

Effects of nedocromil sodium on in vitro induced migration, activation, and mediator release from human granulocytes

Pieter L. B. Bruijnzeel, MD, PhD, Ruud A. J. Warringa, MD, Paul T. M. Kok, Maartje L. Hamelink, Hans Kreukniet, MD, and Leo Koenderman, PhD
Utrecht, The Netherlands

Using the allergen-induced late-phase asthmatic reaction as a working model, we studied the activity of certain inflammatory cells and their reaction to nedocromil sodium. The processes that were examined in vitro included the following: the chemotaxis of purified neutrophils and eosinophils, the early steps of neutrophil and eosinophil activation, and the release of mediators from these cells. Nedocromil sodium strongly inhibited neutrophil mobilization caused by four chemotactic factors (zymosan activated serum, N-formyl-methionyl-leucyl-phenylalanine platelet-activating factor [PAF], and leukotriene B₄ [LTB₄] and eosinophil mobilization caused by two factors (PAF and LTB₄). In vitro treatment of eosinophils from normal subjects with picomolar concentrations of interleukin-3, interleukin-5, or granulocyte-macrophage colony stimulating factor increased the chemotactic responsiveness toward PAF and LTB₄ and induced a chemotactic responsiveness toward N-formyl-methionyl-leucyl-phenylalanine and neutrophil activating factor/interleukin-8. The zymosan activated serum-induced chemotactic responsiveness remained unaltered. Nedocromil sodium inhibited the cytokine-primed chemotactic responsiveness to the various chemotaxins, not the influence of the cytokines on the cells. Activation of granulocytes, as measured by Ca²⁺ influx, was not inhibited by nedocromil sodium. Mediator formation in eosinophils was modified only slightly. These results suggest that inhibiting the mobilization of inflammatory cells in the lung tissue may be an important action of nedocromil sodium. Therefore these effects may be relevant to the treatment of asthma given the role of airway inflammation in this disease process. (J ALLERGY CLIN IMMUNOL 1993;92:159-64.)

Recently evidence has shown that airway inflammation is an important component of both mild and severe asthma. The chain of events leading to airway inflammation can be hypothesized as follows. Inflammatory stimuli activate the primary effector cells present in the airways. On activation these cells release mediators that mobilize secondary effector cells from the circulation. The mediators, which are released from both primary and secondary effector cells, may cause smooth muscle contraction, vasodilation, changes in vascular permeability, epithelial damage, mucus secretion, and efferent nerve stimulation. Granu-

Abbreviations used

fMLP:	N-Formyl-methionyl-leucyl-phenylalanine
GM-CSF:	Granulocyte-macrophage colony-stimulating factor
IC ₃₀ :	30% inhibition concentration
IC ₅₀ :	50% inhibition concentration
IL-3:	Interleukin-3
IL-5:	Interleukin-5
LTB ₄ :	Leukotriene B ₄
LTC ₄ :	Leukotriene C ₄
NAF/IL-8:	Neutrophil activating factor/interleukin-8
OZ:	Opsonized zymosan
PAF:	Platelet-activating factor
ZAS:	Zymosan activated serum

From the Department of Pulmonary Disease, State University Hospital Utrecht, Utrecht, The Netherlands.

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Reprint requests: P. L.B. Bruijnzeel, MD, PhD, Head of Pharmacology MBL/TNO, Lange Kleiweg 139, 2288 GJ Rijswijk, The Netherlands.

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locytes, particularly eosinophils, are considered the most important secondary effector cells in this process.

To study the involvement of inflammatory cells

in asthma, we chose the allergen-induced late-phase asthmatic reaction as a working model.¹ In the beginning of this late-phase asthmatic reaction, there is an influx of inflammatory cells in the bronchioli.²⁻⁴ Bronchial hyperreactivity also increases 24 hours after challenge, and this may be due to the release of mediators from those cells. On the basis of this model we studied the chemotaxis of purified neutrophils and eosinophils, the early steps of neutrophil and eosinophil activation, and the release of mediators from those cells. Supposing that these sequential events should reflect the *in vivo* situation, we investigated whether certain antiasthma drugs, including nedocromil sodium, would interfere with these steps.

