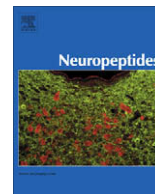




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Bradykinin B₁ receptor antagonist R954 inhibits eosinophil activation/proliferation/migration and increases TGF- β and VEGF in a murine model of asthma

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ABSTRACT

In the present study the effects of bradykinin receptor antagonists were investigated in a murine model of asthma using BALB/c mice immunized with ovalbumin/alum and challenged twice with aerosolized ovalbumin. Twenty four hours later eosinophil proliferation in the bone marrow, activation (lipid bodies formation), migration to lung parenchyma and airways and the contents of the pro-angiogenic and pro-fibrotic cytokines TGF- β and VEGF were determined. The antagonists of the constitutive B₂ (HOE 140) and inducible B₁ (R954) receptors were administered intraperitoneally 30 min before each challenge. In sensitized mice, the antigen challenge induced eosinophil proliferation in the bone marrow, their migration into the lungs and increased the number of lipid bodies in these cells. These events were reduced by treatment of the mice with the B₁ receptor antagonist. The B₂ antagonist increased the number of eosinophils and lipid bodies in the airways without affecting eosinophil counts in the other compartments. After challenge the airway levels of VEGF and TGF- β significantly increased and the B₁ receptor antagonist caused a further increase. By immunohistochemistry techniques TGF- β was found to be expressed in the muscular layer of small blood vessels and VEGF in bronchial epithelial cells. The B₁ receptors were expressed in the endothelial cells. These results showed that in a murine model of asthma the B₁ receptor antagonist has an inhibitory effect on eosinophils in selected compartments and increases the production of cytokines involved in tissue repair. It remains to be determined whether this effects of the B₁ antagonist would modify the progression of the allergic inflammation towards resolution or rather towards fibrosis.

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1. Introduction

Asthma is a chronic inflammatory disease of the airways characterized by an eosinophil infiltration and activation which is orchestrated by Th2 lymphocytes. Eosinophils proliferate in the bone marrow from CD34⁺ progenitors and differentiate in the presence of IL5. The eosinophil trafficking from the bone marrow to the lung tissues and airway spaces is directed by complex interactions between cytokines, chemokines, lipid mediators and adhesion molecules. Once infiltrated into the lungs, eosinophils participate in the early events of allergic inflammation such as airway hyper-reactivity, mucus hypersecretion and lymphocyte infiltration (Kay et al., 2004). The subsequent sustained and progressive inflammation triggered by Th2 lymphocytes can induce structural changes in the airway walls, such as increased thickness of the

basement membrane, increased collagen deposition, sub-epithelial fibrosis, changes in bronchial microcirculation, and smooth muscle hypertrophy and hyperplasia (Chiappara et al., 2001). During this late phase, eosinophils persist in the lung tissues which seems to contribute to the progression of the Th2 inflammation. Besides, eosinophils produce pro-angiogenic and pro-fibrotic cytokines such as transforming growth factor (TGF- β) (Mauviel, 2005) and vascular endothelial growth factor (VEGF) (Horiuchi and Weller, 1997) and may thus contribute to the lung structural changes following allergic inflammation. TGF- β is a pleiotropic factor produced by a variety of cells, the actions of which are dependent on the context of its release. In normal lungs, TGF- β mRNA is expressed in small amounts in bronchial epithelium, mesenchymal cells, vascular endothelium and alveolar cells including macrophages (Letterio and Roberts, 1998). In several studies, TGF- β has been shown to be a marker of tissue repair and remodeling and its increase is considered to precede abnormalities in lung functions (Coker et al., 1996). The release of this cytokine in experimental asthma was reported to occur early (hours) after the antigen challenge of sensitized animals (Bartram and Speer,

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2004). TGF- β is a potent inducer of fibronectin, proteoglycans and collagen from fibroblasts. It also induces fibroblast migration and stimulates the synthesis of other growth factors (Khalil et al., 1989; Kingston et al., 2001). VEGF is a multifunctional cytokine with potent actions on vascular endothelial cells. It can increase vascular permeability, induce endothelial cell mitogenesis, enhance cell migration and is required for endothelial cell survival (Chavakis and Dimmeler, 2002; Ferrara et al., 2003). In asthma, the increased vascularization of the airway walls correlated with increased VEGF expression in the airways (Hoshino et al., 2001), and direct evidence for VEGF involvement in lung tissue remodeling has been brought up by Lee et al. (2004).

