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Expression of nitric oxide synthases and in vitro migration of eosinophils from allergic rhinitis subjects

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Abstract

The expression of nitric oxide (NO) synthases and the role of the NO cyclic GMP pathway on the migration of eosinophils from untreated patients with allergic rhinitis were investigated. Inducible NO synthase was strongly expressed in eosinophils from healthy individuals, but not in eosinophils from allergic rhinitis patients. The neuronal isoform was observed in eosinophils from each group studied, whereas no staining for the endothelial isoform was detected in either group. The chemotaxis to *N*-formyl-methionyl-leucyl-phenylalanine (fMLP, 5×10^{-7} M) and eotaxin (100 ng/ml) was significantly potentiated in allergic rhinitis eosinophils. In both groups, N^{ω}-nitro-L-arginine methyl ester (L-NAME, 1.0 mM) or 1*H*(1,2,4)-oxadiazolo(4,3,-a)quinoxalin-1-one (ODQ, 0.2 mM) markedly reduced the chemotaxis. The selective iNOS inhibitor *N*-(3-(aminomethyl)benzyl)acetamidine (1400 W, 0.1–1.0 mM) significantly reduced the chemotaxis of eosinophils from healthy but not from allergic rhinitis subjects. The inhibition by L-NAME was restored by 3-morpholinosydnonimine (SIN-1) and *S*-nitroso-*N*-acetyl-penicillamine, whereas the inhibition by ODQ was restored by dibutyryl cyclic GMP. In conclusion, both endothelial and inducible NO synthase isoforms are absent in allergic rhinitis eosinophils, suggesting that the NO cyclic GMP pathway in this cell type is maintained through the activity of a neuronal isoform.

Keywords: Allergic rhinitis; Eosinophil; Nitric oxide (NO) synthase; 1400 W; Eotaxin; Chemotaxis

1. Introduction

Allergic rhinitis is characterized by the accumulation of inflammatory cells, particularly eosinophils, in tissues (Varney et al., 1992). The participation of eosinophils in this process, however, depends upon their migration from the circulation to sites of inflammation (Gleich and Adolphson, 1986). The mechanisms responsible for the recruitment of these cells are not clearly understood, but it is likely that various mechanisms, which involve cytokine, chemokine and adhesion molecules, operate in order to attain a selective recruitment of eosinophils in these diseases (Lundahl et al., 1998). Eosinophils from atopic subjects (allergic asthma and/or rhinitis) may have been preexposed to cytokines in vivo (Sehmi et al., 1992), and primed eosinophils are reported to present an increased migratory response to different chemotactic agents (Bruijnzeel et al., 1993).

Nitric oxide (NO) is produced in mammalian cells from L-arginine and oxygen by a family of enzymes known as NO synthases (NOS). The three NOS isoforms are the neuronal (nNOS or type I), endothelial (eNOS or type III) and inducible (iNOS or type II) types. The nNOS and eNOS are constitutively expressed, whereas the iNOS can be induced by bacterial lipopolysaccharide or certain cytokines such as tumor necrosis factor-α, interleukin-1 and interferon γ (Moncada et al., 1991). These three NOS isoforms have been observed in rat peritoneal eosinophils (Zanardo et al., 1997), human peripheral blood eosinophils (Del Pozo et al., 1997) and in dermal eosinophilic pustular folliculitis (Maruo et al., 1999).

Nitric oxide is thought to be an important inflammatory mediator in several atopic diseases, and relatively large amounts of NO are continuously produced in the nasal

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airway (Kharitonov et al., 1994). In patients with untreated rhinitis, elevated levels of nasal NO have been found, although the source and role of NO remain to be clarified (Kharitonov et al., 1997). Incubation of rat (Zanardo et al., 1997) and human (Thomazzi et al., 2001) eosinophils with NOS inhibitors attenuates in vitro chemotaxis, suggesting that NO has a major role in cell locomotion. In addition, rats treated chronically with a NOS inhibitor show a marked reduction in in vivo eosinophil migration in nonallergic inflammation (Ferreira et al., 1996). In ovalbumin-sensitized rats, inhibition of eosinophil infiltration into the airways was also observed with NOS inhibitor (Ferreira et al., 1998).

Since the NO cyclic GMP pathway has been shown to modulate eosinophil locomotion, this study was designed to investigate NOS expression in eosinophils purified from both healthy and allergic rhinitis subjects. The contribution of NO to eosinophil migration in both subject groups was also examined by measuring in vitro eosinophil chemotaxis in response to the eosinophil-selective chemotaxin eotaxin and to the nonselective chemotaxin *N*-formyl-methionyl-leucyl-phenylalanine (fMLP).