CHEMOTAXIS OF HUMAN NEUTROPHILS AND EOSINOPHILS

Mobilization of neutrophils and eosinophils most likely takes place through the release of chemotactic factors from primary effector cells that are present in the lung tissue. These chemotactic factors are protein or lipid in nature. In the chemotaxis studies^{5, 6} we used fMLP and ZAS as representatives of protein chemotaxins, and PAF and LTB₄ as representatives of lipid-derived chemotaxins. Neutrophils and eosinophils were purified from peripheral blood of normal subjects,⁷ and chemotaxis was measured according to the Boyden chamber technique with a 48-well microchemotaxis chamber.⁶ The cells were preincubated with the drug for 30 minutes before the studies were performed. In the first series of experiments fMLP, ZAS, PAF (lyso-PAF), and LTB₄ dose-response curves for both isolated neutrophils and eosinophils were constructed.

All compounds except lyso-PAF induced significant chemotaxis. In the case of eosinophils all compounds except lyso-PAF and fMLP induced significant chemotaxis. The optimal chemotactic concentrations for neutrophils were 10 nmol/L for fMLP, a dilution of 1:3 for ZAS, 1 μ mol/L for PAF, and 1 μ mol/L for LTB₄. The optimal chemotactic concentrations for eosinophils were a dilution of 1:3 for ZAS, 1 μ mol/L for PAF, and 1 μ mol/L for LTB₄. The optimal chemotactic concentrations for eosinophils were a dilution of 1:3 for ZAS, 1 μ mol/L for PAF, and 1 μ mol/L for LTB₄. For neutrophils the order of potency of chemotactic stimuli was ZAS > PAF = fMLP = LTB₄. For eosinophils the order of potency of the tested chemotactic compounds was ZAS > PAF > LTB₄.

The absolute numbers of migrated cells indi-

cated that in all cases the tested chemotaxins attracted neutrophils more powerfully than they did eosinophils. At the optimal chemotactic concentration the ratio between attracted neutrophils and eosinophils was generally between 2 and 3.

In the second series of experiments we evaluated the inhibitory potency of various concentrations of nedocromil sodium at the optimal chemotactic concentration of fMLP, ZAS, PAF, and LTB₄ for both neutrophils and eosinophils (Fig. 1). The results indicate that neutrophil-induced chemotaxis could be inhibited significantly by relatively low concentrations (between 7 μ mol/L and 70 nmol/L) of nedocromil sodium. In the case of eosinophils nedocromil sodium significantly inhibited only the chemotaxis to PAF and LTB₄. It did not inhibit ZAS-induced chemotaxis. Cromolyn sodium inhibited the ZAS-induced chemotaxis but was not effective against PAF- and LTB₄-induced chemotaxis. Nedocromil sodium was as potent as the PAF antagonist BN52021 in inhibiting PAF-induced chemotaxis of both neutrophils and eosinophils. Thus at relatively low concentrations of nedocromil sodium the chemotactic response of neutrophils to fMLP, ZAS, PAF, and LTB₄ is significantly inhibited. With eosinophils the chemotactic response to PAF and LTB₄ is significantly inhibited by nedocromil sodium.

Recently we concentrated on the chemotactic activity of eosinophils toward the cytokines IL-3, IL-5, and GM-CSF, and their modulatory role in the chemotactic response to the chemoattractants PAF, ZAS (C5a), LTB₄, fMLP, and NAF/IL-8.^{8, 9} In these investigations we demonstrated that at nanomolar concentrations IL-3, IL-5, and GM-CSF were capable of attracting eosinophils. At picomolar concentrations they were capable of up-regulating the chemotactic response toward PAF and LTB₄ and inducing a chemotactic response toward fMLP and NAF/IL-8. They were not capable of altering the C5a (ZAS)-induced chemotactic response.^{8, 9} This proved to be valid for eosinophils from the circulation of normal subjects. Eosinophils from asthmatic subjects showed a spontaneous increased chemotactic response toward PAF, LTB₄, fMLP, and NAF/IL-8 similar to the response of eosinophils from normal subjects after cytokine pretreatment *in vitro*. Therefore this could indicate that eosinophils from asthmatic persons have had previous *in vivo* contact with these cytokines (i.e., "priming"). Nedocromil sodium did not interfere with this priming mechanism; it seemed to restrict its mode of action to the chemoattractant-induced mobili-

