Role of nerve growth factor in a mouse model of allergic airway inflammation and asthma

Armin Braun1, Elena Appel2, Rina Baruch3, Udo Herz1, Vladimir Botchkarev3, Ralf Paus4, Chaya Brodie2 and Harald Renz1

1 Department of Laboratory Medicine and Pathobiochemistry, Charité-Virchow Clinic of the Humboldt University, Berlin, Germany
2 Department of Life Sciences, Bar-Ilan University, Ramat-Gan, Israel
3 Department of Dermatology, Charité-Virchow Clinic of the Humboldt University, Berlin, Germany

The role of nerve growth factor (NGF), a potent mediator acting in the development and differentiation of both neuronal and immune cells, was examined in a mouse model of allergic asthma. NGF-positive cells were detected in the inflammatory infiltrate of the lung and enhanced levels of NGF were detected in serum and broncho-alveolar lavage fluids. Mononuclear cells in inflamed airway mucosa as well as broncho-alveolar macrophages were identified as one source of NGF production. Splenic mononuclear cells from allergen-sensitized mice produced NGF in response to allergen. They responded to exogenously added NGF with a dose-dependent increase in IL-4 and IL-5 production and augmented IgE and IgG1 synthesis. In contrast, IFN-γ and IgG2a levels remained unaffected. The effects were NGF specific, since they could be blocked by an anti-NGF-antibody. Nasal application of anti-NGF to allergen-sensitized mice significantly reduced IL-4 and prevented development of airway hyperreactivity. These results show that allergic airway inflammation is accompanied by enhanced local NGF production that acts as an amplifier for Th2 effector functions and plays an important role in the development of airway hyperreactivity. Therefore it is suggested that NGF may serve as a link between the immune and nerve system.

Key words: Neurotrophin / Allergy / Mouse / Th2 immune response / Airway hyperreactivity

1 Introduction

Allergic bronchial asthma (BA) is characterized by elevated serum IgE antibody titers and inflammation of the airways with high levels of IL-4 and IL-5 in broncho-alveolar lavage (BAL) fluids and airways mucosa [1–3]. Airways smooth muscle constriction and development of airway hyperresponsiveness are hallmarks of BA that are controlled by sympathetic and parasympathetic nerves as well as by the non-adrenergic non-cholinergic (NANC) system. The inflammatory response is triggered by neuropeptides, and has been termed “neurogenic inflammation” [4]. However, little is known about the complex interactions of the nervous system with these inflammatory processes.

Nerve growth factor (NGF) is a mediator with functions on both immune and nerve cells [5]. It is a well-studied example of a target-derived neurotrophic factor that is essential for the survival, development, differentiation and maintenance of peripheral sympathetic and neural crest-derived sensory nerve cells [6]. NGF up-regulates expression of neuropeptides in sensory neurons [7] and contributes to inflammatory sensory hypersensitivity [8]. In the central nervous system, NGF is a trophic factor for basal forebrain cholinergic neurons. The biological effects of NGF are mediated by binding either to the high-affinity (Kd \( \sim 10^{-11} \)) glycoprotein receptor trk A or the low-affinity (Kd \( \sim 10^{-9} \)) pan-neurotrophin receptor p75 (NTR). Recent studies indicate, that NGF has specific effects on cells of the immune system. NGF receptors have been described on a variety of immune cells. Trk A is present of human monocytes, B lymphocytes and T lymphocytes [9–12]. All resting peripheral blood and tonsillar B lymphocytes express low-affinity NTR [13] as do murine CD4+ T cells [10]. NGF signal transduction in human B lymphocytes is mediated by trk A [14, 15], and it has been suggested that expression of trk A is an important prerequisite for any NGF effect.
NGF enhances vascular permeability in rat skin [16], increases the number of mast cells in neonatal rats [17], stimulates rapid degranulation of mast cells [18], promotes differentiation of granulocytes and macrophages [19–22], promotes proliferation of B and T cell subsets [23, 24] and induces differentiation of activated B cells into Ig-secreting plasma cells [25–27]; for review see [28]).

Considering these multidirectional effects, it is not surprising to find increased levels of NGF in both acute inflammatory diseases [8] and chronic ones [29] including allergic disorders and BA. In a recent study, high NGF values were found in patients with severe allergic BA, bronchial hyperreactivity, and high levels of IgE and eosinophilic cationic protein. Furthermore, NGF serum levels correlated with total IgE antibody titers [30]. What then is the contribution of NGF to the inflammatory immune response in allergic diseases?