2. Materials and methods

2.1. Patients

All subjects gave their written consent for participation in the study and the study protocol was approved by the local Ethics Committee and was conducted in accordance with the Declaration of Helsinki.

Peripheral blood leukocytes were obtained from two groups of volunteers grouped primarily on the basis of their atopic status: (1) 25 normal healthy nonatopic subjects with a peripheral blood eosinophilia ranging between 2% and 5% of total leukocytes; and (2) 15 allergic rhinitis patients with a peripheral blood eosinophilia ranging between 15% and 48% of total leukocytes and who had been prescreened for development of allergen-induced rhinitis symptoms (sneezing, rhinorrhea and nasal congestion). Atopy was defined on the basis of positive skin prick test reactions to a panel of common aeroallergens, including house dust mite, animals danders and grass pollen. None of the patients were receiving antihistamines, steroids or nonsteroidal antiinflammatory drugs at the time of the study.

2.2. Eosinophil isolation

Eosinophils were isolated from peripheral blood as described by Hansel et al. (1991), with minor modifications. Briefly, 60 ml of heparinized (20 U/ml) blood from healthy or allergic rhinitis subjects was diluted 1:1 with phosphate-buffered saline (PBS) and 30 ml of diluted blood was overlaid onto a 15-ml Percoll gradient (1.089 g/ml, pH 7.4, 340 mosM/kg H_2O). Gradients were centrifuged at 700*g* for 20 min, 4 °C (Jouan, Saint-Herblain, France) and the red cell pellet was collected. Red cells in the granulocyte pellet were lysed with lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA). Washed granulocytes were incubated with anti-CD16 immunomagnetic microbeads (Miltenyi Biotec, Aubum, USA) before being passed through a steel-matrix column in a magnetic field (Miltenyi Biotec). CD 16-negative eosinophils were collected. The final suspension contained 97–99% eosinophils and contaminating cells were neutrophils. Cell viability (>93%) was assessed by the Trypan blue dye exclusion test. Before testing, eosinophils were resuspended in Eagle's minimum essential medium (MEM), pH 7.2.

2.3. Chemotaxis assay

Eosinophil migration assays were performed using a 48-well microchemotaxis chamber (Richards and McCullough, 1984). Fifty-microliter aliquots of eosinophil suspension (7×10^6 cells/ml) were added to the upper compartment of the chamber and separated from the chemotactic agent in the lower chamber by a polycarbonate filter (5-μm pore size, Nucleopore, Peasanton, CA). The chemotactic agents used were eotaxin (10–100 ng/ml, dissolved in PBS containing 0.1% bovine serum albumin) and fMLP (5×10^{-8}–5×10^{-7} M, dissolved in MEM). The chemotactic agent was substituted for MEM to measure random migration. The loaded chambers were incubated at 37 °C in a 5% CO_2 atmosphere (NuAire, Plymouth, MN, USA). Following incubation, the filters were removed, fixed in methanol for 2 min, stained with Diff-Quick (Baxter Healthcare, USA) and mounted on a glass slide. Chemotaxis was quantified by counting eosinophils that migrated completely through the filter in five random high-power field (1000×) per well. Each experiment was carried out in triplicate.

2.4. Treatment of isolated eosinophils before chemotaxis assay

Eosinophil suspensions were incubated with the NOS inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME, 0.1 and 1 mM), its inactive enantiomer D-NAME (0.1 and 1 mM) or the selective iNOS inhibitor *N*-(3-(aminomethyl)-benzyl)acetamidine (1400 W, 0.1–1 mM) for 30 min at 37 °C, 5% CO_2. In another set of experiments, eosinophils were coincubated with L-NAME (1 mM) and the NO donors *S*-nitroso-*N*-acetyl-penicillamine (1.0 mM) or 3-morpholino-sydnonimine (SIN-1, 0.1 mM). The role of soluble guanylyl cyclase was verified by coincubating the eosinophils with a soluble guanylyl cyclase inhibitor, 1*H*(1,2,4)-oxadiazolo(4,3,-a)quinoxalin-1-one (ODQ, 0.2 mM), and the cyclic GMP mimetic, dibutyryl cyclic GMP (dcGMP, 1.0 mM).

2.5. Eosinophil fixation

Eosinophils (2×10^5 cells/slide) were collected on gelatine-chromalum-coated slides using a cytospin centrifuge

(Bioresearch, São Paulo, Brazil), and then fixed with 4% (w/v) paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 15 min. Cells were washed with 0.05 M Tris–HCl buffer, pH 7.4, containing 0.15 M NaCl, dipped in water and dried. All operations were carried out at room temperature.