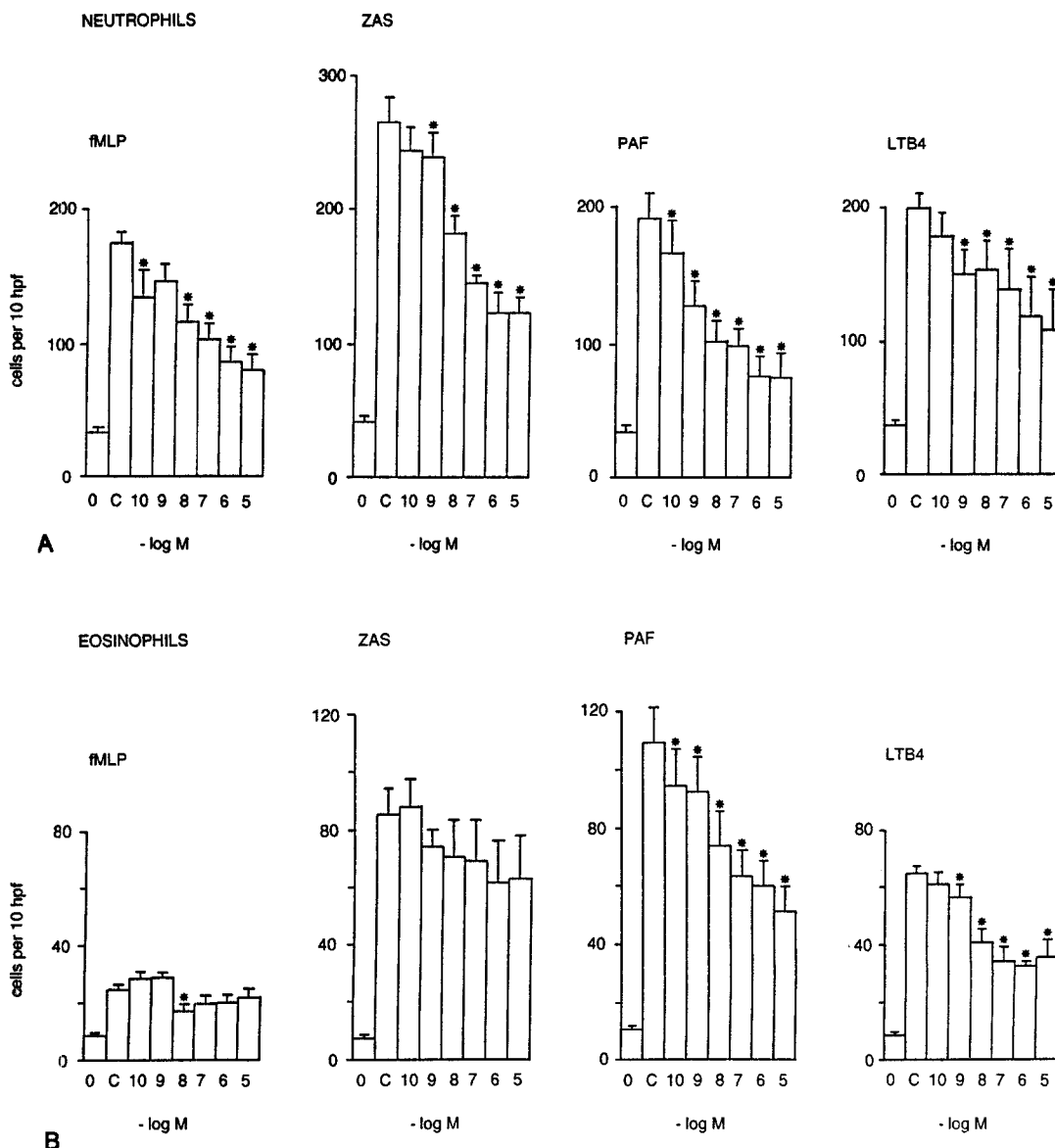


FIG. 1. Inhibition of fMLP-, ZAS-, PAF-, and LTB₄-induced chemotaxis of isolated neutrophils (**A**) and eosinophils (**B**) of normal subjects by increasing concentrations of nedocromil sodium. O; Buffer; C; chemotaxis obtained with optimal chemotactic concentration (see text). Mean values $\pm$ SEM are presented. Cell purities were $\geq 98\%$ for neutrophils and $\geq 85\%$ for eosinophils. IC₅₀ values were calculated with polynomial fit program. For neutrophils IC₅₀ values were 2.4×10^{-7} mol/L (fMLP, $n = 6$); 7.8×10^{-8} mol/L (ZAS, $n = 6$); 7.3×10^{-9} mol/L (PAF, $n = 11$); 1.1×10^{-6} mol/L (LTB₄, $n = 6$). For eosinophils IC₅₀ values were 1.3×10^{-6} mol/L (PAF, $n = 11$); 9.4×10^{-8} mol/L (LTB₄, $n = 6$). * $p < 0.05$ when compared with control value C (Student t test for paired data).

zation, probably interfering with specific receptor sites for the chemoattractants.¹⁰

EARLY STEPS OF NEUTROPHIL AND EOSINOPHIL ACTIVATION

