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Differential modulation of murine lung inflammation by bradykinin B_1 and B_2 selective receptor antagonists

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Abstract

The effect of bradykinin receptor antagonists was studied in a mouse (C57Bl/6) model of allergic lung inflammation. Bradykinin B_2 receptor antagonist HOE-140 (D-Arg-[Hyp^3,Thi^5,Dtic^7-Oic^8]bradykinin) or bradykinin B_1 receptor antagonist R-954 (Ac-Orn[Oic^2,αMePhe^5,βD-Nal^{7}Ile^8]des-Arg^9-bradykinin) were given i.p. to ovalbumin sensitized mice 30 min before antigen challenge. After 24 h, bronchoalveolar lavage was performed for cell analysis and the lungs were removed for evaluation of airway hyperreactivity and histopathology. Treatment with HOE-140 caused a significant increase in bronchoalveolar lavage cell number: eosinophils (182%), neutrophils (98%), lymphocytes CD_4^+ (192%), CD_8^+ (236%), B220 (840%), $T\gamma\delta^+$ (194%) and $NK1.1^+$ (246%). Hyperreactivity and mucus secretion were not significantly affected in this group. Treatment with R-954 significantly reduced eosinophil (79%) and neutrophil (83%) but has no effect on lymphocytes number in bronchoalveolar lavage fluid. Airway hyperreactivity and mucus secretion were reduced by this treatment (84% and 35%, respectively). These results show important modulatory effect of bradykinin B_1 and B_2 receptors on allergic lung inflammation.

Keywords: Bradykinin; Bradykinin receptor antagonist; Airway inflammation; Eosinophil; Asthma

1. Introduction

Bradykinin is an endogenous nonapeptide (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) with vasoactive properties, generated during inflammatory reactions. It is released from tissue and plasma kininogens by the proteolytic action of kallikrein. High molecular weight kininogens are present in the plasma, whereas the low molecular weight is also present in tissues (Nakanish, 1987). Bradykinin is generated from the high molecular weight kininogen, whereas lysil-bradykinin (kalidin) is generated from the low molecular weight kininogen. Kallidin is rapidly converted to bradykinin by the aminopeptidase-N (Proud and Kaplan, 1988). Bradykinin is transformed by kininase I to its active metabolite des-Arg^9-bradykinin.

Two subtypes of bradykinin receptors have been identified, B_1 and B_2 (reviewed by Regoli and Barabé, 1980; Marceau et al., 1998). The bradykinin B_2 receptor is expressed constitutively in several tissues and is considered to mediate the majority of bradykinin effects such as hypotension, vasodilation, increased vascular permeability and inflammatory pain. The bradykinin B_1 receptor is generally absent in normal tissues but is expressed in some inflammatory conditions such as septic shock (Eich-Rathfelder et al., 1997), rheumatoid arthritis (Cassim et al., 1997) and lung inflammation (Perron et al., 1999).

The bradykinin B_1 receptors are preferentially activated by des-Arg^9-bradykinin and des-Arg^{10}-kallidin, whereas the bradykinin B_2 receptors are optimally stimulated by bradykinin and kallidin.

Experimental evidence suggests that bradykinin and its biologically active metabolite des-Arg^9-bradykinin play a role in experimental models of asthma although its role in human airways is not well established. Elevated levels of bradykinin were found in bronchoalveolar lavage fluid from asthmatic patients (Christiansen et al., 1992). Inhalation of bradykinin by asthmatic patients caused strong bronchoconstriction (Barnes, 1992) but was essentially inactive in non-asthmatics (Fuller et al., 1987).

Bronchoconstriction induced by bradykinin in humans is mediated by bradykinin B_2 receptors (Molimard et al., 1994; Hulsmann et al., 1994). The bradykinin B_1 receptor

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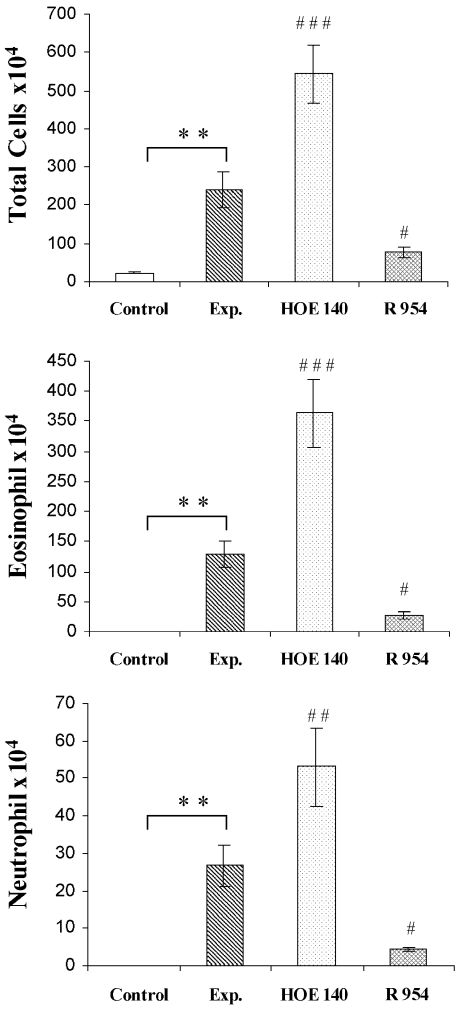

