Effects of Reactive Oxygen and Nitrogen Metabolites on RANTES- and IL-5-Induced Eosinophil Chemotactic Activity *in Vitro*

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Eosinophils and increased production of nitric oxide (NO) and superoxide, components of peroxynitrite, have been implicated in the pathogenesis of a number of allergic disorders including asthma. Peroxynitrite induced protein nitration may compromise enzyme and protein function. We hypothesized that peroxynitrite may modulate eosinophil migration by modulating chemotactic cytokines. To test this hypothesis, the eosinophil chemotactic responses of regulated on activation, normal T cell expressed and secreted (RANTES) and interleukin (IL)-5 incubated with and without peroxynitrite were evaluated. Peroxynitrite attenuated RANTES and IL-5 induced eosinophil chemotactic activity (ECA) in a dose-dependent manner ($P < 0.05$) but did not attenuate leukotriene B₄ or complement-activated serum ECA. The reducing agents deferoxamine and dithiothreitol reversed the ECA inhibition by peroxynitrite, and exogenous L-tyrosine abrogated the inhibition by peroxynitrite. PAPA-NONOate, a NO donor, or superoxide generated by lumazine or xanthine and xanthine oxidase, did not show an inhibitory effect on ECA. The peroxynitrite generator, 3-morpholinodisodium nitrite, caused a concentration-dependent inhibition of ECA. Peroxynitrite reduced RANTES and IL-5 binding to eosinophils and resulted in nitrotyrosine formation. These findings are consistent with nitration of tyrosine by peroxynitrite with subsequent inhibition of RANTES and IL-5 binding to eosinophils and suggest that peroxynitrite may play a role in regulation of eosinophil chemotaxis. (*Am J Pathol* 1999, 155:591–598)

One of the hallmarks of allergic inflammation is the infiltration of eosinophilic granulocytes into the affected tis-

sues. The importance of eosinophils has been emphasized by the finding that the degree of eosinophilia in both blood and bronchoalveolar lavage fluid (BALF) is associated with the clinical severity of asthma as well as with the degree of bronchial hyperresponsiveness.^{1–3} In tissues, eosinophils releasing toxic proteins and oxidants that have been hypothesized to be responsible for the pathological changes observed in allergic diseases of the airways.⁴ Although the mechanism(s) accounting for the recruitment of eosinophils to the airway is not fully understood, it seems likely to involve several chemotactic cytokines.

Nitric oxide (NO) is an important messenger molecule released from a variety of cell types in the airways and the concentration of NO in exhaled air of asthmatic patients is increased.⁵ High levels of superoxide anion have also been found in the BALF of asthmatic patients.^{6,7} Moreover, superoxide dismutase activity is reduced in the leukocytes of asthmatics,⁷ and increased formation of peroxynitrite, a potent oxidant, is found in the airways of asthmatic patients.⁸

Peroxynitrite, an oxidant generated by the interaction between superoxide and NO, is known to nitrate several amino acids including cysteine,⁹ methionine,¹⁰ tryptophan,¹¹ and tyrosine.¹² Peroxynitrite nitrates free or protein-associated tyrosine to form the stable product nitrotyrosine by addition of a nitro group to the 3-position adjacent to the hydroxyl group of tyrosine. Several studies have shown that peroxynitrite-induced protein nitration may alter protein function. Peroxynitrite inactivates manganese superoxide dismutase¹³ and surfactant¹⁴ and inhibits protein phosphorylation by tyrosine kinases, thus interfering with signal transduction mechanisms.¹⁵ It has been also suggested that myeloperoxidase (MPO)-catalyzed nitration or reaction of MPO-generated HOCl with NO₂[–] to form nitrating intermediates as an alternative mechanism of protein nitration independent of peroxynitrite.¹⁶ In addition, asthmatic airways are rich in peroxidases that can not only nitrate, but also chlorinate and brominate, tyrosines.¹⁷

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Current concepts suggest that chemokines lead to eosinophil locomotion by binding to receptors. Alteration of critical chemokine sequences may alter chemokine binding and inactivate chemotactic function. For example, mutation of amino acids 13–15 from the rabbit (His-Ser-Thr) to the human sequence (Tyr-Ser-Lys) confers the high affinity of human IL-8 for the human IL-8A receptor.¹⁸ Consistent with the concept that tyrosine is important in binding to receptor, point mutations of Tyr-13 greatly lowered monocyte chemoattractant protein-1 receptor binding and activity,¹⁹ and changing Tyr-28 to aspartate essentially abolished the monocyte chemoattractant activity of monocyte chemoattractant protein-1.²⁰