In an effort to explain nedocromil sodium's inhibition of the chemotactic responses of neutrophils and eosinophils to the previously mentioned

agents, we attempted to perform binding studies with PAF and LTB₄. Because of high rates of nonspecific binding, we terminated these studies.⁵ Instead, we decided to study the ZAS- and PAF-induced intracellular Ca²⁺ mobilization of both neutrophils and eosinophils. These stimuli were chosen as representatives for the chemotactic factors of protein and lipid nature described earlier.

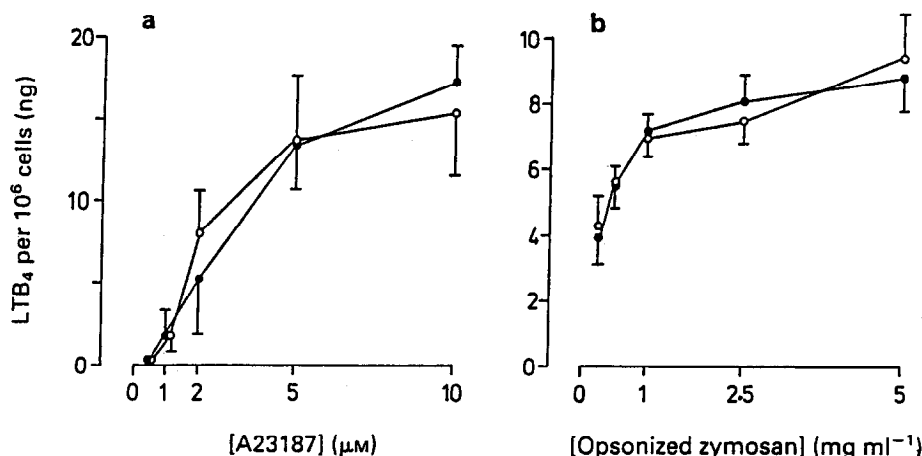


FIG. 2. Concentration dependency of A23187-induced (A) and OZ-induced (B) LTB₄ formation by purified human neutrophils in absence (●) or presence (○) of nedocromil sodium (10 μmol/L). Cells were preincubated with drug for 5 minutes before stimulation. Points shown are mean values ($n = 3$). Vertical lines indicate SEM. (From Bruijnzeel PLB, et al. *Biochem Biophys Res Commun* 1988;153:676-82.)

