

Inhibition of eosinophil density change and leukotriene C₄ generation by nedocromil sodium

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Nedocromil sodium (NS) has been shown to inhibit the late asthma response to inhaled antigen and to control symptoms in chronic asthma; in both processes the eosinophil is thought to be an important contributor. To understand the antiinflammatory actions of NS in asthma, its effects on three eosinophil functions were evaluated: (1) change in cell density during in vitro culture, (2) synthesis of leukotriene C₄ (LTC₄), and (3) generation of superoxide anion. In these studies normal density (>1.095 gm/ml) purified human peripheral blood eosinophils were cultured for 24 hours in 50% conditioned medium from cow pulmonary arterial endothelial cells. After incubation, 45.2% ± 8.0% of the eosinophils had a density less than 1.085 gm/ml. In the presence of NS (10 μmol/L), only 32.0% ± 7.3% became less dense (p = 0.0393). In contrast, NS had no effect on changes in cell density after a 20-minute exposure of eosinophils to functional activators N-formyl-methionyl-leucyl-phenylalanine (0.1 μmol/L) or platelet activating factor (0.1 μmol/L). Furthermore, calcium ionophore-activated LTC₄ secretion was found to be significantly inhibited by NS (3.8 ± 0.6 ng/ml vs 2.4 ± 0.3 ng/ml with 1 μmol/L NS or 1.7 ± 0.6 ng/ml with 10 μmol/L NS, p < 0.025). However, NS did not significantly alter eosinophil superoxide anion generation. The effects of NS on eosinophil function suggest a mechanism by which this medication may be effective in asthma, particularly in the regulation of the late asthmatic response. Furthermore, the selective regulatory effects of NS may also provide insight into the biologic activities of eosinophils. (J ALLERGY CLIN IMMUNOL 1992;90:202-9.)

Key words: Eosinophil, leukotrienes, nedocromil sodium, cell density, asthma, superoxide generation

Nedocromil sodium (NS) is an antiasthma medication whose pharmacologic properties include inhibition of adenosine- and exercise-induced bronchospasm.¹⁻³ Like cromolyn, NS also inhibits antigen-dependent mediator release from lungs, an action that may contribute to its regulation of antigen-provoked asthma.⁴⁻⁶ However, there is reason to believe that NS has other potentially important antiinflammatory actions as well. For example, NS blocks both the im-

Abbreviations used

NS:	Nedocromil sodium
CPAE:	Cow pulmonary arterial endothelial cells
FMLP:	N-formyl-methionyl-leucyl-phenylalanine
PAF:	Platelet activating factor
PMA:	Phorbol myristate acetate
LTC ₄ :	Leukotriene C ₄
GM-CSF:	Granulocyte-macrophage colony stimulating factor
LAR:	Late asthmatic response
HBSS:	Hanks' balanced salt solution
FCS:	Fetal calf serum
Cal:	Calcium ionophore A23187
O ₂ ⁻ :	Superoxide anion

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mediate and late asthmatic response (LAR) to inhaled antigen.⁴⁻⁸ Moreover, NS is effective in the treatment of chronic asthma, reduces associated symptoms, and decreases bronchial hyperresponsiveness.^{7, 9} Collec-

tively these actions suggest that NS has antiinflammatory properties in addition to its ability to modulate mediator release.

Although the mechanisms of LAR are not fully established, leukocyte-dependent inflammation is likely an important factor. In this perspective, NS has been shown to suppress a variety of granulocyte-associated inflammatory responses, including granulocyte-macrophage colony stimulating factor (GM-CSF) and tumor necrosis factor stimulated antibody-dependent cellular cytotoxicity,¹⁰ degranulation,^{11, 12} damage to epithelium,¹³ chemotactic factor-induced enhancement of C3b and Fc rosettes,¹⁴ eosinophil-dependent cytotoxicity,^{8, 15} and eosinophil and neutrophil chemotaxis.¹⁶ Because the eosinophil is pivotal in the development of LAR and chronic asthma, we elected to examine the effect of NS on three proinflammatory features of this cell that may relate to the development of airway injury: change in cell density, generation of leukotriene C₄ (LTC₄), and superoxide anion (O₂⁻) production.