Experimental evidence suggests that bradykinin receptors are involved in asthma. Bradykinin is an endogenous nonapeptide with vasoactive properties released in blood and tissues by the proteolytic action of kallikrein on plasma kininogens. Bradykinin and des-Arg⁹-bradykinin have broad effects mediated by two related G-protein-coupled receptors termed the bradykinin receptors B₁, strongly regulated during inflammation, and B₂, constitutively expressed in many cell types (Marceau and Regoli, 2004). The B₂ receptor antagonist HOE140 was shown to have different effects on bronchoalveolar lavage fluid eosinophilia depending on the model of asthma employed: it decreased the eosinophils in guinea pigs (Farmer et al., 1992; Perron et al., 1999) and rats (Pasquale et al., 1991), increased eosinophils in mice (Landgraf et al., 2003, 2004) and had no effect in rabbits (Woisin et al., 2000). The inducible B₁ receptors are usually associated with pro-inflammatory effects since selective antagonists inhibited eosinophils, both in guinea pig models reported by Farmer et al. (1992) and Perron et al. (1999) and in a murine model (Landgraf et al., 2004).

In the present study we investigated the effect of the antagonists of B₁ and B₂ receptors, R954 and HOE 140, respectively, in eosinophil proliferation in the bone marrow, their activation (lipid body formation) and migration into the lung parenchyma and airways. We also investigated their effect on the airway levels of TGF- β and VEGF and on the localization of these cytokines in the lungs.

2. Methods

2.1. Animals

Male BALB/c mice weighing 20–25 g, 6–8 weeks old, from our own animal facilities were housed in a room with 12 h light/dark cycle with water and food *ad libitum*. Animal care and research protocols were in accordance with the principles and guidelines adopted by the Brazilian College of Animal Experimentation (COBEA) and approved by the Biomedical Sciences Institute/USP-Ethical Committee for Animal Research (CEEA).

2.2. Induction of allergic inflammation

Mice were immunized on days 0 and 7 by an intraperitoneal injection of a mixture containing 50 μ g of ovalbumin (OVA) and 1 mg of Al₂O₃ (alum) in saline. Fourteen and 21 days after the first immunization, the animals were challenged by exposure to aerosolized OVA (grade III, 2.5% wt/vol in saline) generated by an ultrasonic nebulizer delivering particles of 0.5–10 μ m diameter at approximately 0.75 cm³/min for 30 min. Animals were sacrificed 24 h later.

The control group consisted of animals immunized as described and exposed to aerosols of isotonic saline solution. In the treated groups, the animals received either the B₁ antagonist R954 (Ac-Orn[Oic², α MePhe⁵, D⁶-Nal⁷, Ile⁸]des-Arg⁹-bradykinin, 100 μ g/kg, i.p.), or the B₂ antagonist HOE140 (D-Arg-[Hyp³-Thi⁵-D-tic⁷-Oic⁸]-bradykinin, 100 μ g/kg i.p.) 30 min before each challenge. The dose

of each antagonist has been previously shown to abolish the responses to either the selective B₁ receptor agonist, des-Arg⁹-bradykinin or the B₂ agonist, bradykinin in selected animal models (Perron et al., 1999; Gabra et al., 2006).

2.3. Harvesting of bone marrow and bronchoalveolar cells

The animals were killed by an intraperitoneal injection of ketamine/xylazine (50 μ L of a 100 mg/ml solution) at day 22. For collecting bronchoalveolar lavage (BAL) fluid cells, a tracheal cannula was inserted via a midcervical incision and the airways were washed three times with 1 ml of phosphate-buffered saline (PBS, 10 mM; pH 7.4 at 4 °C). Bone marrow cells were harvested after dissection of both femurs by flushing the marrow cavity three times with 1 ml of PBS.

2.4. Differential cell counts

The BAL fluid and bone marrow cell suspensions were centrifuged at 170g for 10 min at 4 °C, the supernatant was removed and the cell pellet was re-suspended in 0.5 ml of PBS. One volume of a solution containing 0.5% crystal violet dissolved in 30% acetic acid was added to nine volumes of the cell suspension. The total number of cells was determined by counting in a hemacytometer. Differential cell counts were performed in cytospin or histological lung preparations stained with hematoxylin/eosin.