Both stimuli induced a Ca²⁺ influx at relatively low concentrations (1:81 dilution and 0.7 μmol/L, respectively). Although nedocromil sodium concentrations up to 0.1 mmol/L were used, no significant inhibition of ZAS- and PAF-induced Ca²⁺ influx was observed in either cell type.^{5, 6} This finding is interesting in light of recent results suggesting that nedocromil sodium does inhibit increase in intracellular Ca²⁺ levels in platelets after stimulation with PAF.¹¹

RELEASE OF MEDIATORS FROM NEUTROPHILS AND EOSINOPHILS

Neutrophils and eosinophils are believed to contribute actively to the pathogenesis of asthma through the release of inflammatory mediators including leukotrienes. Previous studies revealed the almost exclusive synthesis of LTC₄ by human eosinophils and of LTB₄, 20-OH-LTB₄, and the nonenzymatically formed LTB₄ isomers by human neutrophils when stimulated in vitro with the calcium ionophore A23187 or OZ.^{12, 13} Similar findings were obtained after stimulation with PAF and arachidonic acid^{14, 15} and under certain conditions with fMLP.¹⁶ Under optimal incubation conditions, purified neutrophils and eosinophils were challenged by these stimuli, with or without a 5-minute preincubation in the presence of nedocromil sodium (10 μmol/L). The results indicated that leukotriene synthesis induced in the granulocytes of normal and asthmatic subjects by A23187 and OZ was similar. However, the leuko-

triene formation induced in granulocytes of asthmatic subjects by PAF, fMLP, and arachidonic acid seemed to be less. This finding may be due to the fact that granulocytes of asthmatic persons have an increased ability to generate reactive oxygen metabolites when stimulated with fMLP or PAF.¹⁷ These metabolites may degrade the leukotrienes or interfere with the formation of their progenitors. Because nedocromil sodium inhibited only the A23187- and OZ-induced leukotriene formation in these studies, this formation was studied in more detail (see Figs. 2 and 3). The results demonstrated that nedocromil sodium significantly inhibited the A23187- and OZ-induced LTC₄ formation by eosinophils, whereas the synthesis of LTB₄ by neutrophils was not inhibited.

In subsequent experiments the A23187 (5 μmol/L)- and OZ (0.5 mg/ml)-induced LTC₄ formation by eosinophils was inhibited by nedocromil sodium in a concentration-dependent manner (mean 30% inhibition concentration for A23187, 5.6×10^{-5} mol/L; and for OZ, 6.3×10^{-5} mol/L) (see Fig. 4).¹⁸ An explanation for the mode of action of nedocromil sodium in these experiments has not yet been found.

In another series of experiments we investigated whether nedocromil sodium was capable of inhibiting the luminol-dependent chemiluminescence induced in both neutrophils and eosinophils by A23187, OZ, PAF, fMLP, and phorbol-myristate-acetate. At a concentration of 10 μmol/L nedocromil sodium did not appear to

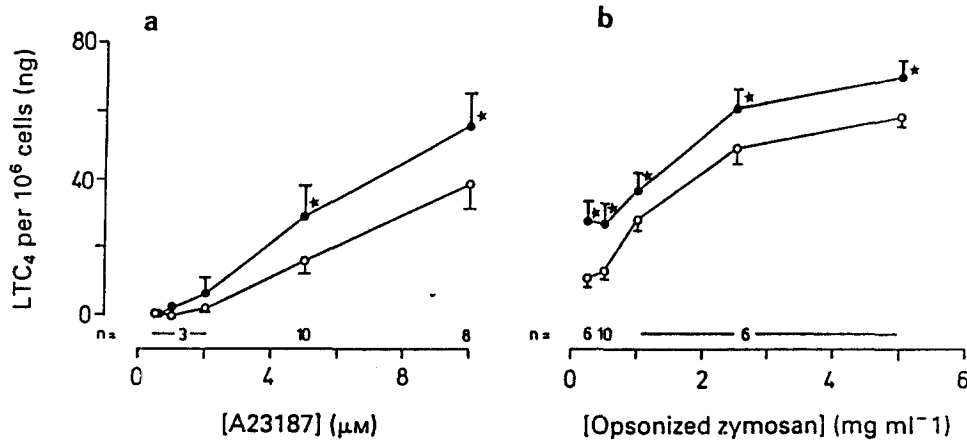


FIG. 3. Concentration dependency of A23187-induced (A) OZ-induced (B) LTC₄ formation by purified human neutrophils in absence (●) or presence (○) of nedocromil sodium (10 μmol/L). Cells were preincubated with drug for 5 minutes before stimulation. **p* < 0.05 (Student *t* test for paired data). Points shown are mean values; vertical lines indicate SEM. (From Bruijnzeel PLB, et al. *Biochem Biophys Res Commun* 1988;153:676-82.)