The BALB/c mouse is a well-characterized model to study allergic immune responses [3, 31]. Local and systemic sensitization to OVA induces allergen-specific IgE and IgG1 production. Allergen-sensitized mice develop immediate-type hypersensitivity responses to allergen challenges of the airway. The consequence is airway hyperresponsiveness and mucosal inflammation with influx of lymphocytes, neutrophil and eosinophils [32]. These reactions are accompanied by a local Th2 type immune response with elevated levels of IL-4 and IL-5 in BAL fluid [31, 33]. We used this model to evaluate the role of NGF as a mediator between inflammation and airway hyperreactivity (AHR), and specifically to establish whether local and systemic NGF production contributes to the immune response in allergen-sensitized mice.

2 Results

2.1 Increased NGF production in allergen-sensitized mice

BALB/c mice sensitized to OVA and challenged via the airways develop in allergen-specific IgE and IgG1 antibody with airway inflammation and increased airway responsiveness [31]. This model was used to assess the contribution of NGF in this type of immune response. First, NGF was measured in serum of OVA-sensitized and challenged mice. The NGF levels in the sensitized mice were about fourfold higher than in non-sensitized mice (Fig. 1). To examine the site of NGF production, immunohistochemistry was performed on sections of the lung and on cytospin preparations from BAL fluid (Fig. 2). NGF-positive cells were detected within the inflammatory mononuclear cell infiltrate of airway mucosa of OVA-sensitized and allergen-challenged animals (Fig. 2 B).
stimulated with OVA, increased NGF production was detected on the protein level in cell culture supernatants (Fig. 5) as well as on the mRNA level (data not shown). In contrast, addition of OVA to MNC from non-sensitized mice had no effect on NGF production. In addition, Con A stimulation was more effective to induce NGF production in sensitized than in non-sensitized mice (Fig. 5).

2.2 Effect of NGF on Th2 immune response

When splenic MNC from allergen-sensitized mice were stimulated in vitro with different concentrations of OVA, there was a Th2-mediated immune response, characterized by elevated IL-4 and IL-5 production as well as increased IgE and IgG1 synthesis. Peak levels of IL-4 and IL-5 were detected at an OVA concentration of 25 μg/ml; these were not further enhanced by NGF. At lower OVA concentrations, however, NGF augmented allergen-induced IL-4 and IL-5 production (data not shown). These allergen concentrations were used for further experiments. When NGF was added to OVA-stimulated MNC, production of Th2 cytokines IL-4 and IL-5, but not the Th1 cytokine IFN-γ was augmented in a dose-dependent fashion (50–200 ng/ml NGF) (Fig. 6). Increased production of IL-4 corresponded with aug-
Figure 3. NGF levels in BAL fluids from non-sensitized and OVA-sensitized mice. NGF levels were measured in BAL fluids from non-sensitized or OVA-sensitized mice 24 h after allergen challenge. * \( p < 0.05 \) statistically significant. In each group three to eight mice were analyzed.

2.3 Effect of anti-NGF treatment in AHR

To assess the contribution of NGF to development of AHR, we examined the effect of anti-NGF treatment on AHR. AHR was assessed \textit{in vitro} by electrical field stimulation (EFS) of tracheal segments. The frequency that caused 50% of maximal airway smooth muscle constriction (\( ES_{50} \)) was at 4.2 Hz in non-sensitized animals after OVA aerosol challenge and nasal PBS treatment. Nasal treatment with anti-NGF or control antibody had no influence in these control mice. In contrast, tracheal segments from sensitized animals already reacted to lower frequencies of electrical stimulation, \( i.e. \) they reached their half-maximal contraction at a mean of 2.2 Hz indicating development of AHR. In contrast, anti-NGF treatment partly prevented this decrease in \( ES_{50} \) (Fig. 8). Nasal treatment with PBS or control antibody had no effect on AHR.