Fig. 1. Effect of Bradykinin B_1 and B_2 receptor antagonists on the bronchoalveolar lavage cells. C57Bl/6 were immunized with an i.p. injection of ovalbumin/alumen, one booster injection 7 days later, and two ovalbumin aerosol challenges (2.5%, 20 min) on days 14 and 21 post-immunization. Bradykinin receptor antagonists were given i.p. (100 μg kg^{-1}) 30 min before each aerosol challenge. Bronchoalveolar lavage was performed 24 h after the second challenge. Eosinophils and neutrophils were counted in cytocentrifuge preparation of bronchoalveolar lavage cells stained with hematoxylin/eosin. Results are the mean ± S.E.M. of 6–8 animal groups. $^{**}P<0.01$ in comparison with the control group and $\#P<0.05$, $\#\#P<0.01$, $\#\#\#P<0.001$ in comparison with the experimental group.

agonist des-Arg^9-bradykinin did not have any effect on the airways of asthmatic patients (Polosa and Holgate, 1990). In animal models, injection of bradykinin in airways induces eosinophilia in several species including guinea pig (Fechter et al., 1986; Farmer et al., 1992) and rat (Pasquale et al., 1991; Ferreira et al., 1996). Allergen induced airway eosinophilia in guinea pig was inhibited by a bradykinin receptor antagonist (Barnes et al., 1998; Perron et al., 1999).

Preliminary results from our group showed that bradykinin and des-Arg^9-bradykinin receptor antagonists inhibit the recruitment of mononuclear cells and eosinophils to the airways of sensitized mice, which suggested the presence of both B_1 and B_2 bradykinin receptors in murine airways (Eric et al., 2000).

Clearly, more studies are needed to elucidate the role of bradykinin and its receptor subtypes in asthma. The aim of the present study was to investigate the role of the bradykinin receptor antagonists R-954 and HOE-140, respectively B_1 and B_2 receptor antagonists, in eosinophil infiltration, increased airway reactivity and mucus production in a murine model of asthma. The effect of these antagonists on the lymphocyte subpopulations (CD_4^+, CD_8^+, B, $T\gamma\delta^+$, $NK1.1^+$) in bronchoalveolar lavage, analyzed by flow cytometry, was also investigated.

2. Materials and methods

2.1. Animals

Male C57Bl/6 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. Immunization protocol

Mice were sensitized on days 0 and 7 by intraperitoneal injection of a mixture containing 50 μg of ovalbumin and 1 mg of Al(OH) in saline (a total volume of 0.6 ml). At 14 and

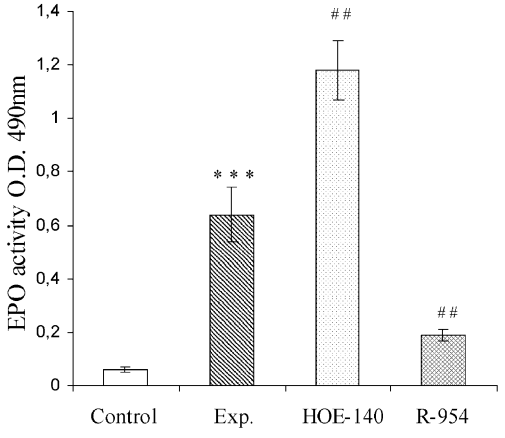

Fig. 2. Effect of Bradykinin B_1 and B_2 receptor antagonists on the bronchoalveolar lavage cell eosinophil peroxidase activity. C57Bl/6 were immunized with an i.p. injection of ovalbumin/alumen, one booster injection 7 days later, and two ovalbumin aerosol challenges (2.5%, 20 min) on days 14 and 21 post-immunization. Bradykinin receptor antagonists were given i.p. (100 μg kg^{-1}) 30 min before each aerosol challenge. Bronchoalveolar lavage was performed 24 h after the second challenge. Results are the mean ± S.E.M. of 7–8 animal groups. $^{***}P<0.001$ in comparison with the control group and $\#\#P<0.01$ in comparison with the experimental group.

21 days after first immunization, the animal were challenged by exposure to an aerosol of ovalbumin (grade III, Sigma) generated by an ultrasonic nebulizer (ICEL US-800, SP, Brazil) delivering particles of 0.5–10 μm diameter at approximately 0.75 cm^3 min^{-1} for 20 min. The concentration of ovalbumin in the nebulizer was 2.5% wt. $vol.^{-1}$.

The control group consisted of animals immunized as described and challenged with saline solution, or non-immunized animals challenged with ovalbumin aerosol as above. In the treated groups, the animals received the bradykinin receptor antagonists R-954 or HOE-140 (i.p.), 30 min before each challenge.

2.3. Bronchoalveolar lavage

The animals were killed by injection of ketamine/xylasine (50 μl of a 100 mg ml^{-1} solution, i.p.) 24 h after exposure to the second aerosol challenge. A tracheal cannula was inserted via a midcervical incision and the airways were lavaged twice with 1 ml of phosphate buffered saline (PBS, pH 7.4 at 4 °C).