2.6. Immunostaining

Nitric oxide synthase expression was detected using the affinity-purified monoclonal antibodies anti-iNOS (clone 3) and III (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, 5% (w/v) defatted dry milk and 15% (v/v) normal goat serum]. The nNOS isoform was detected using an affinity-purified polyclonal antibody (Transduction Laboratories, Lexington, KY) diluted 1:10 (v/v) in blocking buffer, and an anti-nNOS antibody (Schmidt et al., 1992) diluted 1:100 (v/v) in blocking buffer. The latter was kindly supplied by Dr. Ferid Murad (University of Texas Medical School).

NOS-like immunoreactivity was enhanced by boiling the hydrated cells in 10 mM sodium citrate buffer, pH 6.0, in a microwave oven (Sharp) for 7 min. After cooling, cells were rinsed and incubated sequentially in 0.1 M Tris–Gly, pH 7.4 for 30 min, and in blocking buffer, for 4 h (Martins et al., 1999).

Cells were incubated with anti-NOS antibodies diluted as above. Anti-nNOS antibody was detected using a biotinylated swine anti-rabbit immunoglobulin G (IgG) antibody. Endogenous biotin was blocked using the Dako Biotin Blocking System, as indicated by the manufacturer. Anti-NOS II and III antibodies were detected using a biotinylated rabbit anti-mouse IgG antibody. All secondary antibodies were detected using the alkaline phosphatase Vectastain kit. After each immunohistochemical step, cells were washed with 0.02 M sodium phosphate buffer, pH 7.4, containing 0.45 M sodium chloride and 0.2% (w/v) Triton X-100. The alkaline phosphatase reaction was carried out using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate in the presence of 2 mM levamisole, for 30 min. Cells were counterstained with eosin, dehydrated, cleared with xylene and coverslipped with Permount. All operations were carried out at room temperature. The specificity of the anti-nNOS and anti-iNOS antibodies was verified using human cerebral cortex and human tuberculous granuloma as positive internal controls, respectively. The anti-nNOS antibodies stained neurons in the cerebral cortex, but not histiocytes or multinucleated giant cells in tuberculous granuloma. The anti-iNOS antibody stained histiocytes and multinucleated giant cells, but not neurons (not shown).

2.7. Materials

The VarioMACS system complete with columns and microbeads was from Miltenyi Biotec. Eotaxin, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), N^{ω}-nitro-L-arginine methyl ester (L-NAME), D-NAME, dibutyryl cyclic GMP, 1*H*-[1,2,4]-oxadiazolo [4,3-a] quinoxalin-1-one (ODQ), *S*-nitroso-*N*-acetyl-penicillamine and 3-morpholino-sydnonimine (SIN-1) were from Sigma (St. Louis, MO, USA). *N*-(3-(aminomethyl)benzyl)acetamidine (1400 W) was obtained from Alexis (Nottingham, UK). Diff-Quick and Percoll were from Baxter Healthcare and Pharmacia (Uppsala, Sweden), respectively. Polycarbonate filters (5 μm) were from Nucleopore. 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 (Glostrup, Denmark). The alkaline phosphatase Vectastain kit was from Vector (CA, USA). Nitroblue tetrazolium and

