Nedocromil sodium inhibits the A23187- and opsonized zymosan-induced leukotriene formation by human eosinophils but not by human neutrophils

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1 Inflammatory cells such as eosinophils and neutrophils are thought to contribute actively to the pathogenesis of asthma by the release of bronchoconstrictor mediators including leukotrienes. Previous studies have revealed the almost exclusive synthesis of leukotriene C₄ (LTC₄) by human eosinophils and of leukotriene B₄ (LTB₄), 20-OH-LTB₄ and the non-enzymatically formed LTB₄-isomers by neutrophils when stimulated *in vitro* with the calcium ionophore A23187 or opsonized zymosan (OZ). In this study we have investigated whether nedocromil sodium, a new anti-asthma drug, was capable of inhibiting A23187- and OZ-induced leukotriene formation by these cells.

2 Nedocromil sodium inhibited A23187- and OZ-induced LTC₄ formation by eosinophils in a concentration-dependent manner (mean IC₃₀ for A23187: 5.6×10^{-5} M; mean IC₃₀ for OZ: 6.3×10^{-5} M), whereas it did not inhibit A23187- and OZ-induced LTB₄ formation by neutrophils.

3 Extension of the preincubation time of the cells with the drug did not alter the observed inhibitory capacity. The optimal preincubation time was 5 min.

4 The *in vitro* inhibition of LTC₄ formation by eosinophils by nedocromil sodium may be a valuable property of this drug in the treatment of asthma.

Introduction

When allergic asthmatic individuals are challenged with allergen, the early phase asthmatic attack occurring within 30 min after contact with the allergen may be followed by a late phase bronchoconstriction which takes place 4–6 h after challenge (Durham & Kay, 1985; de Monchy, 1986). This late phase asthmatic reaction develops without further contact with the allergen. The cellular events taking place during the late phase response are considered highly relevant to the pathogenesis of asthma (Durham & Kay, 1985; de Monchy, 1986). Bronchoalveolar lavage (BAL) studies to assess the cellular changes during this response have shown that inflammatory cells, particularly eosinophils, infiltrate the bronchioli at the beginning of the reaction (de Monchy *et al.*, 1985; Metzger *et al.*, 1985; Diaz *et al.*, 1987). There is evidence suggesting the involvement of the sulphidopeptide leukotrienes in the late phase bronchoconstriction (Verhagen & Bruijnzeel, 1985; Bruijnzeel *et al.*, 1986). This idea has been further substantiated by the observation that eosinophils specifically synthesize the potent bronchoconstrictor leukotriene C₄ (LTC₄) upon *in vitro* challenge with a

wide variety of stimuli (Verhagen *et al.*, 1984a; Bruijnzeel *et al.*, 1985a, b; 1987b). In the case of neutrophils these stimuli almost exclusively induce the formation of the strongly chemotactic compound leukotriene B₄ (LTB₄), 20-OH-LTB₄ and the non-enzymatically formed LTB₄-isomers.

Since nedocromil sodium is capable of inhibiting allergen-induced early and late phase asthmatic responses when inhaled before allergen challenge (Youngchaiyud & Lee, 1986; Dahl & Pedersen, 1986; Abraham *et al.*, 1987), we have investigated the effect of this drug on leukotriene formation by eosinophils and neutrophils isolated from human peripheral blood.

Methods

Purification of granulocytes

Granulocytes were isolated as follows. Platelet-rich plasma was removed after centrifugation of citrated blood (15 min, 275 *g*_{max}, room temperature). After collection of the buffy coat, granulocytes were separated from lymphocytes and monocytes by centrifugation on Ficoll-Paque (20 min, 1000 *g*_{max}, room

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temperature). The remaining erythrocytes were removed by ammonium chloride lysis at 0°C and subsequent centrifugation. Thereafter the cells were regenerated at 37°C in minimum essential medium/10% foetal calf serum for 30 min. After centrifugation the mixed granulocyte preparation was resuspended in buffer A (phosphate-buffered saline/trisodium citrate/human pasteurized plasma protein = 80/10/10, v/v/v) at a concentration of 75×10^6 cells ml⁻¹.