PATIENTS IN THE STUDY AND METHODS

Reagents

Percoll was obtained from Pharmacia (Piscataway, N. J.). Platelet activating factor (PAF), phorbol myristate acetate (PMA), calcium ionophore A23187 (CaI), and *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) (Sigma Chemical Co., St. Louis, Mo.) were dissolved in dimethylsulfoxide (Sigma) and aliquots stored at -70° C until used. LTC₄ radioimmunoassay kits were obtained from New England Nuclear Company, Boston, Mass. and used as directed. RPMI medium, Hanks' balanced salt solution (HBSS), fetal calf serum (FCS), newborn calf serum, and penicillin/streptomycin (10,000 U/ml and 10,000 µg/ml, respectively) were purchased from GIBCO (Grand Island, N. Y.). NS was provided by the Fison Corporation (Loughborough, Leicestershire, England).

Subjects

Eosinophils were isolated from normal (*n* = 7) and allergic subjects (*n* = 13, six of whom had mild asthma), all of whom ranged in age from 20 to 50 years. The allergic subjects all demonstrated at least one positive immediate skin reaction (as defined by an immediate weal response of greater than 3 mm to a skin prick test) to a battery of antigens including ragweed, house dust mite, grass pollen, cat dander, and dog dander. Allergic patients were used to ensure recovery of adequate number of eosinophils but were asymptomatic and taking no medication at the time of study. Informed consent was obtained before participation in this study as required by the University of Wisconsin Committee on Human Subjects.

Eosinophil separation

Eosinophils were isolated from heparin-anticoagulated blood by multiple discontinuous density Percoll gradients

as previously described.¹⁷ In brief, blood was processed by dextran sedimentation and Hypaque-Ficoll centrifugation. The resulting granulocytes (4×10^7 cells/gradient in HBSS) were layered onto Percoll gradients consisting of five density solutions: 1.080, 1.085, 1.090, 1.095 and 1.100 gm/ml (2.5, 2.5, 3, 3, and 1.5 ml, respectively). After centrifugation (700 g) for 20 minutes at 20° C, eosinophils (>85% pure on a Wright-stained cytocentrifuge preparation) from the 1.095/1.100 gm/ml Percoll interface were collected, defined as the normal density population,¹⁷ and used in all subsequent experiments.

NS effect on eosinophil density changes

Cow pulmonary arterial endothelial cells (CPAE [American Tissue Typing Corporation, Rockville, Md.]) were cultured in RPMI with 10% FCS and 1% each of penicillin/streptomycin (RPMI/FCS). When the CPAE cells reached confluence, they were supplied with fresh medium and cultured for an additional 24 hours. Medium conditioned with CPAE was then collected, centrifuged to remove cell debris, and stored at -70° C until used. Purified normal density eosinophils (5×10^5 /ml) were suspended in RPMI/FCS and 50% CPAE-conditioned medium with, or without, 10 µmol/L NS, and incubated in tissue culture flasks (Falcon, Becton-Dickinson Labware, Lincoln Park, N. J.) for 24 hours at 37° C in a humidified CO₂ incubator. Control cultures were incubated in unconditioned medium with or without 10 µmol/L NS. The cells were then centrifuged, resuspended in room temperature HBSS with 5% newborn calf serum, counted (Coulter Counter, Hialeah, Fla.), and assessed for viability by trypan blue exclusion. Because of the small numbers of eosinophils, minigradients consisting of 1 ml each of the five Percoll solutions were prepared as outlined above. In these experiments, 0.5 to 5×10^6 eosinophils were loaded onto each Percoll minigradient. After centrifugation the five cell bands were carefully collected from the density interfaces and counted. Percent cell recovery and viability were determined at each of the individual density bands. Nonviable eosinophils and cell debris were observed only at the two lowest density Percoll interfaces.