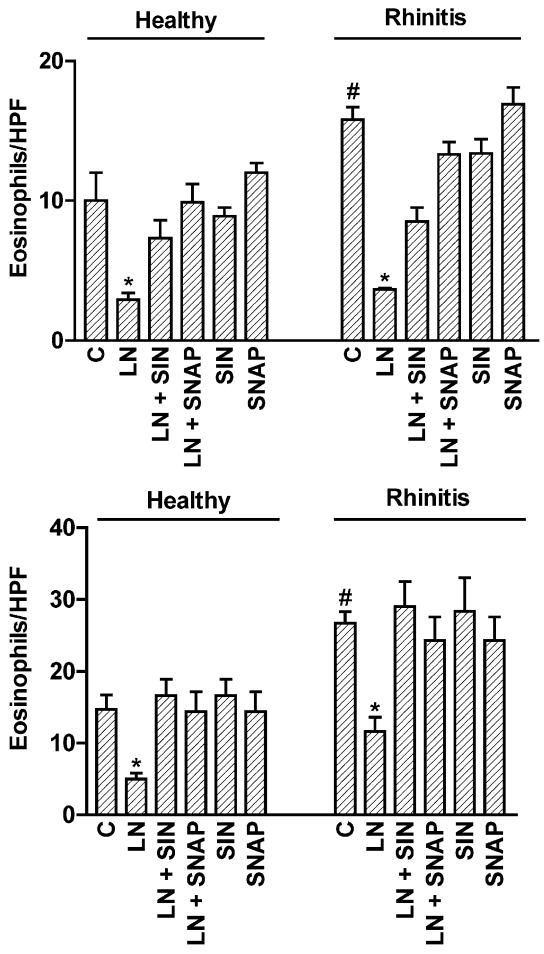

Fig. 1. Role of nitric oxide on the eosinophil chemotaxis in response to fMLP (5×10^{-7} M; upper panel) and eotaxin (100 ng/ml; lower panel). Left columns concern healthy subjects and right columns concern allergic rhinitis subjects. The control value (*C*) is the migration in the absence of both L-NAME and nitric oxide donors. Peripheral blood eosinophils from healthy or allergic rhinitis subjects were preincubated with MEM (*C*), L-NAME (1.0 mM), SIN-1 (0.1 mM) or *S*-nitroso-*N*-acetyl-penicillamine (1.0 mM) alone or combined as shown in the figure. Each column represents the mean ± S.E.M. from five different experiments performed in triplicate. HPF = high-power field. $^{\#}P < 0.05$ compared to healthy subjects (*C*); * $P < 0.05$ compared to respective control (*C*) values.

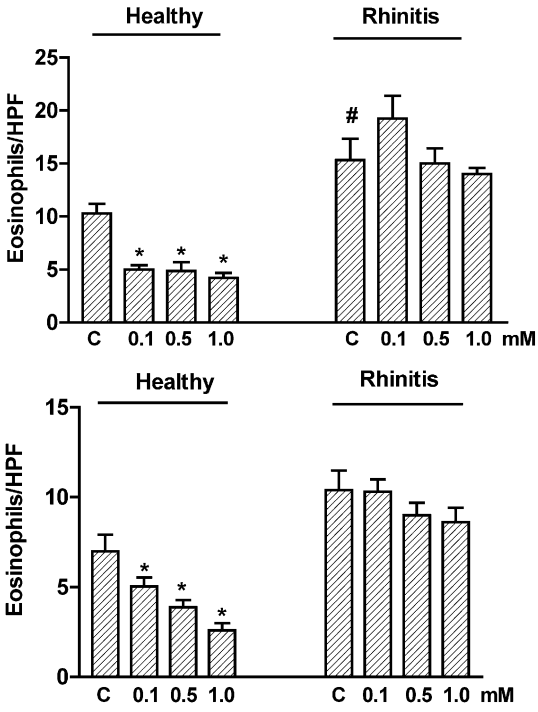

Fig. 2. The effect of the selective iNOS inhibitor 1400 W (0.1–1.0 mM) on eosinophil chemotaxis in response to fMLP (5×10^{-7} M; upper panel) and eotaxin (100 ng/ml; lower panel). Left columns concern healthy subjects and right columns concern allergic rhinitis subjects. The control value (C) is the migration in the absence of 1400 W. Each column represents the mean ± S.E.M. from three different experiments performed in triplicate. HPF = high-power field. * $P < 0.05$ compared to respective control (C) values.

5-bromo-4-chloro-3-indolyl-phosphate were from Promega (Mannheim, Germany). Eosin and Permount were from Merck (Darmstadt, Germany) and Fisher (PA, USA), respectively.

2.8. Statistical analysis

Data are expressed as the means ± S.E.M. of n experiments and were analyzed by Kruskal–Wallis nonparametric analysis of variance (ANOVA) followed by Dunn's multiple comparisons test, or the Mann–Whitney test. A P value of < 0.05 was taken as significant.

3. Results

For the initial experiments, a concentration–response curve for fMLP and eotaxin was made using eosinophils isolated from healthy subjects. A concentration-dependent eosinophil chemotaxis was observed for both fMLP (10.0 ± 0.9, 12.8 ± 0.3 and 16.6 ± 0.2 eosinophils/high-power field for 5×10^{-8}, 1×10^{-7} and 5×10^{-7} M, respectively) and eotaxin (1.6 ± 0.2, 3.2 ± 0.7 and 11.4 ± 1.0 eosinophils/high-power field for 10, 50 and 100 ng/ml, respectively), as compared to random migration (0.9 ± 0.2). In further studies with eosinophils from allergic rhinitis patients, concentrations of fMLP and eotaxin of 5×10^{-7} M and 100 ng/ml, respectively, were routinely used.