We hypothesized that tyrosine nitration by reactive nitrogen species would inhibit cytokine binding and eosinophil migration. To test this hypothesis, the chemotactic responses of human eosinophils to two well known eosinophil chemotaxins, RANTES (regulated on activation, normal T cell expressed and secreted) and interleukin (IL)-5 incubated with peroxynitrite and other compounds were evaluated *in vitro*. We found that peroxynitrite and 3-morpholininosydnonimine (SIN-1), a peroxynitrite donor, significantly attenuated RANTES- and IL-5-induced eosinophil chemotactic activity (ECA). In contrast, activated serum and leukotriene B₄ (LTB₄)-induced ECA was not significantly inhibited by peroxynitrite. These data suggest that peroxynitrite may play a role in eosinophil recruitment and inflammation.

Materials and Methods

Measurement of ECA

Eosinophils were isolated with a modified method of Hansel et al.²¹ Briefly, venous blood anticoagulated with 130 mmol/L trisodium citrate was obtained from normal human volunteers and diluted with phosphate-buffered saline (PBS) in a 1:1 ratio. Diluted blood was overlaid on an isotonic Percoll solution (density 1.082 g/ml; Sigma, St. Louis, MO), then centrifuged at 1000 × g for 30 minutes at 4°C with a Beckman TJ-6 centrifuge. The supernatant and mononuclear cells at the interface were carefully removed, and red blood cells in the sediment were lysed with two cycles of hypotonic lysis (0.1% KHCO₃ and 0.83% NH₄Cl). Isolated granulocytes were washed twice with PIPES buffer (25 mmol/L PIPES, 50 mmol/L NaCl, 5 mmol/L KCl, 25 mmol/L NaOH, and 5.4 mmol/L glucose, pH 7.4) containing 1% defined calf serum (Hyclone Laboratories, Logan, UT), and an approximately equal volume of anti-CD16 antibody conjugated with magnetic particles (Miltenyi Biotec, Bergisch Gladbach, Germany) was added to the cell pellet. After 60 minutes' incubation on ice, 5 ml of PIPES buffer with 1% defined calf serum were added to the cell-antibody mixture. The resuspended cells were loaded onto the separation column positioned in the magnetic cell separation system with a strong magnetic field. The cells were eluted three times with 5 ml of PIPES buffer with 1% defined calf serum. Purity of the eosinophils counted by Randolph's stain was >94%; viability was >98%. The eosinophils were resus-

pended in Gey's solution at 2.0×10^6 cells/ml and used for the chemotaxis assay.

ECA was assayed in 48-well microchemotaxis chambers (Neuroprobe, Inc., Cabin John, MD).²² The bottom wells of the chamber were filled with 25 μ l of the chemotactic stimulus or medium in duplicate. A 10- μ m-thick polyvinylpyrrolidone-free polycarbonate filter with a pore size of 5 μ m was placed over the samples. The silicon gasket and the upper pieces of the chamber were applied and 50 μ l of the cell suspension was placed into the upper wells. The chambers were incubated in humidified air in 5% CO₂ at 37°C for 90 minutes. Nonmigrated cells were wiped away from the filter. The filter was immersed in methanol for 5 minutes, stained with a modified Wright's stain, and mounted on a glass slide. Cells that had completely migrated through the filter were counted using light microscopy. ECA was expressed as the mean number of migrated cells per high-power field from duplicate wells.

Effect of Peroxynitrite on RANTES- and IL-5-Induced ECA

Peroxynitrite was evaluated for its capacity to modulate RANTES- and IL-5-induced ECA *in vitro*. RANTES or IL-5 (R&D Systems, Minneapolis, MN) was incubated for 2 hours at 37°C with each concentration of peroxynitrite (Calbiochem, La Jolla, CA) before the ECA assay. In control experiments, RANTES or IL-5 was incubated with medium alone.

Effect of Peroxynitrite on LTB₄- and Activated Serum-Induced ECA

The capacity of peroxynitrite to modulate LTB₄- and activated serum-induced ECA was compared to RANTES (10^{-7} g/ml) and IL-5 (10^{-8} g/ml). LTB₄ (10^{-6} mol/L, Sigma) or complement-activated serum²³ (1:10 dilution) were incubated with peroxynitrite (10^{-4} mol/L) for 2 hours at 37°C before performing the ECA assay.