2.4. Total and differential cell counts

The bronchoalveolar lavage fluid was centrifuged at $170 \times g$ for 10 min at 4 °C, the supernatant was removed, and the cell pellet was resuspended 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 hematocytometer. Differential cell counts were performed after cytocentrifugation and staining with hematoxylin–eosin (Hema 3).

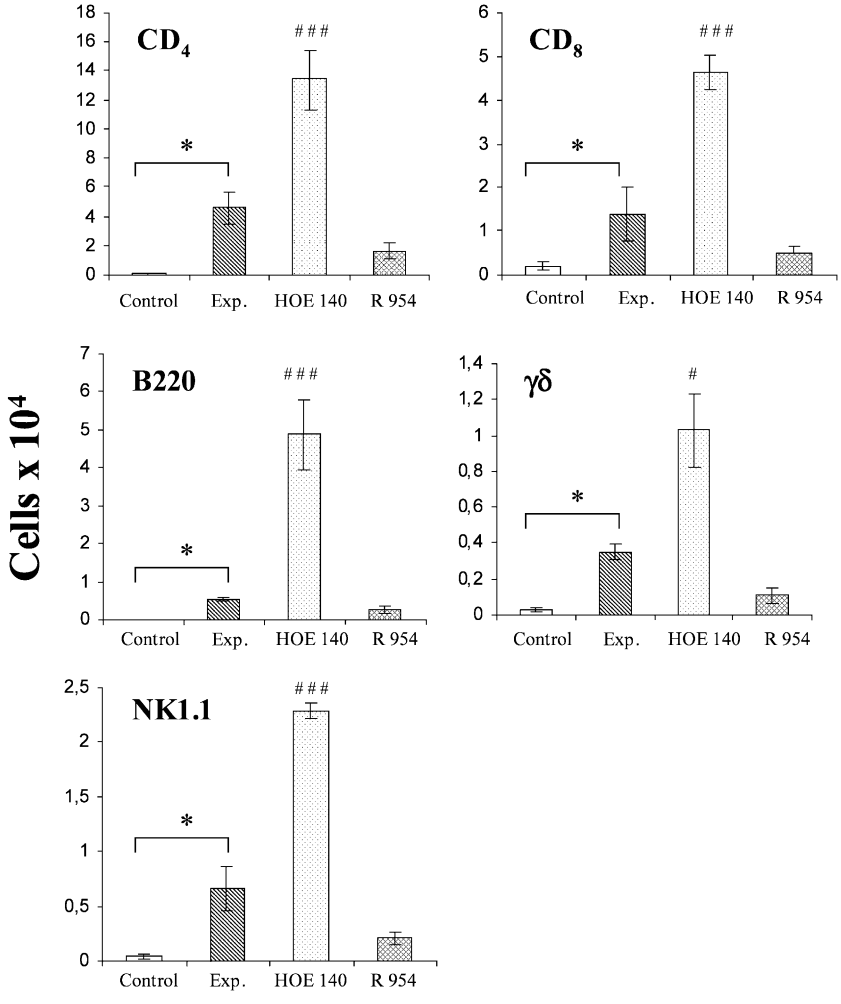

Fig. 3. Effect of Bradykinin B_1 and B_2 receptor antagonists on the bronchoalveolar lavage lymphocyte subpopulations. C57Bl/6 were immunized with an i.p. injection of ovalbumin/alumen, one booster injection 7 days later, and two ovalbumin aerosol challenges (2.5%, 20 min) on days 14 and 21 post-immunization. Bradykinin receptor antagonists were given i.p. (100 μg kg^{-1}) 30 min before each aerosol challenge. Bronchoalveolar lavage was performed 24 h after the second challenge. The cells were incubated with fluorochrome-labeled monoclonal antibodies to CD_4, CD_8, B220, Tγδ, NK1.1 and submitted to FACS analysis. Results are the mean ± S.E.M. of 6–8 animal groups. *$P<0.05$ in comparison with control group and #$P<0.05$, ###$P<0.001$ in comparison with experimental group.

2.5. Flow cytometric analyses of lymphocytes

Phenotypic analysis of lymphocyte subpopulations were assessed by three color FACS, using a Facscalibur Cytometer equipped with Cell Quest software (Becton and Dickinson, San Jose, CA, USA), using gates defined by forward and side light scatter properties. Broncho-alveolar lavage cells were incubated with Fluorescein Isothiocyanate (FITC), R-Phycoerythrin (PE) or Cy-chrome-labeled monoclonal antibodies anti-CD_4 (clone H129.19), anti-CD_8 (clone 53–6.7), anti-CD45R/B220 (clone RA3-6B2), anti-Tγδ (clone GL3) or anti-NK1.1 (clone PK136) and adjusted to 5×10^5 cells ml^{-1} in PBS supplemented with 5% fetal bovine serum and sodium azide (0.1%).