3.1. Chemotactic response to fMLP and eotaxin of eosinophils isolated from allergic rhinitis subjects

The eosinophil chemotaxis in response to fMLP was significantly higher ($P < 0.05$) in eosinophils from allergic rhinitis patients (16.3 ± 0.4 eosinophils/high-power field) than in eosinophils from healthy subjects (10.8 ± 0.5 eosinophils/high-power field). A similar response was observed with eotaxin (22.7 ± 3.0 and 12.8 ± 4.4 eosinophils/high-power field for rhinitis and healthy subjects, respectively; $P < 0.05$). The random migration did not differ significantly between eosinophils from healthy and rhinitis subjects (1.3 ± 0.2 and 1.5 ± 0.4 eosinophils/high-power field, respectively).

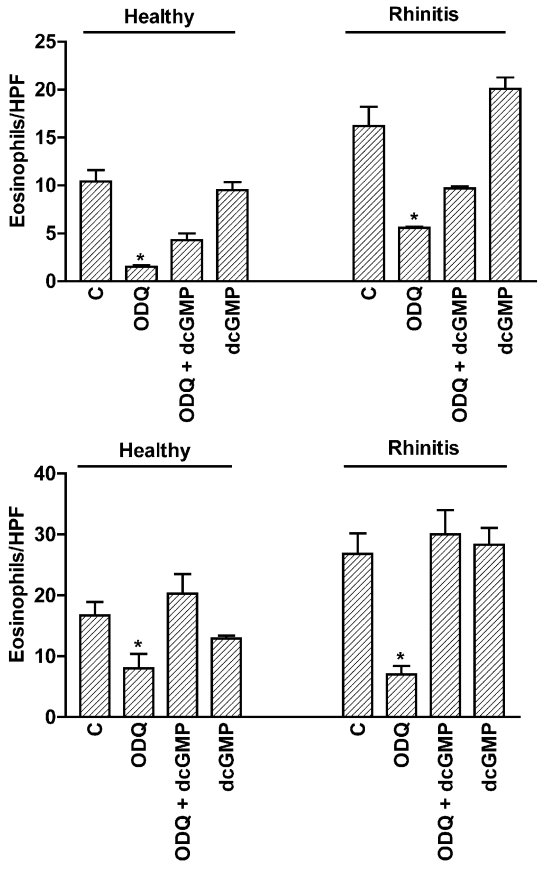

Fig. 3. Role of cyclic GMP on eosinophil chemotaxis in response to fMLP (5×10^{-7} M; upper panel) and eotaxin (100 ng/ml; lower panel). Left columns concern healthy subjects whereas right columns concern allergic rhinitis subjects. The control value (C) is the migration in the absence of both ODQ and dcGMP. Peripheral blood eosinophils from healthy or allergic rhinitis subjects were preincubated with MEM (C), ODQ (0.2 mM), dcGMP (1.0 mM) alone or with ODQ in the presence of dcGMP. Each column represents the mean ± S.E.M. from five different experiments performed in triplicate. HPF = high-power field. * $P < 0.05$ compared to respective control (C) values.

3.2. Influence of NO on fMLP-induced eosinophil chemotaxis

Incubation of eosinophils from healthy individuals with the NO synthesis inhibitor L-NAME significantly ($P < 0.05$) decreased the fMLP-induced chemotaxis (4.8 ± 0.8 and 2.8 ± 0.6 cells/high-power field, for 0.1 and 1.0 mM L-NAME, respectively) as compared with that of untreated cells (9.3 ± 2.2 cells/high-power field). As with eosinophils from healthy individuals, treatment of eosinophils from allergic rhinitis patient with L-NAME (1.0 mM) caused a significant reduction of the fMLP-induced chemotaxis (Fig. 1). At the same concentrations, the inactive enantiomer D-NAME (0.1 and 1.0 mM) did not significantly affect the fMLP-induced chemotaxis of eosinophils from either healthy or rhinitis subjects (not shown). In addition, the NO donor compounds SIN-1 (0.1 mM) and *S*-nitroso-*N*-acetyl-penicillamine (1.0 mM) significantly reversed the inhibition by L-NAME of the migratory response to fMLP of eosinophils from both healthy and allergic rhinitis subjects (Fig. 1). At the concentrations used above, SIN-1 and *S*-nitroso-*N*-acetyl-penicillamine, incubated in the absence of L-NAME, had no significant effect on fMLP-induced chemotaxis of eosinophils form either healthy or rhinitis subjects (Fig. 1).

Incubation of eosinophils from healthy subjects with the highly selective iNOS inhibitor 1400 W (0.1–1.0 mM) decreased by approximately 50% ($P < 0.05$) the fMLP-induced chemotaxis as compared with that of untreated cells (Fig. 2). In contrast, 1400 W failed to affect the chemotaxis of eosinophils from allergic rhinitis patients (Fig. 2).