Purification of eosinophils and neutrophils

Method A Eosinophils and neutrophils were separated from each other by two subsequent centrifugations of a mixed granulocyte preparation over isotonic Percoll solutions with densities of 1.082 and 1.085 g ml⁻¹. One millilitre of mixed granulocytes (containing 75×10^6 cells) was carefully layered on 4 ml of an isotonic Percoll solution (density: 1.082 g ml⁻¹) and centrifuged (10 min, 1000 g_{max} , room temperature). Neutrophils (over 98% pure) migrated to the top of the 1.082 g ml⁻¹ Percoll solution, whereas the eosinophils and some neutrophils were present as a small pellet on the bottom. After resuspension of the eosinophil pellets in a 1.085 g ml⁻¹ Percoll solution (cell concentration $\leq 75 \times 10^6$ cells per 4 ml) 4 ml of this suspension was layered on 1 ml of a Percoll solution with a density of 1.100 g ml⁻¹. After centrifugation (10 min, 1000 g_{max} , room temperature), eosinophils (over 90% pure) were concentrated at the interface of both Percoll layers. This isolation procedure was used for the purification of neutrophils from normal individuals and also for the purification of eosinophils and neutrophils from individuals with moderate eosinophilia (e.g. allergic asthmatics). Since the isolation of eosinophils from normal individuals with this method was often disappointing, we developed an alternative isolation method (method B).

Method B The mixed granulocyte preparation (containing 75×10^6 cells ml⁻¹ buffer A) was first incubated with FMLP (10 nM) in buffer A for 10 min at 37°C before 1 ml of cell suspension was layered on 4 ml of an isotonic Percoll solution (density: 1.082 g ml⁻¹). To prevent contamination of the eosinophils with cell debris and remaining erythrocytes, 1 ml of Percoll (density: 1.100 g ml⁻¹) was brought under the Percoll layer of 1.082 g ml⁻¹. After centrifugation (10 min, 1000 g_{max} , room temperature) eosinophils with a purity of over 85% were present at the interface of both Percoll layers. These eosinophils were collected, washed with buffer A and resuspended in Dulbecco's salt solution.

The density of the eosinophils obtained by method B was identical with the density of the eosinophils obtained by method A (i.e. 1.085 g ml⁻¹; see Koen-

derman *et al.*, 1988). Furthermore, the use of FMLP at a concentration of 10 nM did not influence the LTC₄ generation by eosinophils. Eosinophils from the same normal individuals isolated according to methods A and B synthesized similar amounts of LTC₄ upon challenge with A23187 (10 μ M): 32 ± 6 ng per 10^6 eosinophils (method A) versus 26 ± 3 ng per 10^6 eosinophils (method B) or with opsonized zymosan (OZ, 0.5 mg ml⁻¹): 30 ± 3 ng per 10^6 eosinophils (method A) versus 36 ± 6 ng per 10^6 eosinophils (method B) (mean $\pm$ s.e.mean, $n = 6$).

Incubation procedure

Purified eosinophils and neutrophils were suspended in Dulbecco's salt solution (pH 7.4) at a concentration of 1×10^6 cells ml⁻¹, preincubated at 37°C for the indicated period of time and then stimulated with A23187 or OZ. Eosinophils were stimulated with A23187 for 20 min or with OZ for 60 min. Neutrophils were stimulated with A23187 for 5 min or with OZ for 15 min. Each experiment was performed in the presence of 1 mM CaCl₂ and 5 mM reduced glutathione. Reactions were stopped by the addition of an equal volume of ice-cold radioimmunoassay (RIA) kit buffer to the incubation mixture. The cells were then sedimented (20 min, 2000 g_{max} , 4°C) and the supernatants collected and stored at -70°C until analysis (within 1–2 months after the incubation).

Analysis of leukotriene formation

LTC₄ formation was analysed by means of a commercially available RIA (New England Nuclear, Boston, MA, U.S.A.). LTB₄ formation was also analysed by means of a commercially available RIA (Amersham, Buckinghamshire, U.K.). Leukotriene formation was occasionally analysed by reversed phase-h.p.l.c. (Verhagen *et al.*, 1984b). Leukotriene formation was expressed in ng per 10^6 cells.

Statistical analysis

Statistical analysis was performed by use of Student's *t* test for paired data.