2.6. Assay of eosinophil peroxidase activity

The eosinophil peroxidase activity present in bronchoal-veolar lavage cells was determined with a colorimetric assay as described by Strath et al. (1985) with slight modifications. In brief, the cell suspensions were collected as described above, centrifuged and the cells exposed to Tris–NH_4Cl buffer to lyse erythrocytes. The cells were then washed once with PBS and adjusted to 10^5 cells ml^{-1}. Aliquots of 100 μl of the cell suspension (in duplicate) were transferred to 96-well micro-plates, which were then centrifuged at $150 \times g$ at 4 °C for 10 min. The supernatants were carefully withdrawn and 100 μl of substrate solution containing 2.4 mM of *o*-phenylenediamine in 50 mM Tris–HCl, pH 8.0 and 6.6 mM of H_2O_2 was added to each well, and the plates were incubated at room temperature for 15 min. The reaction was stopped by addition of 50 μl of 4 M H_2SO_4 and the absorbance of the samples determined at 490 nm.

2.7. Evaluation of airway reactivity

Mice were immunized and submitted to two antigen aerosol exposures as described above. Twenty-four hours after the second antigen challenge, mice received an injection of ketamine/xylasine (50 μl of a 100 mg ml^{-1} solution, i.p.), the peritoneal cavity was cut open and animals were exsanguinated by section of the abdominal aorta. The thoracic cavity was then opened; the pulmonary artery was cannulated and perfused with 10 ml Kreb's solution at 10 ml min^{-1}. A cannula was then inserted in the trachea; the lungs were removed carefully and perfused (5 ml min^{-1}) through the trachea with Kreb's (37 °C, 95% O_2 and 5% CO_2) solution. A small incision was made in the lower end of each lobe to permit the outflow of the solution. The perfusion pressure was recorded in a Beckman R511A using Gould P23DB pressure transducers. Increases over basal levels of perfusion pressure following bolus injection of methacholine were taken as a measure of constriction of the airways. Increase in perfusion pressure (cmH_2O) versus dose (μg methacholine) was measured for the entire recording period; areas under the curve were calculated and results expressed as mean area under the curve (mm^2).

2.8. Histopathologic analysis

Lungs were removed after bronchoalveolar lavage collection, perfused via the right ventricle with 10 ml PBS to remove residual blood, immersed in 10% phosphate-buffered formalin for 24 h and then in 70% ethanol until embedding in paraffin. Tissues were sliced and 5 μm sections were stained with hematoxylin–eosin for light microscopy examination or with periodic acid-Schiff (PAS)/hematoxylin for evaluation of mucus-producing cells. The intensity of mucus production was evaluated in each preparation and scores from 0 to 3 were attributed: 0 when none of the bronchi show any sign of mucus; 1, 2 or 3 when 25%, 50% or more than 50% of the bronchi epithelium was covered by mucus. Values represent the sum of 10 bronchi scored randomly at ×250 magnification.

2.9. Drugs and reagents

The antagonists of bradykinin receptors, R-954 (Ac-Orn [Oic^2, αMePhe^5, βD-Nal^{7}Ile^8] des-Arg^9-bradykinin) and HOE-140 (D-Arg-[Hyp^3, Thi^5, Dtic^7-Oic^8]-bradykinin) were synthesized in the Department of Pharmacology, Faculty of Medicine, University of Sherbrooke, Quebec, Canada. Ovalbumin grade III, methacholine, fetal bovine

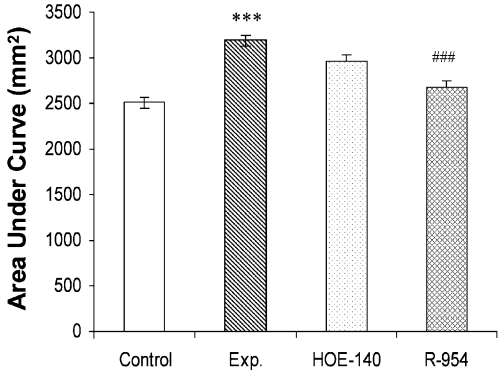

Fig. 4. Effect of Bradykinin B_1 and B_2 receptor antagonists on airway reactivity to methacholine. C57Bl/6 were immunized with an i.p. injection of ovalbumin/alumen, one booster injection 7 days later, and two ovalbumin aerosol challenges (2.5%, 20 min) on days 14 and 21 post-immunization. Bradykinin receptor antagonists were given i.p. (100 μg kg^{-1}) 30 min before each aerosol challenge. Twenty-four hours after the second challenge, the lungs were removed, perfused via the trachea and increases in perfusion pressure to bolus injection of methacholine were recorded. Results are expressed as mean area under the curve ± S.E.M. of 7–8 animal groups. ***$P < 0.001$ in comparison with the control group and ###$P < 0.001$ in comparison with the experimental group.

serum, Tris (hydroxymethyl-aminomethane) and *o*-phenylenediamine dihydrochloride were purchased from Sigma (St. Louis, MO, USA); the monoclonal antibodies to lymphocyte markers from BD Pharmingen (San Diego, CA, USA); the Hema 3 from Biochemical Sciences (Swedesboro, NJ) and aluminum hydroxide gel (Rehydragel) from Reheis (Berkley Heights, NJ, USA).

2.10. Statistical analysis

Data are expressed as the means ± S.E.M. Statistical evaluation of the data was carried out by analysis of variance (ANOVA) and sequential analysis of differences among means was done by Tukey's contrast analysis. A P value lower than 0.05 was considered to be significant.

