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Modulation of eosinophil migration from bone marrow to lungs of allergic rats by nitric oxide

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Abstract

Chronic blockade of nitric oxide (NO) synthesis attenuates the eosinophil infiltration into airways of allergic rats. This study was designed to investigate whether the inhibition of eosinophil influx to the lung of allergic rats reflects modifications in the pattern of cell mobilization from the bone marrow to peripheral blood and/or to lung. Male Wistar rats were treated with N^{ω}-nitro-L-arginine methyl ester (L-NAME; 20 mg/rat per day) for 4 weeks and sensitized with ovalbumin (OVA). In control rats, the pulmonary OVA-challenge promoted an early (24 h) increase in the bone marrow eosinophil population that normalized at 48 h after OVA-challenge, at which time the eosinophils disappeared from the blood and reached the lungs in mass. In L-NAME-treated rats, an accumulation of eosinophils in bone marrow was observed at 24 and 48 h post-OVA-challenge. No variation in this cell type number was observed in peripheral blood and bronchoalveolar lavage throughout the time-course studied. In control rats, the adhesion of bone marrow eosinophils to fibronectin-covered wells was significantly increased at 24 h after OVA-challenge, whereas in L-NAME-treated rats the increased adhesion was detected at 48 h. A 32% decrease in the expression of inducible nitric oxide synthase (iNOS) (but not endothelial nitric oxide synthase; eNOS) in eosinophils from L-NAME-treated rats was observed. The levels of IgE, IgG_1 and IgG_{2a} were not affected by the L-NAME treatment. Our findings suggest that inhibition of NO synthesis upregulates the binding of eosinophils to extracellular matrix proteins such as fibronectin, producing a delayed efflux of eosinophils from bone marrow to peripheral blood and lungs.

Keywords: Allergic inflammation; Eosinophil adhesion; Bone marrow; Immunoglobulins; Nitric oxide; Fibronectin

1. Introduction

Accumulation of eosinophils cells into tissues is a feature of a variety of allergic diseases, including asthma and nasal allergy [1]. The eosinophils are recruited from the bone marrow where progenitor cells proliferate and differentiate within the microenvironment, consisting of several types of stromal cells and extracellular matrix [2]. The molecular mechanisms leading to mobilization of mature bone marrow eosinophils to peripheral blood and the sequence of events from their activation to recruitment into inflammatory tissue sites are poorly understood [2]. Apparently these processes are regulated by cytokines including interleukin 5 and eotaxin [3–6], and by the adhesion molecules very late antigen 4 (VLA-4) and vascular cell adhesion molecule 1 (VCAM) [3,7].

Nitric oxide (NO) has been recognized as an important immunomodulatory mediator of inflammatory responses in the lung, modulating the development of pulmonary

Abbreviations: BAL, bronchoalveolar lavage; EPO, eosinophil peroxidase; Ig, immunoglobulin; L-NAME, N^{ω}-nitro-L-arginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; eNOS, endothelial nitric oxide synthase; bNOS, brain nitric oxide synthase; OVA, ovalbumin; PBS, phosphate buffered saline; VLA-4, very late antigen-4

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eosinophilia in allergic animal models [8–11]. Nitric oxide is enzymatically synthesized from L-arginine through the action of NO synthase (NOS) isoforms [12]. At least three isozymes of NO synthase exist, each of which is derived from a different gene. Endothelial (eNOS) and neural NOS (bNOS) are constitutive and calcium-activated isozymes [13,14] whereas the inducible (iNOS) is calcium-independent and expressed in a wide variety of cells and tissues following stimulation by endotoxin or cytokines [15]. Both iNOS and eNOS are expressed in rat peritoneal eosinophils [16], whereas human circulating eosinophils express iNOS and bNOS [17,18]. Recently, the NO-cyclic GMP pathway has been shown to inhibit the adhesion of human eosinophils to the extracelular matrix [19]. In rats, the chronic blockade of NO synthesis by N^{ω}-nitro-L-arginine methyl ester (L-NAME) suppresses the pulmonary eosinophil infiltration in allergic [9] and non-allergic inflammation [20].

Although not entirely clear, it has been suggested that NO modulates mechanisms involved in cell locomotion [16,21]. This study attempts to clarify whether the inhibition of eosinophil influx to the lung of allergic rats, by NO blockade, reflects modifications in the pattern of cell mobilization from the bone marrow to the peripheral blood and/or to the lungs. We, therefore, evaluated the time-course of eosinophil population in bone marrow, peripheral blood and bronchoalveolar lavage (BAL) after the pulmonary antigen challenge in actively sensitized rats treated chronically with L-NAME. The adhesion of eosinophils to fibronectin-coated wells and the expression of NOS isoforms in bone marrow eosinophils were also evaluated.

