the BMMC but is slowly returning to basal level.

Discussion

Considerable evidence exists for a consistent anatomical association between mast cells and nerves in tissues throughout the body (4, 20). Several studies *in vivo* show a functional and morphological relationship between mast cells and capsaicin-sensitive sensory neurons (8-10, 12). *In vivo* studies only demonstrated possible interactions between mast cells and tachykinins (15). In the present report, we show that substance P *in vivo*, at low dose, has profound stimulatory effects on pulmonary mast cells (figure 1). Electrical stimulation of nerves has been shown to either cause ultrastructural changes in associated mast cell granules or actual degranulation. Dimitriadou and colleagues (21) found that electrical nerve stimulation caused mast cell degranulation in the dura matter. Gottwald and coworkers (22) have presented morphological and histochemical evidence of mast cell activation in the gut following electrical stimulation *in vivo*. These data provide evidence for the functional importance of communication between nerves and mast cells. Yet, electrical stimulation is not always associated with mast cell activation (23, 24).

It proved to be very difficult to directly link substance P release to mast cell activation and release of bioactive mediators *in vivo*. Although several studies suggest direct communication between mast cells and neurons, they do not exclude the possibility of an intermediate cell facilitating this functional cross-talk. Using an *in vitro* co-culture system, we examined direct communication between mast cells and neurites. We have shown that scorpion venom stimulates the neurite followed by activation of the mast cell, possibly induced by substance P release from the neurite.

Previously, we have demonstrated that substance P caused mast cell degranulation at least partly via the NK-1 receptor (chapter 7). Enhanced expression of the NK-1 receptor was induced by short time treatment with IL-4 and SCF and resulted in a significantly increase in responsiveness to substance P. The enhanced sensitivity to substance P could be decreased by blockade or deficiency of the NK-1 receptor. In conclusion, substance P can stimulate BMMC by a receptor (NK-1) dependent route.

We proceeded to assess the role of substance P and its NK-1 receptor in neuron-to-mast cell communication in this tissue culture system. First, BMMC, which were not treated with IL-4 and SCF, were studied in our tissue culture model. Neurite activation did not show to affect these mast cells (preliminary results, data not shown). IL-4 and SCF treatment of BMMC, knowing to enhance the expression of NK-1 receptors on mast cells, therefore seems necessary to achieve communication from neuron-to mast cell. Secondly, BMMC cultured from NK-1 receptor knockout mice were studied for their response to neurite stimulation. In preliminary experiments, we demonstrated that mast cells cultured from the NK-1 receptor knockout mice (treated with IL-4 and SCF) did not respond to neurite activation as elicited by scorpion venom (data not shown). These data are in line with the data in chapter 7 of this thesis and suggest an NK-1 receptor dependent pathway. We have demonstrated that mast cell activation can occur as a direct response to the possible release of substance P from neurites.

Acknowlegements

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Summarizing discussion

1. Introduction

Mast cells are located in close proximity to neurons in the peripheral and central nervous system (1-4). In various studies, tissue mast cells invariably show ultrastructural evidence of activation even in normal healthy conditions, suggesting that these cells are constantly providing information to the nervous system. Neuronal mechanisms are involved in mast cell activation and mast cells act as principle transducers of information between peripheral nerves and local inflammatory events (5).

In virtually all tissues of the body, mast cells are closely associated with nerve fibers. Besides activation in normal healthy conditions, the mast cell-nerve communication pathway has been implicated as an important element in the pathology of various diseases including asthma (6). Mast cells lining the mucosal layer of the respiratory tract, have been found in close vicinity of substance P- and CGRP-immunoreactive nerves of rat trachea and peripheral lung tissue (3).

Asthma is characterized by a chonic inflammatory reaction in the airways. Roughly, asthma can be subdivided into atopic asthma involving elevated levels of serum IgE and a less familiar form, non-atopic asthma. Non-atopic asthma, accounting for 5% to 15% of all new cases of asthma (7), is an increasing problem in the developed world. Low molecular weight substances (<5000 Da) are the most common agents causing occupational asthma without producing specific IgE (7). Although the number of non-atopic asthmatics is increasing, the mechanisms involved in the induction and on-going respiratory impairments associated with non-atopic asthma are unknown and poorly investigated. From previous studies, it is recognized that mast cells and sensory neurons may play a role in non-atopic asthma (8-10). It is our aim to gain a deeper understanding of this immune-nerve interaction in non-atopic asthma. In this thesis mast cell and nerves were studied *in vitro* using primary cultured mast cells and neurons and *in vivo*, in a murine model for non-atopic asthma.

2. *In vivo* studies

Non-atopic asthma was induced in mice by skin-sensitization with the low molecular weight compound dinitrofluorobenzene (DNFB) followed by intranasal intra-airway challenge with a water-soluble dinitrobenzene derivate, dinitrobenzene sulphonic acid (DNS). Features of this pulmonary reaction included acute bronchoconstriction and mast cell activation shortly after challenge, and tracheal hyperreactivity, mononuclear and neutrophilic cell infiltration and an increase in mucosal exudation in the alveolar lumen 24 to 48 h after the challenge. This hypersensitivity reaction was not associated with an increase in hapten specific IgE (8, 11).

Mast cells

In the employed model for non-atopic asthms, the early phase reaction is associated with mast cell activation (chapter 2). mMCP-1 is present in the serum of DNFB-sensitized mice directly after challenge (chapter 2-5). Very recently, it is demonstrated that during sensitization mast cells are armed with hapten-specific immunoglobulin kappa light chains

(IgLC) (12). Upon challenge, the hapten will multivalent crosslink IgLC bound to the mast cell, causing activation and subsequent release of mediators starting the whole inflammatory response (chapter 2).

