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Inhibition of platelet-activating factor- and zymosan-activated serum-induced chemotaxis of human neutrophils by nedocromil sodium, BN 52021 and sodium cromoglycate

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1 Inflammatory cells such as eosinophils and neutrophils are thought to contribute actively to the pathogenesis of asthma since they infiltrate into the lung tissue. These cells are mobilized by lipid-like and protein-like chemotactic factors. As illustrative examples of both groups, platelet-activating-factor (Paf) and zymosan-activated-serum (ZAS) were used in this study. The inhibitory effects of nedocromil sodium, the Paf antagonist BN 52021 and sodium cromoglycate on Paf- and ZAS-induced neutrophil chemotaxis were evaluated.

2 All tested drugs inhibited Paf-induced neutrophil chemotaxis with approximately the same potency ($IC_{50} \approx 1$ nM).

3 Nedocromil sodium and sodium cromoglycate were equally potent in inhibiting ZAS-induced neutrophil chemotaxis ($IC_{50} = 0.1$–$1\,\mu$M), whereas BN 52021 was considerably less potent ($IC_{30} = 10\,\mu$M).

4 To find out whether the drugs tested could inhibit early events in cell activation, their capacity to inhibit Paf- and ZAS-induced cytosolic free Ca^{2+}-mobilization was investigated. BN 52021, at a concentration of $100\,\mu$M, completely inhibited Paf-induced Ca^{2+}-mobilization and inhibited ZAS-induced Ca^{2+}-mobilization by about 50%. Nedocromil sodium and sodium cromoglycate were ineffective.

Introduction

When allergic asthmatic individuals are challenged with allergen, an early phase asthmatic reaction (within 30 min after challenge) and a late phase asthmatic reaction (4–6 h after challenge) may develop (Durham & Kay, 1985; de Monchy, 1986). This late phase asthmatic reaction develops without further exposure to the allergen. The late phase asthmatic reaction has a different pattern compared to the early phase reaction; it has a slower onset of action and it leads to a more prolonged bronchoconstriction. In general the late reaction is clinically more severe (Verhagen & Bruijnzeel, 1985). The cellular events taking place during this late phase asthmatic reaction are considered highly relevant to the pathogenesis of asthma (Durham & Kay, 1985; de Monchy, 1986; Bruijnzeel & Verhagen, 1989). Bronchoalveolar lavage (BAL) studies of the cellular changes during this response have demonstrated increased numbers of inflammatory cells, particularly eosinophils and neutrophils (de Monchy *et al.*, 1985; Metzger *et al.*, 1985; Diaz *et al.*, 1987), indicating active migration of those cells into the bronchial lumen. Inflammatory cells may be mobilized by chemotactic agents of protein- or lipid-nature such as neutrophil chemotactic factor (NCF), eosinophil chemotactic factor (ECF), complement fragments as C5a, leukotriene B_4, and platelet-activating factor (Paf) (Shaw *et al.*, 1981; Atkins & Wasserman, 1983).

Here, we describe the inhibitory effects of nedocromil sodium, the Paf-antagonist BN 52021 and sodium cromoglycate on the chemotactic response of human neutrophils *in vitro*, induced by Paf and ZAS.

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Furthermore, the effects of these drugs on the Paf- and ZAS-induced cytosolic free calcium mobilization have been investigated.

Methods

Purification of neutrophils

Blood of normal volunteers was collected in one-tenth volumes of sterile trisodium citrate (138 mM; pH 7.0). Platelet-rich plasma was removed after centrifugation (15 min, 275 g_{max}, room temperature). The buffy coats were collected, diluted four times (v/v) with buffer A (phosphate-buffered saline/trisodium citrate/human pasteurized plasma protein = 80/10/10, v/v/v) and 16 ml of Ficoll-paque (density 1.077 g ml^{-1}) were syringed carefully under 35 ml of this cell suspension. Tubes were centrifuged (20 min, 1000 g_{max}, room temperature), the upper layers of mononuclear cells removed and granulocytes with contaminating erythrocytes were collected from the bottom of the tube. The remaining erythrocytes were removed by ammonium chloride lysis at 0°C and subsequent centrifugation. Thereafter the cells were washed with buffer A and regenerated at 37°C in minimum essential medium/10% foetal calf serum for 30 min. By this isolation procedure neutrophils were obtained with a recovery over 85%, a purity over 97% and a viability over 99%. The viability was tested with the vital stains fluorescein diacetate and ethidium bromide (Edidin, 1970; Takasugi, 1971). After isolation the neutrophils were resuspended in Geys buffer (Geys balanced salt solution/human serum albumin (5%, w/v)/heparin (10 iu ml^{-1}) and glucose (1 mg ml^{-1})).