2. Material and methods

2.1. Drugs

Anti-NOS antibodies were from Transduction Laboratories. Biotinylated swine anti-rabbit IgG and biotinylated rabbit anti-mouse IgG antibodies as well as the biotin blocking system kit were from Dako. The alkaline phosphatase Vectastain ABC kit was from Vector. Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate, were from Promega. Eosin and Permount were from Merck and Fisher, respectively. All monoclonal antibodies used for ELISA tests were kindly provided by Dr. H. Bazin (Unité d'Immunologie Experimentale, Université Catholique de Louvain, Bruxelles, Belgique). Chloral hydrate was obtained from Quimibrás Indústria Química. All other products were bought from Sigma Chemical Co.

2.2. Chronic treatment with N^{ω}-nitro-L-arginine methyl ester (L-NAME)

The chronic treatment with L-NAME was performed as previously described [22]. Briefly, male Wistar rats (80–100 g at the beginning of the study), provided by the Biology Institute Laboratory Animal Center (CEMIB) of the State University of Campinas (UNICAMP), received L-NAME dissolved in the drinking water at a concentration of 1.2 mM to provide a daily intake of approximately 20 mg/rat per day for up to 4 weeks. Another group of animals was treated with the inactive enantiomer, D-NAME (20 mg/rat per day). Control animals received tap water alone.

2.3. Immunization and antigen challenge

Two weeks after the start of treatment with L-NAME (or D-NAME or vehicle) the rats were actively sensitized with ovalbumin (OVA) as previously described [9]. Briefly, a mixture of 200 μg of OVA and 8 mg $Al(OH)_3$ (0.15 ml), prepared in sterile saline, was subcutaneously injected in the animals. Non-sensitized rats received only 8 mg $Al(OH)_3$. Two weeks later, both sensitized and non-sensitized animals were anaesthetized with chloral hydrate (300 mg/kg, i.p.), and OVA (0.4 ml of 0.25% (w/v) solution) was injected through the tracheal wall into the airways. At 24, 48 and 72 h after challenge, the animals were again anaesthetized with chloral hydrate (300 mg/kg, i.p.), after which a sample of peripheral blood was collected from abdominal aorta, bronchoalveolar lavage (BAL) was performed and the femur was isolated from the rats to obtain the bone marrow. Thus, for each time-point after challenge, our protocols resulted in four experimental groups: (1) control, non-sensitized; (2) control, OVA-sensitized; (3) L-NAME, non-sensitized; (4) L-NAME, OVA-sensitized.

2.4. Bronchoalveolar lavage (BAL)

The trachea of anaesthetized animals was exposed and cannulated with a polyethylene tube (1 mm diameter) connected to a syringe. The lungs were washed by flushing with phosphate buffered saline (PBS) solution containing heparin (20 IU/ml) and 0.03% (w/v) serum albumin. The PBS buffer was instilled through the tracheal cannula in one 10-ml aliquot followed by three 5-ml aliquots. The fluid recovered after each aliquot instillation was combined and centrifuged (1000 × g for 10 min at 20 °C). The cell supernatant was discarded and the cell pellet was resuspended in 2 ml of PBS buffer.

2.5. Bone marrow leukocytes

Femurs were removed from rats immediately after killing. The epiphyses were cut transversely and bone marrow cells were flushed out with PBS containing heparin (20 IU/ml).

2.6. Total and differential leukocyte counts

Total cell counts were performed using an automated cell counter (CELL-DYN, 1600). Differential *leukocyte*

counts were carried out on a minimum of 400 cells using cytospin preparation of bone marrow cells suspension stained with Leishman. The peripheral blood differential counts were carried out on air-dried smears stained with Leishman. Both bone marrow and blood eosinophils were evaluated as the mixture of mature and immature forms, as recognized by the intensely eosinophilic granules.

2.7. Immunohistochemical assays

To perform the immunohistochemical experiments, bone marrow cells (2×10^5 cells per slide) were collected on gelatin–chromatum stubbed slides and fixed with 4% (w/v) paraformaldehyde in sodium phosphate buffer (0.1 M, pH 7.4) for 15 min. The cells were washed with Tris–HCl buffer (0.05 M, pH 7.4) containing 0.15 M sodium chloride, dipped in water and dried. All operations were carried out at room temperature.

Expression of NOS was carried out as previously described [16]. Briefly, expression of both iNOS and eNOS were detected using affinity purified mouse mAbs anti-iNOS (clone 3) and eNOS (clone 6), diluted 1:10 (v/v) and 1:100 (v/v), respectively, in blocking buffer (0.02 M sodium phosphate buffer, pH 7.4, containing 0.45 M sodium chloride, 0.2% (w/v) Triton X-100 (New England Nuclear), 5% (w/v) defatted dry milk, and 15% (v/v) normal goat serum). bNOS was detected by using an affinity purified rabbit polyclonal antibody diluted 1:10 (v/v) in blocking buffer.