3.3. Influence of NO on eotaxin-induced eosinophil chemotaxis

Treatment of eosinophils from either healthy or allergic rhinitis subjects with L-NAME (1.0 mM) caused a significant reduction of the eotaxin-induced chemotaxis ($P < 0.05$). The NO donor compounds SIN-1 (0.1 mM) and *S*-nitroso-*N*-acetyl-penicillamine (1.0 mM) significantly reversed the inhibition by L-NAME of the migratory response to eotaxin of eosinophils from both healthy indi-

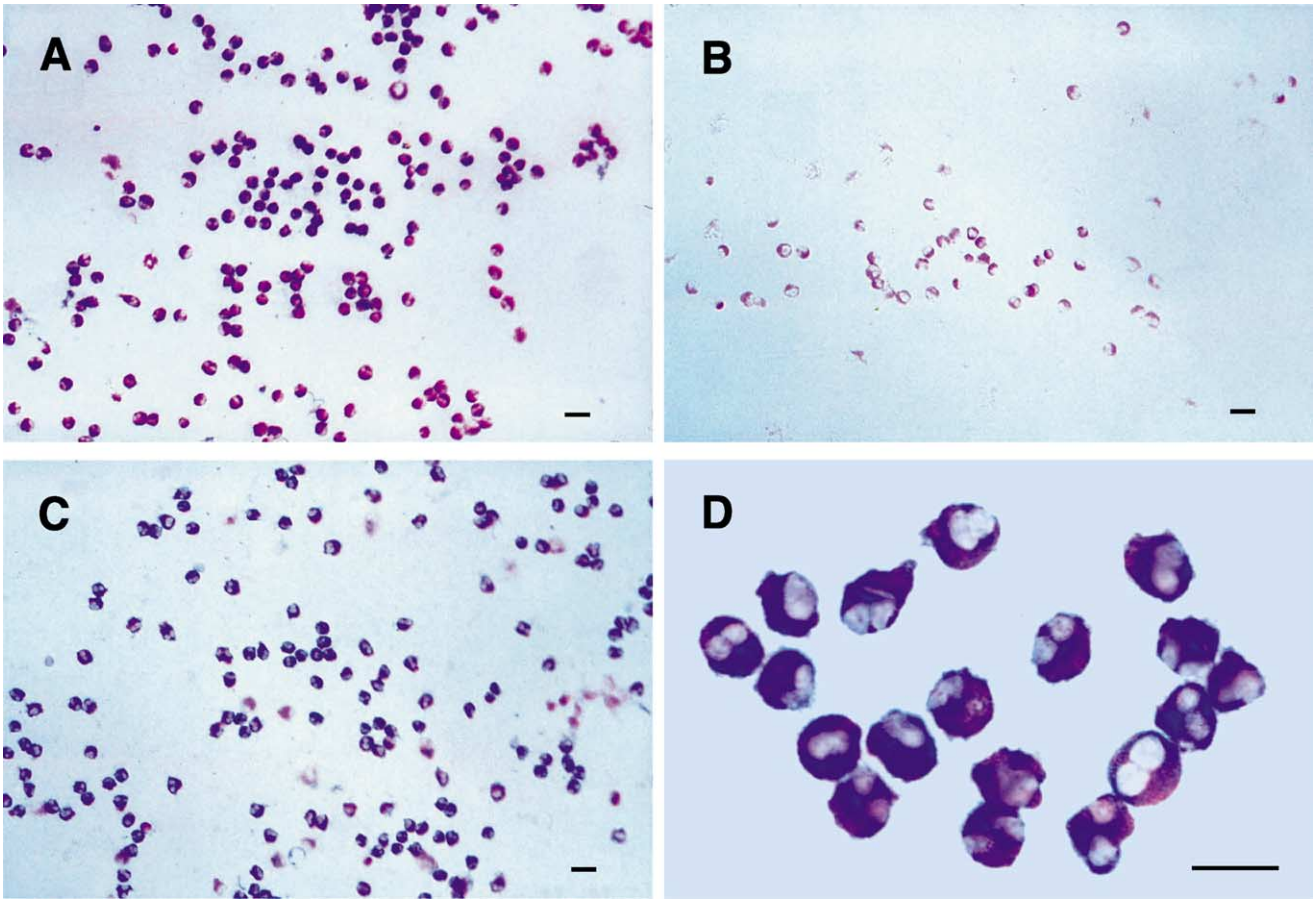

Fig. 4. Expression of NO synthase isoforms in eosinophils purified from healthy volunteers and untreated allergic rhinitis patients. The NO synthase isoforms were detected using the alkaline phosphatase technique with NBT as substrate (blue reaction color). All sections were counterstained with eosin. (Panel A) Immunolocalization of the neuronal isoform in eosinophils from an untreated allergic rhinitis patient. (Panel B) Control of iNOS expression, which was obtained by omitting the primary antibody. (Panel C) The majority of eosinophils from healthy volunteers were stained with the anti-iNOS antibody. (Panel D) iNOS immunoreactivity was colocalized with eosinophilic granules. Nomarski optics. Bar = 20 μm in all panels.

viduals and allergic rhinitis patients (Fig. 1). At the concentrations used above, SIN-1 and *S*-nitroso-*N*-acetyl-penicillamine, incubated in the absence of L-NAME, had no significant effect on the eotaxin-induced chemotaxis of eosinophils from either healthy or rhinitis subjects (Fig. 1).