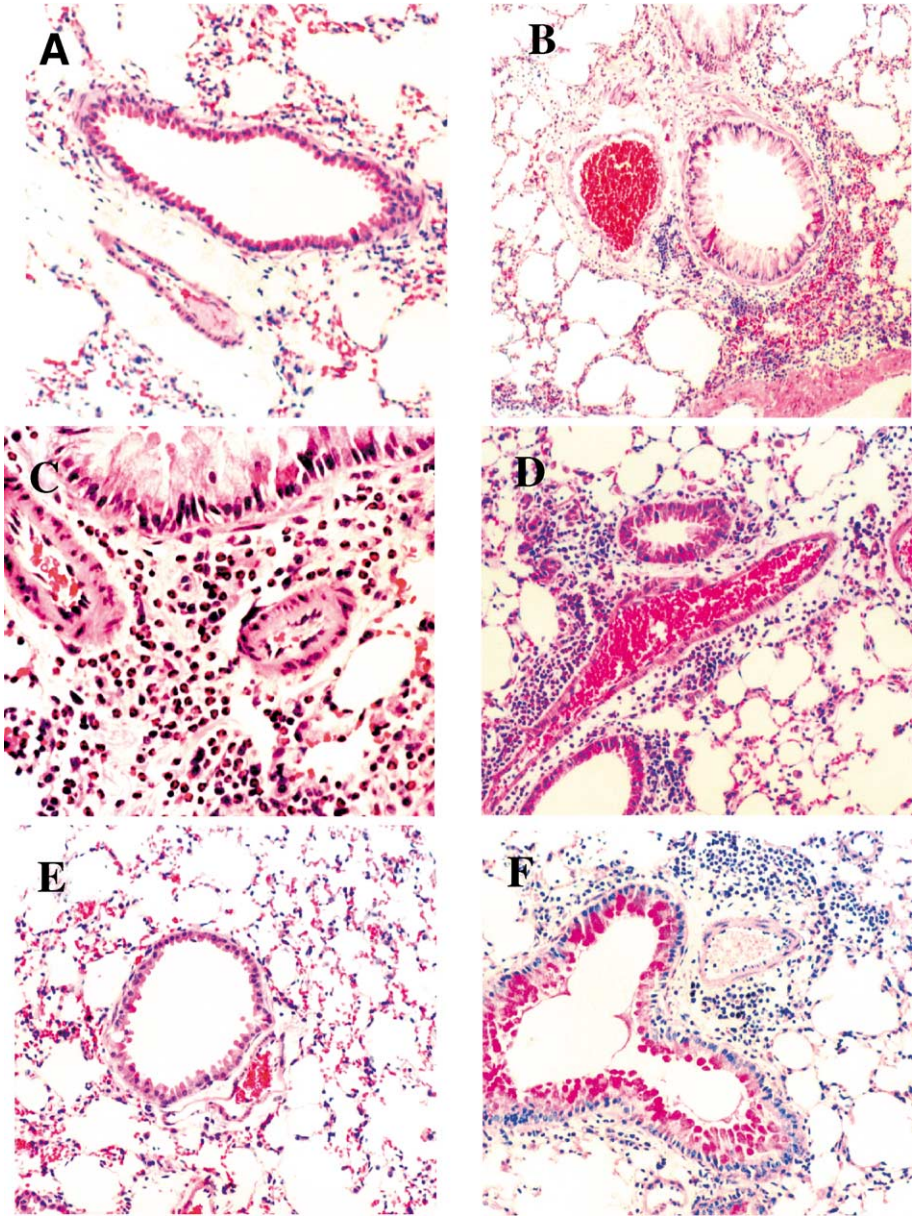

Fig. 5. Photomicrography of pulmonary parenchyma from C57Bl/6 mice 24 h after induction of allergic lung inflammation. Panel A: Normal histological appearance of the lung parenchyma of control animals; hematoxylin/eosin staining, 400 × . Panel B: Lungs from sensitized mice that received two antigen aerosol challenges (experimental group), peribronchial infiltration of polymorphonuclear cells, hematoxylin/eosin, 400 × . Panel C: Higher magnification of the same lung preparation as in (B) showing that eosinophils are abundant in peribronchial infiltrate; hematoxylin/eosin, 800 × . Panel D: Lungs of sensitized mice pretreated with the bradykinin B_2 receptor antagonist (HOE-140, 100 μg kg^{-1}) before each challenge—note the more intense peribronchial infiltrate compared to the non-treated lung (B); hematoxylin/eosin, 400 × . Panel E: Lungs of sensitized mice treated with the bradykinin B_1 receptor antagonist (R-954, 100 μg kg^{-1}) before each challenge—note the marked inhibition of the peribronchial infiltrate; hematoxylin/eosin, 400 × . Panel F: Lungs of sensitized and challenged mice—note the presence of mucus inside bronchial epithelial cells (mucus score 3); periodic acid-Schiff staining, 400 ×.

$P<0.05$, $P<0.01$ and $P<0.001$ were marked with one, two or three asterisks, respectively.

3. Results

3.1. Cells in the bronchoalveolar lavage

3.1.1. Eosinophils and neutrophils

Mice immunized with ovalbumin were submitted to two ovalbumin aerosol challenges and bronchoalveolar lavage was performed 24 h after the second aerosol challenge. A significant increase in total cell number in bronchoalveolar lavage fluid (from 23.6 to 241.7×10^4 cells) was observed in this group compared to the control group (ovalbumin immunized mice submitted to saline aerosol). The increase in cell number observed in the experimental group was due to infiltration of eosinophils and neutrophils, which averaged 53% and 12% of the bronchoalveolar lavage cells, respectively (Fig. 1).

