

Key Role for Mast Cells in Nonatopic Asthma¹

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The mechanisms involved in nonatopic asthma are poorly defined. In particular, the importance of mast cells in the development of nonatopic asthma is not clear. In the mouse, pulmonary hypersensitivity reactions induced by skin sensitization with the low-m.w. compound dinitrofluorobenzene (DNFB) followed by an intra-airway application of the hapten have been featured as a model for nonatopic asthma. In present study, we used this model to examine the role of mast cells in the pathogenesis of nonatopic asthma. First, the effect of DNFB sensitization and intra-airway challenge with dinitrobenzene sulfonic acid (DNS) on mast cell activation was monitored during the early phase of the response in BALB/c mice. Second, mast cell-deficient *W/W^v* and *Sl/Sl^d* mice and their respective normal (+/+) littermate mice and mast cell-reconstituted *W/W^v* mice (bone marrow-derived mast cells→*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 bone marrow-derived mast cell→*W/W^v* mice sensitized with DNFB and DNS-challenged exhibited tracheal hyperreactivity 24 and 48 h after the challenge when compared with 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 nonatopic asthma. *The Journal of Immunology*, 2002, 169: 2044–2053.

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). Nonatopic 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. Considerably less information is available about the pathologic characteristics of nonatopic asthma (7). Very recently, Amin et al. (8) have compared the cellular pattern and structural changes in the airways of atopic and nonatopic asthmatic patients. 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 nonatopic 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 nonatopic 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-m.w. compounds such as picryl chloride, toluene diisocyanate, and dinitrofluorobenzene (DNFB)³ as models for nonatopic 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 nonatopic 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 (24–48 h) phases of the hapten-induced pulmonary reaction were found to depend on the presence of T lymphocytes, because in athymic mice airway hyperreactivity and cellular accumulation were suppressed (10, 12, 15).

Studies in mice contact-sensitized and locally challenged with low-m.w. 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 nonatopic asthma. Furthermore, a role of the mast cell in hapten-induced pulmonary hypersensitivity reactions, the murine model of nonatopic asthma, has also been suggested (16–19). We hypothesized

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³ Abbreviations used in this paper: DNFB, dinitrofluorobenzene; DNS, dinitrobenzene sulfonic acid; mMCP-1, mouse mast cell protease 1; BMMC, bone marrow-derived mast cell; GMA, glycol methacrylate; TB, Heath's aluminum-toluidine blue; CAE, chloro-acetate esterase; PO, peroxidase; BAL, bronchoalveolar lavage; PenH, enhanced pause.

that mast cells may be critical in the development of nonatopic 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. First, 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 nonatopic asthma.

Materials and Methods

Materials

DNFB, olive oil, carbachol, and *o*-phenylenediamine dihydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). Dinitrobenzene sulfonic acid (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 (München, Germany) and heparin was from Leo Pharmaceutical Products (Ballerup, Denmark). Monastral blue dye was a generous gift of P. Baluk (Cardiovascular Research Institute, University of California, San Francisco, CA). Histamine radioimmunoassay was purchased from Immunotech (Marseilles, France). The mouse mast cell protease 1 (mMCP-1) ELISA was from Moredun Scientific (Midlothian, U.K.). Maxisorp surface 96-well plates were purchased from Nunc Immunoplate (Roskilde, Denmark).

Animals

Male BALB/c mice were supplied by the central animal laboratory (Gezinschappelijk Dier Laboratorium), Utrecht University (Utrecht, The Netherlands). Male mast cell-deficient mice (WBB6F₁ W/W⁻) and their respective normal littermates (WBB6F₁ +/+) and mast cell-deficient mice (WCB6F₁ Sl/Sl⁻) and their respective normal littermates (WCB6F₁ +/+) were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were used at 6–8 wk of age, with the exception of mast cell-reconstituted W/W⁻ mice and age-matched +/+ and W/W⁻ mice that were used at 40 wk of age. All the mice were housed in groups not exceeding 10 per cage and were maintained under standard conditions. The Animal Care Committee at Utrecht University approved all experiments.