Bone marrow cells were hydrated with sodium citrate buffer (10 mM, pH 6.0) for 30 min. NOS-like immunoreactivity was retrieved by boiling the hydrated cells in sodium citrate buffer (10 mM, pH 6.0) in a microwave oven (Sharp, model RB4A33) for 15 min. Microwave settings were adjusted to obtain maximum power. The slides were cooled for 20 min at room temperature, and the cells were then rinsed and incubated sequentially in 0.1 M Tris-glycine, pH 7.4, and in blocking buffer, for 30 min each. Subsequently, the cells were incubated with anti-NOS antibodies diluted as described above. In control sections, primary antibodies were substituted with blocking buffer. After incubation, the cells were washed with sodium phosphate buffer (0.02 M, pH 7.4, containing 0.45 M sodium chloride, 0.2% (w/v) Triton X-100; buffer A). Detection of bNOS antibody was carried out using an alkaline phosphatase-conjugated goat anti-rabbit IgG, diluted 1:500 (v/v) in blocking buffer. Detection of iNOS and eNOS antibodies was carried out using a biotinylated goat anti-mouse IgG, followed by incubation with streptavidin-alkaline phosphatase. After each immunochemical incubation step, the cells were washed with buffer A. The alkaline phosphatase reaction was developed using nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate in the presence of 2 mM levamisole, for 30 min [23]. The reaction was stopped with water. The cells were then counterstained with eosin or chromotrope-2R [24], dehydrated, cleared with xylene, and coverslipped with Permount. All operations were carried out at room temperature.

2.8. Eosinophil cell adhesion assays

Ninety-six-well plates were prepared by coating individual wells with 60 μl of rat fibronectin (20 μg/ml) overnight at 4 °C. Wells were then washed twice with PBS before blocking non-coated sites with 0.1% (w/v) albumin, bovine (BSA) for 60 min at 37 °C. Wells were washed twice again with PBS before allowing plates to dry. Eosinophils were added in a volume of 50 μl of minimum essential media/ovalbumin (7×10^4 cells/ml) to the coated wells of a 96-well plate. Cells were allowed to adhere to wells for 15 min at 37 °C, 5% CO_2. After incubation, non-adhered cells were removed and the remaining cells were washed twice with PBS. Fifty microliters of varying concentrations of the original cell suspension in minimum essential media was added to empty wells to form a standard curve. Eosinophil adhesion was calculated by measuring residual eosinophil peroxidase (EPO) activity of adherent cells [25]. Fifty microliters of EPO substrate (1 mM H_2O_2, 1 mM *o*-phenylenediamine and 0.1% Triton X-100 in Tris buffer pH 8.0) was added to each well. After 30 min incubation at room temperature, 25 μl of 4 M H_2SO_4 were added to each well to stop the reaction and absorbance measured at 49.0 nM in a microplate reader (Multiscan MS, Labsystems). Adherence was calculated by comparing absorbance of unknowns to that of the standard curve.

2.9. Quantification of OVA-specific antibodies

To evaluate the sensitization efficacy, blood samples from both non-sensitized ($n = 5$) and OVA-sensitized ($n = 5$) rats were collected at day 14 after immunization with OVA. The serum was then separated and stored at −20 °C for quantification of rat OVA-specific immunoglobulin (Ig) E (IgE), IgG_1 and IgG_{2a}. Serum OVA-specific antibodies were determined in both untreated and L-NAME animals. The serum levels of rat IgG_1 and IgG_{2a} anti-OVA antibodies were determined by standard sandwich ELISA. Briefly, 96-well microplates were coated overnight at 4 °C with 100 μl (100 μg/ml) of OVA 2× crystallized. Plates were washed five times with phosphate buffered saline (PBS) 0.1% (v/v) Tween 20 solution and blocked for 1.5 h at 37 °C with skimmed milk. A volume of 100 μl of serial 0.5 dilutions of sera was applied for 2 h at 37 °C. Peroxidase labeled mouse monoclonal antibodies against rat γ 1 or γ 2a heavy chain (1 μg/ml) were then added for 1 h at 37 °C. Finally, 0.4 mg/ml of *o*-phenylenediamine diluted in citrate buffer pH 5.5 and H_2O_2 was applied. The reaction was stopped with 50 μl of H_2SO_4 (30%) and optical densities were read at 492 nm. Quantification of specific IgE against OVA was made using capture ELISA.

Microplates were coated with 100 μl of mouse anti-rat IgE monoclonal antibodies in borate buffer, then blocked. One hundred microliters of serial dilution of serum samples were applied for 2 h. Finally, peroxidade-labeled OVA (10 μg/ml) was used. Results were expressed as mean ± S.E.M. ($n = 4$–5).

2.10. Statistical analysis

Data are presented as the mean ± S.E.M., and were analyzed by analysis of variance (ANOVA) followed by Tukey test for multiple comparisons or unpaired Student's *t*-test for single comparison. A P value of less than 0.05 was considered to indicate significance.