To determine the effect of NS on activator-induced changes in cell density, purified normal density eosinophils (1.095 to 1.100 gm/ml, $\leq 5 \times 10^6$ cells/ml) were first incubated with 10 µmol/L NS (15 minutes at 37° C) and then exposed to either 0.1 µmol/L FMLP or 0.1 µmol/L PAF (20 minutes at 37° C). After this treatment the eosinophils were placed directly on discontinuous density Percoll minigradients and centrifuged as described above. The cell bands at the various densities were collected, counted, and viability ascertained. Eosinophil viability was not altered after this procedure.

Effect of NS on eosinophil LTC₄ generation

Eosinophils (5×10^5 cells/0.5 ml) were pretreated with NS for 15 minutes at 37° C and then activated with 2.5 µmol/L CaI. The reaction was terminated after a 20-minute incubation (37° C) by the addition of 0.5 ml reaction buffer

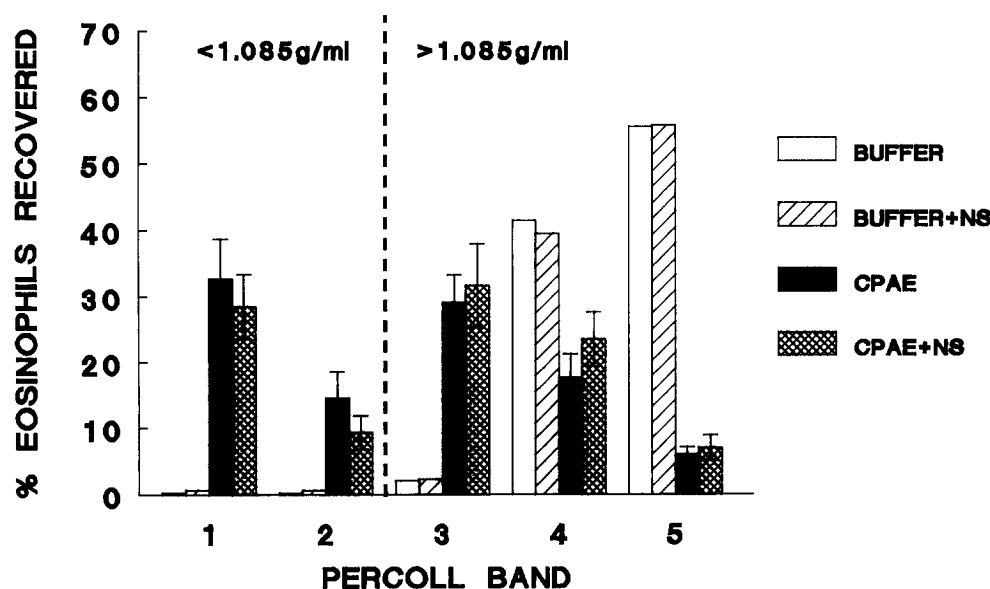


FIG. 1. Effect of NS on eosinophil density. Purified normal density eosinophils (≥ 1.095 gm/ml) were incubated with 50% CPAE-conditioned medium for 24 hours in the presence or absence of $10 \mu\text{mol/L}$ NS ($N = 5$). As a control, eosinophils were incubated with RPMI/FCS and NS ($N = 2$).

(New England Nuclear); and LTC_4 levels were quantitated by radioimmunoassay kit (New England Nuclear).

NS effect on eosinophil O_2^- generation

Eosinophil O_2^- generation was measured as previously described.¹⁸ In brief, 1×10^5 eosinophils were activated in 96-well plates (Immunolon II, Dynatech, Inc., Alexandria, Va.) by $0.1 \mu\text{mol/L}$ FMLP, $0.1 \mu\text{mol/L}$ PAF, or 1 ng/ml PMA in the presence of $0.1 \mu\text{mol/L}$ cytochrome C. The reaction mixture was incubated at $5\% \text{ CO}_2$, 37°C and absorbance at $550 \mu\text{mol/L}$ was measured at 10-minute intervals (Microplate Autoreader, Biotek, Winooski, Vt.). Superoxide dismutase ($20 \mu\text{g/ml}$) was added to duplicate reactions and served as controls. With use of the extinction coefficient of $21.1 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$, the results were expressed as nanomoles of cytochrome C reduction per 5×10^5 cells per time. Since O_2^- generation for all three activators reached its maximal response by 30 minutes, data at this time point are presented.