Thus far no direct proof for the involvement of mast cells in hapten-induced pulmonary hypersensitivity reaction in the mouse has been presented. In chapter 3, genetically mast cell-deficient and congenic normal mice were used to further establish the role of the mast cell in this murine model for non-atopic asthma. DNFB-sensitization was applied to mast cell-deficient *W/W^v* and *Sl/Sl^d* mice, their respective normal (+/+) littermate mice and mast cell-reconstituted *W/W^v* mice (BMMC→ *W/W^v)*. In BALB/c and +/+ mice, an early phase mast cell activation was found accompanied by bronchoconstriction and increased vascular permeability. The late phase was associated with mucosal exudation, infiltration of neutrophils in bronchoalveolar lavage fluid and tracheal hyperreactivity. Both mast cell-deficient strains failed to show any features of this hypersensitivity response. Mast cell reconstitution restored the acute bronchoconstriction, tracheal vascular hyperpermeability, BAL neutrophilia and tracheal hyperreactivity observed after DNFB sensitization and intranasal DNS challenge. These findings clearly demonstrate a key role for mast cells in the regulation of pulmonary hypersensitivity responses associated with non-atopic asthma.

Tumor necrosis factor alpha

TNF-α is a multifunctional proinflammatory cytokine that is produced and released by a variety of cells such as alveolar macrophages, fibroblasts, monocytes, endothelial cells, neutrophils and mast cells (13-17). TNF-α levels in BAL fluid of non-atopic mice were significantly increased shortly after challenge (chapter 5). This suggests that TNF-α is released by cells that contain prestored TNF-α leaving the mast cell as potential source (18). This hypothesis is confirmed by a another study performed in our laboratory (19) demonstrating that mast cell-deficient mice failed to show an increase in TNF-α BAL levels 30 minutes after challenge in the murine model presented in this thesis. These data point to an important role of mast cell-derived TNF-α in mice undergoing a non-atopic asthmatic reaction.

An important role for TNF-α in pulmonary hypersensitivity reactions is demonstrated in chapters 5 and 6. Intra-airway application of TNF-α to non-treated mice resulted in the infiltration of a significant number of neutrophils (chapter 6). Other animal models confirmed this role for TNF-α in the recruitment of neutrophils into inflammatory tissue (20, 21). However, TNF-α per se is a weak chemoattractant. The chemotactic effect of TNF-α is dependent on the induction of cell adhesion molecules and the release of chemokines (22, 23). Intra-airway application of TNF-α also results in the development of tracheal hyperreactivity (chapter 6). Neutrophil depletion prevented this induction. Furthermore, in the employed model, DNFB-sensitization and intra-airway DNS challenge causes neutrophil infiltration and tracheal hyperreactivity, both strongly reduced by neutralizing anti-TNF-α antibodies (chapter 5). Together, these results provide evidence of a relationship between the migration of neutrophils into BAL fluid and the development of tracheal hyperreactivity. Furthermore, TNF-α seems a crucial mediator released by mast cells after challenge.

Substance P

A role for substance P was reported in animal models for airway inflammation (24, 25). In the employed model for non-atopic asthma, blockade of the NK-1 receptor with a specific antagonist or the genetic absence of the NK-1 receptor prevented the development of tracheal hyperreactivity and cellular (neutrophil) accumulation in the mouse airways (chapter 2). Since mast cell activation was still observed in these mice, the role for the NK-1 receptor/substance P is downstream from mast cell activation. It is hypothesized that upon DNFB-sensitization and DNS challenge, mast cells will be activated and mast cell mediators such as TNF-α will affect NANC nerves to release their tachykinins. In turn, tachykinins will activate NK-1 receptors possibly on endothelial cells inducing vasodilatation, vascular exudation and upregulation of adhesion molecules leading to the infiltration of leukocytes, especially neutrophils. This cellular infiltration leads to the induction of tracheal hyperreactivity. Indeed, in chapter 6 it is demonstrated that TNF-α-induced tracheal hyperreactivity is diminished after neutrophil depletion. Furthermore, unpublished observations (van Houwelingen et al., submitted) also showed inhibition of tracheal hyperreactivity in the DNFB model after neutrophil depletion.

In chapter 6 it is shown that intra-airway application of substance P in non-treated mice results in the development of tracheal hyperreactivity. This suggests a causal relation between substance P and the induction of tracheal hyperreactivity. In contrast to the results in chapter 2, no neutrophil accumulation was detected in these mice. This suggests that substance P can induce tracheal hyperreactivity via different pathways depending on the local microenvironment in the tissue. For instance, injection of substance P in normal rat and mouse skin did not induce neutrophil infiltration (26), but in inflamed skin the NK-1 receptor was involved in mediating neutrophil accumulation (27). This could explain why neutrophil infiltration is found in DNFB-sensitized mice and is not significantly increased in animals treated with substance P intranasally.

We do not exclude a direct role of substance P on tracheal smooth muscle cells inducing tracheal hyperreactivity to carbachol. Substance P has a facilitatory role in the release of acetylcholine from cholinergic nerves in guinea pigs (28) and rabbits (29). In mice, electrical field stimulation induced a significant lower contraction of the trachea in NK-1 receptor knockout mice compared to wild type (30). This provides evidence for a direct role of the NK-1 receptor in the augmentation of cholinergic neurotransmission in the mouse trachea.

3. *In vitro* mast cell studies

NK-1 receptor expression

Mast cells are ubiquitous in the body and often found close to neurons. They can be activated by a number of neuropeptides, including tachykinins such as substance P. There are different opinions about the pathways involved in mast cell activation by non-immunological stimuli like neuropeptides. Although it has been suggested that functional NK-1 receptors are expressed on mast cells (31-33), most investigators support non-receptor mediated activation induced via direct interaction with pertussis toxin-sensitive

G proteins though the N-terminal domain located in the inner surface of the plasma membrane (34, 35).