Chemotaxis

Chemotaxis was measured with a modified Boyden chamber technique. In the chamber the lower filter had a pore width of 0.45 μm (Millipore) and the upper filter a pore width of 8 μm (Sartorius). The cells as well as the reagents used were suspended in Geys buffer. The cell suspension, 5×10^5 cells in 250 μl, was added to the upper compartment and 220 μl of the chemotactic agent was added to the lower compartment of the chamber. Concentration ranges of the chemotactic agents Paf, lyso-Paf and ZAS were tested with the buffer as a control. At optimal concentrations of these chemotactic agents dose-response inhibition curves for nedocromil sodium, BN 52021 and sodium cromoglycate were constructed. The cells were preincubated for the indicated periods of time with the drugs before the chemotaxis was carried out. The Boyden chambers were incubated for 1.5 h at 37°C. Thereafter the filters were removed, fixed in butanol/ethanol (20/80, v/v) for 10 min and stained with Weigert solution (1% haematoxylin (v/v) in 95% ethanol (v/v) and an acidic $FeCl_3$-solution (70 mM) in a volume ratio of 1:1). The filters were dehydrated with ethanol, made transparent with 100% xylene (v/v) and fixed upside down. The number of cells per high power field (hpf) was determined with light microscopy (magnification 400 ×). In this way, the number of cells that have passed the upper filter was determined.

Measurement of cytosolic free calcium concentration

For cytosolic free calcium measurements indo-1 was used as the fluorescent indicator, described previously (Koenderman et al., 1988). The cells were loaded with the acetoxy-methyl ester of indo-1 (Grynkiewicz et al., 1985); control cells were incubated with 0.1% DMSO. Calibration of indo-1 fluorescence as a function of cytosolic free calcium was determined essentially as described for quin-2 fluorescence (Pozzan et al., 1983), with the following modifications. To saturate all trapped indo-1 with Ca^{2+}, digitonin (5 μM) was added to the cell suspension. Subsequently, the indo-1 signal was quenched by adding 0.5 mM Mn^{2+}. Calculations (Bijsterbosch et al., 1986) were made using a K_d of 250 nM for the indo-1/Ca^{2+} complex (Grynkiewicz et al., 1985). Dye content in the cells was determined by adding indo-1 free acid to the cell suspension. Cells were preincubated for 30 min with the indicated concentrations of the various drugs before they were challenged with Paf or ZAS.

Statistical analysis

Statistical analysis was performed by use of Student's t test for paired data. P values of <0.05 were considered to be significant.

Materials

Zymosan A was purchased from Sigma (St. Louis, MO, U.S.A.). 1-O-hexadecyl/octadecyl-2-O-acetyl-sn-glycero-3-phosphoryl-choline (Paf) and 1-O-hexadecyl-sn-glycero-3- phosphorylcholine (lyso-Paf) were purchased from Bachem (Bubendorf, Switzerland) and were stored at −20°C in a toluene/ethanol (1:1, v/v) solution under N_2. The acetoxy-methyl ester of indo-1 and indo-1 free acid were purchased from Molecular Probes (Junction City, OR, U.S.A.). Digitonin was obtained from Merck (Darmstadt, F.R.G.). Ficoll-Paque (1.077 g ml^{-1}) was obtained from Pharmacia (Uppsala, Sweden). Nedocromil sodium and sodium cromoglycate were provided by Fisons plc (Loughborough, U.K.). BN 52021 was a kind gift of Dr P. Braquet (Institut