Mast cell reconstitution

Selective reconstitution of mast cells in mast cell-deficient W/W⁻ mice was conducted by the methods earlier described by Karimi et al. (20) and Williams and Galli (21), with several modifications. Bone marrow-derived mast cells (BMMCs) were obtained from WBB6F₁ +/+ mice. Bone marrow was aseptically flushed from femurs of +/+ mice and cultured for 4–5 wk in complete RPMI (RPMI 1640 medium, which contained 10% FCS, 4 mM 1-glutamine, 0.5 μ M 2-ME, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.1 mM nonessential amino acids). 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 per 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- to 5-wk culture. No stem-like cell was detected in the mast cell preparation. We suspect that the <10% of the c-Kit-negative cells are immature mast cells.

Mast cell-deficient W/W⁻ mice were injected via the tail vein with 5 $\times$ 10⁶ cultured BMMC cells, and the recipients were studied 20 wk later. Age-matched mast cell-deficient W/W⁻ mice and congenic normal mice were used when examining the mast cell-reconstituted mast cell-deficient mice (BMMC→W/W⁻). BMMC→W/W⁻ mice had 7.70 $\pm$ 1.33 mast cells/mm trachea vs none or 8.04 $\pm$ 1.46 mast cells/mm trachea for W/W⁻ 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⁻ 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 glycol methacrylate

(GMA) (22). Serial sections were cut at 3 μ m and stained with Heath's aluminum-toluidin blue (TB) or using chloro-acetate esterase (CAE) and peroxidase (PO) procedures, respectively (23–25).

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 μ l) and all four paws (50 μ l). On day 1, DNFB or vehicle control (50 μ l) was applied to the thorax alone. DNFB- and vehicle-sensitized mice were intranasally challenged with DNS on day 5 (50 μ l, 0.6% in PBS, pH 7.2). 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 (BAL) fluid.

Measurement of acute bronchoconstriction

Bronchoconstriction was measured as reported previously (26). In short, 5 min before intranasal DNS challenge, unrestrained conscious mice were placed in a whole-body plethysmographic chamber (Buxco Electronics, Shanon, CT) to analyze the respiratory wave forms and obtain basal line. After 4 min and 30 s, 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 $\times$ PEP/PIP, where PEP stands for peak expiratory pressure and PIP for peak inspiratory pressure. Pause is defined as $(T_e - T_i)/T_i$, where T_e stands for time of expiration and T_i 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 s, 5 min, 7 min 30 s, 10 min, 15 min, and 20 min.

Mast cell activation in vivo

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 14,000 rpm at 4°C for 10 min. Supernatants were separated and frozen at –70°C until assay. Histamine radioimmunoassay was performed according to the manufacturer's instructions (Immunotech). Resulting cpm from 0.5 to 150 nmol/L (assay standard enclosed with kit) and 0.1–50.0 ng/ml histamine standard solutions in plasma were used to construct standard curves. Histamine values for unknown samples were determined from the standard curve.

Mouse MCP-1 ELISA in serum and tissue samples. Mouse MCP-1 is a protease specific for mouse mast cells and will appear in the blood of mice after mast cell activation (27). 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°C until assay. In addition, 30 min after the challenge and after perfusing the mice with 5 ml of PBS (37°C) via the right ventricle, the lungs were isolated and homogenated in 1.5 M KCl at 4°C. The lung homogenates were centrifuged for 10 min at 10,000 $\times$ g and the supernatants were stored at –70°C until assay. A commercially available mMCP-1 ELISA was used for the measurements of mMCP-1 in the sera and tissue homogenates. Serum and tissue homogenate supernatants were diluted 1:1 before assaying the samples using two anti-mMCP1 Abs. Ninety-six-well flat-bottom micro ELISA plates (Nunc Immunoplate, maxisorp surface) were coated with sheep anti-mMCP-1 capture Ab (2 μ g/ml) and kept for 24 h at 4°C in a humid chamber before use. The coated plates were washed six times before loading standard mMCP-1 and samples for 24 h. After another wash step, plates were incubated with rabbit anti-mMCP-1-HRP conjugate for 1.5 h at room temperature. HRP activity was assessed by adding orthophenylenediamine/H₂O₂ (0.4 mg/ml). After stopping the reaction with 2.5 M H₂SO₄, OD was measured at a wavelength of 490 nm using a microplate reader (Benchmark plate reader). Results were expressed as nanograms mMCP-1 per milliliter serum or nanograms mMCP-1 per gram tissue wet weight.