Materials

Calcium ionophore A23187, reduced glutathione, N-formyl-methionyl-leucyl-phenylalanine (FMLP) and zymosan A were purchased from Sigma (St. Louis, MO, U.S.A.). Ficoll-Paque (1.077 g ml⁻¹) and Percoll (1.129 g ml⁻¹) were obtained from Pharmacia (Uppsala, Sweden). Nedocromil sodium was provided by Fisons plc (Loughborough, U.K.). All other materials were reagent grade. Opsonized zymosan (OZ) was prepared as previously described (Goldstein *et al.*, 1975). Human blood was obtained from healthy volunteers at the Red Cross Bloodbank

Foundation (Utrecht) and from extrinsic allergic asthmatic patients visiting our Outpatient Department. These patients were classified as described elsewhere (Bruijnzeel *et al.*, 1987a).

Results

Leukotriene formation by eosinophils and neutrophils from normal individuals and allergic asthmatics

The optimal conditions for A23187- and OZ-induced leukotriene formation by both eosinophils and neutrophils were determined in previous studies (Bruijnzeel *et al.*, 1985a,b). Under these conditions (A23187 $10\text{ }\mu\text{M}$; incubation time: 20 min for eosinophils/5 min for neutrophils; OZ 2.5 mg ml^{-1} ; incubation time: 60 min for eosinophils/15 min for neutrophils) eosinophils and neutrophils of normal individuals and allergic asthmatic individuals did not synthesize statistically significantly different amounts of leukotrienes (see Table 1). Since no differences were observed between leukotriene formation of eosinophils and neutrophils from normal individuals and allergic asthmatic individuals when cells with identical density were stimulated, it was decided to study only cells from normal individuals in subsequent experiments.

A23187 and opsonized zymosan concentration ranges

LTC₄ formation by eosinophils The concentration-dependency of A23187- and OZ-induced LTC₄ formation by eosinophils and the inhibitory effects of nedocromil sodium ($10\text{ }\mu\text{M}$) are shown in Figure 1. These figures suggest that at a concentration of $5\text{ }\mu\text{M}$ A23187 and of 0.5 mg ml^{-1} OZ almost optimal inhibition by nedocromil sodium of induced LTC₄ formation occurs.

LTB₄ formation by neutrophils The concentration-dependency of A23187- and OZ-induced LTB₄ formation by neutrophils is shown in Figure 2.

Figure 1 Concentration-dependency of A23187 (a)- and opsonized zymosan (b)-induced leukotriene C₄ (LTC₄) formation by purified human eosinophils in the absence (●) or presence (○) of nedocromil sodium ($10\text{ }\mu\text{M}$). The cells were preincubated for 5 min with the drug before stimulation. * $P < 0.05$, Student's *t* test for paired data. Points shown are mean values and vertical lines indicate s.e.mean.

Table 1 A23187 ($10\text{ }\mu\text{M}$)- and opsonized zymosan (OZ, 2.5 mg ml^{-1})-induced leukotriene C₄ (LTC₄) formation (ng per 10^6 cells) by eosinophils and leukotriene B₄ formation (ng per 10^6 cells) by neutrophils from normal individuals (N) and allergic asthmatic individuals (A)

Cell type	Stimulus	Time (min)	LTC ₄ or LTB ₄ formation	
			N	A
Eosinophils	A23187	20	57 ± 12^a	36 ± 7^b
	OZ	60	40 ± 8	46 ± 15
Neutrophils	A23187	5	16 ± 4^c	11 ± 4^d
	OZ	15	7 ± 1	6 ± 2

Results shown are means $\pm$ s.e.mean ($n = 5$).

Eosinophils and neutrophils of the same density for both N and A were studied.

Cell purities: ^a $86 \pm 3\%$, ^b $97 \pm 3\%$, ^c $96 \pm 1\%$, ^d $95 \pm 1\%$.

No statistically significant differences between N and A were observed (Student's *t* test for paired data).

Figure 2 Concentration-dependency of A23187 (a)- and opsonized zymosan (b)-induced leukotriene B₄ (LTB₄) formation by purified human neutrophils in the absence (●) or the presence (○) of nedocromil sodium (10 μM). The cells were preincubated for 5 min with the drug before stimulation. Points shown are mean values ($n = 3$) and vertical lines indicate s.e.mean.

Nedocromil sodium (10 μM) did not inhibit induced LTB₄ formation.