Effect of PAPA-NONOate on RANTES- and IL-5-Induced ECA

To evaluate the effect of NO to modulate RANTES and IL-5 induced ECA, we used PAPA-NONOate (Alexis, San Diego, CA) as a NO donor.^{24–27} RANTES (10^{-7} g/ml) or IL-5 (10^{-8} g/ml) was incubated with PAPA-NONOate (10^{-3} – 10^{-6} M) for 2 hours at 37°C before performing the ECA assay. The samples were dialyzed overnight at 4°C against Hanks' balanced salt solution using tubing with a molecular weight cutoff of 3 kd. The half-life of PAPA-NONOate is 15 minutes in physiological buffer at 37°C and two moles of NO are released per mole of PAPA-NONOate.

Effect of Superoxide on RANTES and IL-5 ECA

To evaluate the effect of superoxide on RANTES- and IL-5-induced ECA, lumazine (10^{-4} mol/L, Sigma) or xan-

thine (10^{-6} , 10^{-5} , 10^{-4} , 10^{-3} mol/L, Sigma) was combined with xanthine oxidase (3.4×10^{-6} , 3.4×10^{-5} , 3.4×10^{-4} , 3.4×10^{-3} U/ml, Sigma) to produce superoxide.^{28,29} Xanthine was combined with xanthine oxidase in descending order of concentration. RANTES (10^{-7} g/ml) or IL-5 (10^{-8} g/ml) was incubated with lumazine and xanthine oxidase or xanthine and xanthine oxidase for 2 hours at 37°C before performing the ECA assay.

Effect of 3-Morpholinosydnonimine (SIN-1) on RANTES- and IL-5-Induced ECA

To confirm the results with the peroxynitrite, 3-morpholinosydnonimine (SIN-1) (Alexis), a slow peroxynitrite generator,^{30,31} was used. RANTES- and IL-5-induced ECA was evaluated by incubating RANTES (10^{-7} g/ml) or IL-5 (10^{-8} g/ml) with SIN-1 (10^{-4} , 10^{-5} , 10^{-6} , 10^{-7} mol/L) for 2 hours at 37°C before performing the ECA assay.

Effect of Reducing Agents on Peroxynitrite-Induced Attenuation of ECA by RANTES and IL-5

The capacity of the reducing agents dithiothreitol and deferoxamine to attenuate the effect of peroxynitrite on RANTES and IL-5 induced ECA by peroxynitrite was assessed. Dithiothreitol (1 mmol/L, Sigma), deferoxamine (50 μ mol/L, Sigma), and peroxynitrite (10^{-4} mol/L) were added to RANTES (10^{-7} g/ml) or IL-5 (10^{-8} g/ml) and incubated for 2 hours at 37°C before evaluating for ECA.

Effect of L-Tyrosine on Peroxynitrite-Induced Attenuation of ECA by RANTES and IL-5

The capacity of L-tyrosine to reserve the attenuation of ECA induced by peroxynitrite was assessed by addition of L-tyrosine (10^{-3} , 10^{-4} , 10^{-5} mol/L, Sigma) to RANTES (10^{-7} g/ml) or IL-5 (10^{-8} g/ml) before exposed to peroxynitrite (10^{-5} mol/L).

Detection of Nitrotyrosine on RANTES and IL-5 Incubated with Peroxynitrite

Nitrotyrosine on RANTES and IL-5 incubated with peroxynitrite was detected using modifications of previously described techniques.³² RANTES (10^{-7} g/ml) or IL-5 (10^{-8} g/ml) was incubated with peroxynitrite (100 μ mol/L) or media as above and frozen until assayed. Goat anti-human RANTES or IL-5 IgG (R&D Systems) was dissolved in Voller's buffer (1.59 g sodium carbonate, 2.93 g sodium bicarbonate, 0.2 g sodium azide in 1 L distilled water, pH 9.6) at the final concentration of 200 ng/ml. Two hundred microliters were added to flat-bottomed 96-well plates (Costar, Cambridge, MA) and allowed to adsorb to the plastic overnight at 4°C. After washing the flat-bottomed plate 3 times with PBS-Tween,

200 μ l of RANTES or IL-5 with and without peroxynitrite incubation were added to their respective antibody-coated plate and incubated for 60 minutes at room temperature. After washing 3 times with PBS-Tween, 200 μ l of a 1:400 dilution of rabbit polyclonal anti-nitrotyrosine (Calbiochem) were added to the wells and incubated for 90 minutes. After again washing 3 times with PBS-Tween, 200 μ l of a 1:500 dilution of peroxidase-conjugated anti-rabbit IgG were added to the wells and incubated for 90 minutes. Two hundred microliters of o-phenylenediamine (100 μ g/ml, Sigma) in 0.003% H_2O_2 were added and visually monitored. The reaction was terminated by addition of 25 μ l of 8N H_2SO_4 and the absorbance read at 490 nm.