Groups of immunized mice received intraperitoneal injections of the bradykinin antagonists of B_1 or B_2 receptors, R-954 or HOE-140, respectively, 30 min before each of the antigen aerosol challenges. Fig. 1 shows that treatment with HOE-140 increased significantly the number of eosinophils (from 129.11 to 364.12×10^4 cells) and neutrophils (from 26.75 to 53.11×10^4 cells) in the bronchoalveolar lavage, whereas treatment with R-954 significantly reduced the numbers of the eosinophils (from 129.11 to 27.08×10^4 cells) and neutrophils (from 26.75 to 4.47×10^4 cells).

Eosinophil peroxidase activity measured in bronchoalveolar lavage cell suspensions (Fig. 2) was increased in the experimental group. Pre-treatment of the mice with HOE-140 further increased eosinophil peroxidase levels (84.38%) in bronchoalveolar lavage cells, whereas pre-treatment with R-954 caused a significant inhibition (75.31%).

3.1.2. Lymphocytes

Bronchoalveolar lavage cells were incubated with monoclonal antibodies to the cell markers CD_4^+, CD_8^+, B220, $T\gamma\delta^+$, $NK1.1^+$ as described in Materials and methods and submitted to flow cytometric analysis. Fig. 3 shows that whereas these cell populations are either undetectable or found in very low numbers in the control group, a significant increase in all cell populations is observed in the sensitized and challenged group, 24 h after the second challenge.

Sensitized mice that received HOE-140 i.p. 30 min before each of the ovalbumin challenges showed a significant increase in the CD_4^+, CD_8^+, $B220^+$, $T\gamma\delta^+$ and $NK1.1^+$ cells in the bronchoalveolar lavage. Treatment with R-954 as above had no significant effect on lymphocytes migration to the bronchoalveolar space.

We also observed that in the experimental group, a significant percentage of CD_4^+, CD_8^+ and $NK1.1^+$ cells presented larger size and/or different granulosity than cells from the control group. These changes are indicative of cell activation. When we compared the experimental group with the group treated with the bradykinin B_2 receptor antagonist, these cell changes were even more evident. This was not observed in the group treated with the bradykinin B_1 receptor antagonist (data not shown).

3.2. Reactivity of the airways to methacholine

Groups of immunized mice were submitted to two ovalbumin aerosol challenges (experimental group) or saline (control group) and the lungs were removed and perfused through the trachea 24 h later as described in Materials and methods. Bolus injection of increasing doses of methacholine (0.1 to 100 μg) caused bronchoconstriction measured as increase in perfusion pressure. In the experimental group, the reactivity of the airways to methacholine was significantly higher than that of the control group (around 30% higher comparing areas under the curves). Airway hyperreactivity was significantly reduced by pre-treatment of the mice with compound R-954. The group treated with HOE-140 developed the same level of hyperreactivity as the non-treated group (Fig. 4).

3.3. Histopathology of the lungs

As can be seen in Fig. 5, lungs of the experimental group showed a marked inflammatory infiltrate around small vessels and bronchi (Fig. 5B) as compared to the control

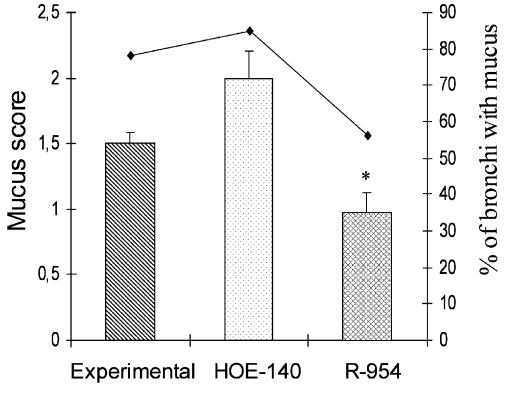

Fig. 6. Effect of bradykinin B_1 and B_2 receptor antagonists on mucus production. C57Bl/6 were immunized with an i.p. injection of ovalbumin/alumen, one booster injection 7 days later, and two ovalbumin aerosol challenges (2.5%, 20 min) on days 14 and 21 post-immunization. Bradykinin receptor antagonists were given i.p. (100 μg kg^{-1}) 30 min before each aerosol challenge. Lungs were removed 24 h after the second antigen challenge, embedded in paraphin, sliced and stained with PAS/hematoxylin for evaluation of mucus. To quantify the intensity of mucus production, 10 bronchi in each preparation were examined for presence of mucus and scores from 0 to 3 were attributed: 0 when none of the bronchi show any sign of mucus 1, 2 and 3 when 25%, 50% and more than 50% of the bronchi epithelium was taken by mucus. The percentage of bronchi with mucus is also shown. Results are the mean ± S.E.M. of 7–8 animal groups. *$P<0.05$ in comparison with the experimental group.

group (Fig. 5A). Neutrophils and eosinophils were found in high numbers among the infiltrated cells (Fig. 5B and C). Treatment with HOE-140 did not affect leukocyte infiltration (Fig. 5D) but treatment with R-954 strongly inhibited cellular infiltrate (Fig. 5E). PAS staining showed several bronchi with mucus in the experimental group (Fig. 5F).