3. Results

3.1. Effect of chronic L-NAME treatment on bone marrow, peripheral blood and bronchoalveolar lavage (BAL) eosinophil number

The non-sensitized and OVA-sensitized rats received an intratracheal injection of OVA (0.4 ml of 0.25% solution), after which the eosinophil counts in bone marrow, peripheral blood and bronchoalveolar lavage (BAL) fluid were evaluated at 24, 48 and 72 after OVA-challenge. These analyses were carried out in both control and L-NAME groups.

In the control group, the OVA-challenge in sensitized animals promoted a significant increase ($P < 0.05$) in the eosinophil bone marrow number at 24 h post-OVA-challenge that normalized from 48 to 72 h, compared to non-sensitized rats (Fig. 1A). In the L-NAME group, the OVA-challenge in sensitized animals also promoted a significant increase ($P < 0.05$) in the eosinophil bone marrow number at 24 h post-OVA-challenge (Fig. 1A). However, the eosinophil number in this group was still significantly elevated at 48 h, returning to normal values only at 72 h post-OVA-challenge (Fig. 1A).

In non-sensitized rats, the eosinophil number in peripheral blood was significantly higher in the L-NAME group compared to the control animals ($P < 0.05$; Fig. 1B). In the OVA-sensitized rats of the control group, the number of peripheral eosinophils did not change at 24 h compared to non-sensitized rats, but at 48 h after OVA-challenge these cells were nearly absent in peripheral blood, being significantly detected again at 72 h (Fig. 1B). In the OVA-sensitized rats of the L-NAME group, the blood eosinophil number did not significantly change at any of the times evaluated (24, 48 and 72 h), compared to the respective non-sensitized animals (Fig. 1B).

In the control group, the eosinophil number in BAL fluid was markedly elevated ($P < 0.01$) only at 48 h post-OVA-challenge, compared to non-sensitized rats (Fig. 1C). In contrast, in the L-NAME group, no significant increase in eosinophil number in BAL fluid was observed at any studied time.

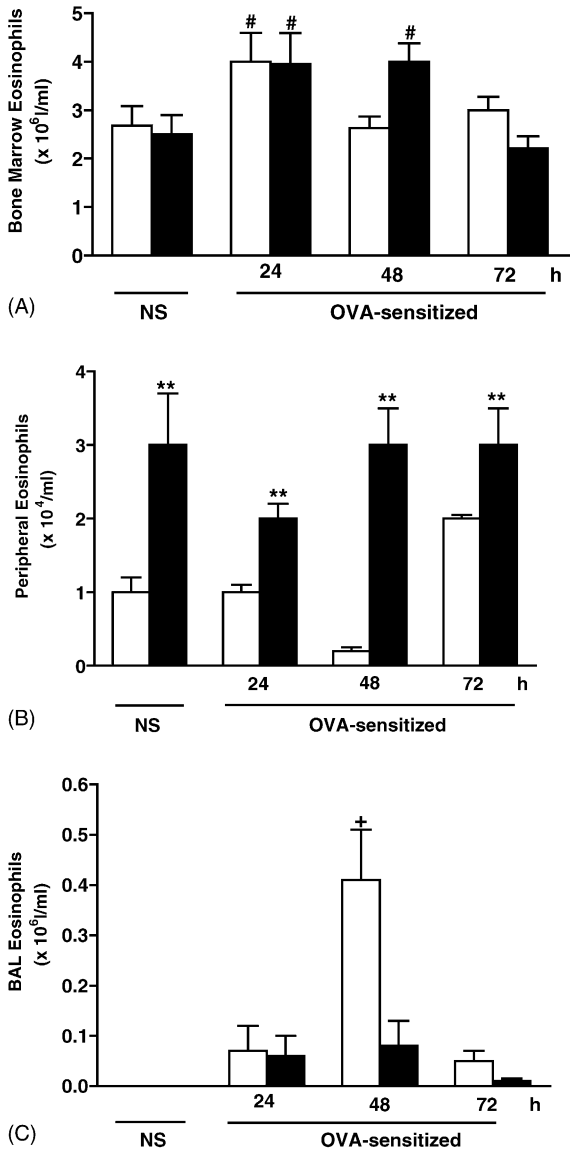

Fig. 1. Effect of L-NAME on the eosinophil number in the bone marrow (A), peripheral blood (B) and bronchoalveolar lavage (BAL; C) of ovalbumin (OVA)-sensitized and challenged rats. Open columns represent non-treated (control) animals whereas closed columns represent L-NAME-treated animals. Bone marrow eosinophils, peripheral blood and BAL were obtained from non-sensitized (NS; $n = 18$) or sensitized rats at 24 h ($n = 13$), 48 h ($n = 18$) and 72 h ($n = 12$) after intratracheal injection of OVA. L-NAME was administrated in drinking water (20 mg/rat per day) during 4 weeks whereas control animals received tap water alone. Non-sensitized rats represent a pool of eosinophil counts at 24, 48 and 72 h. Each column represents the mean ± S.E.M. $^{\#}P < 0.05$ compared to non-sensitized group. $^{**}P < 0.05$ compared to respective control rats; $^{+}P < 0.05$ compared to other groups.