2.5. Lipid body analyses

While still moist, leukocytes on cytospin slides were fixed in 3.7% formaldehyde in Ca²⁺ Mg²⁺-free HBSS (pH 7.4), rinsed in 0.1 M cacodylate buffer (pH 7.4), stained with 1.5% OsO₄ (30 min), rinsed in distilled water, immersed in 1% thiocarbohydrazide (5 min), rinsed in 0.1 M cacodylate buffer, stained again with 1.5% OsO₄ (3 min), rinsed in distilled water and then dried and mounted. The morphology of fixed cells was observed and lipid bodies were counted under light microscopy with a 100 $\times$ objective lens in 25 consecutively scanned eosinophils.

2.6. Histopathologic analyses

Lungs were dissected after BAL fluid collection, immersed in 10% phosphate-buffered formalin for 24 h and then in 70% ethanol until embedding in paraffin. Tissues were sliced in 5 μ m sections and stained with hematoxylin/eosin for evaluation of the number of tissue eosinophils. Eosinophils around bronchi were enumerated by light microscopy at 250 $\times$ magnification and divided by the number of bronchi present in the slide.

2.7. Immunohistochemistry techniques

Tissue slices were deparaffinated by washing in xylene (3 $\times$ 15 min) and then rehydrated by a series of 5 min wash in graded ethanol (100%, 95%, 80%, 70% and 50% in water) followed by water and 10 mM PBS, pH 7.4 (2 $\times$ 10 min). Non-specific sites were blocked by incubation in TN buffer (100 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween 20) containing 0.5% blocking solution for 10 min. The tissues were then incubated overnight at 4 °C with one of the primary antibodies diluted in TN buffer: anti-B₁ receptor, anti-VEGF or anti-TGF- β (1:250). The slices were washed with TN buffer (3 $\times$ 10 min) and incubated for 1 h with an anti-IgG Alexa 488 or an anti-IgG alkaline phosphatase conjugated secondary antibody, detected with Fast Red. After washing with TN buffer (3 $\times$ 10 min), slides were air-dried and mounted with pro-long anti-fade with DAPI. Control slides were made using goat IgG as primary antibody.

2.8. VEGF and TGF- β analyses

The VEGF and TGF- β contents of BAL fluid supernatants were measured by specific enzyme-linked immunoassay kits according to the manufacturer's instructions.

2.9. Drugs and reagents

Anti-B₁ receptor, anti-VEGF and anti-TGF- β (1:250, Santa Cruz Biotechnology, Santa Cruz, CA, USA); blocking solution (Biogenex, San Ramon, CA, USA); anti-IgG Alexa 488, anti-IgG alkaline phosphatase conjugated secondary antibody and DAPI (Molecular Probes, Foster City, CA, USA); Ovalbumin and Fast Red (Sigma, St. Louis, MO, USA); VEGF and TGF- β (R&D Systems (Minneapolis, MN, USA), ROE 140 and R; aluminium hydroxide gel (Reheis Inc., Berkley Heights, NJ, USA).

The antagonists of bradykinin receptors, R-954 and HOE-140 were synthesized in the Dept. of Pharmacology, University of Sherbrooke, Quebec, Canada.

2.10. Statistical analyses

Results are means $\pm$ SEM of 5–6 determinations. Statistical differences between groups were determined by analysis of variance (ANOVA), complemented with Neuman–Keul's test. Values of $P < 0.05$ were considered indicative of a significant difference between means.

3. Results

3.1. Bradykinin receptor antagonists modulate eosinophils in selected compartments

Mice immunized with OVA and submitted to two OVA aerosol challenges (experimental group) or saline aerosol (control group) were either pretreated with one of the antagonists of bradykinin receptors or with the vehicle before each challenge. The non-treated and treated control groups were not statistically different and only the non-treated controls were presented in the figures. As shown in Fig. 1, the number of eosinophils in the experimental group increased in the three compartments analyzed 24 h after the last challenge: Bone marrow (Fig. 1A), lung parenchyma (Fig. 1B) and BAL fluid (Fig. 1C). Pre-treatment with the B₁ receptor antagonist R954 before each challenge reduced the eosinophil proliferation in the bone marrow by 67% and their migration into the BAL fluid by 57%, but did not affect the number of eosinophils in the parenchyma. The B₂ antagonist HOE140 neither affected the eosinophil proliferation in the bone marrow nor their infiltration into the parenchyma. However it increased the number of eosinophils in the BAL fluid by 42%.