In vivo, several investigators have discussed the increased expression of the NK-1 receptor in inflamed tissue (36-38). Therefore, it can be proposed that NK-1 receptor expression on immune cells such as mast cells is influenced by environmental inflammatory factors such as cytokines. In previous work, Karimi et al (39) demonstrated the increased sensitivity of BMMC to substance P, the preferred ligand for the NK-1 receptor, after a short co-culture with the cytokines IL-4 and SCF. In other studies, it is shown that elevated levels of SCF and IL-4 both have been implicated to play a role in the pathogenesis of inflammatory diseases including asthma (40). Indeed, NK-1 receptors are significantly expressed on BMMC shortly co-cultured with IL-4 and/or SCF in contrast to non-treated BMMC that barely express NK-1 receptors (chapter 7). Although IL-4 by itself can promote the expression of the NK-1 receptor on BMMC, it mainly functioned as an efficient cofactor for SCF to induce the expression of NK-1 receptors on BMMC. This is in line with literature data demonstrating that optimal murine mast cell proliferation and differentiation in response to SCF may require cofactors such as IL-4 (41-43). The increase in NK-1 receptor expression induced by IL-4 and SCF is time dependent. The number of positive cells increases with the length of co-culture although a plateau level is reached.

The traditional classification of mast cells into two main subsets, CTMC and MMC, based on different phenotypical and functional characteristics is unsufficient (44). Mast cells are highly influenced by their environment resulting in functionally different subtypes. *In vivo* and *in vitro* studies have shown that mast cells can fully transform their phenotype, if transplanted into a different environment (45, 46). Indeed, a short-term culture of BMMC with IL-4 and SCF resulted in a mast cell population with an altered phenotype (47). The cell environment has been suggested as a critical regulatory factor in the responsiveness of mast cells to basic secretagogues (48). The data in chapter 7 show that mast cells increase their responsiveness to substance P by an increased expression of NK-1 receptors.

Nerve-mast cell interaction *in vitro*

The effect of a single mediator, substance P, on mast cells was investigated in chapter 7. *In vitro*, substance P is able to activate mast cells. Furthermore, by electron microscopy we were able to show that substance P *in vivo* is capable of inducing mast cell activation (chapter 8). To directly link substance P release to mast cell activation, the direct communication of BMMC and neurite-sprouting SCG was examined. It is shown that mast cell activation can occur as a direct response to the possible release of substance P from neurites (chapter 8). In general, relatively high concentration of substance P ($>10^{-5}$ M) are required to induce mast cells degranulation *in vitro*. Researchers have argued that these concentrations are not physiological relevant. However, mast cells and neurites are closely related in the body. It is postulated that under inflammatory conditions given the close contact, it is possible that such concentrations of neuropeptides can be obtained locally within the tissue. In chapter 8, in an *in vitro* experimental setup, we provide clear evidence for that mast cell degranulation can occur as a response to neurite activation.

Priming

Besides direct activation, there is the concept of priming. Priming appears to be a broadly based biological process and has been reported in several cell types. Mast cells have been reported to be primed by different cytokine growth factors for activation by different agonists (49). SCF, for instance, can act as a priming agent in some circumstances (50, 51). In this thesis, we show that SCF and IL-4 prime BMMC to induce increased responsiveness to substance P. Mast cells can also be primed by substance P itself. Although relatively high levels of substance P are to directly induce mast cell degranulation ($>10^{-5}$M), it has been demonstrated that repeated doses of very low concentrations (picomolar) of substance P can induce mast cell degranulation (52). In other words, very low concentrations of substance P showed to be able to prime mast cells and lower their thesholds to subsequent activation (52). The concept of priming also applies to neurons. TNF-α may exert a priming, rather than a direct stimulatory effect on sensory activity (19, 53).

Mast cell activation versus mast cell degranulation

Exocytosis is the most obvious event associated with secretion of the mediator molecules contained in granules. However, secretion can occur without evidence of degranulation, and even molecules stored within the same granules can be released and secreted in a discriminatory pattern.

Serotonin can be released separately from histamine (54). Differential synthesis and release of arachidonic acid metabolites, prostaglandins and leukotrienes have been reported (55). Ultrastructurally, a study by Ratliff et al (56) showed mast cells in close proximity to unmyelinated nerve fibers. These mast cells contained granules showing ultrastructural features of activation or piecemeal degranulation, which have been associated with differential secretion.

In the present thesis, mast cell degranulation was measured *in vivo* via mMCP-1 release and *in vitro* by the release of β-hexosaminidase. Degranulation might be not the best marker to monitor mast cell activation but in most cases it is the best available tool.

The observation that mediator release can occur without degranulation is very interesting, especially in situations where mast cell degranulation was not found and mast cells were likely to be involved. In chapter 6, mice received substance P and TNF-α on two consecutive days. Both substance P and TNF-α can cause degranulation of mast cells. However, no increase in mMCP-1 release was found in these animals (data not shown). Marshall and coworkers showed that low doses of substance P can cause synthesis and secretion of TNF-α from mast cells, in the absence of degranulation (57). Therefore, TNF-α and substance P both could have affected mast cells in this study.

Enhancement mast cell-nerve interaction

Mast cell mediators can cause the release of tachykinins from sensory nerve endings (6, 58, 59). The release of neurotransmitters may exacerbate the inflammatory response. Neurogenic inflammation has been documented in a number of inflammatory diseases including asthma. The mast cell-nerve communication pathway has been implicated as an important element in neurogenic inflammation.

Mast cells and nerve are in constant contact with each other. An enhanced interaction can lead to neurogenic inflammation. Inflammatory models have shown a significant increase in the number of mast cells resulting in the increased release of inflammatory mediators upon degranulation. This could lead to an enhancement of the mast cell-nerve association.