The compound 1400 W significantly ($P<0.05$) reduced the eotaxin-induced chemotaxis of eosinophils from healthy subjects at concentrations of 0.1–1.0 mM, but had no significant effect on eosinophils from rhinitis subjects at any of the concentrations tested (Fig. 2).

3.4. Involvement of soluble guanylyl cyclase in eosinophil locomotion

To verify whether soluble guanylyl cyclase was involved in the eosinophil chemotaxis induced by fMLP or eotaxin, cells were incubated with ODQ, a selective inhibitor of this enzyme. Fig. 3 shows that ODQ (0.2 mM) significantly reduced the fMLP-induced chemotaxis of eosinophils from both healthy and allergic rhinitis subjects. This inhibition was partly, but significantly, restored by dibutyryl cyclic GMP (1.0 mM). Similarly to fMLP, the eotaxin-induced chemotaxis was markedly inhibited by ODQ (0.2 mM) in eosinophils from both healthy and allergic rhinitis subjects, an inhibition completely restored by dibutyryl cyclic GMP (1.0 mM; Fig. 3). Dibutyryl cyclic GMP, incubated in absence of ODQ, had no significant effect on the fMLP- or eotaxin-induced chemotaxis of the eosinophils from healthy or rhinitis subjects (Fig. 3).

3.5. Differential expression of nitric oxide synthase isoforms in healthy and allergic rhinitis subjects

To investigate the expression of NOS in eosinophils from healthy and allergic rhinitis subjects, immunohistochemical staining for NOS isoforms was performed. Neuronal NOS immunoreactivity was detected in 70% of the eosinophils from healthy individuals and in 50% of the eosinophils from allergic rhinitis patients (Fig. 4A). Inducible NOS immunoreactivity was strongly expressed and apparently colocalized with eosinophilic granules in the majority of human eosinophils purified from all of the healthy individuals (Fig. 4D). In contrast, this isoform was not found in eosinophils purified from patients with untreated allergic rhinitis. The eNOS isoform was detected neither in eosinophils from healthy volunteers nor in eosinophils from allergic rhinitis patients (not shown). NOS immunoreactivity was not detected in controls carried out by omitting the primary antibody (shown for iNOS in Fig. 4B).

4. Discussion

We herein demonstrate that the mechanisms controlling the in vitro chemotaxis of human eosinophils through the NO cyclic GMP pathway in allergic rhinitis subjects are identical to those of healthy subjects, as evaluated using the eosinophil-selective chemotaxin eotaxin and the nonselective chemotaxin fMLP. Nevertheless, in contrast to eosinophils from healthy subjects, eosinophils from allergic rhinitis patients do not express the iNOS isoform, whilst no differences between both groups of subjects were found for the nNOS and eNOS isoforms.

Eosinophils isolated from patients with allergic disease are supposed to be preexposed to cytokines such as interleukin-3, interleukin-5 and granulocyte/macrophage-colony-stimulating factor, and this preexposure may be important for the pathogenesis of such diseases (Bruijnzeel et al., 1993; see also Giembycz and Lindsay, 1999). Our results showed that fMLP-induced chemotaxis was significantly increased in eosinophils from allergic rhinitis patients as compared to healthy subjects, confirming previous studies that primed eosinophils from atopic dermatitis patients show an increased migratory response to different chemotactic agents, including fMLP (Bruijnzeel et al., 1993). With regard to eotaxin, this eosinophil-selective chemoattractant has been implicated in in vivo eosinophil tissue infiltration in allergic rhinitis (Hanazawa et al., 2000) and asthmatic patients (Lilly et al., 1999; Conroy et al., 1997). However, no studies have focussed on the in vitro chemotactic activity of this agent on eosinophils from atopic patients. In agreement with a previous report (Elsner et al., 1996), our results showed that eotaxin caused concentration-dependent eosinophil chemotaxis in healthy subjects. Furthermore, the chemotactic activity of eosinophils from allergic rhinitis patients was significantly increased compared to that of healthy subjects. Although the mechanisms that enhance the chemotactic response of primed eosinophils to exogenously applied agents are not established yet, it is speculated that priming by cytokines may be associated with the induction of high-affinity receptors for chemoattractants (Weisbart et al., 1986). Primed eosinophils may also exert pathobiologic effects by responding easily to stimulation by soluble ligands and by releasing toxic metabolites (reviewed by Giembycz and Lindsay, 1999).