The chronic treatment with D-NAME had no effect upon eosinophil number in bone marrow, peripheral blood and BAL, as evaluated at 48 h post-OVA-challenge (Table 1). Among the non-sensitized groups, no statistical differences were found in eosinophil counts in bone marrow, blood or BAL at any time after OVA-challenge (not shown).

Table 1
Lack of effect of D-NAME on the number of eosinophils in bone marrow, peripheral blood, bronchoalveolar lavage (BAL) and adhesion to fibronectin-coated wells

Group	Eosinophil			
	Bone marrow ($\times 10^6$ ml^{-1})	Blood ($\times 10^4$ μl^{-1})	BAL ($\times 10^6$ ml^{-1})	Adhesion (%)
Control	2.5 ± 0.2	0.2 ± 0.05	0.4 ± 0.08	17.0 ± 0.8
D-NAME	2.0 ± 0.3	0.3 ± 0.1	0.3 ± 0.05	15.5 ± 1.0

The animals received 20 mg/rat of D-NAME per day during 4 weeks whereas control animals received water alone. Bone marrow, blood and BAL eosinophils were obtained from sensitized rats at 48 h after intratracheal challenge with ovalbumin (OVA; $n = 6$–12).

3.2. Effect of L-NAME treatment on bone marrow eosinophil adhesion to fibronectin-coated wells

To examine the adhesion of bone marrow eosinophils to fibronectin, a suspension of bone marrow cells obtained at 24, 48 and 72 h after OVA-challenge was allowed to adhere to fibronectin-coated wells. Eosinophil adhesion was determined by measuring the EPO activity.

As shown in Fig. 2, in the control group, the eosinophil adhesion peaked at 24 h post-OVA-challenge, normalizing at 48 and 72 h thereafter. In the L-NAME group, a delay in the eosinophil adhesion was observed, where adhesion peaked at 48 h post-OVA-challenge. At 24 and 72 h, the adhesion values did not significantly differ from respective non-sensitized rats (Fig. 2). The chronic treatment with D-NAME had no effect on the adhesion of bone marrow eosinophils to fibronectin-treated wells, as assessed at 48 h after OVA-challenge (Table 1). Among the non-sensitized groups, no statistical differences were found at any evaluated time (not shown).

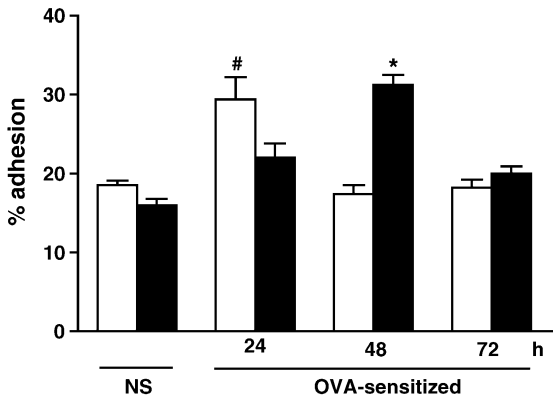

Fig. 2. Effect of L-NAME on adhesion of bone marrow eosinophils to fibronectin-coated wells. Bone marrow eosinophils were obtained from femur of non-sensitized (NS; $n = 18$) or sensitized rats at 24 h ($n = 13$), 48 h ($n = 18$) and 72 h ($n = 12$) after intratracheal injection of OVA. Open and closed columns represent cells obtained from non-treated and L-NAME-treated rats, respectively. Bone marrow cells (7×10^4 cells) were added to the wells and incubated at 37 °C, 5% CO_2. Percentage cell adhesion was calculated by comparing EPO activity of adhered cells to that of a standard curve. L-NAME was administrated in drinking water (20 mg/rat per day) during 4 weeks. Control animals received tap water alone. $^{\#}P < 0.05$ when compared to the other groups excluding L-NAME 48 h. $^{*}P < 0.05$ when compared to other groups excluding control 24 h.

3.3. Effect of L-NAME treatment on immunoglobulin (IgE, IgG_1 and IgG_{2a}) levels

The serum levels of IgE, IgG_1 and IgG_{2a} in control animals were markedly elevated in OVA-sensitized rats compared to non-sensitized ones, thus confirming the efficacy of the sensitization procedure employed here (Fig. 3). The chronic treatment with L-NAME had