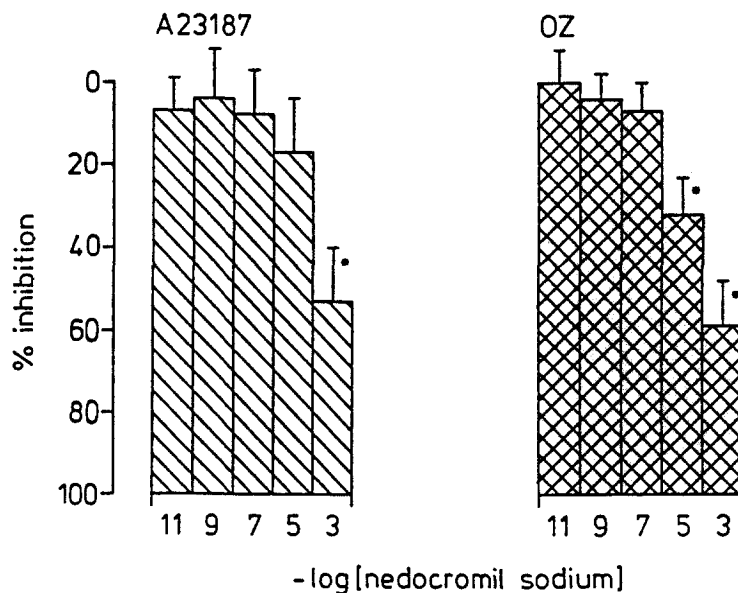


FIG. 4. Inhibition of A23187 (5 μmol/L) and OZ (0.5 mg/ml) induced LTC₄ formation by purified human eosinophils at varying concentrations of nedocromil sodium. Cells were preincubated for 5 minutes with drug before stimulation. Incubation without nedocromil sodium was taken as 100% value (*n* ≥ 7, mean ± SEM; **p* < 0.05).

show significant inhibitory effects. Thus it may not interfere with the myeloperoxidase- or eosinophil peroxidase-dependent generation of extracellular reactive oxygen metabolites.

CONCLUSION

Because inflammatory cells are thought to play an active role in the pathogenesis of asthma, the

inhibitory effect of nedocromil sodium on the mobilization, activation, and mediator release from those cells has been investigated in vitro. Nedocromil sodium strongly inhibited neutrophil mobilization caused by ZAS, fMLP, PAF, and LTB₄, and strongly inhibited PAF- and LTB₄-induced mobilization of eosinophils. In vitro pre-treatment of eosinophils with picomolar concen-

trations of IL-3, IL-5, and GM-CSF could increase and induce chemotactic responsiveness to various stimuli. Nedocromil sodium did not interfere with the influence of the cytokines on the eosinophils; it seemed rather to restrict its mode of action to the receptor site for the chemoattractant. As measured by Ca^{2+} influx, ZAS- and PAF-induced activation of both cell types was not inhibited by nedocromil sodium. On the other hand, A23187- and OZ-induced LTC_4 formation of eosinophils could be slightly but significantly inhibited. The A23187- and OZ-induced LTB_4 formation by neutrophils was not affected by nedocromil. In most cases, when other stimuli were applied to generate leukotrienes from both cells types, no inhibition was observed when these cells had been preincubated with nedocromil sodium. Furthermore, nedocromil sodium exerted no inhibitory action on the generation of extracellular reactive oxygen species when neutrophils and eosinophils were exposed to a variety of stimuli.

Taken together, these in vitro results suggest that inhibiting the mobilization of inflammatory cells into the lung tissue may be an important action of nedocromil sodium. The drug may interfere with a receptor-mediated intracellular signal transduction process. If eosinophils are considered more important than neutrophils in the pathogenesis of asthma, then these results indicate that nedocromil sodium may effectively block the PAF- and LTB_4 -induced mobilization of eosinophils as well as the A23187- and OZ-induced synthesis of LTC_4 . Both effects may be relevant to the treatment of asthma.

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