Inflammatory mediators may modulate sensory nerves in the airways through the activation of receptors on nerve terminals. NANC nerve endings express receptors for histamine (H_1 and H_3) and serotonin (5-HT_{2A}) (60-62). Under inflammatory-like conditions, it has been shown that primary NANC nerves show an upregulation of at least histamine H_1 receptor expression (63). A recent report by Shubabey and Myers (64) provide evidence of TNF-α receptor expression TNFR1 and TNFR2 in DRG neutrons in adult rat. Both receptor subtypes were upregulated in DRG neurons during inflammation. Capsaicin-sensitive nerves can be altered in this way that could result in an increased release of neuropeptides. Allergen/hapten challenge can also lead to substance P production in a subset of sensory nerve fibers that are typically devoid of neuropeptides. In other words, allergen/hapten challenge leads to a phenotypic switch in the sensory neuropeptide innervation in the airways probably via mast cell activation (65, 66) again increasing the interaction between mast cells and substance P-immunoreactive nerves. Thus, mast cell activation is able to increase the excitability of sensory nerves, the neuropeptide production and secretion.

In summary, the mast cell-nerve communication may amplify and spread the inflammatory response that may contribute to the pathogenesis of airway diseases.

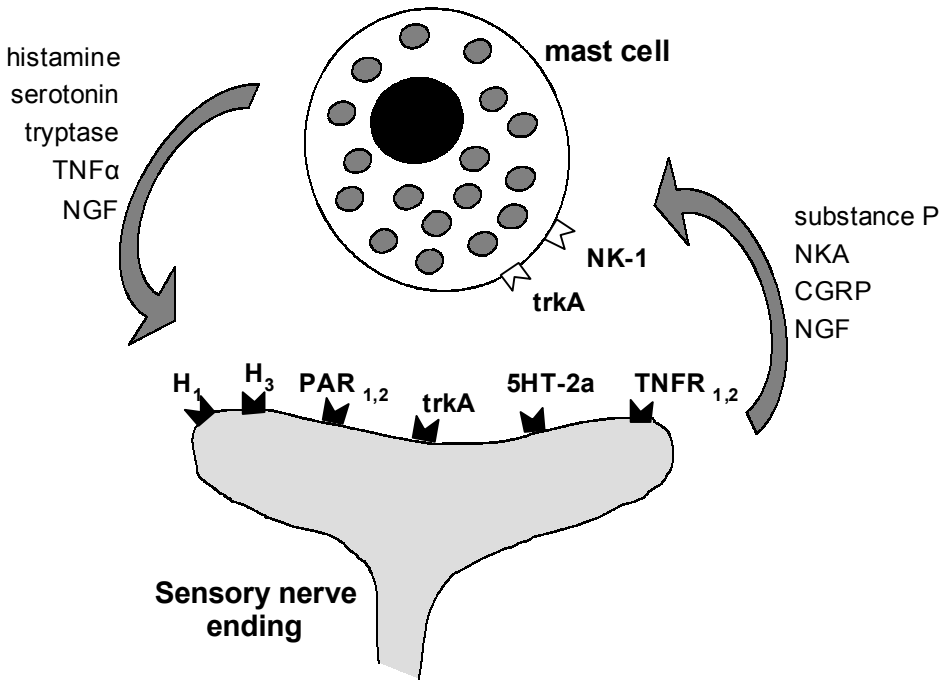

Figure 1: Mast cell-nerve interactions. Inflammatory mediators may modulate sensory nerves in the airways through the activation of receptors on nerve terminals. Neuropeptides can stimulate mast cells via a receptor-dependent and a receptor-independent mechanism. Under inflammatory-like conditions, receptor expression on nerve endings and on mast cells can be upregulated.

Sensory nerves in human airways

Human airways are found to be less densely innervated with substance P-immunoreactive nerves compared to rodent airways (67). However, the pattern of innervation might change during inflammation. A striking increase in substance P-immunoreactive nerves was reported in the airways of patients with fatal asthma (68). Several human studies suggest that tachykinins are present in increased amount in asthmatic airways. (68-70). In addition, tachykinin levels have shown to be elevated during exacerbations of asthma (71). Although human aiwarys are less densely innervated with substance P-immunoreactive nerves, it is clear that tachykinins are involved in the pathogenisis of asthma.

Immune cells as a source of substance P

Although classically neuropeptides are released from autonomic and sensory nerves, there is increasing evidence that they may also be synthesized and released from inflammatory cells, particularly in disease. Immune cells could represent an additional source of tachykinins in inflamed tissues, providing a non-neurogenic tachykininergic contribution to the local inflammatory process (72). Non-neuronal cells of the immune system such as monocytes, macrophages, T lymphocytes and eosinophils have shown to produce endogenous substance P (73-76). This alternative source of substance P, although not further addressed in this thesis, could be involved in the development of both atopic and non-atopic asthma.

Nerve-immune interactions

In this thesis, we mainly discussed nerve-mast cell communication and its relation to asthma. It should be mentioned that similar pathways may, and likely do exist in which other cells of the immune system may have analogous modes of interaction. Besides mast cells, neural contact can also occur between nerves and for instance eosinophils or plasma cells (4, 77). In addition, neuropeptides from sensory nerves can directly modulate the function of langerhans cells. Among these neuropeptides, the tachykinins have been shown to modulate immune functions such as cytokine production and cell proliferation (78, 79).

4. *In vivo* versus *in vitro*

The present thesis describes both *in vivo* and *in vitro* studies. *In vitro* findings can be used to clarify *in vivo* observations and visa versa. However, *in vivo* and *in vitro* observations may not always be complementary.

In this thesis, the most striking difference between the *in vivo* and *in vitro* observations is the role of the NK-1 receptor in mast cell activation. The studies described in chapter 2 show suggest that the NK receptors were not involved since NK-receptor antagonists were not able to block mast cell activation. However, chapter 7 provides clear evidence for the inducible expression of NK-1 receptors on BMMC in response to IL-4 and SCF. Additionally, this NK-1 receptor expression is accompanied by enhanced sensitivity/activation of the mast cell to stimulation by substance P. Furthermore, *in vitro* mast cell activation could be blocked partially by an NK-1 receptor antagonist. The *in vitro* data prompted the hypothesis that proinflammatory conditions could be sufficient to induce NK-1 receptor expression on mast cells *in vivo*. The *in vivo* data, however, do not support this hypothesis. There are several possible explanations for this.

