Nedocromil sodium prevents *in vivo* generation of the eosinophilotactic substance induced by PAF but fails to antagonize its effects

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1 The intrathoracic injection of platelet activating factor (PAF) into rats induced a decrease in the pleural leucocyte numbers within 15 min, accompanied by a marked exudation, maximal 1 h later. After 6 h, concomitantly with the reduction of exudation, a marked increase in the number of mononuclear cells, neutrophils and eosinophils was observed. Within 24 h, the pleural eosinophil accumulation peaked and persisted up to 96 h.

2 Topical treatment with nedocromil sodium affected pleural exudation by PAF under conditions where systemic meclizine was ineffective. Nedocromil sodium blocked, dose-dependently, the increase in the pleural content of mononuclear cells, neutrophils and eosinophils, observed 6 h after PAF administration, as well as the eosinophilia 24 h later. Moreover, the co-incubation of peritoneal eosinophils with nedocromil sodium did not interfere with the migration triggered by PAF.

3 The transfer of the 6 h-PAF pleural washings from donor to recipient rats caused a selective pleural eosinophilia, which was clearly inhibited when nedocromil sodium was administered to donor, but not to recipient animals, showing that this drug interferes with the generation rather than with the expression of the eosinophilotactic activity(ies).

4 These findings indicate that the nedocromil sodium interferes with PAF-induced exudation and leucocyte accumulation, by a mechanism other than its ability to reduce the local effects of histamine and which may relate to suppression of the eosinophilotactic principle generation.

Keywords: Nedocromil sodium; PAF; pleurisy; eosinophils

Introduction

Eosinophils are important participants in human allergy, particularly asthma (Frigas & Gleich, 1986). They are found in excess in blood and in the bronchoalveolar lavage fluid (BALF) after immune provocation in man and animals. Eosinophil invasion also occurs in lungs of guinea-pigs after systemic administration of platelet activating factor PAF or antigen (Lellouch-Tubiana *et al.*, 1988). We have recently demonstrated a marked eosinophil migration following the intrapleural administration of PAF (Silva *et al.*, 1989), allergen (Lima *et al.*, 1990) or compound 48/80 (Martins *et al.*, 1991) to rats. Eosinophilia was independent of acute inflammation, i.e., of early exudation and neutrophil-monocyte invasion of the pleural cavity, since it persisted when the acute inflammatory process was over (Silva *et al.*, 1989). In addition, eosinophil invasion was suppressed by the *in situ* co-injection of protein synthesis inhibitors with (PAF) (Silva *et al.*, 1991), under conditions where the other signs of inflammation including neutrophil invasion, were unaffected (Silva *et al.*, unpublished data). Furthermore, the transfer of pleural washings from PAF-injected rats to the pleural cavity of recipient animals also led to a selective eosinophil invasion, unaccompanied by acute inflammation. Generation of this eosinophilic principle was suppressed by protein synthesis inhibitors although such drugs failed to inhibit the eosinophilia in recipient animals, after the pleural fluid transfer (Silva *et al.*, 1991). This led us to postulate the existence of a protein synthesis-dependent mediator formed locally in the pleural cavity of PAF-injected rats, which was capable of stimulating *in vivo* and *in vitro* the migration of eosinophils (Silva *et al.*, 1989). Since PAF is claimed to participate in allergy and particularly in eosinophil

migration after the i.v. administration of antigen to passively sensitized guinea-pigs (Lellouch-Tubiana *et al.*, 1987), we have investigated whether the anti-allergic drug, nedocromil sodium (Ruggieri & Patalano, 1989) interferes with the effects of PAF and/or with the generation of this eosinophilotactic factor, as nedocromil sodium did inhibit *in vivo* eosinophil recruitment following antigen challenge of sensitized guinea-pigs (Pretolani *et al.*, 1990).

Methods

Animals

Wistar rats of either sex weighing 180–200 g, obtained from Charles River and Oswaldo Cruz Foundation breeding were used.

Induction of pleurisy

The reaction was produced in ether-anaesthetized rats by intrathoracic (i.t.) injections (0.1 ml) of PAF (1 µg per cavity) diluted with 0.9% NaCl solution (saline), containing 0.01% bovine serum albumin (BSA), and histamine (200 µg per cavity) diluted with saline. Control animals received the same volume of the vehicle. After different time intervals, the animals were killed with an overdose of ether, the pleural cavity was opened and rinsed with 3 ml of heparinized saline (5 iu ml⁻¹). The pleural washing was recovered and the volume measured in a graduated syringe. In the rare case in which a haemorrhage was noted in the pleural cavity, the animal was not included in subsequent studies.

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Total protein quantification

The fluid recovered from the pleural cavity was centrifuged for 10 min at 2000 r.p.m., and the total protein content was quantified from the supernatant by the Biuret technique in a spectrophotometer (Shimadzu Corporation).