Statistics

Data are presented as mean $\pm$ SEM. Statistical analysis was by paired Student's t test, and a $p < 0.05$ was concluded to denote significance.

RESULTS

Effect of NS on eosinophil density

A 24-hour incubation with medium conditioned with CPAE caused $45.2\% \pm 8.0\%$ of normal density eosinophils (>1.095 gm/ml) from atopic subjects to become less dense, <1.085 gm/ml (Fig. 1). However, in the presence of $10 \mu\text{mol/L}$ NS, the per-

centage of eosinophils that became less dense, $32.0\% \pm 7.3\%$, was significantly less ($p = 0.0393$). In preliminary experiments, low concentrations of NS were also evaluated on this response but did not alter changes in eosinophil density during CPAE culture. Because only a limited number of eosinophils could be isolated from our subjects with the quantity of blood drawn (200 ml), and a relatively high number of cells were needed for density gradient experiments, it was necessary to limit our evaluation on this response to only one concentration of NS, $10 \mu\text{mol/L}$. The percentage of eosinophils recovered after a 24-hour incubation with medium conditioned with CPAE was similar in medium alone and cultures treated with NS; however, eosinophils treated with NS were significantly less viable, as determined by trypan blue exclusion ($74.6\% \pm 3.6\%$ vs $83.8\% \pm 5.0\%$ for untreated cells, $p = 0.0048$). Eosinophils cultured in RPMI/FCS medium alone also became less dense. However, this change in density was much less than the large shift seen with medium conditioned with CPAE (Fig. 1). The addition of NS to medium cultures not conditioned with CPAE had no effect on eosinophil density or on cell recovery and viability.

Exposure of granulocytes to selected agonists will also decrease granulocyte density.¹⁹⁻²¹ Incubation of eosinophils for 20 minutes with either $0.1 \mu\text{mol/L}$ of FMLP or PAF caused a significant decrease in cell density (Fig. 2). Under these experimental conditions, $10 \mu\text{mol/L}$ NS did not inhibit the agonist-induced change in eosinophil density. Furthermore, the re-