Determination of tracheal vascular permeability

Monastral blue pigment is a tracer to localize leakage at postcapillary venules in the trachea (28). Monastral blue was injected i.v. via the tail vein immediately before the challenge. Thirty minutes 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 BAL fluid

BALs 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 (37°C) was slowly injected into the lung and withdrawn in 4×1 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, $580 \times g$, 10 min, 4°C) to isolate the BAL cells from the supernatant. After measuring the volume of the first milliliter, the supernatant was used to assess mucosal exudation. BAL cell pellets were pooled and resuspended in 150 μ l 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 H&E (DiffQuik; Merz & Dade, Dubingen, Switzerland) to ascertain the percentage of neutrophils, eosinophils, and mononuclear cells (lymphocytes, macrophages, and monocytes) in the airway lumen. One hundred 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 cells per lung.

Mucosal exudation

Mucosal exudation was assessed as previously described (29). At time of challenge or 22 h after the challenge, Evans blue dye (1.25%, 50 μ l 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 milliliter 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 (microliters/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 NaH_2PO_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 (37°C). Isometric measurements were made with a force displacement transducer (Harvard Bioscience, Boston, MA) and a two-channel recorder (Servogor type SE-120; Plato, Diemen, The Netherlands) 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} – 10^{-4} M) were determined 24 or 48 h after the challenge of vehicle- or DNFB-sensitized mice.

Statistical analysis

All experiments were designed as completely randomized factorials with 4–14 mice per group. Maximal response values for the carbachol-induced tracheal contraction for each experimental animal were calculated separately by nonlinear least-square regression analysis (simplex minimization) of the measured contractions vs carbachol concentration, using the

sigmoid concentration-response relationship and including a threshold value.

Histamine content in blood was analyzed using unpaired *t* tests at the two different time points of sampling. The following data obtained from individual animals were analyzed by two-way ANOVA: mMCP-1 content in blood and lung homogenates, mucosal exudation values, and EC_{50} and maximal response values for the carbachol-induced tracheal contractility, followed by a posthoc comparison between groups. In the figures, group means $\pm$ 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 test. The cell data are expressed as medians (minimum-maximum). All data manipulation, non-linear fittings, unpaired *t* test, ANOVA, and posthoc comparisons were conducted with a commercially available statistical package (SYSTAT, version 5.03; Systat, 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 (27). An increase in histamine levels ($\pm 150\%$) was found in the plasma of DNFB-sensitized BALB/c mice 10 and 30 min after intranasal DNS challenge when compared with vehicle-sensitized animals (10 min: vehicle/DNS, 158.4 ± 21.4 nM, and DNFB/DNS, 240.1 ± 20.3 nM, $p < 0.05$, $n = 4$ mice per group; 30 min: vehicle/DNS, 98.4 ± 29.9 and 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, however, was 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 (Fig. 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 (Fig. 1). 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 Fig. 2, intranasal DNS challenge in DNFB-sensitized BALB/c mice resulted in an increase in PenH values when compared with vehicle-sensitized mice (max PenH: saline/DNS = 0.76 ± 0.07 , and DNFB/DNS =

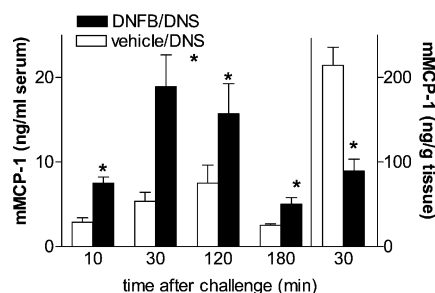


FIGURE 1. Mast cells are activated shortly after intranasal DNS challenge in DNFB-sensitized BALB/c mice. Mast cell protease (mMCP-1) levels in serum and in lung tissue of vehicle- and DNFB-sensitized mice 10, 30, 120, and 180 min after intranasal DNS challenge. Results are expressed as mean (nanograms mMCP-1/milliliter serum or nanograms/gram tissue wet weight) $\pm$ SEM, *, $p < 0.05$; $n = 6$ –12 mice/group.