Nedocromil sodium concentration range

LTC₄ formation by eosinophils Cells were preincubated for 5 min with nedocromil sodium at various concentrations. A23187 (5 μM)- and OZ (0.5 mg ml⁻¹)-induced LTC₄ formation by eosinophils was inhibited by nedocromil sodium in a concentration-dependent manner (Figure 3). The mean concentration of nedocromil sodium (IC₃₀) required to give 30% inhibition of LTC₄ release induced by A23187 was 5.6×10^{-5} M ($n = 7$). Simi-

Figure 3 Inhibition of A23187 (5 μM) (a)- and opsonized zymosan (0.5 mg ml⁻¹) (b)-induced leukotriene C₄ (LTC₄) formation by purified human eosinophils at various concentrations of nedocromil sodium. The cells were preincubated for 5 min with the drug before stimulation. The incubation without nedocromil sodium was taken as the 100% value. Points shown are mean values ($n = 7$) and vertical lines indicate s.e.mean. * $P < 0.05$, Student's *t* test for paired data.

larly, the IC₃₀ for OZ-induced LTC₄ release was 6.3×10^{-5} M ($n = 7$).

LTC₄ formation by neutrophils A23187 (5 μM)- and OZ (0.5 mg ml⁻¹)-induced LTB₄ formation was not inhibited by nedocromil sodium at concentrations of 10^{-9} M– 10^{-3} M.

Variation of the preincubation time of the cells with nedocromil sodium

To exclude any effect of duration of preincubation time with nedocromil sodium, the preincubation time was extended from 5 to 30 min. After preincubation for 10 min with nedocromil sodium (10^{-3} M) the following inhibition percentages were obtained:

A23187 ($5 \mu\text{M}$); $62 \pm 12\%$, OZ (0.5 mg ml^{-1}); $60 \pm 21\%$ (mean $\pm$ s.e.mean, $n = 4$). After preincubation for 30 min with the same nedocromil sodium concentration the following inhibition percentages were obtained: A23187 ($5 \mu\text{M}$); $61 \pm 14\%$, OZ (0.5 mg ml^{-1}); $50 \pm 23\%$ (mean $\pm$ s.e.mean, $n = 3$). In the case of induced LTB_4 formation by neutrophils, no inhibition was obtained by increasing the preincubation time with the drug. These results suggest that a preincubation time of 5 min is sufficient for optimal inhibition of A23187- and OZ-induced LTC_4 formation by eosinophils.

Discussion

We have shown that nedocromil sodium inhibits A23187- and OZ-induced LTC_4 formation by human eosinophils in a concentration-dependent way. In contrast, LTB_4 formation by human neutrophils induced by the same stimuli was not inhibited by this drug. Nedocromil sodium has been demonstrated to inhibit *Ascaris* antigen- and anti-IgE-induced LTC_4 formation by bronchoalveolar mast cells from *Ascaris suum* infected macaque monkeys (Wells *et al.*, 1986). However, A23187- and OZ-induced LTB_4 formation by alveolar macrophages from normal and asthmatic individuals (A23187 induced LTB_4 formation only) were not inhibited by the compound, although there was a tendency towards inhibition of A23187-induced LTD_4 formation (Damon *et al.*, 1986). Here, the mean IC_{30} value obtained for inhibition of A23187-induced LTC_4 formation by eosinophils is one order of magnitude greater than that found for inhibition

of LTC_4 formation by bronchoalveolar mast cells from the macaque monkey, but is the same order of magnitude as that obtained for inhibition of LTD_4 formation by human alveolar macrophages. This may be due to potency differences between the stimuli used.

The inhibition by nedocromil sodium of LTC_4 formation by eosinophils, but not of LTB_4 formation by neutrophils, led us to speculate that nedocromil sodium may inhibit glutathione-S-transferase activity in eosinophils, leading to the formation of non-enzymatically formed LTB_4 -isomers. However, reversed phase-h.p.l.c. analysis of the pooled samples remaining after RIA-analysis showed a complete absence of non-enzymatically formed LTB_4 -isomers. Since it has been found that nedocromil sodium induces phosphorylation of a 78 000 molecular weight mast cell protein which is likely to be involved in the termination of mediator release (Wells & Mann, 1983; Theoharides *et al.*, 1980), it is tempting to suggest as an alternative hypothesis that phosphorylation of such a protein takes place in eosinophils but not in neutrophils. Further studies are required for investigation of this possibility.

The finding that LTC_4 formation by eosinophils may be inhibited by nedocromil sodium indicates that this drug acts not only on mast cells but also on secondary inflammatory cells. Since eosinophilia in the lung is one of the characteristic features of asthma, it is of value that topically applied drugs such as nedocromil sodium can prevent formation of the potent bronchoconstrictor compound LTC_4 by this cell.

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