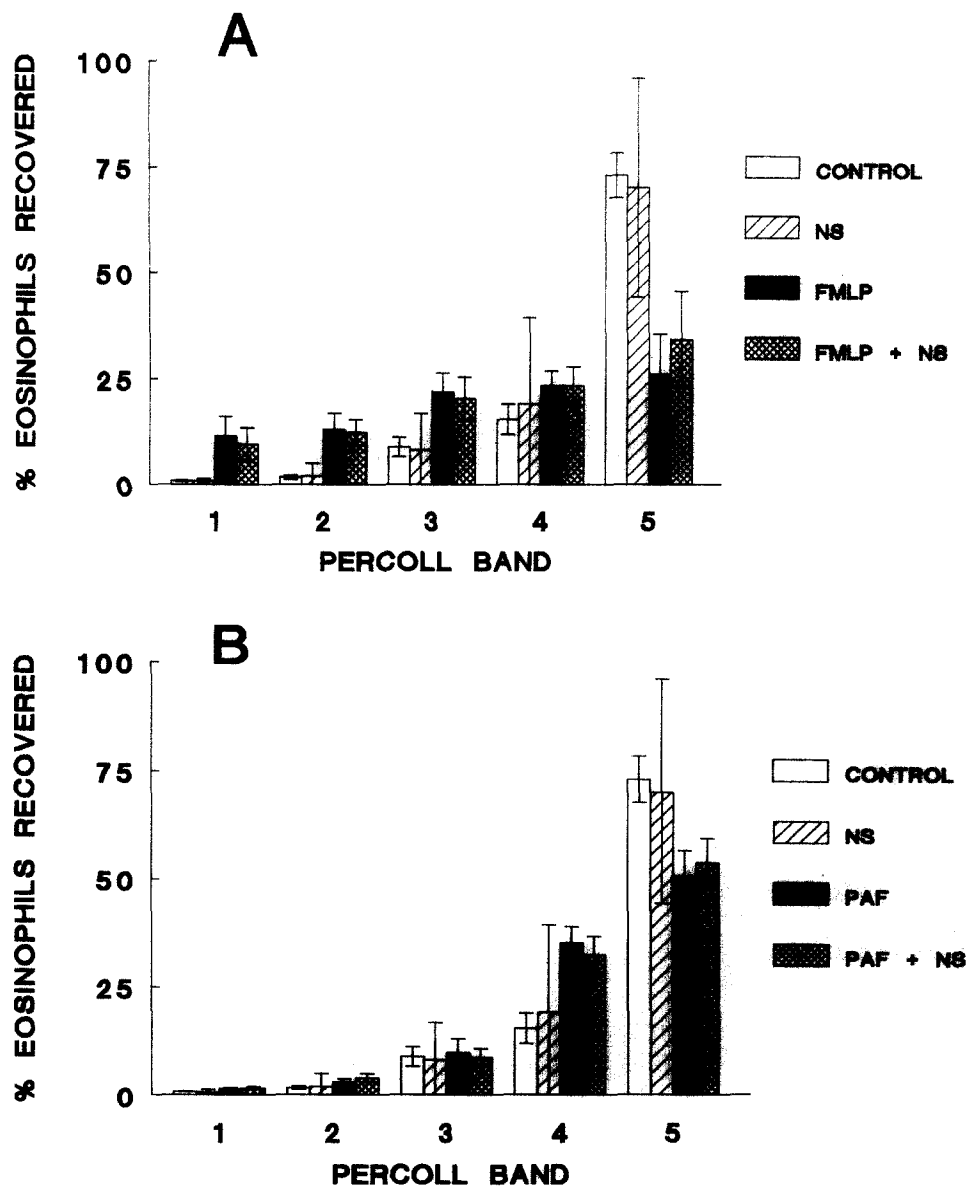


FIG. 2. Effect of NS on eosinophil density in the presence of an activator. Purified normal density eosinophils were preincubated at 37° C for 15 minutes with or without 10 μ mol/L NS followed by a 20-minute 37° C incubation with 0.1 μ mol/L FMLP (A) or 0.1 μ mol/L PAF (B). *N* = 6. **p* < 0.05 versus control eosinophils $\pm$ NS.

duction in eosinophil density, and lack of effect by NS, was similar whether cells were obtained from either normal or allergic subjects (Table I); consequently, the results from these experiments were combined and reported as a single subject group.

Fractionating untreated, normal dense eosinophils over a second Percoll gradient resulted in only slight redistribution of cells to lower density bands as can be seen in Fig. 2, control data. Therefore the density fractionation procedure itself had little effect on eosinophil density. The percent of eosinophils recovered from the second Percoll gradients was dependent on

the duration of in vitro cell culture with fewer cells recovered after 24 hours than after 20 minutes of incubation (Table II). However, cell recovery did not vary between culture conditions in individual experiments.

Effect of NS on eosinophil LTC₄ generation

A 20-minute incubation with 2.5 μ mol/L CaI stimulated eosinophils to generate 3.8 ± 0.6 ng/ml of LTC₄. CaI activated eosinophil generation of LTC₄ was significantly inhibited by 1 and 10 μ mol/L NS (Fig. 3). Although the suppression of LTC₄ generation

TABLE I. Comparison of normal and allergic eosinophil donors; the effect of activator and NS on cell density*

	FMLP (0.1 μmol/L)		PAF (0.1 μmol/L)	
	Normal	Allergic	Normal	Allergic
Control	35.3 ± 12.1†	56.7 ± 6.2	19.8 ± 5.0	9.2 ± 2.0
NS (10 μmol/L)	54.0 ± 15.1	67.7 ± 6.4	16.7 ± 5.6	9.8 ± 2.0
Activator	60.3 ± 18.9	83.7 ± 1.2	60.5 ± 9.6	45.2 ± 6.3
Activator ± NS	67.7 ± 21.5	79.7 ± 2.3	55.8 ± 8.1	42.5 ± 6.4

**p* > 0.05 for all comparisons between normal and allergic by paired *t* test.

†*N* = 3 for each subject group (mean ± SEM).