The intensity of mucus production was evaluated in bronchi ($n = 10$) and scored from 0 to 3 as described in Materials and methods. In the control group, none of the bronchi contained mucus (score 0) in contrast to the experimental group were the mean of scores was 1.5. Mucus plugs were not observed in any group. It can be seen in Fig. 6 that HOE-140 had no significant effect on mucus score, whereas R-954 significantly inhibited mucus production.

When analyzing the percentage of bronchi which contained mucus, it was found that 78% were positive in the experimental group and 85% positive in HOE-140 treated group, whereas only 56% of the bronchi were positive for mucus in the R-954 treated group.

4. Discussion

The model used here for the study of lung allergic inflammation consisted of immunization with ovalbumin using alumen as adjuvant followed 14 days later by two ovalbumin-aerosol challenges, 1 week apart. This model presents many of the characteristic features of allergic asthma such as: eosinophil and lymphocyte infiltration, mucus production and airway hyperreactivity. Eosinophils were very significantly increased in bronchoalveolar lavage fluid and represented 53% of total bronchoalveolar lavage cells. There was also an increase in the levels of eosinophil peroxidase activity, which paralleled the eosinophil influx.

Using this model and selective bradykinin B_1 and B_2 receptor antagonists (R-954 and HOE-140), the participation of kinins in the modulation of leukocyte recruitment and migration into the airways was investigated. The results showed that the bradykinin B_1 receptor antagonist (R-954) administered before each antigen challenge reduced the number of eosinophils and neutrophils in the bronchoalveolar lavage fluid. The decrease of the eosinophils count was associated with a decrease in eosinophil peroxidase activity, a decrease in lung hyperreactivity to methacholine, as well as a significant reduction in the amount of mucus in the bronchi. Farmer et al. (1992) demonstrated that the bradykinin B_1 receptor antagonist [Leu^8] des-Arg^9-bradykinin inhibited the leukocyte migration in airway inflammation of guinea pigs induced by antigen and Perron et al. (1999) showed that another bradykinin B_1 receptor antagonist LysLys [Hyp^3, Igl^5, Digl7, Oic^8] des-Arg^9-bradykinin (code name B9858) reduced the eosinophil count and the eosinophil peroxidase activity in guinea pigs bronchoalveolar lavage fluid.

On the other hand, in our experiments, treatment of the animals with the bradykinin B_2 receptor antagonist (HOE-140) increased bronchoalveolar lavage eosinophil and neutrophil numbers as well as the levels of eosinophil peroxidase activity in bronchoalveolar lavage fluid. However, these changes were not associated with increased hyperreactivity to methacholine or with increased mucus in the bronchi.

Allergic asthma is a chronic inflammatory disease associated with T lymphocytes predominantly of the Th2 profile that produce IL-4 and IL-5 considered to be pivotal for the recruitment/activation of eosinophils to the airways (reviewed by Cohn and Ray, 2000). Biopsy studies have revealed that in human asthma, eosinophilic and lymphocytic infiltrations consistently occur in the epithelium and lamina propria. Most of the lymphocytes in the bronchial mucosa were identified as Th2 cells, a profile that is never seen in normal controls. Furthermore, following in vivo allergen-provocation of asthmatic patients the number of $\gamma\delta^+$ T cells was significantly higher in asthmatics than in controls (reviewed by Spinozzi et al., 1998). Another cell population that is normally present in considerable numbers in human lung interstitium is the NK cells (Weissler et al., 1987). The activity of these cells is increased after bronchial allergen challenge in asthmatic subjects (Vesterinen and Timonen, 1988). Mouse NK1.1$^+$ cells represent a small population of natural killer (NK) cells that express T cell receptor, thus are natural killer T cells (reviewed by Bendelac et al., 1997). A human counterpart of mouse NKT cells has been identified (Prussin and Foster, 1997) but the role of these cells in allergic lung diseases has never been investigated. It has been suggested that the NK1.1$^+$ T cells and $\gamma\delta^+$ T cells may regulate the commitment of CD_4^+ T lymphocytes to the Th1 or Th2 profile (Medzhitov and Janeway, 1997) and thus modulate the development of allergic airway disease. Depletion of NK1.1$^+$ cells before the immunization was shown to inhibit the lung eosinophilia in a mice model of allergic asthma (Korsgren et al., 1999). Studies using $\gamma\delta^+$ deficient mice showed that these cells are required for inducing allergen specific Th2 mediated airways inflammation (Zuany-Amorim et al., 1998).

In our model of lung inflammation, we found a clear increase in the number of T$\gamma\delta^+$ and NK1.1$^+$ as well as of CD_4^+, CD_8^+, and B lymphocytes in the bronchoalveolar lavage of immunized mice following antigen challenge. We also observed that they exhibited characteristics of activated cells. It is interesting to point out that this is the reflection of the inflammation that started 1 week before, following the first challenge, plus the 24 h response to the second challenge. Walker et al. (1991) reported that in individuals with bronchial asthma the CD_4^+ and CD_8^+ lymphocytes in the bronchoalveolar lavage were significantly increased, whereas B, T$\gamma\delta^+$ and NK were not increased.