Lipid bodies are intracellular lipid-rich organelles which have been shown to play a role in eicosanoid production in leukocytes, and their number in the cytoplasm can be used as a marker of cell activation (Bozza et al., 2007). In bone marrow-derived eosinophils the number of lipid bodies increased significantly in the immunized and challenged mice compared to the control mice (Fig. 2A). Pre-treatment with HOE140 did not alter this profile whereas the pre-treatment with R954 reduced by 37% the number of lipid bodies per bone marrow eosinophil. In BAL fluid eosinophils, the number of lipid bodies also increased in the immunized and challenged mice, and pre-treatment with HOE140 increased significantly (29%) the number of lipid bodies whereas pre-treatment with R954 reduced their number by 22% (Fig. 2B).

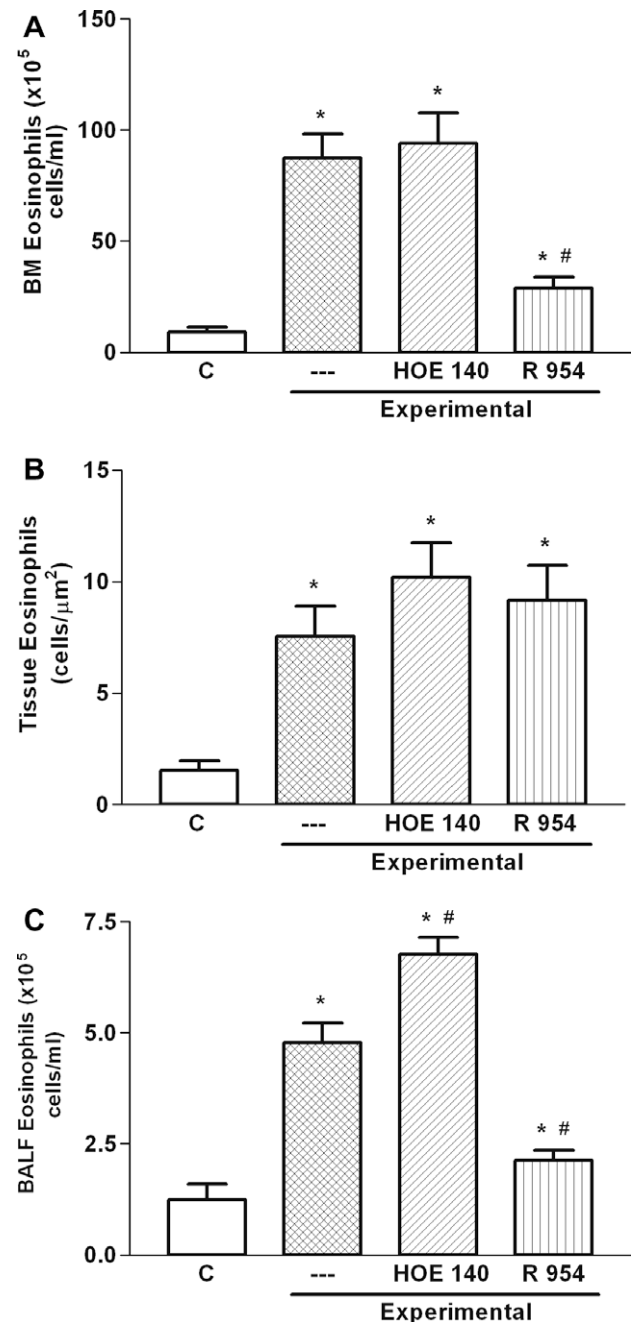


Fig. 1. Effects of bradykinin antagonists on eosinophil counts in selected body compartments. Mice were immunized with an i.p. injection of OVA/alum, one booster injection 7 days later and two OVA aerosol challenges on days 14 and 21 post-immunization. HOE140 or R954 (100 μ g/kg, i.p.) were administered 30 min before each challenge. Cells were harvested 24 h after the second challenge (experimental group). Eosinophils from the bone marrow (A), lung tissue (B) or bronchoalveolar lavage fluid (C) were counted in preparations stained with hematoxylin/eosin. Control groups consisted of sensitized mice given aerosol saline. Results shown are the mean $\pm$ SEM of six animals per group. * $P < 0.05$ comparing the experimental groups with the control group; # $P < 0.05$ comparing the experimental groups, drugs-treated with the non-treated.

3.2. Effect of bradykinin receptor antagonists on TGF- β and VEGF levels in BAL fluid

The levels of TGF- β and VEGF in the BAL fluid of immunized mice given aerosol saline (control) were not significantly different from those found in naïve mice. However, 24 h after the antigen challenge, the levels of both cytokines significantly increased.