TABLE II. Percent eosinophils recovered from second Percoll gradient*

Time of incubation	No NS	10 μmol/L
24 hours: (<i>N</i> = 6)	48 ± 4†	65 ± 10
20 minutes: (<i>N</i> = 13)		
Control	76 ± 5	74 ± 8
Activated	71 ± 5	65 ± 6

**p* > 0.05 for all *t* test comparisons within each incubation period.

†Percent eosinophils (mean ± SEM).

by 10 μmol/L NS was greater than a 1.0 μmol/L concentration, the observed differences did not achieve significance.

Effect of NS on O₂⁻ generation

The measurement of O₂⁻ generation required fewer eosinophils than did experiments that evaluated changes in cell density or generation of LTC₄. This requirement for fewer cells enabled us to assess the effect of NS concentration on O₂⁻ production (Table III). None of the NS concentrations tested altered O₂⁻ generation to any of the three activators, FMLP, PAF, or PMA.

DISCUSSION

We have found that an in vitro incubation of eosinophils with 10 μmol/L NS significantly inhibited two of this cell's properties: change in cell density and generation of LTC₄. Incubation of eosinophils with medium conditioned with CPAE converts this cell to a lower density phenotype. Previous studies have shown that incubation of normal density eosinophils with medium conditioned with endothelial cells, or selected cytokines, will decrease cell density, prolong in vitro survival, and enhance generation of LTC₄ and cytotoxicity responses.²²⁻²⁴ It is hypothesized that this in vitro phenotypic conversion to lower cell density is a step associated with the functional up-

regulation of eosinophils and may reflect in vivo events during the late airway response to antigen.^{23, 24}

In a previous study we have found that low density peripheral blood eosinophils increase only in allergic patients who have LAR to inhaled antigen.¹⁷ Furthermore, we have also found that eosinophils in bronchoalveolar lavage fluid obtained 48 hours after segmental antigen challenge include a cell population with decreased cell density.²⁵ Collectively, these observations suggest that the development of LAR is associated with changes in eosinophil density. Moreover, the increased presence of the low density subpopulation in LAR may represent a phenotypic conversion of eosinophils important in the development of bronchial inflammation. Thus changes in eosinophil density may provide an in vitro model to assess potential mechanisms of this cell's participation in asthma and a method to evaluate selected effects of antiasthma therapeutic agents.

On the basis of this premise, eosinophils cultured with CPAE cells or medium conditioned with CPAE were found to undergo a decrease in cell density and an increase in cell survival.²²⁻²⁴ In the present study NS inhibited the decrease in eosinophil density associated with in vitro incubation with medium conditioned with CPAE. However, the ability of NS to block changes in eosinophil density was not found with all stimuli. The incubation of granulocytes with noncytokine activators causes a rapid (10 to 20 minute) decrease in cell density.¹⁹⁻²¹ In a similar manner we found that the membrane receptor-dependent agonists FMLP and PAF rapidly decrease eosinophil density; NS, however, did not modify the eosinophil response to these stimuli. Although our data do not establish a mechanism by which NS regulates changes in eosinophil density, they do indicate a stimulus-specificity or selectivity for such actions on this particular cell property.

A similar selectivity in the ability of NS to modify certain granulocyte functions has been reported by Rand et al.¹⁰ These investigators found NS inhibited