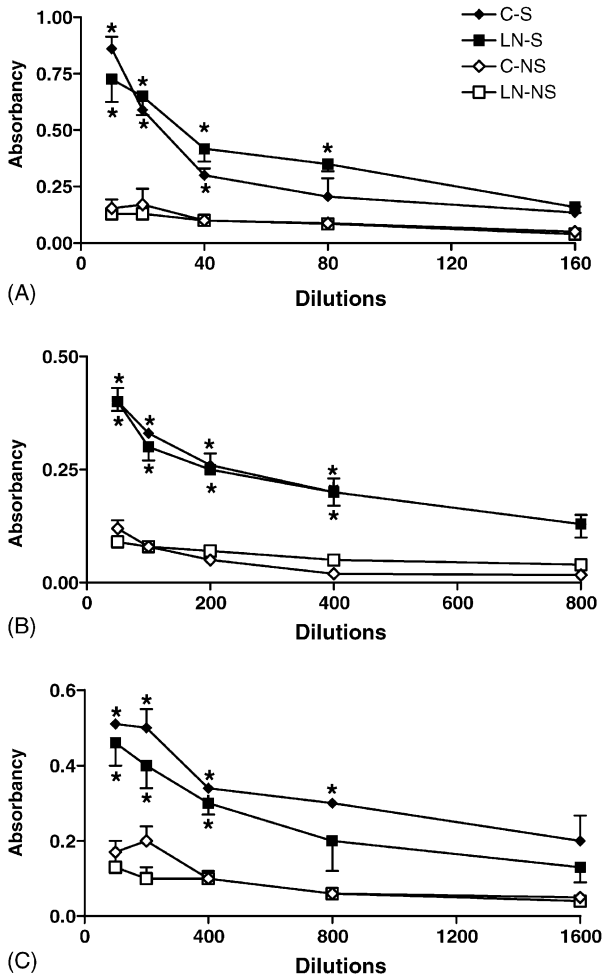

Fig. 3. Levels of immunoglobulin IgE (dilution 1:10 to 1:160; A), IgG_{2a} (dilution 1:50 to 1:800; B) and IgG_1 (dilution 1:100 to 1:1600; C) anti-ovalbumin antibodies in serum of both control and L-NAME-treated rats ($n = 5$ each). $^{*}P < 0.05$ compared to the respective non-sensitized group. C-S, control ovalbumin-sensitized rats; C-NS, control non-sensitized rats; LN-S, L-NAME-treated ovalbumin-sensitized rats; LN-NS, L-NAME-treated non-sensitized rats.