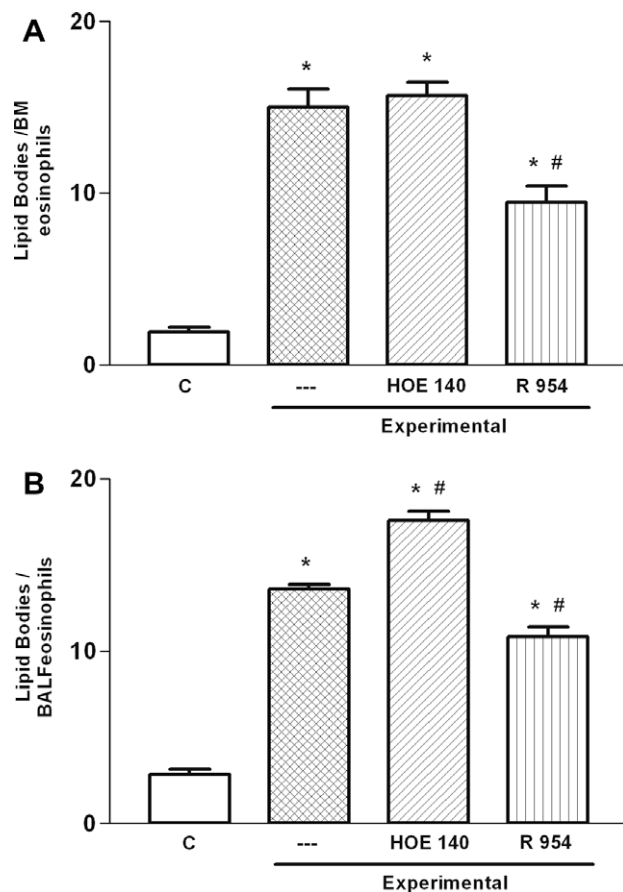


Fig. 2. Effects of bradykinin antagonists on eosinophil lipid bodies. Mice were immunized with an i.p. injection of OVA/alum, one booster injection 7 days later and two OVA aerosol challenges on days 14 and 21 post-immunization. HOE140 or R954 (100 µg/kg, i.p.) were administered 30 min before each challenge. Bone marrow-derived (BM) cells (A) and bronchoalveolar lavage fluid cells (B) were harvested 24 h after the second challenge. Lipid bodies present in eosinophils were counted under light microscopy after osmium staining. Results shown are the mean $\pm$ SEM lipid bodies per eosinophils from 25 eosinophils from each of six animals per group. * P < 0.05 comparing the experimental groups with the control group; # P < 0.05 comparing the experimental groups, drugs-treated with the non-treated.

The pre-treatment with R954 further increased TGF- β and VEGF levels (54% and 37% increase, respectively, compared to the levels found in non-treated mice). HOE140 did not significantly affect the increased levels of TGF- β (Fig. 3A) but it increased the levels of VEGF by 27% (Fig. 3B). Groups of mice were treated with the drug vehicle and the results did not differ from those of the non-treated animals (results not shown).

3.3. VEGF, TGF- β and B_1 receptor expression in lung tissues

Lungs were harvested 24 h after the second challenge, fixed and stained for immunohistochemistry techniques as described in Section 2. Antibodies to VEGF and TGF- β were detected with Alexa 488, producing a green color and the antibody to B_1 was detected with Fast Red, producing a red color. Cell nuclei were stained in blue. Fig. 4A shows a representative slide from a lung of the control group (naïve mice) double stained for TGF β and for the B_1 receptors. Fig. 4B and C shows lungs from the experimental group (immunized mice, 24 h after antigen challenge). The B_1 receptors (Fig. 4B) were found to be expressed in the vascular endothelial cells (arrows). TGF- β is located in the smooth muscle layer (arrows) under the endothelium (Fig. 4C).