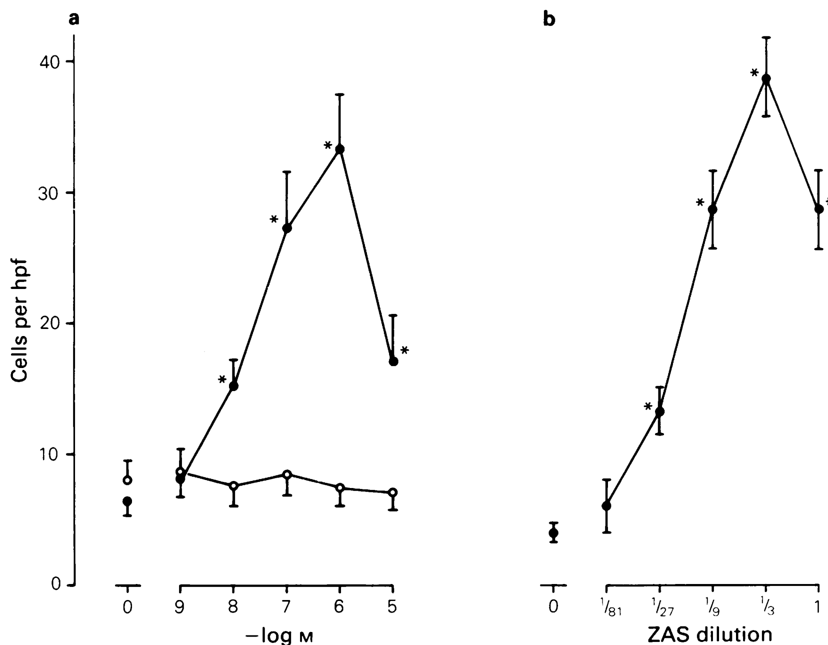

Figure 1 (a) Paf (●), lyso-Paf (○) and (b) zymosan-activated serum (ZAS) (●)-induced chemotaxis of normal human granulocytes using buffer as a control; mean values are shown with vertical bars indicating s.e.mean. (n = 12 (Paf, lyso-Paf); n = 8 (ZAS); $^{*}P < 0.05$).

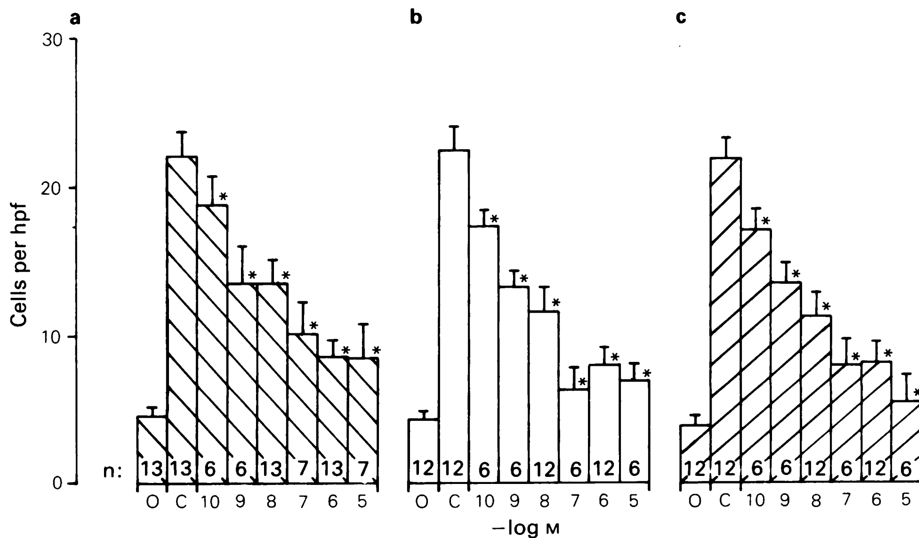

Figure 2 Dose-dependent inhibition of Paf-induced chemotaxis of human granulocytes by (a) nedocromil sodium, (b) BN 52021 and (c) sodium cromoglycate. O: buffer; C: chemotaxis obtained with Paf at a concentration of 1 μM. $^{*}P < 0.05$, when compared with the control value (C).