2.4 Effect of anti-NGF on airway inflammation

To examine whether prevention of AHR by anti-NGF was associated with a reduction in airway inflammation, BAL was performed 24 h after the last airway challenge. As expected in allergen-sensitized mice, OVA-aerosol challenge stimulated a marked inflammatory response mainly characterized by an influx of eosinophiles and T cells into the airways. This response was not inhibited by local anti-NGF treatment (Fig. 9 A). During airway inflammation, enhanced levels of IL-4 but not of IFN-\( \gamma \) were measured in BALF-fluids. Anti-NGF antibody treatment significantly \( \left(p < 0.05\right) \) reduced IL-4 production in sensitized animals, while IFN-\( \gamma \) levels remained unchanged (Fig. 9 B).

3 Discussion

An association between increased NGF production and various inflammatory conditions has been shown in patients as well as in several animal models. These conditions include systemic lupus erythematosus [34], multiple sclerosis [29], psoriasis [35], arthritis [36] and vernal kreatoconjunctivitis [37]. Recently it was demonstrated that NGF levels are also elevated in patients with allergic diseases including bronchial asthma [30]. In this study

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<tr>
<th>Culture condition</th>
<th>NGF</th>
<th>Non-sensitized</th>
<th>OVA-sensitized</th>
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<tr>
<td>Medium</td>
<td>−</td>
<td>1.1±0.8</td>
<td>8.0±0.6</td>
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<tr>
<td>Medium</td>
<td>+</td>
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<tr>
<td>OVA</td>
<td>−</td>
<td>0.9±0.5</td>
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<td>OVA</td>
<td>+</td>
<td>0.8±0.2</td>
<td>18.2±2.2*</td>
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<tr>
<td>Con A</td>
<td>−</td>
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<td>8.05±1.1</td>
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<td>Con A</td>
<td>+</td>
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(MNC from non-sensitized and OVA-sensitized mice were cultured with medium, 5\( \mu \)g/ml OVA or 100 ng/ml Con A for 11 days. When indicated, cells were treated with 100 ng/ml NGF. IgE was measured in cell-free supernatants by ELISA. Shown are mean ± SD of \( n = 4 \) wells. \( \ast \) \( p < 0.05 \).)

Table 1. Effect of NGF on IgE secretion by MNC from non-sensitized and OVA-sensitized BALB/c mice\(^a\)
NGF concentrations in serum correlated with severity of allergic disease and IgE titers.

Since the source as well as the function of enhanced NGF production in allergic disease are still unknown, we investigated the hypothesis that neurotrophins are produced locally during the allergenic reaction and serve as amplifiers of inflammation and airway hyperresponsiveness. In our mouse model of allergic airway inflammation, immunohistological analysis of lungs and airways revealed strong positive NGF protein signals in allergen-sensitized and challenged mice, whereas tissues from various controls were NGF negative. Increased NGF production was always associated with development of airway inflammation, and was dependent on systemic allergen sensitization plus local allergen challenge. Only under these conditions, both airway inflammation and increased NGF production were observed.

Our results indicate that T cells and macrophages represent sources of NGF production, since NGF mRNA was
Figure 6. Effect of NGF on cytokine and immunoglobulin secretion in OVA-sensitized mice. MNC from OVA-sensitized mice were treated with different concentrations of NGF in the presence of 5μg/ml OVA. Supernatants were examined for cytokines (after 5 days) and immunoglobulin production (after 11 days) by ELISA. Shown are mean ± SD of ten wells from five independently performed experiments. Mean cytokine/immunoglobulin production in the absence of NGF was for IL-4: 1255pg/ml, IL-5: 1390pg/ml, IFN-γ: 521pg/ml, IgE: 10.2ng/ml, IgG1: 40.6ng/ml, IgG2a: 100ng/ml. *p < 0.05, **p < 0.001.

NGF acted on pre-activated, allergen-stimulated lymphocytes. Since NGF had no detectable effect on resting MNC from non-sensitized mice and since there is no effect without allergen co-stimulation, we suggest that TCR ligation provides a signal that renders T cells sensitive for NGF. NGF is known to have multiple effects on immune cells. It affects the differentiation of B cells into plasma cells and modulates immunoglobulin synthesis of B cells, with preferential IgG4 production, an isotype that is homologous to the mouse IgG1 and is regulated by a Th2 mechanism [26]. NGF enhances several T and
B cell-mediated immune responses, enhances survival and cytotoxic activity of eosinophils [41], induces mast cell degranulation [42] and monocyte activation [11]. Therefore, we propose that the Th2 response is not initiated under the influence of NGF, but that an existing Th2 immune response can be augmented by NGF. This was shown selectively at the level of IL-4 and IL-5, and at the level of IgE and IgG1, isotype production.