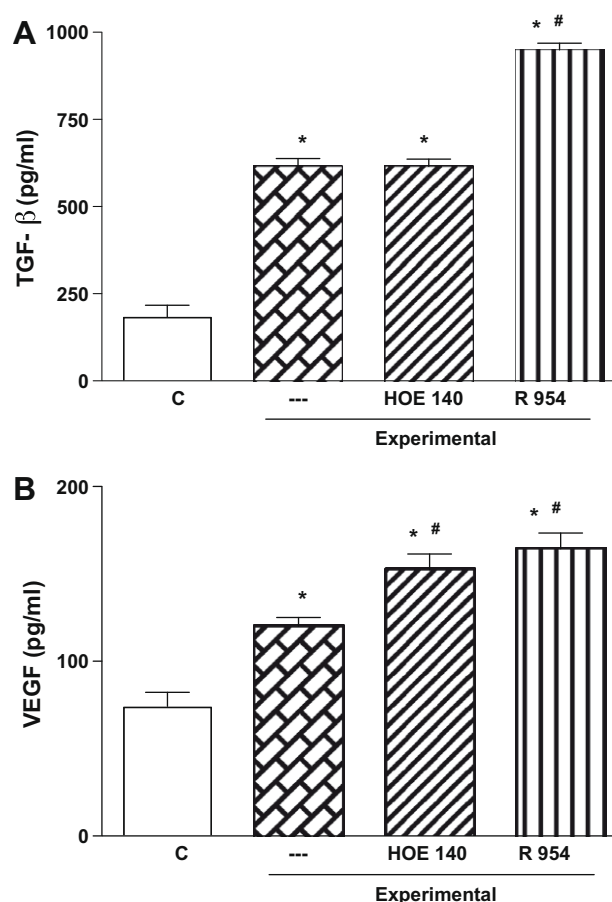


Fig. 3. Effects of bradykinin antagonists on VEGF and TGF- β levels in bronchoalveolar lavage fluid. Mice were immunized with an i.p. injection of OVA/alum ovalbumin/alum, one booster injection 7 days later and two OVA aerosol challenges on days 14 and 21 post-immunization. Control group consisted of immunized mice that received saline nebulization instead of OVA. HOE140 or R954 (100 µg/kg, i.p.) was administered 30 min before each challenge. The contents of TGF- β (A) and VEGF (B) in the BAL fluid were measured 24 h after the antigen challenge by specific enzyme-linked immunoassays according to the manufacturer's instructions. Results shown are the mean $\pm$ SEM from 5 to 6 animals per group. * P < 0.05 comparing the experimental groups with the control group; # P < 0.05 comparing the experimental groups, drug-treated groups with the non-treated.

Fig. 5A shows a representative slide from a lung of the control group (naïve mice) stained for VEGF. In the experimental group (Fig. 5B), VEGF is mostly located in the luminal portion of the bronchial epithelial cells.

VEGF and TGF β staining was not detected in the naïve group, but it was detected in the immunized/saline challenged group (not shown) which suggests that the immunization process by itself induced the expression of these cytokines. This might be explained by the protocol of immunization employed in this study which consisted of two i.p. injections of antigen in alum, 1 week apart.

We also investigated the expression of VEGF and TGF β in mice treated with the BK antagonists. The staining pattern for VEGF and TGF β was apparently not altered by blocking the B_1 or B_2 receptor (not shown).

4. Discussion

To further characterize the role of the bradykinin receptors in a murine model of asthma, compound R954, a selective B_1 antagonist and HOE140, a well known B_2 antagonist which has been more

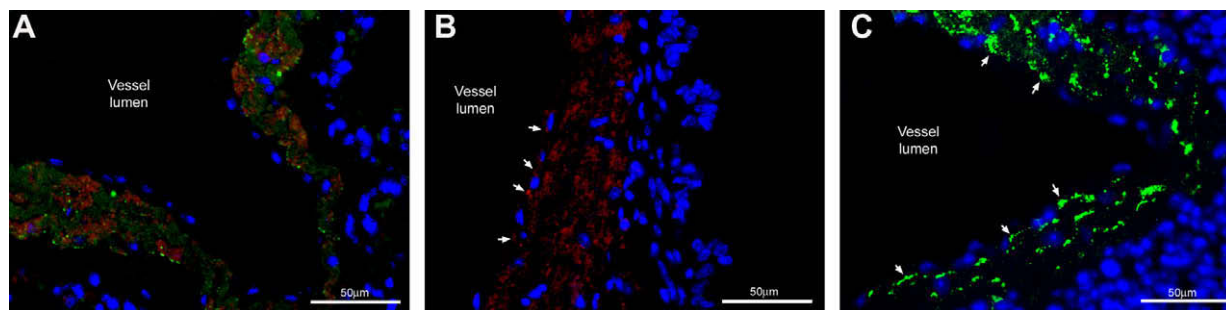


Fig. 4. TGF- β and B₁ receptor localization in lung tissues. Antibody to TGF- β was detected with Alexa 488 which produced a green color, and the antibody to B₁ was detected with Fast Red which produced a red color. In (A) naive lungs double-stained for B₁ receptors and TGF- β . Lungs from immunized mice, 24 h after antigen challenge (B and C). Note that the B₁ receptors (B) are expressed in the endothelial cells (arrows). TGF- β (C) is located in the smooth muscle layer (arrows) under the endothelium (B). Cell nuclei are stained in blue. Black bars = 100 μ m.