Mast cell activation was determined by measuring mMCP-1 in serum and is released by mast cells throughout the whole body. DNS challenge was applied intranasally and changes in mast cell activation were expected to occur predominantly in the airway. NK-1 receptor expression, therefore, might be induced only locally in the inflamed area. It is possible that the mMCP-1 release from the airway mast cells may not be sufficient to significantly increase serum mMCP-1. This implies that NK receptors may be involved in the mast cell activation described in chapter 2 of this thesis, but it was not observed due to a lack of sensitivity in the assay method used.

On the other hand, the NK-1 receptor antagonist could have inhibited the release of mediators other than mMCP-1 from mast cells. Low doses of substance P can cause the secretion of TNF-α from mast cells (57), which may have been prevented by the NK-1 receptor antagonist.

Alternatively, mast cells can exist in various phenotypic states and therefore differ in their response to non-immunological stimuli such as substance P. It is possible that phenotypic heterogeneity between the mast cells present in the airways of DNFB-sensitized, DNS challenged mice and the primary cultured BMMC explains the differences in mast cell responsiveness to substance P. NK-1 receptor expression can be upregulated in inflamed conditions when levels of IL-4 and SCF are elevated. It is possible that IL-4 and SCF levels are not increased in the animal model used for the experiments described in chapter 2.

It is also likely that there are more pathways involved in the *in vivo* studies in chapter 2. Possibly, DNS challenge does not just activate mast cells. For instance, DNS might cause the release of inflammatory mediators from other immune cells or the direct release of neuropeptides other than only substance P from sensory nerve endings. In this way mast cells can also be activated by other stimuli that do not activate the cell via the NK-1 receptor. Therefore, it is postulated that in the employed model, the NK-1 receptor is crucial but parallel to or downstream from mast cell activation.

Summarizing, the increased expression of NK-1 receptors on mast cells, leading to an increased sensitivity to substance P, is an interesting *in vitro* observation that may be very relevant in inflammatory conditions. A more sensitive detection method for determining local mast cell activation *in vivo* is necessary to resolve the discrepancy between in the *in vivo* and *in vitro* observations.

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Samenvatting

Mestcellen en sensorische zenuwuiteinden bevinden zich dicht bij elkaar in het lichaam. Er is echter nog weinig bekend over de communicatie die tussen deze twee deze celtypen plaatsvindt. In dit proefschrift is gekeken naar de rol van mestcellen en zenuwen en hun interactie bij de ontwikkeling van astma.

Astma

Astma is een longaandoening die gepaard gaat met een chronische ontsteking. Dit leidt tot overgevoeligheid voor specifieke en niet-specifieke prikkels (zoals koude lucht en tabaksrook). Astmapatiënten zijn grofweg onder te verdelen in twee groepen. Bij de eerste groep is astma geassocieerd met atopie of allergie. De tweede groep patiënten is niet-atopisch of niet-allergisch. De aan- of afwezigheid van allergeen-specifieke antistoffen (Immunoglobuline E: IgE) in het bloed is het belangrijkste criterium van verschil tussen de twee groepen.

In dit proefschrift is met name gekeken naar de niet-atopische vorm van astma. Van verbindingen als isocyanaten (verfmiddelen), persulfaat (kappersmiddelen) en houtstof is bekend dat zij astma kunnen veroorzaken via een IgE-onafhankelijk mechanisme. Deze verbindingen (zogenaamde haptenen) hebben een laag molecuulgewicht en zijn daardoor te klein om zelf allergeen te zijn. Door binding aan lichaamseigen eiwitten onstaat een complex dat een immuunreactie van het afweersysteem teweegbrengt en in staat is astma te veroorzaken.

Muismodel voor niet-atopisch astma

In ons laboratorium is een muismodel voor astma ontwikkeld op basis van de laag moleculaire verbinding dinitrofluorobenzeen (DNFB). Via een eerste toediening op de huid (sensibilisatie) en een tweede toediening via de longen (challenge) wordt een ontstekingsreactie in de luchtwegen uitgelokt, die veel overeenkomsten vertoont met de situatie in de mens.

De mestcel in het muismodel voor niet-atopisch astma

Mestcellen bevinden zich in alle weefsels van het lichaam en bevatten een groot aantal biologisch actieve stoffen waaronder histamine, serotonine en tumor necrosis factor alpha (TNF-α). In het muismodel vervult de mestcel een centrale rol. Gespecialiseerde witte bloedcellen, de B cellen, produceren vrije lichte ketens nadat het lichaam in aanraking is gekomen met het hapteen (in het model is dit DNFB). Dit proces wordt ook wel sensibilisatie genoemd. Deze vrije lichte ketens hechten aan een specifieke receptor op het celoppervlak van de mestcel. Na een tweede contact (de challenge) wordt het hapteen herkend door de lichte ketens, die gebonden zijn aan de mestcel. De mestcel wordt dan geactiveerd waardoor stoffen worden vrijgezet, die de bloedvaten verwijden (zie figuur 1). Deze biologisch actieve mestcelmediatoren veroorzaken allereerst een vroege reactie en zetten mede een cascade

van reacties in werking die uiteindelijk leiden tot een late ontstekingsreactie 24 tot 48 uur na het tweede contact met het hapteen.