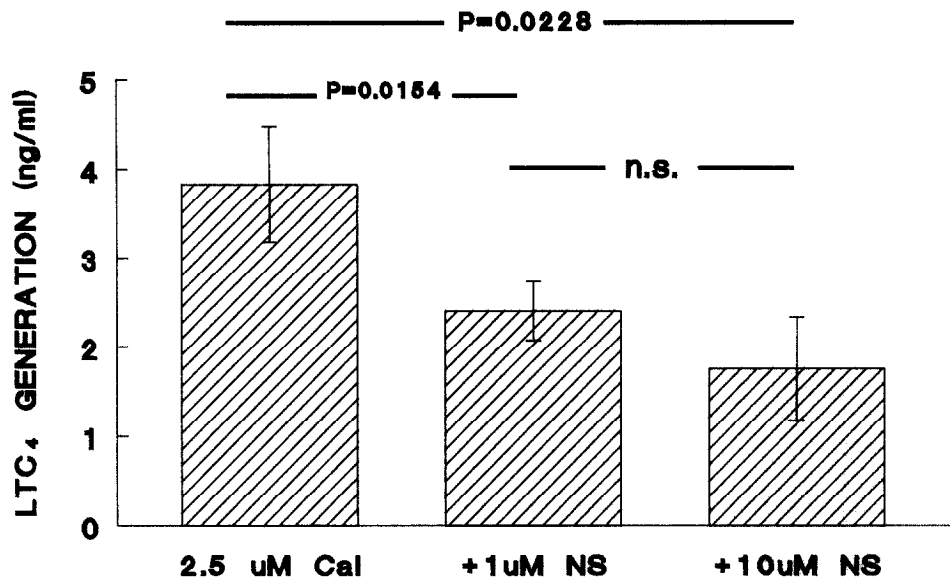


FIG. 3. Effect of NS on eosinophil LTC₄ generation. After a 15-minute preincubation with NS, normal dense eosinophils generated less LTC₄ after 2.5 μ mol/L A23187 stimulation (20 minutes). *N* = 5.

GM-CSF and tumor necrosis factor stimulated antibody-dependent cytotoxicity; in contrast, NS had no effect on either O₂⁻ generation or lysozyme release to soluble and particle-ingestible stimuli or to GM-CSF enhanced FMLP activation. From these observations Rand et al.¹⁰ concluded that NS inhibits the direct effect of cytokines on granulocyte function but has little effect on noncytokine stimuli such as FMLP. Our findings parallel and support these conclusions.

The number of viable eosinophils decreased after a 24-hour CPAE incubation in the presence of NS, an effect similar to that seen with corticosteroids.^{22, 26} This effect may indicate another potential action of NS in modifying the LAR to inhaled antigen: a prevention of cytokine-promoted eosinophil survival. It is interesting to note that this NS-related decrease in eosinophil viability was not reflected in decreased cell density (Fig. 1) or cell recovery off of the second Percoll gradient (Table II). Short-term (20-minute) culture with medium FMLP or PAF had no effect on eosinophil viability, which remained greater than 95%.

Prolonged eosinophil survival is associated with two other cell features: decreased density and increased inflammatory activity.^{23, 24} The cytokines interleukin-3, interleukin-5, and GM-CSF have been proposed as the components of media conditioned with CPAE and human umbilical vein endothelial cells, which prolong eosinophil survival and decrease cell density.²⁷⁻²⁹ Preliminary experiments in our laboratory suggest that NS inhibits eosinophil survival

TABLE III. Effect of NS on O₂⁻ generation

	FMLP (0.1 μ mol/L)	PAF (0.1 μ mol/L)	PMA (1 ng/ml)
<i>N</i>	5	6	6
No. NS	7.3 $\pm$ 1.0*	10.7 $\pm$ 2.1	24.2 $\pm$ 3.5
0.1 μ mol/L NS	5.9 $\pm$ 0.5	11.8 $\pm$ 2.6	30.9 $\pm$ 3.8
0.1 μ mol/L NS	6.2 $\pm$ 1.0	13.4 $\pm$ 2.4	31.9 $\pm$ 4.7
1 μ mol/L NS	5.7 $\pm$ 2.0	10.8 $\pm$ 2.3	29.0 $\pm$ 2.9
10 μ mol/L NS	6.5 $\pm$ 1.6	9.4 $\pm$ 1.8	29.3 $\pm$ 1.9

*nmol/L cytochrome C/5 $\times$ 10⁵ cells/30 minutes.

when the cells are cultured with the cytokine interleukin-5. The observed loss of cell viability does not, in our opinion, represent cell killing by NS, but rather an inhibition of the survival promoting activity caused by some cytokines. Further studies on the effect of NS on cytokine-regulated eosinophil survival are underway.