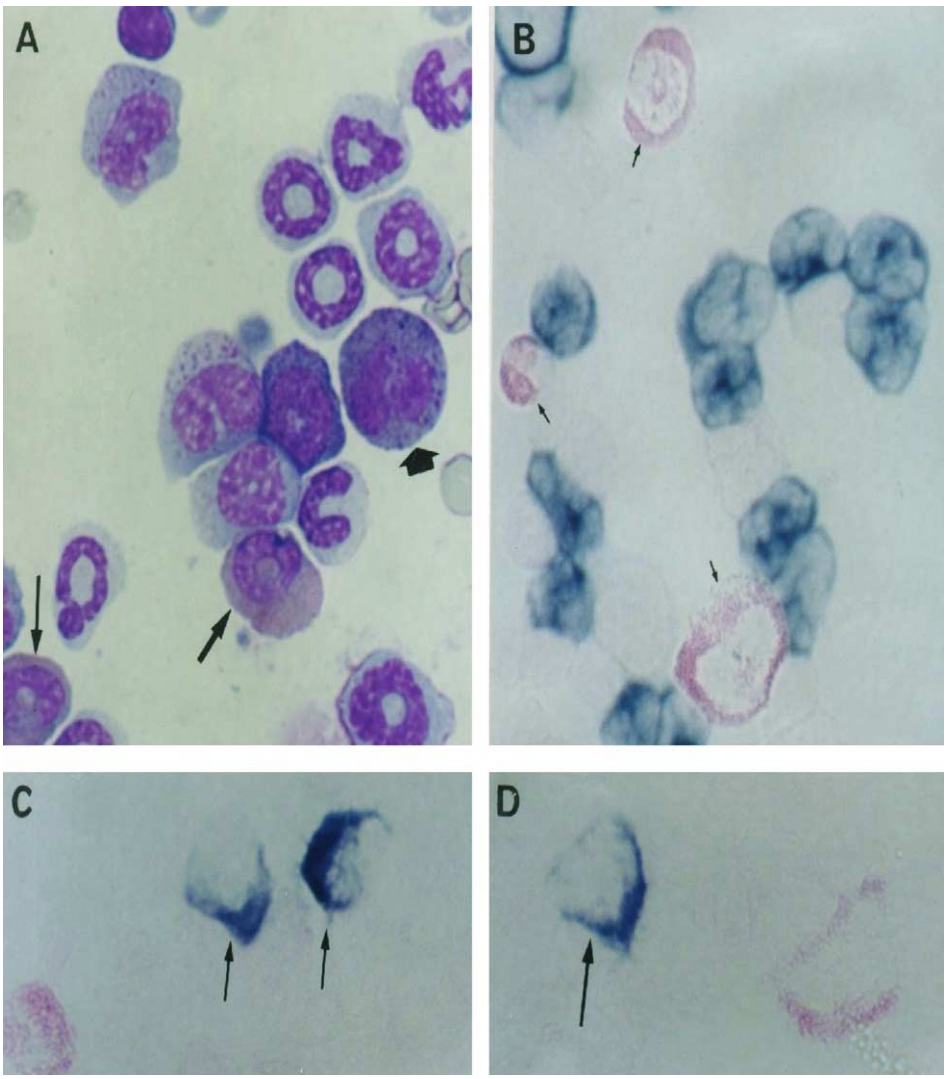

Fig. 4. (A) Bone marrow smear showing mature eosinophils with ring shaped nuclei and red cytoplasmic granules (arrows), a myelocyte eosinophil (arrowhead) and five mature neutrophils (top right) (Leishman, 1000×). Immunolocalization of inducible and endothelial NO synthases are shown in panels B, C and D. NOS were detected using a blue reaction product (NBT) and counterstaining with eosin. In panel B, neutrophils stained positive for bNOS whereas eosinophils were negative (arrows). Panel C shows two positive eosinophils for iNOS (arrows). In panel D, one eosinophil positive for eNOS (arrows) and one negative are shown (1000×).

no significant effect on the levels of IgE, IgG_1 and IgG_2 in neither non-sensitized nor OVA-sensitized rats (Fig. 3).

Table 2
Immunoreactivity-like expression of NOS isoforms in the bone marrow eosinophils from ovalbumin (OVA)-sensitized, challenged-rats

Treatment	Percent positive cells (±S.E.M.)		
	bNOS	iNOS	eNOS
Control	ND	41.0 ± 1.1	14.0 ± 2.6
L-NAME	ND	27.7 ± 1.5*	17.0 ± 0.6

Rats were treated chronically with N^{ω}-nitro-L-arginine methyl ester (L-NAME; 20 mg/rat per day) for 4 weeks or received tap water alone (control animals). Bone marrow cells were obtained from femur of OVA-sensitized rats at 48 h after ovalbumin (OVA) challenge. ND: not detected.
* $P < 0.05$ compared to control.

3.4. Expression of nitric oxide synthase isoforms in bone marrow eosinophils of OVA-sensitized and challenged rats

The immunohistochemical study showed that rat bone marrow eosinophils express immunoreactivity to both iNOS and eNOS (Fig. 4). The expression of iNOS was significantly reduced by the chronic treatment with L-NAME (Table 2), whereas eNOS expression was unchanged by the L-NAME treatment. bNOS was not detected neither in control nor in L-NAME-treated rats.

4. Discussion

This study shows that chronic blockade of the NO synthesis in OVA-challenged rats causes a delay in the

mobilization of bone marrow eosinophils to peripheral blood and/or lung by prolonging the bone marrow eosinophil adhesion to extracellular matrix proteins such as fibronectin. We propose that this mechanism accounts for the attenuation by chronic L-NAME of eosinophil infiltration into lungs of sensitized rats [9].

In certain diseases such as allergic asthma, the antigen-specific immunoglobulins of IgE isotypes are involved in bronchoconstriction and inflammatory reaction in airways. Sensitization of different animal species with antigens such as ovalbumin stimulates an allergic inflammation mediated by IgE and/or IgG antibodies [8,26,27]. In our study, elevated serum IgE and IgG_{2a} levels were found in both sensitized control and L-NAME-treated animals. In agreement with previous studies [10,11], chronic treatment with L-NAME failed to change these OVA-specific serum immunoglobulin profiles, excluding that the increase in bone marrow eosinophils by L-NAME reflects mechanisms involving cell accumulation via anaphylactic immunoglobulin-mediated responses.

Antigen challenge in allergy diseases can activate a systemic response that provokes inflammation cell production by the bone marrow [28]. Eosinophils are derived in the bone marrow from myeloid precursors in response to cytokine activation, and following appropriate stimulus they are released into the circulation and recruited to tissues in atopic individuals [29]. In this study, an examination of bone marrow leukocytes in untreated rats revealed an increase in the number of eosinophils at 24 h after the antigen challenge that could reflect an ongoing differentiation of inflammatory cells in the bone marrow in response to OVA. This is in agreement with OVA-induced airway inflammation in mice [30] and asthmatic subjects after inhaled antigen [28]. The increase in bone marrow eosinophil number was followed by a cell content decrease 48 h after allergen challenge that may reflect the release of bone marrow eosinophils into the circulation. In the L-NAME group, an accumulation of eosinophils in bone marrow is observed for up to 48 h post-OVA-challenge, returning to basal values only at 72 h. This indicates that chronic L-NAME treatment produces a delay in eosinophil mobilization from the bone marrow to peripheral blood. In other animal species such as the mouse, the eosinophil pulmonary infiltration in allergic animals is also significantly attenuated by acute administration of NO inhibitors [8]. Similar results are observed in iNOS-deficient mice [31]. However, it is unclear why the reduced pulmonary cell infiltration showed in both of these studies was not accompanied by changes in eosinophil maturation or efflux from the bone marrow. Nevertheless, these discrepancies may reflect the different animal species used (mice) and a single time-point evaluated after OVA-challenge (48 h).