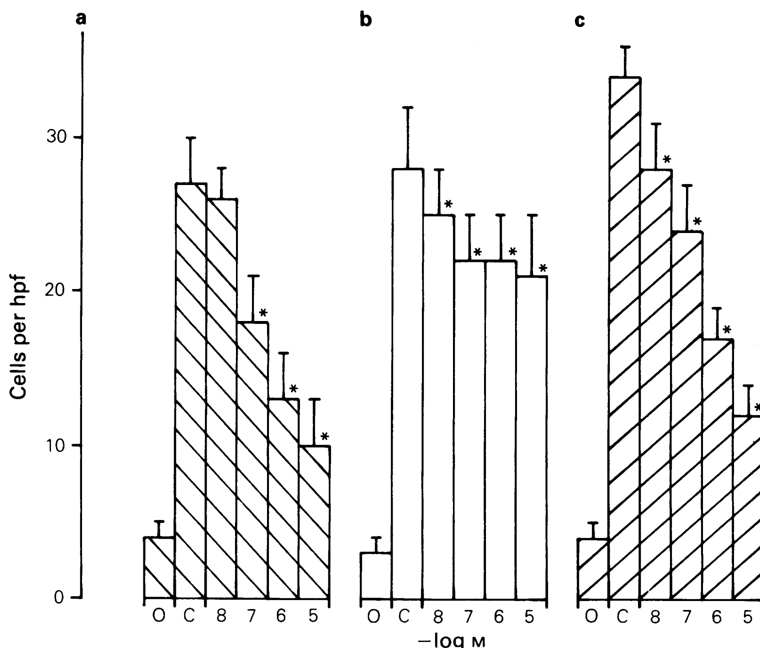

Figure 3 Dose-dependent inhibition of zymosan-activated serum (ZAS)-induced chemotaxis of human granulocytes by (a) nedocromil sodium, (b) BN 52021 and (c) sodium cromoglycate ($n = 6$, mean with s.e.mean shown by vertical bars). O: buffer; C: chemotaxis obtained with ZAS (dilution 1/3). *$P < 0.05$, when compared with the control value (C).

Henri Beaufour, Le Plessis Robinson, France). All other materials were reagent grade. Zymosan activated serum (ZAS) was prepared as previously described (Goldstein *et al.*, 1975).

Results

Chemotaxis of neutrophils

Paf, lyso-Paf and ZAS concentration ranges The concentration dependency of the Paf and ZAS induced chemotaxis by human neutrophils is shown in Figure 1. Lyso-Paf did not induce chemotaxis. The optimal chemotactic concentration for Paf was 1 μM and for ZAS a serum dilution of 1/3. The inhibitory potencies of nedocromil sodium, BN 52021 and sodium cromoglycate were tested at these optimal chemotactic concentrations.

Inhibition of the Paf- and ZAS-induced chemotaxis of human neutrophils by nedocromil sodium, BN 52021 and sodium cromoglycate The inhibition of Paf-induced chemotaxis of human neutrophils by concentration-ranges of nedocromil sodium, BN 52021 and sodium cromoglycate is shown in Figure 2. All drugs appeared to be equally potent in inhibiting Paf-induced chemotaxis of granulocytes. The IC_{50} was approximately 1 nM.

The inhibition of ZAS-induced chemotaxis of human neutrophils by concentration ranges of nedocromil sodium, BN 52021 and sodium cromoglycate is shown in Figure 3. Nedocromil sodium and sodium cromoglycate were almost equally potent in inhibiting ZAS-induced chemotaxis (IC_{50} = 0.1–1 μM), whereas BN 52021 had a very weak inhibitory potency (IC_{30} = 10 μM).

A series of control experiments was carried out to verify that the drug-induced inhibition of chemotaxis was due to an interaction of the drug with the cells and not with the chemotactic agent. Addition of the various drugs to the compartment of the Boyden chamber in which the chemotactic compound had been inserted did not inhibit the chemotactic response, whereas addition of the various drugs to the compartment in which the cells had been inserted did inhibit the chemotactic response.

Variation of the preincubation time of the cells with the inhibiting drugs To exclude an effect of the duration of the preincubation time of the cells with the various drugs tested, the preincubation time was

varied from 5 min up to 120 min. Nedocromil sodium was used as representative for the other drugs tested. After a preincubation time of 30 min about 70–80% inhibition of Paf- and ZAS-induced chemotaxis was obtained, thereafter reaching a plateau. Therefore a preincubation time of 30 min was considered optimal. This preincubation time was used in all experiments described.