Nitric oxide has been implicated as an important mediator in allergic disease, and increased levels of exhaled NO have been detected in asthmatic and allergic rhinitis patients (Alving et al., 1993; Kharitonov et al., 1997). Inducible NOS is found in a variety of cells in the respiratory tract, including macrophages, airway epithelial cells, fibroblasts and endothelial cells (Kobzik et al., 1993). Additionally, an increase in iNOS expression was described in bronchial mucosa and airway epithelium of asthmatic patients (Saleh et al., 1998; Guo et al., 2000), in keratinocytes from patients with acute urticaria (Becherel et al., 1997) and in nasal epithelial cells of allergic rhinitis patients (Kawamoto et al., 1998, 1999). Interestingly, although mRNA transcripts for iNOS in human peripheral blood eosinophils have been described (Del Pozo et al., 1997), no immunohistochemical staining for iNOS in eosinophils infiltrating tissues has been demonstrated. In the present study, we show for the first time

the absence of iNOS expression in eosinophils from patients with an allergic disease, while it was strongly expressed in the majority of eosinophils from healthy individuals. These findings may suggest that the iNOS isoform is downregulated in blood eosinophils from rhinitis allergic subjects, which might be due to the previous exposure of these cells to factors that modulate NOS expression, such as glucocorticoids, a hormone known to inhibit the iNOS expression (Szabó, 1998; De Vera et al., 1997). The elevation of endogenous cortisol in allergic rhinitis subjects may lead to inhibition of iNOS expression in eosinophils, as previously described in other allergic disease such as asthma (Cydulka and Emerman, 1998; Fujitaka et al., 2000). Although a direct effect of steroids on eosinophil chemotaxis is questionable (reviewed by Giembycz and Lindsay, 1999), the plasma level of cortisol would be insufficient to suppress the eosinophil migration into the nasal mucosa seen in allergic rhinitis (Hanazawa et al., 2000). Regarding the other isoforms, our results showed no immunohistochemical staining for eNOS in either group, whereas the nNOS isoform was observed in eosinophils from about one-half of the individuals of each group studied. Although the presence of nNOS in eosinophils is unclear, our results corroborate a previous study where this isoform was detected in infiltrating eosinophils in the nonallergic disease, eosinophilic pustular folliculitis (Maruo et al., 1999).

The role of NO in modulating eosinophil locomotion in both nonallergic and allergic inflammatory models has been clearly described using in vivo and in vitro experimental models (Ferreira et al., 1996, 1998; Feder et al., 1997; Zanardo et al., 1997; Thomazzi et al., 2001). Since eosinophil locomotion is increased in eosinophils isolated from allergic rhinitis patients, and such cells have no immunoreactivity to iNOS, we next examined the involvement of the NO cyclic GMP pathway in the eosinophil chemotactic response. Using a nonselective NOS inhibitor (L-NAME) and a soluble guanylyl cyclase inhibitor ODQ (Garthwaite et al., 1995), we showed that both compounds markedly reduced the chemotaxis of eosinophils from both allergic rhinitis patients and healthy individuals. Furthermore, no remarkable differences between eosinophils from healthy individuals and rhinitis patients were found with regard to the ability of the NO donor compounds SIN-1 and *S*-nitroso-*N*-acetyl-penicillamine to restore the inhibitory effect of L-NAME. These findings confirm that the NO cyclic pathway modulates eosinophil locomotion in both normal and allergic states. Although the inhibition of chemotaxis by L-NAME was similar in eosinophils from healthy and allergic subjects, the highly selective iNOS inhibitor 1400 W (Garvey et al., 1997) reduced the fMLP- and eotaxin-induced chemotaxis in eosinophils from healthy individuals, but not in eosinophils from allergic rhinitis subjects. These data reinforce our immunohistochemical demonstration of the absence of iNOS expression in eosinophils from allergic rhinitis patients.

In conclusion, our findings indicate that eosinophils from patients with allergic rhinitis are present in a preactivated state in the circulation and that this could account for the large influx of eosinophils into the nasal mucosa. Since both eNOS and iNOS isoforms are absent in eosinophils from allergic rhinitis patients, we suggest that the NO cyclic GMP pathway in these cells is maintained through the activity of a nNOS isoform.

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