Effect of Peroxynitrite on RANTES and IL-5 Binding to Eosinophils

To investigate the peroxynitrite effect on RANTES and IL-5 binding to eosinophils, RANTES (10^{-7} g/ml) or IL-5 (10^{-8} g/ml) was incubated with 100 μ mol/L of peroxynitrite for 2 hours at 37°C. In control experiments, RANTES or IL-5 was incubated with medium alone. Subsequently, RANTES or IL-5 with or without peroxynitrite was incubated with eosinophils (5×10^5 cells) at 4°C for 30 minutes. Then supernatants were removed and eosinophils were washed 3 times with Hanks' balanced salt solution. Eosinophils were suspended in 1 ml PBS-Tween, sonicated for 20 seconds (MSE Soniprep, Crawley, UK), and then centrifuged at $20,000 \times g$ for 30 minutes in a refrigerated microcentrifuge to obtain a supernatant (soluble) and particulate fraction. RANTES and IL-5 were measured using a commercially available enzyme-linked immunosorbent assay (R & D Systems).

Statistics

In experiments, the differences between groups were tested using Student's paired *t*-test. In all cases, a *P* value of <0.05 was considered significant. Data in figures are expressed as mean $\pm$ SE.

Results

Effect of Peroxynitrite on ECA by RANTES and IL-5

Various amounts of RANTES (Figure 1A) or IL-5 (Figure 1B) were incubated with peroxynitrite (100 μ mol/L). At each concentration, exposure to peroxynitrite caused a reduction in ECA (Figure 1) ($n = 4$, $P < 0.05$). Incubation of RANTES (Figure 2A, 10^{-7} g/ml) or IL-5 (Figure 2B, 10^{-8} g/ml) with various amounts of peroxynitrite induced a significant, concentration-dependent attenuation of ECA ($n = 4$, $P < 0.05$). The lowest dose of peroxynitrite tested, 10^{-6} mol/L, significantly inhibited ECA induced by RANTES and 10^{-5} mol/L inhibited ECA induced by

Figure 1. Inhibition of ECA by peroxynitrite. ECA is on the ordinate and RANTES (A) or IL-5 (B) concentration is on the abscissa. RANTES or IL-5 was incubated for 2 hours with medium alone or 100 μ M peroxynitrite; $n = 4$ each condition. Open circles represent ECA by RANTES or IL-5 with 100 μ M/L peroxynitrite, and closed circles represent ECA without peroxynitrite incubation. * $P < 0.05$ compared with ECA by RANTES or IL-5 incubated with medium alone.

IL-5. Peroxynitrite itself was not chemotactic for eosinophils (data not shown). Similarly, incubation of peroxynitrite (100 μ M/L) with the eosinophils before the chemotaxis assay did not inhibit ECA to RANTES or IL-5 (data not shown).

Effect of Peroxynitrite on LTB₄- and Activated Serum-Induced ECA

To ensure that the effect of peroxynitrite was not a non-specific effect on eosinophil chemotaxis, the effect of peroxynitrite on ECA induced by LTB₄ and complement-activated serum was assessed. Peroxynitrite did not significantly inhibit the ECA of LTB₄ or complement-activated serum (Figure 3) ($n = 4$, $P < 0.05$).

Effect of PAPA-NONOate on RANTES and IL-5 Induced ECA

To investigate the capacity of NO to modulate ECA induced by RANTES (Figure 4A) and IL-5 (Figure 4B), the effect of the NO donor, PAPA-NONOate, was evaluated. PAPA-NONOate did not significantly change ECA induced by RANTES or IL-5 (Figure 4) ($n = 4$).