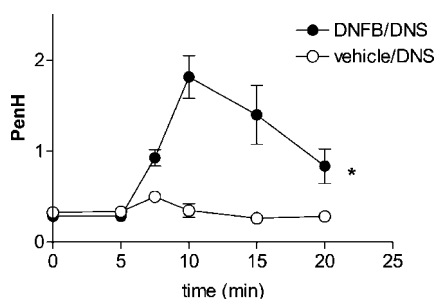


FIGURE 2. Acute bronchoconstriction is found in DNFB-sensitized BALB/c mice after intranasal DNS challenge. Bronchoconstriction is characterized by increases in PenH values, which were recorded in vehicle- and DNFB-sensitized mice 5 min before until 20 min after the challenge. For each mouse, max PenH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

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 DNFB-sensitized BALB/c mice compared with 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 with vehicle-sensitized mice (Table I).

Table I. Mucosal exudation in the airway lumen 1 and 24 h after DNS challenge of vehicle- or DNFB-sensitized BALB/c, W/W^v, SI/SI^d and their respective normal littermate control (+/+), and reconstituted BMMC→W/W^v mice^a

Mouse	n	Time (h)	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*
SI/SI ^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	B ^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

^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 microliters of plasma/lung ± SEM. Significant differences between vehicle- and DNFB-sensitized groups are indicated.

^b The mice examined in these experiments were age-matched (40 wk).

^c B, Basal mucosal exudation in airway of 40-wk-old nontreated +/+ mice.

*, $p < 0.05$.

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

BAL 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 with controls (Table I). 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 with vehicle-sensitized mice (Table II). The increase in total BAL fluid cells was largely attributable to an increase in the number of neutrophils and mononuclear cells (Table II).

At 24 and 48 h, BALB/c mice exhibit a marked and significant tracheal hyperreactivity to carbachol when DNFB sensitized and DNS challenged (Table III). 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.

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-wk-old mast cell-deficient mice (W/W^v and SI/SI^d) and their respective normal (+/+) littermates. Fig. 3a shows that in both strains of normal (+/+) littermates, DNFB/DNS-induced significant increases in mMCP-1 serum levels were found compared with those of 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 with those of vehicle-sensitized animals (Fig. 3a). Moreover, no mucosal exudation response was found 1 h after intranasal DNS challenge in DNFB-sensitized mast cell-deficient mice (Table I).

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 in their respective normal (+/+) littermates. In both strains of mast cell-deficient mice, no significantly different mucosal exudation or total BAL cell numbers were found after DNFB sensitization and DNS challenge when compared with vehicle-sensitized mice (Tables I and II). 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 (Table I and II).

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 III). In both strains of mast cell-deficient mice (W/W^v and SI/SI^d), no enhanced contractile responses to carbachol were found in DNFB-sensitized and DNS-challenged mice compared with control groups (Table III).

Table II. 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 × 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 are lymphocytes, macrophages, and monocytes. Results are expressed as median (minimum–maximum). Significant differences between vehicle- and DNFB-sensitized groups are indicated.

^b The mice examined in these experiments were age-matched (40 wk).

^c B, Basal; leukocyte numbers of 40-wk-old nontreated +/+ mice.

*, *p* < 0.05.

Table III. Maximal tracheal reactivity (*E*_{max}) and *pD*2 (–log *EC*₅₀) values derived from concentration response curves to carbachol (10^{–8}–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 (h)	DNFB Sensitization	<i>E</i> _{max} (mg)	<i>pD</i> 2
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. *EC*₅₀, Effective agonist concentration inducing 50% of the maximal response.

^b The mice examined in these experiments were age-matched (40 wk).

*, *p* < 0.05.