Leucocyte analysis

Total leucocytes were counted in a Coulter counter ZBI and expressed as cells $\times 10^6$ per cavity. Differential analyses were performed in cytocentrifuged smears stained with May-Grunwald-Giemsa dye under oil immersion objective, by means of a light microscope.

Transfer procedure

Six hours after the i.t. injection of PAF into donor animals, the pleural cavity was washed with 0.5 ml of sterile saline, and the pleural fluid was centrifuged (2500 r.p.m.) at 4°C for 15 min (Silva *et al.*, 1991). The supernatant was injected into the pleural cavity of recipient rats in a final volume of 0.2 ml and the analysis of eosinophil counts was made 24 h later.

In vitro eosinophil chemotaxis assay

Eosinophils were obtained from the peritoneal cavity of normal rats and were 'purified' by means of a discontinuous gradient of metrizamide, according to Martins *et al.* (1989). To test the potential effect of nedocromil sodium on eosinophil migration promoted by PAF (10^{-8} and 10^{-6} M), the cells were pre-incubated with nedocromil sodium (10^{-6} and 10^{-4} M), or its vehicle, for 10 min at 37°C. Migration experiments were performed in 48-Well microchemotaxis chamber and Toyo cellulose nitrate filters (3 μ m pore). The latter were fixed and stained as previously described by Richards & McCullough (1984).

Treatment

Nedocromil sodium (7.5 – 30 mg kg $^{-1}$) and meclizine (30 mg kg $^{-1}$) were given by the i.p. route, 1 h before the provocation. In some experiments nedocromil sodium was administered i.t. at 10 and 30 μ g per cavity. Nedocromil sodium was diluted with sterile saline and meclizine was dissolved in Tween 80 and further diluted with saline. All drugs were prepared immediately before use.

Drugs

PAF (1-O-hexadecyl-2-acetyl- sn-glyceryl-3- phosphorylcholine) was purchased from Bachem (Switzerland); histamine and meclizine were from Sigma (U.S.A.); nedocromil sodium (disodium 6,9-dihydro-9-ethyl-4,6-dioxo- 10-propyl-4H-pyrano [3,2-g]-guinoline-2,8-dicarboxylate) was kindly provided by Dr A. Norris, from Fisons plc (U.K.).

Statistical analysis

Data are expressed as mean $\pm$ standard error mean (s.e.mean) and analysed statistically by means of the analysis of variance (ANOVA) followed by the Newman-Keuls Student's test. In some particular cases the difference in means was analysed by unpaired Student's *t* test. *P* values of 0.05 or less were considered significant.

Results

Inflammatory effects following the intrathoracic administration of PAF

As seen in Table 1, the i.t. administration of PAF (1 μ g per cavity) was followed by a marked exudation within 1 h, which was accompanied by a 6 fold increase in the protein extrava-

Table 1 Temporal kinetics of exudate volume and extravasated protein induced by the i.t. injection of PAF (1 μ g per cavity)

Groups	Time (h)	Exudation volume (ml)	Total protein (mg)
Sal	1 h	0.08 ± 0.03	3.19 ± 0.39
PAF	1 h	$0.68 \pm 0.03^*$	$19.14 \pm 1.53^*$
Sal	6 h	0.04 ± 0.02	3.04 ± 0.23
PAF	6 h	$0.40 \pm 0.06^*$	$6.22 \pm 1.07^*$
Sal	24 h	0.09 ± 0.03	1.91 ± 0.58
PAF	24 h	0.12 ± 0.03	2.32 ± 0.64

Each value represents the mean $\pm$ s.e.mean from at least 7 animals; * *P* < 0.001 statistically significant as compared to saline-injected control group by Student's *t* test.

sation. Fifteen minutes after PAF, a significant reduction in the total leucocyte counts was observed, represented by a decrease of mono- and polymorphonuclear leucocyte counts (Figure 1a,b). The same figure shows that leucocyte numbers were restored to the basal levels 1 h after PAF stimulation. By 6 h, when exudation and protein extravasation were halved (Table 1), the total number of leucocytes recovered from the pleural cavity was at its height (*P* < 0.001) and was represented by an increase of mononuclear cells (*P* < 0.02), eosinophils (*P* < 0.001) and mainly neutrophils (*P* < 0.003) (Figure 1 a,b). Total leucocyte counts (Figure 1) and exudation (Table 1) were back to basal values at 24 h, whereas the eosinophil counts remained significantly increased up to 96 h (*P* < 0.001, d.f. = 81) (Figure 1b).