Inhibition of the Paf- and ZAS-induced cytosolic free calcium mobilization by nedocromil sodium, BN 52021 and sodium cromoglycate.

To determine whether the investigated drugs would interfere with early events of cell activation, the possible inhibition of Paf- and ZAS-induced cytosolic free calcium mobilization in neutrophils was investigated.

In the case of the Paf-induced transient rise in cytosolic free Ca^{2+} concentration, only the Paf antagonist BN 52021 at a concentration of 100 μM was capable of completely blocking this transient rise, whereas nedocromil sodium and sodium cromoglycate were ineffective at these concentrations (see Figure 4).

In the case of inhibition of the ZAS-induced transient rise in cytosolic free Ca^{2+} concentration, only the Paf antagonist BN 52021 at a concentration of 100 μM proved effective (50% inhibition), whereas the other drugs at the same concentration were ineffective (see Figure 4).

When in the above-mentioned experiments the drug concentrations were decreased to 10 and 1 μM respectively the inhibitory potency of BN 52021 against Paf stimulation gradually decreased; in the case of ZAS stimulation, the inhibitory effects had already disappeared at a concentration of 10 μM. The other drugs remained ineffective.

Discussion

The mobilization of inflammatory cells, particularly neutrophils and eosinophils, in the lung tissue *in vivo* may be caused by chemotactic factors of lipid or protein nature. As representatives of those groups of chemotactic factors Paf and ZAS were chosen for our studies. Both these factors induced chemotaxis of human neutrophils in a concentration-dependent way. Nedocromil sodium, BN 52021 and sodium cromoglycate all induced concentration-dependent

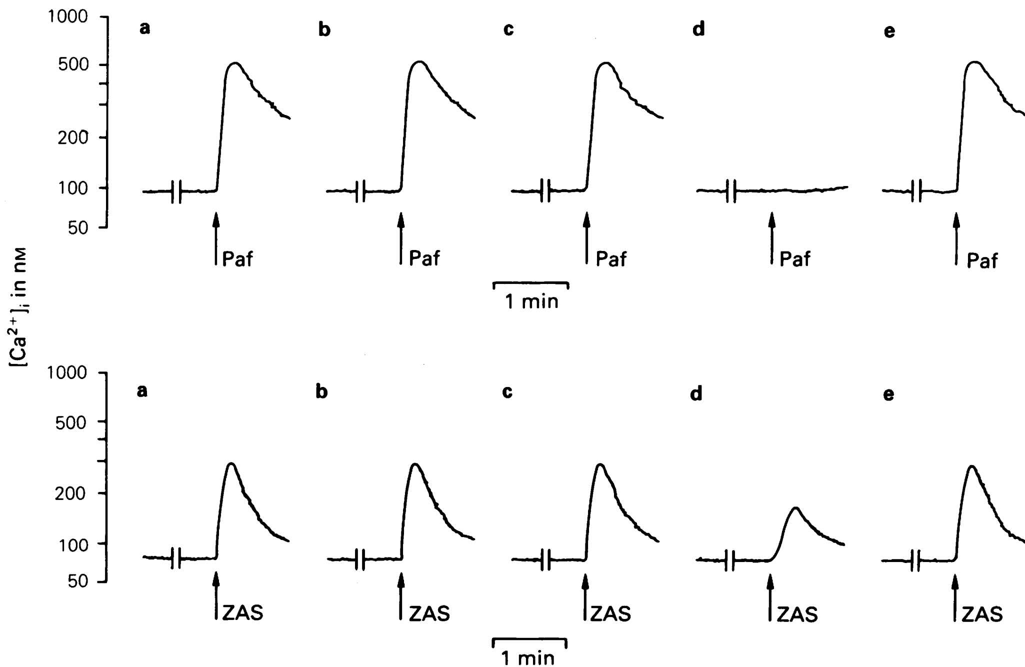

Figure 4 Intracellular free Ca^{2+} concentrations in neutrophils upon stimulation with Paf (1 nM, upper panel) and zymosan-activated serum (ZAS) (1/1000 dilution, lower panel). Effects are shown of (a) buffer, (b) dimethyl sulphoxide (0.2%), (c) nedocromil sodium (100 μM), (d) BN 52021 (100 μM), (e) sodium cromoglycate (100 μM), representative of three experiments. The cells were preincubated for 30 min with the various drugs.