The Th2 immune response is characterized by the development of IL-4 and IL-5-producing effector T cells. These cells contribute to the allergic response in several aspects. They control production of IgE and IgG1 [43], up-regulate endothelial adhesion molecules necessary for the recruitment of inflammatory cells into the airways [44], and promote development, maturation and recruitment of eosinophils [45]. These cells represent a hallmark of allergic inflammation and eosinophils are to a great extent responsible for tissue damage during allergic reactions [46]. Thus, many events of the allergic response are directly or indirectly dependent on Th2 cell functions.

AHR represents a hallmark of bronchial asthma and indicated a lowered threshold of airway smooth muscle contractility. The mechanisms that regulate the airway smooth muscle tone in bronchial asthma are still unclear. Data from human studies and animal experiments suggest a close link between AHR and airway inflammation. In our study, anti-NGF treatment prevented the development of increased AHR. This finding indicates that NGF plays an important role in the regulation of AHR. Our data clearly indicate that NGF productions is up-regulated during airway inflammation and that locally recruited immune cells represent one important source for NGF. These results lead us to the conclusion that NGF produced by immune cells during airway inflammation contributes to the development of AHR. On the other hand, anti-NGF treatment had no effect on the recruitment of inflammatory cells into the airways. However, IL-4 production, which is elevated during airway inflammation, was significantly reduced in anti-NGF-treated mice. This result suggests that NGF modulates the activity of locally recruited cells to some degree rather than the recruitment process per se.

Airway smooth muscle constriction and development of AHR are controlled by sympathetic and parasympathetic nerves as well as by the NANC system and NGF was
Figure 9. Effect of intranasal anti-NGF treatment in airway inflammation. Animals were treated 3 h before each allergen challenge with 50 μl anti-NGF (1:50) or PBS (n = 5 mice/group). BAL was performed 24 h after the last allergen challenge as described in Sect. 4.5. (A) Cell differentiation performed on cytospin preparations. (B) Cell-free BAL fluids were analyzed for cytokine contents. Results are expressed as the means ± SD. lym: lymphocytes, neu: neutrophils, eos: eosinophils, macr: macrophages. Statistical significance: *p < 0.05, n.s. not significant.

shown to increase sensitivity and excitability of sensory neurons [8]; a role for NGF in the regulation of airway smooth muscle tone in asthma is likely. Neutrophotrans are potent inducers of neuropeptide expression (e.g. tachykinis) in sensory neurons [7, 8] and contribute to inflammatory sensory hypersensitivity. Neuropeptides which belong to the tachykinin family are elevated in BAL fluids of asthmatic patients [47] and mediate important biological activities during acute inflammatory responses, including smooth muscle contraction, dilatation of blood vessels and increased vascular permeability [4], all of which contribute to the severity of asthmatic bronchoconstriction. Thus, it can be speculated that neurotrophins that are up-regulated following allergic inflammation induce neuropeptide production and nerve hypersensitivity. Unspecific stimuli like EFS or cold air would cause enhanced release of this preformed neuropeptides, which then induce AHR.

In conclusion, we have demonstrated local up-regulation of NGF production in allergic inflammation. Sources of NGF may be locally infiltrating lymphocytes and macrophages in addition to fibroblasts, nerve cells and resident mast cells. The important effect of NGF is in augmenting the allergic local inflammatory immune response and in regulating development of increased AHR, a hallmark of bronchial asthma. The finding of NGF production in various other clinical conditions suggests that NGF production is not restricted to allergies, but is a phenomenon in many inflammatory conditions. Since NGF augments the Th2 immune response and mediates AHR, development and use of specific inhibitors and antagonists will reveal whether inhibition of NGF production or neutralization of produced NGF will reduce airway inflammation and AHR. These data support the hypothesis that NGF is an important mediator between immune systeme and nervous system and plays a key role in the development of AHR and asthma.

4 Materials and methods

4.1 Animals

BALB/c mice were obtained from Bomholtgard, Ry, Denmark, and maintained under pathogen-free conditions.