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

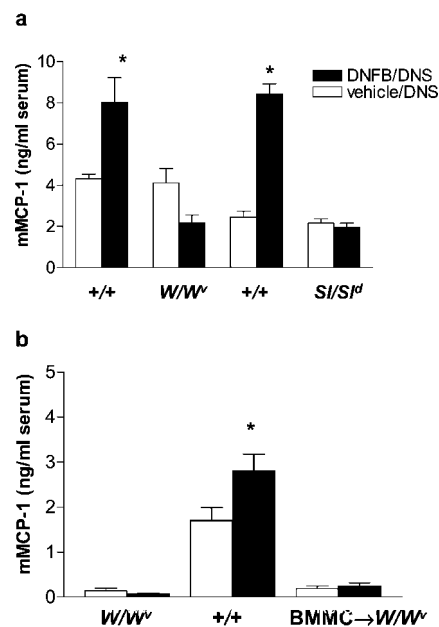


FIGURE 3. Mast cells are activated shortly after intranasal DNS challenge in DNFB-sensitized +/+ mice, but not in two strains of mast cell-deficient mice. Mast cell protease (mMCP-1) levels in serum of vehicle- and DNFB-sensitized mast cell-deficient W/W^v, mast cell-deficient Sl/Sl^d, and their respective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

determined the effect of reconstitution of W/W^v mice with mast cells cultured from bone marrow of $+/+$ mice (BMMC $\rightarrow$ W/W^v mice). An age-matched study was performed because the mast cell reconstitution took 20 wk.

To assess the establishment of mast cells in mast cell-reconstituted mice, trachea of $+/+$, W/W^v , and BMMC $\rightarrow$ 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 (Fig. 4*a*). In trachea of mast cell-reconstituted mice, TB did not stain any mast cells (Fig. 4*d*). Because CAE staining is an accurate manner for identifying 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 $\rightarrow$ W/W^v mice using CAE staining (Fig. 4, *b*, *c*, *e*, and *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—being located in the mucosa, being smaller in size, and having lower granular density (Fig. 4).

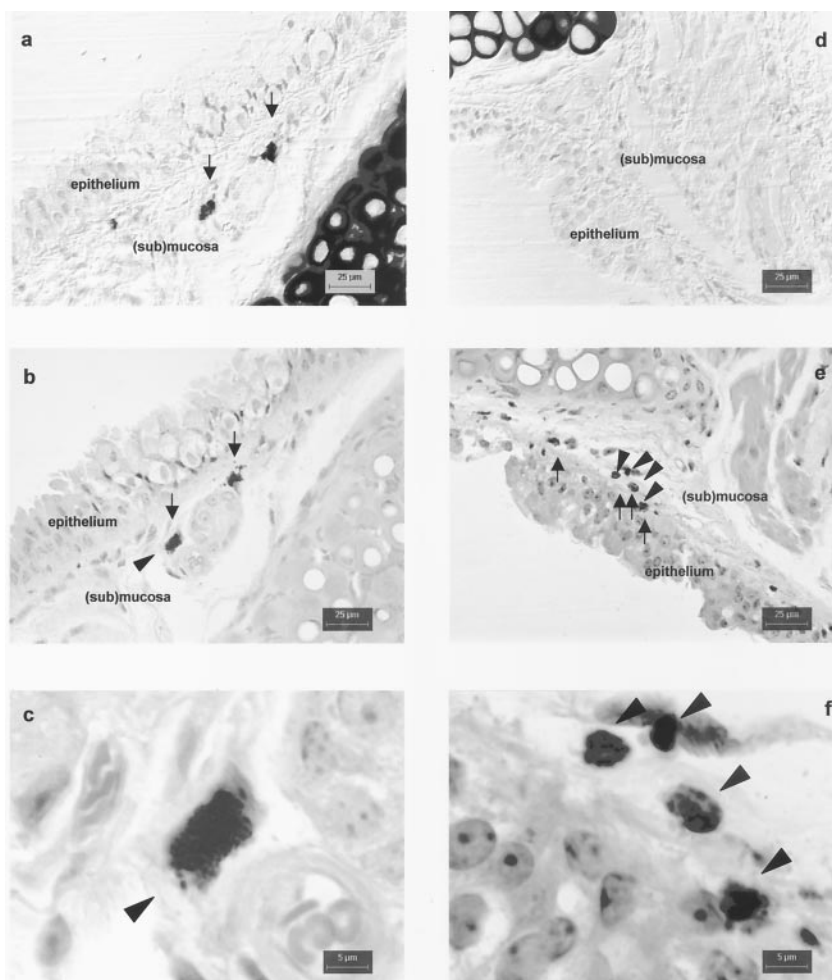
As shown in Fig. 5, DNFB sensitization and DNS challenge