inhibition of Paf-induced chemotaxis of human neutrophils ($IC_{50} \approx 1$ nM), demonstrating that they are very potent inhibitors. Since BN 52021 has been shown (Kurihara *et al.*, 1988) to antagonize specific Paf binding to neutrophils, our results suggest that this drug at least may act by interfering with the cell surface interaction of Paf with the neutrophil. The results described here contrast with those of Kurihara *et al.* (1988), who found that Paf-induced chemotaxis of neutrophils and eosinophils could only be inhibited by BN 52021 and not by nedocromil sodium and sodium cromoglycate. These authors also found a rather high IC_{50} (around 10 μM), which in our opinion does not necessarily support the idea that BN 52021 is a potential anti-allergic agent. When ZAS was used instead of Paf, nedocromil sodium and sodium cromoglycate were capable of inhibiting the chemotaxis to the same extent with an $IC_{50} = 0.1$–1 μM, whereas BN 52021 was hardly capable of inhibiting this chemotaxis ($IC_{30} = 10$ μM). This could indicate that nedocromil sodium and sodium cromoglycate may also interfere with the C5a receptor on neutrophils, although less powerfully than with the Paf receptor on the same cells.

To obtain more evidence of a possible interaction between the tested drugs and the above-mentioned receptor sites on neutrophils, binding studies with labelled Paf and C5a have been performed. So far, these binding studies have not yielded any conclusions, mainly because of high non-specific binding. Therefore, we decided to investigate whether an early step in cell activation, such as the induction of a transient rise in the cytosolic free Ca^{2+} concentration by Paf and ZAS, could be blocked. At low drug concentrations (1 μM) no inhibition was observed. However, BN 52021 at a concentration of 100 μM effectively blocked both Paf- and ZAS-induced Ca^{2+} mobilization (100 and 50% respectively), whereas nedocromil sodium and sodium cromoglycate were ineffective. Our data are in contrast with those of Skedinger *et al.* (1987), who found a 50% inhibition of the ZAS-induced Ca^{2+} mobilization by sodium cromoglycate at a concentration of 100 μM. However, they did not measure the intracellular Ca^{2+} concentration continuously, but sampled every minute after addition of the stimulus and therefore used a ZAS concentration 100 times higher than ours. Because the fast and transient rise of the Ca^{2+} concentration takes place within 20 s of addition of the stimulus, we believe that our method is more reliable.

Taken together, these data support the idea that BN 52021 acts as a Paf-receptor antagonist. Since nedocromil sodium and sodium cromoglycate were ineffective in inhibiting the Paf- and ZAS-induced Ca^{2+} mobilization, the effect of these drugs for inhibition of Paf- and ZAS-induced chemotaxis is most likely to be at a later event in the activation mechanism, for example, inhibition of protein kinase C as suggested for rabbit peritoneal neutrophils (Bradford & Rubin, 1986), rat peritoneal mast cells (Kay, 1987) and reptilian pigment cells (Lucas & Shuster, 1987).

Although the effects described here were obtained *in vitro*, they might be relevant to the *in vivo* situation. Since inflammatory cells such as neutrophils are actively involved *in vivo* in the pathogenesis of asthma, then our *in vitro* results might in part explain how neutrophil migration into the lung tissue can be prevented effectively by nedocromil sodium and sodium cromoglycate (Hutson *et al.*, 1988; 1989).

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References

ATKINS, P.C. & WASSERMAN, S.I. (1983). Chemotactic mediators. *Clin. Rev. Allergy*, **1**, 385–397.

BIJSTERBOSCH, M.K., RIGLEY, K.P. & KLAUS, G.G.B. (1986). Cross-linking of surface immunoglobulin on B-lymphocytes induces both intracellular Ca^{2+}-release and Ca^{2+}-influx: analysis with indo-1. *Biochem. Biophys. Res. Commun.*, **137**, 500–506.