4.2 Protocol of allergic sensitization

Mice were sensitized to OVA (10 μg/injection) (Sigma, Deisenhofen, Germany) adsorbed to 1.5 mg/injection Al(OH)3 (Pierce, Rockford, IL) by i.p. injection on days 1, 14 and 21. Prior to analysis, animals received two consecutive local allergen challenges of 1% OVA (w/v) diluted in PBS and delivered by aerosolization of 20 min on days 28 and 29 as previously described [3]. Non-sensitized mice were injected with PBS alone and challenged in days 28 and 29 with OVA. BAL was performed 24 h after the last challenge (day 30).

4.3 Intranasal anti-NGF treatment

Intranasal application of 50 μl polyclonal rabbit anti-mouse NGF antibody (biological activity: 1:4000 dilution blocks bioactivity of 5 ng/ml NGF) (Sigma), sterile PBS or isotype-matched antiserum was performed 3 h before each airway allergen challenge. Two times 25 μl of anti-NGF (1:50) were used for each animal. Mice were slightly anesthetized with 2.6 mg/mouse ketaminhydrochloride (Ketanest®), Parke
4.4 Assessment of airway smooth muscle responsiveness

Airway muscle smooth muscle responsiveness was assessed by EFS as described before [31]. Mice were killed 24 h after the last allergen challenged and tracheal smooth muscle segments (~0.5 cm) were removed and hung between stainless steel wire triangular supports. The contraction in response to EFS stimulus (12 V, 200 mA, 0.5–30 Hz) was measured via an isometric force transducer (Grass Instruments, Quincy, MA). The frequency that caused 50 % of the maximal contraction was calculated from logarithmic plots of the contractile response versus the frequency of EFS, and expressed as the ES50.

4.5 BAL

Animals were killed 24 h after the last allergen challenge, the trachea was cannulated and airways were lavaged with 0.8 ml ice-cold PBS. Total cell numbers in pooled lavage fluid were counted and cytospins were evaluated as previously described [31]. Similar volumes of BAL fluid were recovered in each study group (1.4 ± 0.2 ml). Cell-free supernatants were frozen at −20 °C until measurement of cytokine content.

4.6 Cell preparation and culture conditions

Spleens were prepared at indicated time points under sterile conditions. MNC were purified by density gradient centrifugation (Lympholyte M, Cedarline Laboratories, Hornby, Canada; 1000 × g, 20 min), washed twice and suspended in RPMI 1640 culture medium supplemented with 10 % heat-inactivated FCS (Gibco-BRL, Eggenstein, Germany), 2 mM L-glutamine (Biochrom), 1.25 μg/ml amphotericin B (Gibco-BRL), 100 U/ml penicillin (Biochrom) and 100 μg/ml streptomycin (Biochrom). For NGF, cytokine and immunoglobulin production 3 × 10⁶ MNC/well were incubated in 24-well tissue culture plates for 8 h. Cells were stimulated with 5 μg/ml LPS, 10 μg/ml Con A or 10 μg/ml OVA. Cells were washed twice and pellets were frozen in liquid nitrogen until mRNA extraction.

4.7 Preparation of mRNA and PCR analysis

Total RNA was extracted from primary cultures of mouse MNC with TRI reagent according to the manufacturer’s manual (Sigma). Five micrograms of total RNA were transcribed into cDNA with an Expand™ Reverse Transcriptase (Boehringer Mannheim, Mannheim, Germany), using 50 pmol of the oligo (dT) 15, according to the protocol provided by the manufacturer. Relative levels of NGF mRNA were estimated by a semi-quantitative PCR in comparison to the ribosomal protein S12 mRNA level. The cDNA product, 1 mg for PCR with NGF primers and 0.25 mg for PCR with S12 primers, was resuspended in a total volume of 50 ml containing: 1 U Taq DNA polymerase (Appligene, Gaithersburg, MD), 200 mM each of dATP, dCTP, dGTP, dTTP, reaction buffer provided by the manufacturer and 50 pmol of both forward and reverse primers. The 658-bp NGF cDNA fragment corresponding to nucleotides 284–942 of mouse cDNA [48] was amplified by semi-quantitative PCR, using forward primer: 5’ – CATAGCGTAATGTCATGTGTTCT; and reverse primer: 5’ – CCTCTCATCTGTTGTCACCGC – 3’. The 368-bp S12 cDNA fragment was amplified with the primers: forward: 5’ – GGAAGGCTATTGTGCAGTG – 3’; reverse: 5’ – CTCTAATGA-CATCCTTG – 3’. Primers for NGF and S12 are spanning exon-intron junctions to avoid amplification of contaminating genomic DNA. Reaction mixture without template was processed for PCR and is referred to as “O” control. Amplification steps consisted of 95 °C for 3 min and 40 cycles (for NGF) or 30 cycles (for S12) of 95 °C for 30 s, then 55 °C for 1 min and 70 °C for 1 min. A linear range of PCR products was established by serial amplification (25, 30 and 40 cycles). PCR products were size-fractionated by electrophoresis in 1.5 % agarose gels and stained with ethidium bromide. As a molecular mass marker we used a 50-bp DNA ladder (MWXIII, Boehringer Mannheim). The intensity of the bands was quantified with the Image-Pro Plus program. Values are calculated as the ratio of densitometric scores for NGF and S12 PCR products.