resulted in a profound bronchoconstriction in 40-wk-old $+/+$ and BMMC $\rightarrow$ W/W^v mice, but mast cell-deficient W/W^v mice failed to exhibit a bronchoconstrictive response directly after the 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 $\rightarrow$ W/W^v mice (Fig. 3*b*). Nevertheless, an early phase-associated increase in tracheal vascular permeability observed in $+/+$ mice, which was absent in DNFB-sensitized W/W^v mice 30 min after challenge, could be completely restored after mast cell reconstitution (Fig. 6).

Examination of BAL fluid of age-matched W/W^v , $+/+$, BMMC $\rightarrow$ W/W^v mice indicated that the presence of mast cells in the lung is important for the infiltration of neutrophils (Table II). Note that the total cell number of BAL cells is markedly increased in 40-wk-old mice compared with that of 8-wk-old mice (Table II). We were unable to assess mucosal exudation upon DNFB sensitization in 40-wk-old $+/+$ and BMMC $\rightarrow$ W/W^v mice 24 h after intranasal challenge. The mucosal exudation values observed in $+/+$ W/W^v mice and BMMC $\rightarrow$ W/W^v mice after DNFB sensitization and DNS challenge did not significantly differ from basal values in nontreated $+/+$ mice (Table I).

Finally, reconstitution of mast cells in W/W^v mice restored the DNFB-induced tracheal hyperreactivity found 48 h after the challenge (Fig. 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 III).

FIGURE 4. BMMC transplantation reconstitutes mast cells in trachea of mast cell-deficient W/W^v mice. BMMCs were injected i.v. into W/W^v mice. After reconstitution, mice were housed for 20 wk before being subjected to DNFB sensitization and intranasal DNS challenge. At the time of experiment, all mice were 40 wk old. Serial sections of GMA-embedded trachea from vehicle-sensitized $+/+$ (*a–c*) and BMMC $\rightarrow$ W/W^v mice (*d–f*) were stained with TB (*a* and *d*) and the CAE reaction (*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*).