Interference of nedocromil sodium with the effects of PAF and histamine

As illustrated in Table 2, the local treatment with nedocromil sodium (30 μ g per cavity) significantly inhibited the pleural exudation and the increase in the total protein content induced by PAF and histamine (200 μ g per cavity), 1 h later. In addition, the H $_1$ anti-histaminic, meclizine, failed to modify PAF-induced exudation under conditions where pleurisy

Figure 1 Time course of pleurisy induced by PAF (1 μ g per cavity). Total leucocyte and mononuclear cells (a) as well as neutrophil and eosinophil counts (b) are represented in number of cells per pleural cavity. Each point represents the mean from at least 7 animals; vertical bars show s.e.mean. The closed symbols are the mean of all values (at least 40) obtained from saline-injected animals. Statistically significant differences are indicated by an asterisk (* *P* < 0.01).

Table 2 Interference of nedocromil sodium (30 µg per cavity, Ned) and meclizine (30 mg kg⁻¹, Mec) with the exuded volume and extravasated protein caused by PAF and histamine

Treatment	Exudate volume (ml)		Total protein (mg)	
	PAF	Hist	PAF	Hist
None	0.68 ± 0.03†	0.76 ± 0.07†	19.14 ± 1.53†	20.08 ± 2.32†
Ned	0.42 ± 0.03*	0.38 ± 0.06*	7.31 ± 0.67*	10.88 ± 1.47*
Mec	0.72 ± 0.04	0.11 ± 0.06*	19.51 ± 2.23	4.44 ± 0.84*

Values obtained from saline-injected rats of 0.08 ± 0.03 for exudate volume and of 3.19 ± 0.39 for total protein were considered as basal. Each value represents the mean $\pm$ s.e.mean from at least 7 animals. † $P < 0.001$ from the saline-injected group and * $P < 0.001$ from PAF or histamine groups by Student's *t* test.

induced by histamine was markedly suppressed by its respective antagonist.

Treatment with nedocromil sodium was also effective in inhibiting the increased number of mononuclear cells ($P < 0.001$, d.f. = 21, Figure 2b, neutrophils ($P < 0.001$, d.f. = 17, Figure 2c) and eosinophils ($P < 0.005$, d.f. = 21, Figure 2d) observed 6 h after PAF injection. In addition, as shown in Table 3, nedocromil sodium given i.p., unlike the H₁ blocking agent meclizine, also abolished in a dose-dependent manner the late eosinophil accumulation caused by PAF.

Figure 2 Interference of topical administration of nedocromil sodium (10–30 µg per cavity) with the leucocyte accumulation 6 h after PAF stimulation. Each column represents the mean from at least 7 animals with s.e.mean shown by vertical bars; † $P < 0.001$ from the saline-injected group by Student's *t* test and * $P < 0.01$ from PAF-injected group by Newman-Keuls Student's test.

Interference of nedocromil sodium with in vitro eosinophil chemotaxis

As shown in Table 4, the pre-incubation of rat peritoneal eosinophils with nedocromil sodium (10^{-6} and 10^{-4} M), for

Table 3 Interference of nedocromil sodium with the eosinophil infiltration (24 h) induced by PAF (1 µg per cavity)

Stimulus	Treatment	Dose (mg kg ⁻¹)	Eosinophils per site ($\times 10^{-6}$)	Inhibition (%)
Sal	None	—	0.84 ± 0.06	—
PAF	None	—	$1.98 \pm 0.12†$	—
PAF	Ned	7.5	1.72 ± 0.09	22.8
PAF	Ned	15.0	$1.48 \pm 0.08*$	43.8
PAF	Ned	30.0	$1.09 \pm 0.06*$	78.0
PAF	Mec	30.0	1.81 ± 0.09	14.9

Nedocromil sodium (Ned) and meclizine (Mec) were administered i.p., 1 h before the lipid, at the indicated doses. Each value represents the mean $\pm$ s.e.mean from at least 8 animals; † $P < 0.001$ from the saline-injected group by Student's *t* test and * $P < 0.001$ from PAF-injected group by Newman-Keuls Student's test.

Table 4 Interference of nedocromil sodium with the in vitro eosinophil chemotaxis induced by PAF

Stimulus	Treatment (M)	Eosinophils/15HPF
Vehicle	—	25.0 ± 3.5
PAF (10^{-8} M)	—	82.3 ± 2.7
	10^{-6}	78.5 ± 2.4
	10^{-4}	92.3 ± 4.6
PAF (10^{-6} M)	—	115.4 ± 8.6
	10^{-6}	113.5 ± 5.3
	10^{-4}	114.3 ± 3.6

The eosinophils were pre-incubated with nedocromil sodium, for 10 min at 37°C. Each value represents the mean $\pm$ s.e.mean from 3 assays. HPF = high power field.