In animals treated with the bradykinin B_1 receptor antagonist, which presented a significant reduction in lung inflammation, there was a tendency to decrease the numbers of NK1.1$^+$ and $\gamma\delta^+$ T cells although it did not reach statistical significance. Same pattern was observed with the

other T lymphocytes analyzed, CD_4^+, CD_8^+ and B lymphocytes. Animals treated with the bradykinin B_2 receptor antagonist, who showed increased eosinophil infiltration, showed increased levels of $NK1.1^+$ and $\gamma\delta^+$ T cells, as well as of the other lymphocytes. However, these changes were not associated with a significant increase of airway reactivity or mucus production.

We speculate that at the beginning of the lung inflammatory reaction induced by antigen challenge, the predominant receptor expressed in local airway cells or blood vessels should be the bradykinin B_2 receptor and we showed here that its activation produced a down-regulation of cellular infiltration. Later on, bradykinin B_1 receptors would also be expressed on local airway cells or migrated cells and their activation would potentiate cellular infiltration as well as airway hyperreactivity and mucus production. This is in agreement with molecular and pharmacological evidence that supports a role for bradykinin B_2 receptors in the acute phase of the inflammatory and pain response, whereas bradykinin B_1 receptors most likely intervene in the chronic phase of inflammatory processes (reviewed by Couture et al., 2001). However, it is important to mention that inhalation of bradykinin causes bronchoconstriction in asthmatic but not in normal individuals, whereas the selective bradykinin B_1 receptor antagonist (des-Arg^9-bradykinin) has no effect on airway function in asthmatic patients (Polosa and Holgate, 1990).

Data from the literature, using bradykinin B_2 receptor antagonists in experimental allergic lung inflammation usually show inhibition of both cell infiltration and hyperreactivity. Farmer et al. (1992), Perron et al. (1999) and Mashito et al. (1999), using a guinea pig model, found that the bradykinin B_2 receptor antagonists D-Arg-[Hyp^3, Thi$^{5-8}$, D-Phe^7]-bradykinin (code name NPC-349) or the D-Arg-[Hyp^3, Thi^5, D-tic^7, Oic^8]-bradykinin (code name HOE-140) inhibited antigen-induced eosinophilia and neutrophilia and decreased eosinophil peroxidase activity. Another bradykinin B_2 receptor antagonist D-Arg-[Hyp^3, D-Phe^7] bradykinin (code name NPC 567) abolished the bronchial hyperresponsiveness and reduced the airway inflammation in sheep with natural hypersensitivity to *Ascaris suum* (Soler et al., 1990). On the other hand, Woisin et al. (2000), using a rabbit model of allergic lung inflammation, found that pretreatment with a potent and selective bradykinin B_2 receptor antagonist (D-Arg-Arg-Pro-Hyp-Gly-Thi-Ser-Dtic-*N*Chg-Arg) (code name CP-0597) did not affect antigen-induced acute bronchoconstriction, airway hyperresponsiveness nor pulmonary eosinophilia.

Our results obtained with the bradykinin B_2 receptor antagonist (HOE-140) differ from those obtained previously by others, where this antagonist showed inhibitory effect in allergic lung inflammation in guinea pigs, sheep and rabbit models. Species differences were indicated by Regoli et al. (1997), particularly between human, rabbit and mouse bradykinin B1 receptors and between human, rabbit and guinea pig bradykinin B2 receptors. Moreover, the type of lung inflammation, immunization protocol or treatment schedules employed could also account for the discrepancies observed. In the studies with HOE-140, reported by Perron et al. (1999) and Mashito et al. (1999), the animals were treated with the antagonist before a single antigen challenge and inflammation evaluated 24 h later. In contrast, in our protocol, the mice were treated with the antagonist before the first and the second antigen challenge and inflammation was evaluated 24 h after the second challenge. The protocol of immunization and challenges can modify the production of inflammatory mediators, cellular infiltration and expression of bradykinin receptors. Indeed, when we used our immunization protocol but analyzed cell infiltration after the first challenge, we found that HOE-140 given 30 min before significantly reduced bronchoalveolar eosinophils (18.5 ± 1.0 to $0.23 \pm 0.04 \times 10^4$ cells) and neutrophils (5.85 ± 1.35 to $0.14 \pm 0.06 \times 10^4$ cells) infiltration (data not shown). We believe that our protocol could better mimic the situation found in chronic asthma where several inflammatory episodes are superimposed.

In conclusion, our results with the bradykinin receptor antagonists showed that bradykinin receptor subtypes are involved in the recruitment of polymorphonuclear leukocytes and lymphocytes, in airway hyperreactivity and in mucus production. Our data suggest that in this model of lung allergic inflammation, activation of the constitutive bradykinin B_2 receptor would down-regulate the inflammation, while the activation of inducible bradykinin B_1 receptor would be responsible for pro-inflammatory effects. A better knowledge of bradykinin receptor subtypes in lung cells and tissues at different stages of the inflammation process would be important to better understand the development of this complex disease. It is believed that bradykinin B_1 selective receptor antagonists can be of relevance for treatment of asthma.

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