Figure 2. Dose-responsive inhibition of ECA by peroxynitrite. RANTES (A; 10^{-7} g/ml) or IL-5 (B; 10^{-8} g/ml) was incubated for 2 hours in the presence with medium alone or peroxynitrite at the concentration indicated; $n = 4$ each condition. ECA is on the ordinate and the concentration of peroxynitrite is on the abscissa. * $P < 0.05$ compared with RANTES or IL-5 incubated with medium alone.

Figure 3. Effect of peroxynitrite on ECA induced by RANTES, IL-5, activated serum, and LTB₄. RANTES, IL-5, activated serum and LTB₄ were incubated for 2 hours in the presence of medium alone or 100 μ M/L peroxynitrite; $n = 4$ each condition. ECA is on the ordinate and the experimental groups are on the abscissa. * $P < 0.05$ compared with RANTES or IL-5 incubated with medium alone.

Effect of Lumazine or Xanthine/Xanthine Oxidase on RANTES- and IL-5-Induced ECA

To evaluate the effect of superoxide on ECA, RANTES (Figure 5A) or IL-5 (Figure 5B) were incubated with lumazine or xanthine and xanthine oxidase (Figure 5) ($n = 4$). None significantly altered ECA to RANTES or IL-5.

Effect of SIN-1 on RANTES- and IL-5-Induced ECA

SIN-1, a nitrovasodilator, spontaneously decomposes under aqueous conditions, generating first O_2^- and then NO at comparable rates. SIN-1 induced a significant, concentration-dependent attenuation of ECA by RANTES (Figure 6A) ($n = 4$, $P < 0.05$) and IL-5 (Figure 6B) ($n = 4$, $P < 0.05$). The lowest dose of SIN-1 to inhibit ECA was 10^{-5} mol/L. SIN-1 itself was not chemotactic for eosinophils (data not shown).

Figure 4. Effect of PAPA-NONOate on ECA. RANTES (A; 10^{-7} g/ml) or IL-5 (B; 10^{-8} g/ml) was incubated for 2 hours in the presence of medium alone or PAPA-NONOate at the concentration indicated; $n = 4$ each condition. ECA is on the ordinate and the PAPA-NONOate concentration is on the abscissa. * $P < 0.05$ compared with RANTES or IL-5 incubated with medium alone.

Figure 5. Effect of lumazine (solid bars) or xanthine (stippled bars) plus xanthine oxidase on ECA. RANTES (A) or IL-5 (B) was incubated for 2 hours in the presence of medium alone, lumazine and xanthine oxidase, or xanthine and xanthine oxidase at the concentrations indicated; $n = 4$ each condition. ECA is on the ordinate and the experimental groups are on the abscissa.

Effect of Dithiothreitol and Deferoxamine on Peroxynitrite-Induced Attenuation of ECA by RANTES and IL-5

The reducing agents dithiothreitol and deferoxamine were added to RANTES (Figure 7A) or IL-5 (Figure 7B) before incubating with peroxynitrite. Each attenuated the inhibition of ECA induced by peroxynitrite (Figure 7) ($n = 4$, $P < 0.05$). Dithiothreitol or deferoxamine alone was not chemotactic for eosinophils (data not shown).

Figure 6. Effect of SIN-1 on ECA induced by RANTES (A) or IL-5 (B). RANTES or IL-5 were incubated for 2 hours in the presence of medium alone or SIN-1 at the concentration indicated; $n = 4$ each condition. ECA is on the ordinate and SIN-1 concentration is on the abscissa. * $P < 0.05$ compared with RANTES or IL-5 incubated with medium alone.

Figure 7. Effect of dithiothreitol and deferoxamine on peroxynitrite-induced attenuation of ECA by RANTES (A) or IL-5 (B). RANTES or IL-5 were incubated with peroxynitrite for 2 hours in the presence of dithiothreitol and deferoxamine at the concentration indicated; $n = 4$ each condition. ECA is on the ordinate and the experimental groups are on the abscissa. * $P < 0.05$ compared with RANTES or IL-5 incubated with peroxynitrite.

Figure 8. Effect of L-tyrosine on peroxynitrite-induced attenuation of ECA by RANTES (A) or IL-5 (B). RANTES or IL-5 was incubated with 100 μmol/L of peroxynitrite for 2 hours in the presence of medium alone or L-tyrosine at the concentration indicated; $n = 4$ each condition. ECA is on the ordinate and the experimental groups are on the abscissa. * $P < 0.05$ compared with RANTES or IL-5 incubated with peroxynitrite.