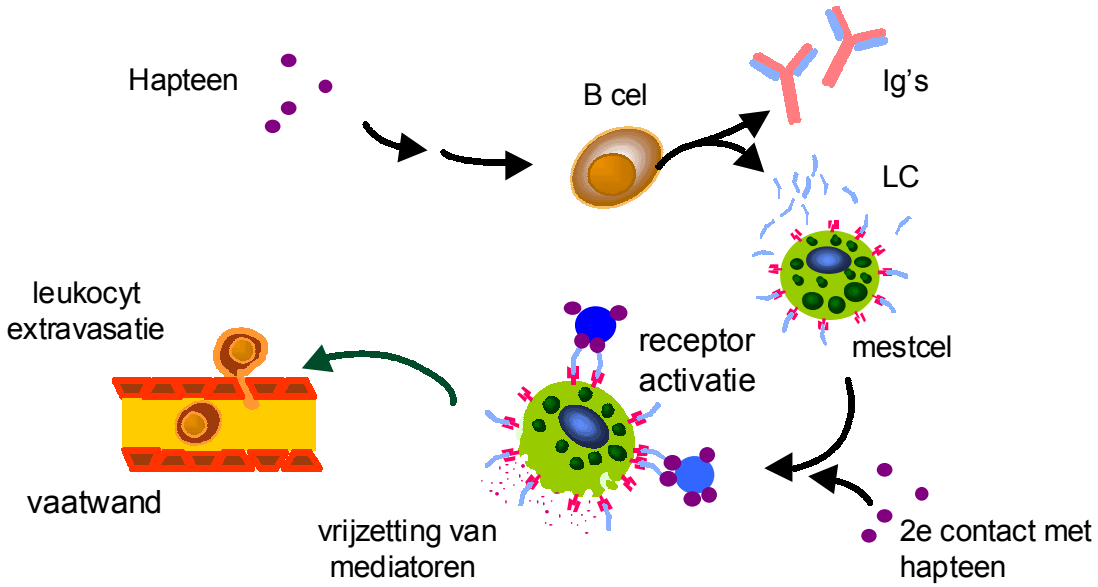

Figure 1: Mestcelactivatie via Ig lichte ketens

In hoofdstuk 2 zijn de reacties op een rij gezet die optreden in het dier na sensibilisatie en challenge met DNFB. Een niet-specifieke hyperreactiviteit (overgevoeligheid) van de trachea (luchtpijp) en een ontsteking van de luchtwegen worden waargenomen. Het laatst genoemde wordt gekarakteriseerd door een toename van het aantal ontstekingscellen, met name neutrofiele granulocyten.

Vervolgens is gekeken naar deze overgevoeligheidsreacties in mestcel-deficiënte muizen (deze muizen hebben van nature geen mestcellen). Hoofdstuk 3 laat zien dat in mestcel-deficiënte muizen geen luchtwegovergevoeligheidsreacties te meten zijn. Door het inspuiten van mestcellen in deze muizen keert deze overgevoeligheid weer terug. Samenvattend wordt in hoofstuk 3 aangetoond dat de mestcel een centrale rol speelt in ontwikkeling van niet-atopisch astma.

Tumor necrosis factor alpha (TNF-α)

TNF-α is een ontstekingscytokine dat geproduceerd en vrijgezet wordt door verschillende cellen, waaronder de mestcel. TNF-α spiegels zijn verhoogd in de longspoeling van niet-atopische muizen kort na challenge (hoofdstuk 5) en dit TNF-α is zeer waarschijnlijk afkomstig van de mestcel. Op verschillende manieren is aangetoond dat TNF-α een belangrijke mediator is, die betrokken is bij de pathogenese van astma. Allereerst leidt rechtstreekse toediening van TNF-α via de neus in de luchtwegen van muizen, tot infiltratie van neutrofielen en de ontwikkeling van tracheale hyperreactiviteit (hoofdstuk 6). Daarnaast is gebleken dat toediening van antilichamen tegen TNF-α aan muizen behandeld met DNFB, de ontwikkeling van astmatische karakteristieken sterk kan reduceren. Deze twee studies geven duidelijk aan dat TNF-α een cruciale mediator is, die betrokken is bij de pathogenese van astma.

Substance P

Sensorische zenuwen geven signalen van de luchtwegen door aan de hersenen. Een bepaald soort sensorische zenuwen brengen specifieke neuropeptiden tot expressie, de tachykininen. Substance P is een belangrijk tachykinine waaraan in dit proefschrift aandacht is besteed. Substance P werkt voornamelijk via de activatie van de neurokinine 1 receptor (NK-1 receptor). Tachykininen kunnen direct luchtwegovergevoeligheid veroorzaken (hoofdstuk 6) of daaraan bijdragen via een indirect mechanisme bijvoorbeeld door de vaatdoorlaatbaarheid te verhogen. Hoofdstuk 4 laat zien dat blokkade dan wel genetische afwezigheid van de NK-1 receptor de ontwikkeling van tracheale hyperreactiviteit en de infiltratie van neutrofielen in de luchtwegen remt. Verder wordt aangetoond dat tachykininen receptoren niet betrokken zijn bij het activeren van mestcellen.

Na DNFB sensibilisatie en challenge worden mestcellen geactiveerd en mestcelmediatoren, zoals TNF-α, zouden mogelijk sensorische zenuwen kunnen prikkelen tot de vrijzetting van tachykininen (substance P). Substance P activeert vervolgens NK-1 receptoren die onder andere aanwezig zijn op endotheelcellen. Endotheelcellen vormen de bekleding van de bloedvaten. Door het activeren van de NK-1 receptor wordt een cascade van reacties in gang gezet. Uiteindelijk ontstaan er zowel gaatjes in als tussen endotheelcellen waardoor plasma zich kan verplaatsen richting het weefsel (vasculaire oedeemvorming) en ontstekingscellen, zoals neutrofielen, makkelijker infiltreren in het weefsel. Ook verhogen substance P en TNF-α de aanwezigheid van adhesiemoleculen waardoor de infiltratie van neutrofielen wordt versterkt. Neutrofielen spelen een belangrijke rol bij de inductie van tracheale hyperreactiviteit. Wanneer je deze cellen uit het lichaam verwijdert, leidt dit tot een duidelijke afname in hyperreactiviteit (hoofdstuk 6).