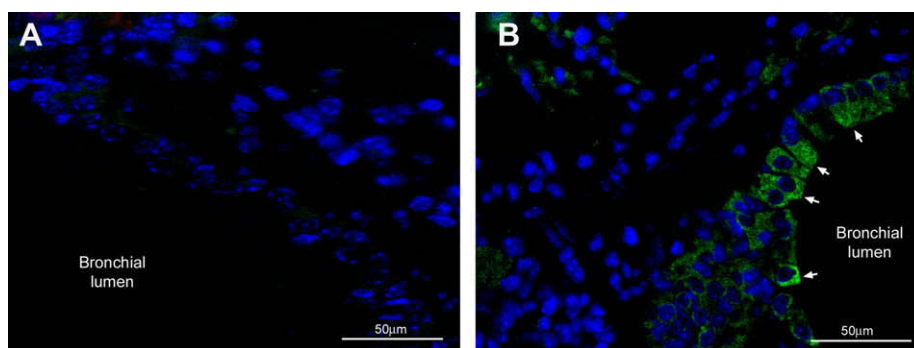


Fig. 5. VEGF localization in lung tissues. VEGF was detected with Alexa 488 (green color). Naive lungs presented no staining for VEGF (A). In (B) lungs from an immunized mouse 24 h after antigen challenge. A clear and strong fluorescence inside the epithelial cells on the bronchial wall (arrows) can be seen which is not present in the naive group. Cell nuclei are stained in blue. Black bars = 100 μ m.

extensively studied in asthma were used. R954 is a peptide developed for human use with non-natural amino acid replacements that protects it from amino peptidases M and P action as well as from the angiotensin converting enzyme, thus enhancing its bio-availability (Kay et al., 2004).

In previous articles from our group, we used the same protocol of immunization and challenges as in the present study to induce asthma in C57Bl/6 (Landgraf et al., 2003) and in BALB/c mice (Landgraf et al., 2004). These mouse strains are used in asthma research as prototypic strains, C57Bl/6 being more susceptible than BALB/c regarding the development of asthma symptoms. In our studies cited above, they differed markedly in eosinophil infiltration into the BALF (around 13×10^5 eosinophils in C57Bl/6 and 3×10^5 in BALB/c). Despite this difference we found that in both strains R954 inhibited whereas HOE140 increased the number of eosinophils in BALF. In the present study we used BALB/c mice and found that the eosinophils that migrated into BALF were activated (evaluated by the increased number of lipid bodies). Both the number of BALF eosinophils and the number of lipid bodies/eosinophil were reduced by R954 while increased by HOE 140. When we examined the number of eosinophils in bone marrow, they increased after antigen challenges and presented augmented number of lipid bodies/eosinophil. Both events were significantly reduced by R954. It is generally believed that the number of lipid bodies present in the leukocyte cytoplasm represents a marker of cell activation. Lipid bodies are specialized, inducible intracellular entities that function as signaling platforms in the inflammatory mediator production (Bozza et al., 2007). Our results clearly showed that the antagonism of the B₁ receptors reduced two related cell events elicited by mouse sensitization and challenge, the bone marrow eosinophil proliferation and their migration into the airways as

well as the cell activation as underlined by the decreased numbers of lipid bodies in the cytoplasm of cells from both the bone marrow and the BAL fluid.

After antigen challenges the eosinophils infiltration around bronchi were not affected by R954 treatment. This is in apparent conflict with our previous study where we reported reduction of the peribronchovascular leukocyte infiltration by R954 (Landgraf et al., 2003). However, in that study we measured the size of the peribronchovascular cell infiltrate area which consisted of mononuclear and polymorphonuclear cells, whereas in the present work we counted the number of eosinophils around bronchi only.

The finding that the treatment with compound R954 reduced the number of eosinophils in the bone marrow but did not affect the eosinophil infiltration in parenchyma could possibly be due to eosinophil progenitor proliferation in lung tissues as a result of antigen challenge. Robinson et al. (1999) previously showed that progenitor cells committed to the eosinophilic lineage (CD34⁺/IL5R α ⁺) were identified in the lungs of asthmatic subjects and were able to proliferate locally after antigen challenge. It would be likely that these progenitor cells have not expressed the inducible B₁ receptors which would explain the lack of effects of R954, the B₁ receptor antagonist, in lung tissues.