Table 5 Interference of nedocromil sodium (30 µg per cavity) with eosinophil influx in the pleural cavity of recipient, after the treatment of donor or recipient rats

Stimulus	Treatment	Treated groups	
		Donor	Recipient
SPW	Saline	0.58 ± 0.09	0.50 ± 0.06
PPW	Saline	$1.54 \pm 0.18†$	$1.20 \pm 0.07†$
PPW	Nedocromil	$0.57 \pm 0.07*$	1.12 ± 0.16

Numbers represent eosinophil counts ($\times 10^6$) per cavity. The analyses were performed in recipient animals 24 h after the transfer of saline pleural washings (SPW) or PAF pleural washings (PPW). Each value represents the mean $\pm$ s.e.mean from at least 7 animals; † $P < 0.05$ from the SPW group and * $P < 0.007$ from PPW group by Student's *t* test.

10 min at 37°C, did not inhibit the eosinophil migration promoted by PAF (10^{-8} and 10^{-6} M), as shown by 48-Well microchemotaxis chamber assay.

Interference of nedocromil sodium with the generation or expression of eosinophilia from PAF-injected rats to recipient animals

When pleural washings from PAF prestimulated rats were transferred to normal recipient animals, the number of eosinophils recovered from their pleural cavity was doubled within 24 h ($P < 0.05$, Table 5). The topical administration of nedocromil sodium into the pleural cavity of donor rats, followed within 6 h by the transfer of the washing to recipients, suppressed the late eosinophilia (Table 5). In contrast, when nedocromil sodium was administered to recipients, no inhibition of the eosinophil infiltration was observed (Table 5).

Discussion

Nedocromil sodium is the disodium salt of a pyranoquinoline dicarboxylic acid which, though being structurally quite different, shares several properties with the classic anti-allergic drug, sodium cromoglycate (Auty & Holgate, 1989). It has been demonstrated that nedocromil sodium impairs the release of inflammatory mediators from activated mast cells (Riley *et al.*, 1987), and accordingly its efficacy has been attributed to this ability. It has also been shown that nedocromil sodium prevents early and late allergic reactions and allergen-induced bronchial hyperresponsiveness in experimental animals and man (Altounyan *et al.*, 1986; Hutson *et al.*, 1988; Pretolani *et al.*, 1990).

Our findings confirm those of Tarayre *et al.* (1986) and Martins *et al.* (1989), that the intrathoracic injection of PAF into rats leads to acute protein extravasation, maximal at 1 h, declining thereafter. This phenomenon was accompanied by an initial reduction in the pleural leucocyte content, which was over within 6 h. At this time, a significant increase in the number of mononuclear cells, eosinophils and mainly neutrophils was noted. In contrast, 24 h after PAF stimulation, only the eosinophil content was augmented, remaining elevated for up to 96 h.

The topical administration of nedocromil sodium significantly reduced the acute cellular exudate (40%) and protein

extravasation (61.8%) induced by PAF, under conditions where the H_1 -antagonist, meclizine, was ineffective. Nedocromil sodium thus inhibits PAF-induced exudation by a mechanism other than antagonism of histamine receptors or of inhibition of amine release. Furthermore, the cellular influx into the pleural cavity at 6 h, as well as the late eosinophilia induced by PAF, were significantly suppressed by topical treatment with nedocromil sodium. Since the latter's efficacy has also been demonstrated against cellular activation (Moqbel *et al.*, 1986), we hypothesized that nedocromil sodium might inhibit the cellular influx by acting directly upon the leucocytes. As previously reported (Wardlaw *et al.*, 1986; Martins *et al.*, 1989), PAF is also chemotactic for eosinophils *in vitro*. However, in our experiments, the preincubation of the eosinophils with nedocromil sodium at 10^{-6} and 10^{-4} M, did not prevent the chemotaxis induced by the lipid. These results rule out the possibility that the effectiveness of nedocromil sodium in abrogating the late eosinophil recruitment might be related to a direct effect on eosinophils.

We have recently demonstrated that the eosinophil accumulation triggered by PAF, at 24 h, depends on the synthesis of an intermediate factor, probably a protein which is completely abrogated by its co-administration with protein synthesis inhibitors (Silva *et al.*, 1991). After the transfer of the pleural washing from donors stimulated with PAF to recipients, the latter presented a 2 fold increase in the pleural eosinophil content after 24 h (Silva *et al.*, 1989). In order to clarify the mode of action of nedocromil sodium, we pretreated donor or recipient animals with this drug before transferring the pleural fluid. Under these conditions, nedocromil sodium inhibited the transferred eosinophilia when administered to the donor, and no protection was observed when the recipient animals were treated. These results are consistent with the interpretation that the efficacy of nedocromil sodium relates to its ability to inhibit the generation of the eosinophilotactic factor rather than the effects of this material.

In conclusion, our findings indicate that nedocromil sodium prevents PAF-induced pleurisy in rats by a mechanism independent of its recognized property of antagonizing H_1 -histamine receptors and possibly via its ability to impair the formation of the eosinophilotactic activity generated by PAF.

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