NK-1 receptor expressie *in vitro*

Substance P kan mestcellen activeren. Door mestcellen kort in kweek te brengen met de cytokines interleukine 4 (IL-4) en stamcelfactor (SCF) wordt hun gevoeligheid voor substance P verhoogd. Verschillende onderzoekers hebben laten zien dat substance P direct aan kan grijpen op G-eiwitten, die zich bevinden aan de binnenkant van de celmembraan en op deze manier stimulatie van de cel teweeg kan brengen zonder tussenkomst van een receptor. In hoofdstuk 7 wordt echter beschreven, dat onder invloed van IL-4 en SCF de expressie van de NK-1 receptor op de mestcel toeneemt. Het aantal NK-1 positieve cellen neemt toe met de tijdsduur van de cocultuur. De verhoogde gevoeligheid van de mestcel voor substance P zou dus een gevolg zijn van de toename in NK-1 receptoren. Blokkade van de NK-1 receptor of genetische afwezigheid van de NK-1 receptor leidt tot een lagere gevoeligheid van de mestcel voor substance P. Deze observatie versterkt de bewering dat de NK-1 receptor van belang is bij het activeren van de mestcel door substance P.

Interactie *in vitro:* van zenuw naar mestcel

Met behulp van electronenmicroscopie is aangetoond dat substance P ook *in vivo* in staat is om mestcellen te activeren (hoofdstuk 8). Mestcellen en neuronen zijn vervolgens samen gekweekt om hun directe interactie te onderzoeken. Na korte tijd samen in kweek te zijn (± 2 dagen), hechten de twee aan elkaar. Wanneer de neuron wordt gestimuleerd tot de afgifte van zijn neuropeptiden, waaronder substance P, reageert de mestcel hierop door

ook zijn mediatoren vrij te zetten (hoofdstuk 8). Dit bewijst een directe interactie van de neuron naar de mestcel zonder de tussenkomst van een andere cel of ander mechanisme. Een belangrijk gegeven is dat de mestcel niet geactiveerd wordt wanneer deze niet in contact is geweest met de cytokines IL-4 en SCF. Ook vindt er geen activatie plaats in mestcellen die geen NK-1 receptor bezitten. Ook uit deze studie blijkt dat de NK-1 receptor belangrijk is bij de interactie tussen mestcellen en sensorische zenuwen.

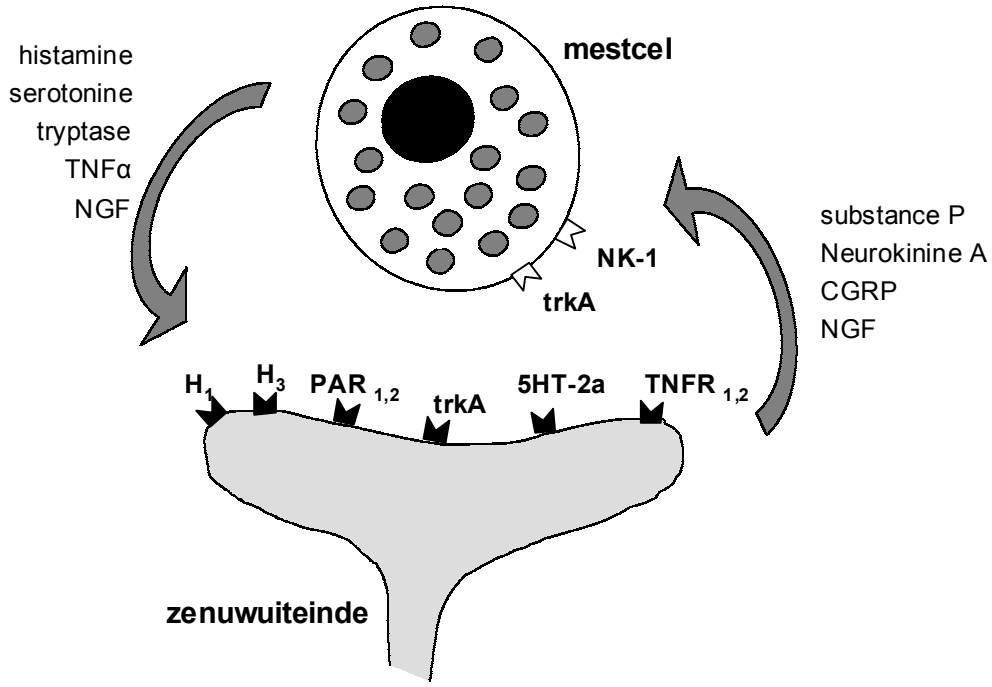

Figure 2: Interactie tussen de mestcel en de sensorische zenuw. Ontstekingsmediatoren uit de mestcel kunnen sensorische zenuwen stimuleren via de activatie van receptoren op de zenuwuiteinden. Neuropeptiden vrijgezet door sensorische zenuwen kunnen mestcellen activeren via een receptor-onafhankelijk en een receptor-afhankelijk mechanisme. Tijdens ontsteking kan het aantal receptoren op mestcellen en sensorische zenuwen toenemen waardoor de interactie tussen de twee wordt versterkt.

Conclusie

Samengevat is in dit proefschrift uiteengezet dat de mestcel en de sensorische zenuwen beide een belangrijke rol spelen in de pathogenese van niet-atopisch astma. TNF-α is een belangrijke mestcelmediator gebleken, die betrokken bij de ontwikkeling van niet-atopisch astma. De NK-1 receptor, de receptor voor substance P, is cruciaal bij de ontwikkeling van deze vorm van astma. De NK-1 receptor is ook belangrijk gebleken bij de interactie tussen de mestcel en de zenuw. *In vitro* is aangetoond dat de mestcel NK-1 receptoren tot expressie brengt onder invloed van de cytyokinen IL-4 en SCF. Dit resulteert in een toename in gevoeligheid van mestcellen voor de stimulus substance P. Daarnaast is in een *in vitro* systeem duidelijk geworden dat de NK-1 receptor op de mestcel belangrijk is bij de rechtstreekse interactie van de zenuw met de mestcel.