Finally, NS suppressed eosinophil LTC₄ production to Cal. Our observations corroborate the findings of Bruijnzeel et al.³⁰ who showed that NS inhibits Cal and opsonized zymosan-induced release of eosinophil LTC₄. We and Bruijnzeel et al.³⁰ used 2.5 μ mol/L Cal and obtained similar inhibitory results with NS. Similarly, Moqbel et al.¹⁵ found NS reduced immunoglobulin G-dependent release of LTC₄ by eosinophils after stimulation with FMLP. However, Burke et al.³¹ were unable to inhibit 5.0 μ mol/L Cal-dependent release of LTC₄ with NS. This discrepancy

possibly relates to the concentrations of CaI used by the various investigative groups, 2.5 versus 5.0 $\mu\text{mol/L}$; higher concentrations of CaI likely overcome the regulating actions of NS. Thus it appears that the suppressive effect of NS on eosinophil function is also dependent on the concentration of the stimulus.

NS does not modulate all granulocyte inflammatory responses. As reported for neutrophils by Rand et al.,¹⁰ we found that NS does not affect O_2^- generation by eosinophils. The difference in the effects of NS on LTC_4 and O_2^- generation suggest that NS may have specific cell-site actions that are reflected in only selected responses. This selectivity may provide insight into the mechanism of activity of NS.

A number of practical limitations to this study occurred that curtailed our ability to be more definitive in characterizing the effect of NS on eosinophil functions. We were able to evaluate only one concentration of NS in some of our experiments. This limitation was imposed for a number of reasons. First, we obtained eosinophils from nonhypereosinophilic subjects and were consequently limited by the number of cells available for study. To obtain sufficient numbers of eosinophils to assess multiple concentrations of NS, either large (500 ml) volumes of blood would need to be drawn from our subjects or patients with hyper-eosinophilia would be necessary. Since eosinophils from hypereosinophilic subjects may not be "normal" cells,³² we elected to study only control and atopic subjects with normal peripheral eosinophil values (0 to 400/ mm^3), which limited our evaluation to one concentration of NS for experiments requiring high numbers of eosinophils (density measurements). Second, no significant difference in NS effects was observed in preliminary experiments at lower drug concentrations (O_2^- and LTC_4 generation). The 10 $\mu\text{mol/L}$ concentration of NS was chosen because it has been reported by other investigators to have maximal effects on cell function.^{10, 14, 16, 30} Whether such concentrations are achieved in vivo is hard to determine; however, because 4 mg of NS is the usual inhaled dose, it is possible that a 10 $\mu\text{mol/L}$ concentration can be achieved in airway tissues or humoral cells.

We were also limited in our experiments to normal density eosinophils. Airway eosinophils increase after antigen challenge, and include a large proportion of lower density cells.^{25, 33, 34} One interpretation of these observations is that the appearance of the LAR is associated with conversion of eosinophils to a low density. The in vitro conversion to lower eosinophil density and increased survival during culture suggests a linkage of these two processes and the possibility

that the low density phenotype is the principal cell of interest. Therefore the effect of NS on these cells would be very interesting. However, because of poor purity (<80%) of the low density blood eosinophils (1.085 to 1.095 gm/ml) from atopic subjects, the effect of NS on these cells could not be compared with normal density eosinophils from the same donor.

A number of in vitro models have been used to evaluate eosinophil function and its contribution to asthma. These include changes in cell density and generation of the bronchoconstricting LTC_4 . These same responses can be used as an in vitro target for antiasthma medication. Therefore our observation that NS blocks the eosinophil's phenotypic conversion to a lower density, longer surviving subpopulation may, in part, explain its inhibitory actions on the LAR. The protective effects of NS in asthma may be further amplified by its ability to inhibit eosinophil generation of LTC_4 . Consequently, NS has antiinflammatory effects on at least two eosinophil properties. Future studies will be necessary to establish the cellular mechanism of NS on eosinophils and other inflammatory cells. Nonetheless, our data to show NS regulation of selected human eosinophil functions may help our understanding of the protective effect of this drug on chronic airway inflammation, the LAR, and asthma itself.

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