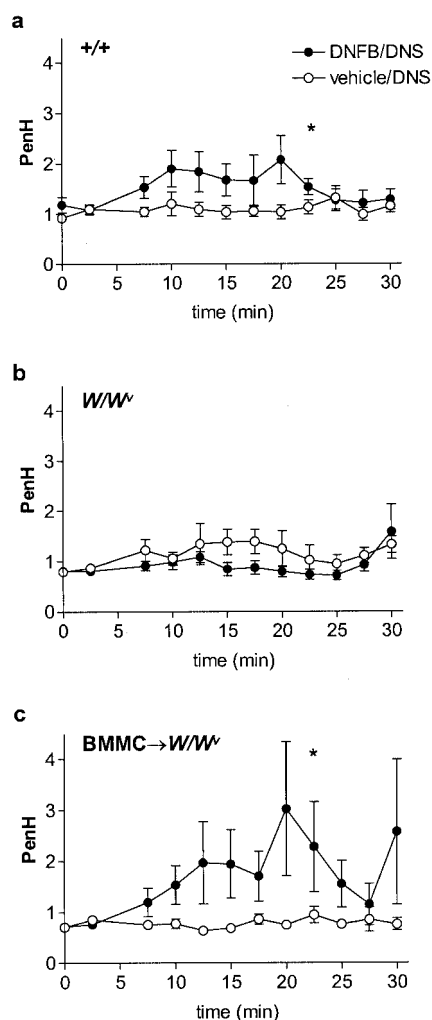


FIGURE 5. Acute bronchoconstriction is found in DNFB-sensitized $+/+$ and BMMC $\rightarrow$ W/W^v mice, but not in mast cell-deficient W/W^v mice. After reconstitution, mice were housed for 20 wk before being subjected to DNFB sensitization and intranasal DNS challenge. At the time of experiment, all mice were 40 wk old. Bronchoconstriction is characterized by increases in PenH values, which were recorded in vehicle- and DNFB-sensitized mice from 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 s, 5 min, 7 min 30 s, 10 min, 12 min 30 s, 15 min, 17 min 30 s, 20 min, 22 min 30 s, 25 min, and 30 min. *, $p < 0.05$, $n = 5$ –6 mice/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 nonatopic asthmatic patients (8). The presence of increased mast cell numbers in nonatopic 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 nonatopic asthma and examine the putative role of mast cells. Cutaneous sensitization with the low-m.w. 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 with normal congenic $+/+$ mice. Similar results were found for the late-phase responses of this murine model for nonatopic asthma, such as mucosal exudation, cellular infiltration, and tracheal hyperreactivity. Moreover, DNFB-induced pulmonary hypersensitivity responses in mast cell-reconstituted BMMC $\rightarrow$ W/W^v mice were indistinguishable from those of age-matched normal $+/+$ mice.

These data extend previous work performed by Garssen et al. (16) and other investigators that indicated that mast cells are involved in the elicitation of hapten-induced T cell-mediated responses in the airways of actively immunized mice (18, 19). There are several lines of evidence that favor a role for the mast cell in the early phase of hapten-induced hypersensitivity reactions. First, these reactions are elicited preferentially at sites enriched with mast cells, such as lung, gastrointestinal tract, buccal mucosa, and skin (12, 30–32). Studies have reported the release of the mast cell mediator serotonin during contact sensitization in skin and lung early after the challenge (12, 33–35). This vasoactive amine can act locally by increasing vascular permeability and inducing vasodilatation, thereby facilitating cellular infiltration (33, 35, 36). 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, 33, 35–37). 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 (38). It was suggested that other cell types, such as the platelet, could be involved. However, very recently Biedermann et al. (39) 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, increased vascular permeability, late-phase infiltration of inflammatory cells into the airways, or tracheal hyperreactivity at 24–48 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 nonatopic 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