Curriculum vitae

De schrijfster van dit proefschrift werd geboren op 16 april 1973 in Utrecht. Na het behalen van het VWO diploma aan het Mgr. Zwijsen College in Veghel, is zij in 1991 begonnen aan de opleiding Gezondheidswetenschappen, faculteit der Geneeskunde aan de Rijksuniversiteit Maastricht. Na het behalen van haar propaedeuse Gezondheidswetenschappen in 1992, begon zij met de bovenbouwstudie milieugezondheidkunde aan dezelfde faculteit. Keuzevakken in het kader van deze studie werden gevolgd aan de Universiteit van Wageningen. Als onderdeel van haar studie liep zij stage van augustus 1996 tot april 1997 bij de divisie Toxicologie, vakgroep Immuno- Inhalatie- en In vitro Toxicologie, TNO Voeding te Zeist. Tijdens deze stage deed zij onderzoek naar de ontwikkeling en validatie van orale diermodellen voor onderzoek naar en screening op allergeniciteit van voedingseiwitten onder begeleiding van Dr. Leon Knippels. Na haar afstuderen begon zij in februari 1998 als assistent in opleiding aan het project 'The involvement of mast cells in an animal model for occupational asthma; a role for the tachykinins' bij de afdeling Farmacologie en Pathofysiologie van de faculteit Farmacie aan de Universiteit Utrecht. Dit project werd uitgevoerd onder leiding van Dr. Frank Redegeld, Dr. Aletta Kraneveld en Prof. Dr. Frans Nijkamp. Een deel van het project werd gedaan bij 'The department of Molecular Medicine' van de McMaster University in Hamilton, Canada, in samenwerking met Prof. Dr. John Bienenstock.

List of publications

The NK-1 receptor is important in the delayed type hypersensitivity-induced tracheal hyperreactivity in the mouse.
J.P.M. van der Kleij, A.D. Kraneveld, F.A.M. Redegeld, F.P. Nijkamp. Immunology Letters, 1999, 69: 136. *Abstract*

Comparison of antibody responses to hen's egg and cow's milk proteins in orally sensitized rats and food-allergic patients.
Knippels L.M., van der Kleij J.P.M., Koppelman S.J., Houben G.F., Penninks A.H.
Allergy 2000; 55(3): 251-258

The NK-1 receptor is important in the delayed type hypersensitivity-induced tracheal hyperreactivity in the mouse.
J.P.M. van der Kleij, A.D. Kraneveld, F.A.M. Redegeld, F.P. Nijkamp.
The Faseb Journal, 2000, 14: 6. *Abstract*

Key role for mast cells in nonatopic asthma
Aletta D. Kraneveld, Hanneke P.M. van der Kleij, Mirjam Kool, Anneke H. van Houwelingen, Andrys C.D. Weitenberg, Frank A.M. Redegeld, Frans P. Nijkamp
Journal of Immunology 2002; 169: 4

The neurokinin-1 receptor is crucial for the development of non-atopic asthma.
J.P.M. van der Kleij, A.D. Kraneveld, F.A.M. Redegeld, Norma P.Gerard, Olivier Morteau and F.P. Nijkamp.
Journal of Allergy and Clinical Immunology 2002; 109: 1. *Abstract*

Nerve-mast cell interactions – Partnership in Health and Disease.
Hanneke P.M. van der Kleij, Michael Blennerhassett and John Bienenstock.
In: Autonomic Neuroscience Series, 2002; Volume 15.
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Functional expression of neurokinin 1 receptors on mast cells induced by IL-4 and SCF.
Hanneke P.M. van der Kleij, Donglai Ma, Frank A.M. Redegeld, Aletta D. Kraneveld, Frans .P. Nijkamp and John Bienenstock. *Submitted*

The neurokinin-1 receptor is crucial for the development of non-atopic asthma
Hanneke P.M. van der Kleij Aletta D. Kraneveld, Frank A.M. Redegeld, Norma P. Gerard, Olivier Morteau and Frans .P. Nijkamp. *submitted*

TNF-α plays a central role in the development of airway hypersensitivity reactions associated with non-atopic asthma
Hanneke P.M. van der Kleij, Anneke H. van Houwelingen, Aletta D. Kraneveld, Frank A.M. Redegeld and Frans .P. Nijkamp. *Submitted*

Vascular Endothelial Growth Factor plays a key role in a murine model for non-eosinophilic asthma: regulation by neutrophils
Anneke H.van Houwelingen, Saskia C.A. de Jager, Panagiota Iliopoulou, Mirjam Kool, Hanneke P.M. van der Kleij, Aletta D. Kraneveld and Frans P. Nijkamp. *Submitted*

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Frank, co-promotor

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Natuurlijk **W019**: Mijn kamergenoten....
Anneke Moulin Rouge Rijnierse.
Mijn persoonlijke styliste en kledingadviseuse (you've got a HUGE talent!).
Inge ik heb zoiets van ik heb een hypo Valstar
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Abbreviations

BAL	bronchoalveolar lavage
BMMC	bone marrow-derived mast cell
BMMC→*W/W^v* mice	mast cell reconstituted mast cell-deficient mice
CGRP	calcitonin gene-related peptide
CTMC	connective tissue type mast cells
DNFB	dinitrofluorobenzene
DNS	dinitrobenzene sulfonic acid
EC50	concentration agonist that induces 50% of the maximal response
ELISA	enzyme-linked immunosorbent assay
Emax	maximal response
FACS	fluorescent adhesion cell sorter
IFN	interferon
Ig	immunoglobulin
IL	interleukin
LC	light chain
MMC	mucosal mast cells
mMCP-1	mouse mast cell protease 1
NANC	non-adrenergic non-cholinergic
NGF	nerve growth factor
NK	neurokinin
pD2	-log EC50
PenH	enhanced pause
SCF	stem cell factor
SEM	standard error of the mean
TNF-α	tumor necrosis factor alpha
$TNFR_{1,2}$	tumor necrosis factor alpha $receptor_{1,2}$