Our results also show that the B₂R antagonist, HOE140, increased eosinophils migration and activation in the BALF but did not affect eosinophils in the other compartments analyzed (bone marrow and parenchyma). This is in agreement with previous data from our group in the same model of murine asthma (Landgraf et al., 2003). However, in other animal species the results are different: B₂ antagonists were reported to inhibit cell infiltration in allergic lung inflammation in guinea pig (Farmer et al., 1992; Peron et al., 1999) or to have no effect on antigen-induced pulmonary

eosinophilia in rabbit (Woisin et al., 2000). This discrepancy could be explained by the variable extent of bradykinin receptor expression among species as reported by Marceau and Regoli (2004) or by the different experimental immunization protocols employed. A number of studies have shown that the protocols of immunization and challenge play a prominent role on the production of mediators and cytokines as well as on the expression of bradykinin receptors in the lungs (Smith and Broadley, 2007; Bertram et al., 2009). Indeed, in our previous work the B₂R antagonist HOE140 increased BALF eosinophilia when measured after the two antigen challenges (HOE140 being given before each antigen challenge) whereas it has an inhibitory effect when eosinophils counts were performed after the first challenge (Landgraf et al., 2003).

The cytokines VEGF and TGF- β were previously shown to have a wide range of biological activities which include the induction of angiogenesis and of extravascular matrix protein synthesis, two characteristic features of tissue remodeling (Fattouh and Jordana, 2008). In the present study, increased levels of VEGF and TGF β were detected in the BAL fluid of sensitized mice after antigen challenge and treatment with R954 caused a further increase. The increased levels of these cytokines after treatment with the B₁R antagonist does not necessarily exclude its beneficial effects. TGF- β is a multifunctional cytokine known to possess anti-inflammatory and immuno-suppressive properties (Heijink et al., 2009; Thomas et al., 2009). In allergic airway diseases it has several effects on airway epithelium and it has been suggested that it may have a beneficial role in the treatment of nasal polyposis and sinus scar tissue formation (Salib and Howarth, 2009).

When we analyzed the expression of these cytokines in the lung by immuno-histochemistry TGF- β was found expressed by cells of the muscular layer of small blood vessels and VEGF in bronchial epithelial cells. Interestingly we found these cytokines also expressed in the immunized/saline challenged group but, since we did not quantify the intensity of the fluorescence, we are unable to say if the expression is different among the immunized/antigen challenged and immunized/saline challenged groups. In the lungs from naïve mice however, expression of these cytokines was not detected. These results suggest that the immunization protocol used in this study, which consisted of two intraperitoneal injections of the antigen, 1 week apart, could already induce an allergic reaction in the lung, of low intensity since we did not find eosinophils infiltration in this group. It seems that the expression of these cytokines in the lung is either very sensitive to allergic stimulation and/or the assay of these cytokines has very high sensitivity.

When we investigated the expression of VEGF and TGF β in mice treated with the BK antagonists we observed that the staining pattern for both cytokines was apparently not altered by blocking the B₁ or B₂ receptors. But we do not know if the treatments modified the quantity expressed since our assay was not quantitative. We can conclude that TGF β and VEGF are produced in this model of asthma and that at least in the BALF the production is regulated by the B₁ receptor.

Finally, we analyzed by immunohistochemistry the expression of the B₁R and found that it is expressed in the lung endothelial cells and it could be detected only in the immunized/antigen challenged group. However, because of the low specificity of the antibody to B₁R employed in this study, it is not possible to conclude that its expression is restricted to the lung endothelial cells.

In summary, the antigen administration into the airways of sensitized mice has local and systemic consequences which are partially dependent of the bradykinin inducible receptor expression and activation. Our results showed that the eosinophil proliferation in the bone marrow, their migration into the airways and their activation measured by the increased lipid bodies formation were reduced by treatment with a B₁ receptor antagonist. The production of TGF- β and VEGF was increased by this treatment. Increased

levels of these cytokines could accelerate tissue repair after the allergic injury or contribute to the fibrosis that is a characteristic feature of the chronic phase of asthma. Although the B₁ receptor antagonist clearly modulated the early phase of allergic lung inflammation, more studies are needed before its possible benefit in the treatment of asthma could be confirmed.

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