4.8 Determination of cytokines by ELISA

IL-4, IL-5 and IFN-γ production were measured by ELISA as described [31]. The limits of detection were 50 pg/ml for IL-4, 50 pg/ml for IL-5 and 50 pg/ml for IFN-γ.

4.9 Determination of immunoglobulins by ELISA

Total IgG₁, IgG₂a and IgE were measured by ELISA using the same protocol as described above. The concentrations for the primary rat anti-mouse mAb were: IgG₁ (2 μg/ml), IgG₂a (2 μg/ml) and IgE (5 μg/ml) (Pharmingen). Biotin-labeled rat anti-mouse antibodies IgG₁ (2 μg/ml), IgG₂a (2 μg/ml) and IgE (2 μg/ml) (Pharmingen) were used as capture antibodies. The detection limits were 15 ng/ml for IgG₁, 15 ng/ml for IgG₂a and 0.5 ng/ml for IgE.
4.10 Determination of NGF by ELISA

NGF was measured in cell-free BAL fluid, cell culture supernatant or serum using a commercial ELISA kit according to the manufacturer’s instructions (Progema, Madison, WI). The detection limits was 4 pg/ml NGF.

4.11 Immunohistochemistry of NGF

Lungs were removed, cryo-fixed in liquid nitrogen and stored at –80 °C. Cryostat sections (14 μm) or cyto spin preparations were mounted in 3-aminopropyltriethoxysilane-coated slides, air-dried (1 h), fixed in acetone (10 min, –20 °C) and placed in Tris-buffered saline (TBS; 0.05 M Tris in 0.9 % NaCl, pH 7.6). Sections were preabsorbed for 15 min with 10 % normal bovine serum (NBS) in TBS, and then incubated overnight at room temperature with rat anti mouse β-NGF mAb (Pharmingen), diluted 1:100 in TBS containing 2 % NBS. After repeated washing in TBS (4 × 5 min), sections were incubated with rabbit anti-rat Ig (Dako, Hamburg, Germany, 1:70 in TBS) containing 5 % mouse and 2.5 % normal rabbit serum for 30 min, followed by incubation with rat alkaline phosphatase-anti-alkaline phosphatase complex (Dako, diluted 1:70 in the same buffer as the secondary antibody). For intensification, the last two steps were repeated for 10 min each. For development of the color reaction, a buffer solution of 0.035 M Tris-HCl containing 0.1 M NaCl, 0.05 M 2-amino-2-methyl-1,3-propandiol (Merck, Darmstadt, Germany) and 1.7 M levamisol was prepared. To obtain a coloring mixture, 300 ml of a New Fuchsin stock solution (Chroma, Stuttgart, Germany) (5 % in 2 N HCl, final concentration 0.1 g/l) was slowly dissolved in a solution of 0.03 g sodium nitrite in 750 ml distilled water. This was added to the buffer, and was complemented with the substrate, 0.75 g naphtol-AS-BI-phosphoric acid (final concentration 1 mM) dissolved in 900 ml N,N-dimethylformamide (Merck). After adjusting to pH 8.8, the sections were incubated, counterstained (hemalum) and covered by coverslips.

4.12 Statistical analysis

Results are presented as mean values ± SD, unless otherwise stated. Student’s t-test was used to determine the level of difference between animal groups.

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5 References


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**Correspondence:** Harald Renz, Charité-Campus Virchow-Klinikum, Department of Laboratory Medicine and Pathobiocchemistry, Augustenburger Platz 1, D-13353 Berlin, Germany
Fax: +49-30 45 06 99 00
e-mail: abraun@ukrv.de