Effect of L-Tyrosine on Peroxynitrite-Induced Attenuation of ECA by RANTES and IL-5

One mechanism of peroxynitrite inhibition may be through nitrating tyrosine residues. Therefore, the effect of L-tyrosine addition to RANTES (Figure 8A) and IL-5 (Figure 8B) before incubating with peroxynitrite was investigated. Addition of L-tyrosine to RANTES or IL-5 abrogated the attenuation of ECA induced by peroxynitrite (Figure 8) ($n = 4$, $P < 0.05$). L-tyrosine itself was not chemotactic for eosinophils (data not shown).

Detection of Nitrotyrosine on RANTES and IL-5 Incubated with Peroxynitrite

Optical density of RANTES (Figure 9A) or IL-5 (Figure 9B) with peroxynitrite incubation was significantly higher than RANTES or IL-5 without peroxynitrite incubation. Peroxynitrite resulted in nitrotyrosine formation on RANTES and IL-5 (Figure 9) ($n = 6$, $P < 0.05$).

Figure 9. Nitrotyrosine detection on RANTES (A) or IL-5 (B) after incubation with peroxynitrite. RANTES or IL-5 was incubated with 100 μmol/L of peroxynitrite for 2 hours. RANTES or IL-5 with and without peroxynitrite incubation was allowed to adsorb to the RANTES or IL-5 antibody coated plate. Optical density is on the ordinate and the experimental groups are on the abscissa. * $P < 0.05$ compared with RANTES or IL-5 incubated without peroxynitrite.

Figure 10. Effects of peroxynitrite on RANTES (A) or IL-5 (B) binding to eosinophils. RANTES or IL-5 were incubated with and without 100 μ M/L of peroxynitrite for 2 hours at 37°C. Subsequently, the treated RANTES or IL-5 was incubated with eosinophils (5×10^5 cells) at 37°C for 30 minutes. RANTES or IL-5 binding to eosinophils was measured by ELISA. RANTES or IL-5 concentration is on the ordinate and the experimental groups are on the abscissa. * $P < 0.05$ compared with RANTES or IL-5 incubated with medium alone.

Effect of Peroxynitrite on RANTES and IL-5 Binding to Eosinophils

RANTES (Figure 10A) and IL-5 (Figure 10B) induce chemotactic activity by binding to eosinophils. Addition of peroxynitrite to RANTES and IL-5 resulted in an inhibition of their binding to eosinophils. (Figure 10) ($n = 4$, $P < 0.05$).

Discussion

The results of this study show that the peroxynitrite significantly attenuated RANTES- and IL-5-induced ECA *in vitro*. The inhibitory effects of peroxynitrite were not significant on ECA induced by LTB₄ and activated serum. Deferoxamine, dithiothreitol, or tyrosine attenuated the inhibition. NO or superoxide did not cause the reduction in RANTES- and IL-5-induced ECA, because PAPA-NONOate and lumazine/xanthine oxidase did not show an inhibitory effect. The peroxynitrite donor, SIN-1, induced a significant, concentration-dependent inhibition of ECA by RANTES and IL-5. Nitrotyrosine was detected on RANTES or IL-5 incubated with peroxynitrite by ELISA. Furthermore, peroxynitrite reduced RANTES and IL-5 binding to eosinophils. These data suggest that peroxynitrite plays an important role in regulating human eosinophil locomotion by modulating chemotactic cytokines.

In vitro, NO production has been demonstrated from a variety of cell types, and various inflammatory mediators are reported to enhance production of NO and superoxide, leading to the *in vivo* formation of peroxynitrite. Inflammatory cell chemotactic factors, including RANTES and IL-5, are likely to encounter high local concentrations of NO, superoxide, and peroxynitrite in inflammatory sites. Several studies have examined the effects of NO on neutrophil, monocyte, and eosinophil chemotaxis,^{33–38} but few have examined the effects on chemotactic factors.

The concentration of peroxynitrite *in vivo* is not known. Peroxynitrite is a transient intermediate in free radical chemistry and is highly reactive at physiological pH. For

this reason, peroxynitrite cannot be considered as a pharmacological drug that has steady-state concentrations. Thom et al³⁹ reported finding concentrations of nitrotyrosine, an end product of peroxynitrite, of 30 to 60 ng/mg protein in lung homogenates. Assuming that peroxynitrite nitrated tyrosine residues one-to-one, these levels would seem comparable to doses we used to affect chemokine function as reported in this manuscript.