The molecular mechanisms implicated in the trafficking of eosinophils from the bone marrow to blood are unclear but seem to involve IL-5 and eotaxin, two cytokines believed to selectively regulate eosinophil migration in asthma and allergic diseases [4]. Additionally, adhesion molecules expressed at both eosinophil and endothelial cell surface as well as in the extracellular matrix have shown to play a pivotal role [32]. Eosinophils constitutively express VLA-4 ($\alpha4\beta1$) that binds to the CS-1 region of the extracellular matrix fibronectin by means of this receptor [32]. Furthermore, VLA-4 and VLA-5 integrins mediate hematopoietic progenitor cell attachment to bone marrow stroma, interacting with fibronectin and VCAM-1 that is constitutively expressed by bone marrow stromal cells [33].

Cytokines have an essential role in this process and can modulate the VLA-4 expression. For instance, IL-5 acts synergistically together with eotaxin in the process of mobilization of the eosinophil from the bone marrow [5]. IL-5 stimulates selective release of eosinophils from the bone marrow and these eosinophils mobilized by IL-5 had an increase in β_2 integrin expression, which may be necessary for eosinophil migration within the hematopoietic compartment or their transmigration through the bone marrow endothelium. In contrast, an inhibition of $\alpha4$ expression was observed, reducing the VLA-4 adhesion to VCAM-1 on endothelium cells, that could represent a stage of adhesive interaction before the eosinophils leave the bone marrow in response to IL-5 [6]. Eotaxin seems to be involved in the chemotaxis process by promoting de-adhesion of CS-1 adherent eosinophils in the extracellular matrix by modulating $\alpha4$ integrin CS-1 interaction [34]. Furthermore, the NO precursor L-arginine enhances both IL-5 expression and eosinophil pulmonary infiltration [33] whereas L-NAME reduces eotaxin levels in pulmonary granulomatous inflammation in murines [35]. However, the suppression of allergic inflammation in iNOS-deficient mice was not accompanied by reductions in the secretion of IL-5 [31]. Thus, it is plausible to suggest that an inhibition of IL-5 or eotaxin production by NO synthesis blockade could indeed impair the de-adhesion process of eosinophils $\alpha4$ integrin to fibronection in the extracellular matrix, producing their delayed migration from the bone marrow. Accordingly, our results showed that the eosinophil number in the peripheral blood of L-NAME-treated rats did not change at any of the times studied, indicating that cells did not move from the bone marrow to peripheral blood nor did they migrated to tissues. On the other hand, in the control group, the population of eosinophils in the peripheral blood nearly disappeared from the circulation, reaching the lungs in mass by this time. This same profile is observed in asthmatic patients where allergen inhalation challenge caused a significant decrease eosinophils in the circulation at 5 h after challenge, returning to values pre-challenge at 24 h [28].

In our study, the incubation of eosinophils in the fibronectin-covered wells demonstrated an increase in cell adhesion 24 h after OVA-challenge when the cells were obtained from control rats. However, an enhancement in

fibronectin adhesion was verified 48 h after antigen challenge when eosinophils were collected from L-NAME-treated rats. It is interesting to note that increased adhesion preceded the reduction in eosinophil bone marrow content, i.e., 48 h in control group and 72 h in L-NAME group. This finding suggests that NO performs a temporal modulation of eosinophil adhesion molecules functions, since L-NAME treatment could be delaying the eosinophil bone marrow emigration.

Additionally, the ability of VLA-4 to modulate its binding affinity to VCAM-1 has previously been demonstrated. In the presence of GM-CSF, the functional state of eosinophil-expressed VLA-4 is altered from a low- to a high-affinity state [36]. Recently, Conran et al. [19] reported that in vitro treatment of human peripheral blood eosinophil with L-NAME increased the adhesion to both fibronectin and serum accompanied by an increase in Mac-1 expression. L-NAME also increased the VLA-4 function, which is believed to be due to conformational changes in the VLA-4 receptor, leading to changes in integrin avidity or affinity.

According to a previous study with peritoneal eosinophils [16], our present study shows that bone marrow eosinophils from allergic rats express iNOS and eNOS. A 32% decrease in the expression of iNOS, but not eNOS, was observed in eosinophils from L-NAME-treated rats. These findings suggest that inhibition of NO synthesis upregulates the binding of eosinophils to fibronectin by enhancing the affinity of VLA-4 for the CS-1 region of fibronectin, producing the delayed efflux of eosinophil from bone marrow.

In conclusion, we suggest that the suppression of eosinophil infiltration into the lungs and the concomitant increase of bone marrow eosinophils in rats with chronic blockade of NO synthesis can reflect impaired emigration to peripheral blood and hence to lung as consequence of an upregulation in adhesion molecule function or expression.

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