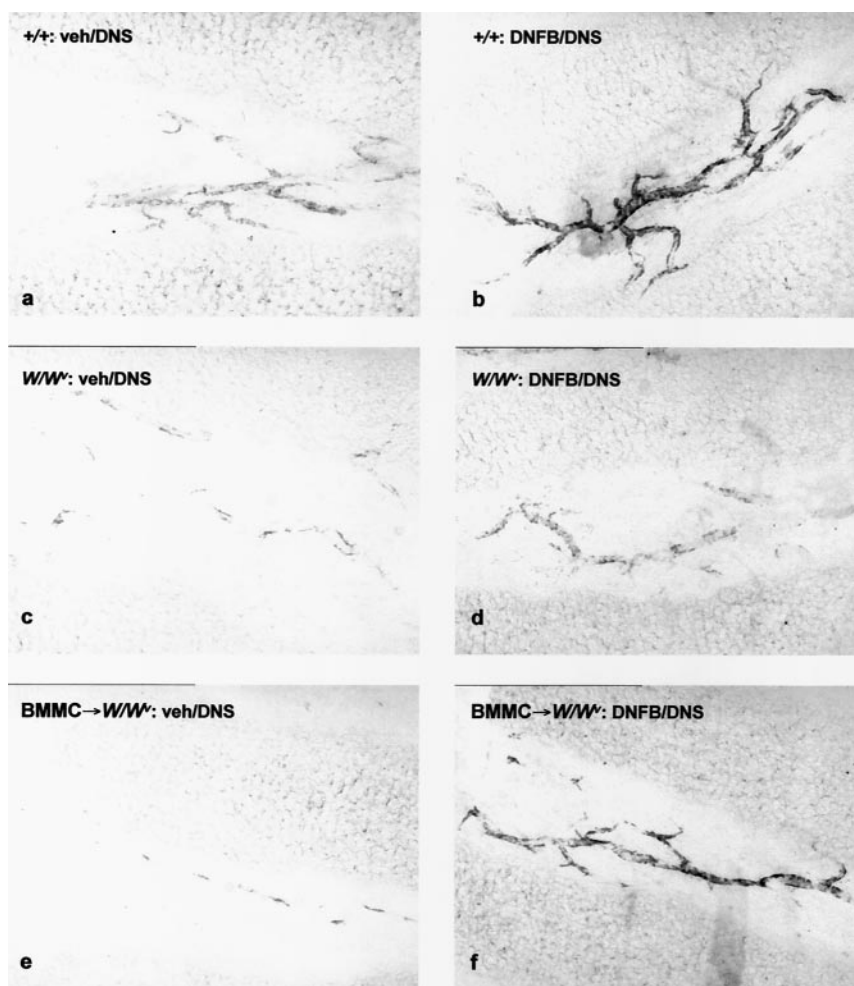


FIGURE 6. Tracheal vascular hyperpermeability is found in DNFB-sensitized $+/+$ (*a* and *b*) and BMMC $\rightarrow$ W/W^v mice (*e* and *f*), but not in mast cell-deficient W/W^v mice (*c* and *d*). After reconstitution, mice were housed for 20 wk before being subjected to DNFB sensitization and intranasal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

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 $\rightarrow$ 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 BMMCs, the density of pulmonary mast cells is 5- to 15-fold lower than in age-matched $+/+$ mice, suggesting minimal reconstitution (40). 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 (41). It could be possible that the number of mast cell activated in reconstituted mice is too low to detect mMCP1 levels in serum. In our study, however, tracheal mast cell counts did not indicate differences in the number of mast cells comparing $+/+$ and BMMC $\rightarrow$ 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 (42) stressed that appropriate studies should be done to assess the number, phenotype, and anatomical distribution of mast cells that develop in W/W^v recipients of BMMCs. Indeed, histological examination of mast cells in trachea obtained from $+/+$ and BMMC $\rightarrow$ 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 do an in-depth investigation of 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, which are not detectable in serum, could result in mast cell sensitization. However, we have demonstrated that upon contact sensitization with low-m.w. haptens, hapten-specific proteins are produced. These hapten-specific proteins were devoid of IgG, IgE, and IgM (F. A. M. Redegeld, M. W. van der Heijden, M. Kool, A. D. Kraneveld, and F. P. Nijkamp, 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 (43). 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, 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, 17, 31, 36, 39, 44, 45). Evidence from this study is 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 nonatopic asthma.

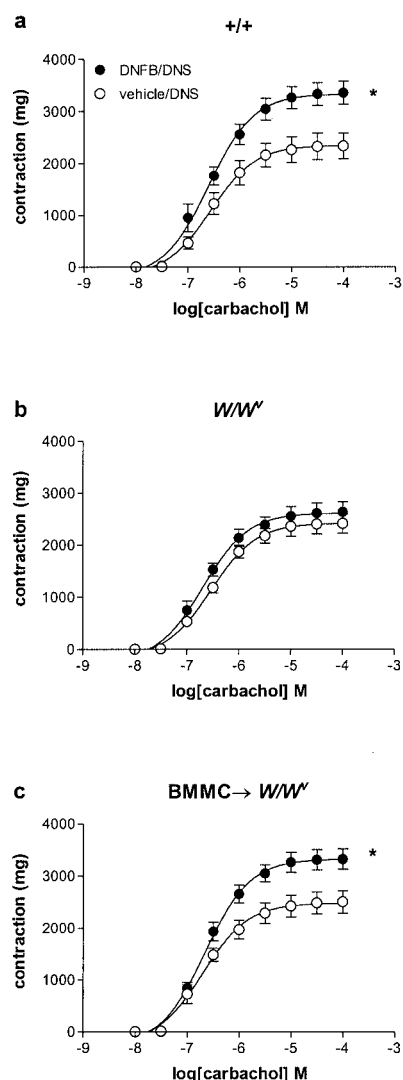


FIGURE 7. Tracheal hyperreactivity is found in DNFB-sensitized $+/-$ (a) and $BMMC \rightarrow W/W^m$ (c) mice, but not in mast cell-deficient W/W^m mice (b). After reconstitution, mice were housed for 20 wk before being subjected to DNFB sensitization and intranasal DNS challenge. At the time of experiment, all mice were 40 wk 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 (milligrams contraction) $\pm$ SEM. *, $p < 0.05$; $n = 5-6$ mice/group.

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