Several lines of evidence suggest that RANTES, an 8-kd member of the C-C group of chemokines, may also play an important role in eosinophil recruitment during allergic inflammation.⁴⁰ Large amounts of RANTES are found in nasal polyp tissues, which are rich in infiltrating eosinophils.⁴¹ In addition, injection of RANTES into dog skin has been shown to induce a large eosinophil infiltrate *in vivo*.⁴² IL-5 is also the key cytokine involved in regulating the production and many of the specialized functions of mature eosinophils, including priming,⁴³ adhesion,⁴⁴ survival,⁴⁵ and migration.⁴⁰

The amino acid sequence on RANTES and IL-5 that binds to its receptors is unknown. However, the five tyrosine residues in RANTES and two tyrosine residues in IL-5 are rather evenly distributed through the molecule, and disulfide bonds are predicted to occur between cysteines.^{46,47} Although the mechanism(s) of peroxynitrite induced inhibition of RANTES and IL-5 ECA is not clear, there is strong evidence to support oxidative damage to the chemokines as a mechanism. Coincubation of RANTES and IL-5 with several peroxynitrite scavengers ameliorated ECA inhibition. Dithiothreitol prevented peroxynitrite-mediated nitration of tyrosine.⁴⁸ The iron chelator deferoxamine is also scavenger of peroxynitrite reaction independent of iron chelation.⁴⁹ In addition, L-tyrosine abrogated the peroxynitrite ECA inhibition. These results are consistent with tyrosine nitration by peroxynitrite as a mechanism for RANTES and IL-5 inhibition.

Current concepts suggest that the mechanism that leads to eosinophil locomotion in response to chemotactic cytokines is binding of the chemotactic factor to receptors on eosinophils. Consistent with this concept, our results demonstrate that peroxynitrite-treated RANTES and IL-5 exhibited decreased binding to eosinophils. Although the binding sites of RANTES and IL-5 to eosinophil receptors are not fully known, several studies have reported that alteration of amino acid residue inactivates chemokine function. Schraufstatter et al¹⁸ reported the importance of the Tyr¹³ and Lys¹⁵ sequence of IL-8 in binding to its receptor. Mutation of amino acids 13–15 from the rabbit (His-Ser-Thr) to the human (Tyr-Ser-Lys) sequence confers the high affinity of human IL-8 for the human IL-8A receptor to this mutated form of rabbit IL-8.¹⁸ Consistent with the concept that tyrosine is important in binding to receptor, Steitz et al¹⁹ reported that point mutations of Tyr-13 greatly lowered monocyte chemoattractant protein-1 receptor binding and activity. Our findings with RANTES and IL-5 after peroxynitrite incubation are consistent with these observations and suggest that tyrosine nitration by peroxynitrite on RANTES and IL-5 may be a mechanism altering their binding and chemotactic function. However, peroxynitrite may potentially affect protein function by other mechanisms, including ni-

tration of methionine⁵⁰ and tryptophan¹¹ or formation of S-nitroso-thiol groups on cysteines.¹⁵

Although NO and peroxynitrite are physiological regulators, they have been shown to alter respiration^{51,52} and induce cell death.⁵³ To estimate the effect of peroxynitrite on eosinophils, we incubated eosinophils with peroxynitrite for 90 minutes at 37°C before chemotaxis experiments. It induced no significant cytotoxicity as assessed by trypan blue exclusion in comparison to medium alone, and it had no significant effect on ECA by RANTES and IL-5.

The evidence for a role of peroxynitrite *in vivo* is based on detection of 3-nitrotyrosine in injured tissues; however, an additional mechanism of tyrosine nitration independent of peroxynitrite has lately been demonstrated.¹⁶ NO₂⁻ promotes tyrosine nitration through formation of nitryl chloride (Cl-NO₂) and nitrogen dioxide (•NO₂) by reaction with the inflammatory mediators hypochlorous acid (HOCl) or MPO. Peroxidases may potentially affect protein function by not only nitrating but also chlorinating or brominating tyrosine residues.¹⁷ Therefore, it cannot be stated definitively that the formation of nitrotyrosine *in vivo* is due to peroxynitrite.

In summary, we found that peroxynitrite nitrates tyrosine residue and modulates RANTES- and IL-5-induced ECA *in vitro*. These data suggest a role for peroxynitrite in regulating human eosinophil locomotion during inflammation.

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