BRADFORD, P.G. & RUBIN, R.P. (1986). The differential effects of nedocromil sodium and sodium cromoglycate on the secretory response of rabbit peritoneal neutrophils. *Eur. J. Respir. Dis.*, **69** (suppl. 147), 238–240.

BRUIJNZEEL, P.L.B. & VERHAGEN, J. (1989). The possible role of particular leukotrienes in the allergen-induced late phase asthmatic reaction. *Clin. Exp. Allergy*, **19** (suppl. 1), 25–32.

DIAZ, P., GONZALEZ, C., GALLEGUILLOS, F., ANCIC, P., CROMWELL, O. & KAY, A.B. (1987). Leukocytes and mediators in bronchial mucus and broncho alveolar lavage during allergen-induced late phase asthmatic reactions. *J. Allergy Clin. Immunol.*, **79**, 256.

DURHAM, S.R. & KAY, A.B. (1985). Eosinophils, bronchial hyperreactivity and late-phase asthmatic reactions. *Clin. Allergy*, **15**, 411–418.

EDIDIN, M.A. (1970). A rapid, quantitative fluorescence assay for cell damage by cytotoxic antibodies. *J. Immunol.*, **104**, 1303–1306.

GOLDSTEIN, I.M., ROOS, D., KAPLAN, H.B. & WEISSMANN, G. (1975). Complement and immunoglobulins stimulate superoxide production by human leukocytes independently of phagocytosis. *J. Clin. Invest.*, **56**, 1155–1163.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3450.

HUTSON, P.A., HOLGATE, S.T. & CHURCH, M.K. (1988). Inhibition by nedocromil sodium of early and late

phase bronchoconstriction and airway cellular infiltration provoked by ovalbumin inhalation in conscious sensitized guinea-pigs. *Br. J. Pharmacol.*, **94**, 6–8.

HUTSON, P.A., CHURCH, M.K., CLAY, T.P., MILLER, P. & HOLGATE, S.T. (1989). The effect of sodium cromoglycate and salbutamol on early and late phase bronchoconstriction and airway leukocyte infiltration following allergen challenge of non-anaesthetised guinea-pigs. *Am. Rev. Respir. Dis.*, **138**, 1157–1163.

KAY, A.B. (1987). The mode of action of anti-allergic drugs. *Clin. Allergy*, **17**, 153–164.

KOENDERMAN, L., HAMELINK, M.L., KOK, P.T.M., VERHOEVEN, A. & BRUIJNZEEL, P.L.B. (1988). An improved method for the isolation of eosinophilic granulocytes from peripheral blood of normal individuals. *J. Leukocyte Biol.*, **44**, 79–86.

KOK, P.T.M., HAMELINK, M.L., KIJNE, G.M., VERHAGEN, J., KOENDERMAN, L. & BRUIJNZEEL, P.L.B. (1988). Arachidonic acid can induce leukotriene C4 formation by purified human eosinophils in the absence of other stimuli. *Biochem. Biophys. Res. Commun.*, **153**, 676–682.

KURIHARA, K., WARDLAW, A.J., MOQBEL, R. & KAY, A.B. (1988). The ginkgolide (BN 52021) inhibits PAF-induced chemotaxis and specific binding to human eosinophils and neutrophils. *J. Allergy Clin. Immunol.*, **81**, 208.

LUCAS, A.M. & SHUSTER, S. (1987). Cromolyn inhibition of protein kinase C activity. *Biochem. Pharmacol.*, **36**, 562–565.

METZGER, W.J., HUNNINGHAKE, G.W. & RICHERSON, H.B. (1985). Late phase asthmatic responses: inquiry to mechanisms and significance. *Clin. Rev. Allergy*, **3**, 145–165.

DE MONCHY, J.G.R., KAUFFMAN, H.F., VENGE, P., KOËTER, G.H., JANSEN, H.M., SLUITER, H.J. & DE VRIES, K. (1985). Bronchoalveolar eosinophilia during allergen-induced late phase asthmatic reactions. *Am. Rev. Respir. Dis.*, **131**, 373–377.

DE MONCHY, J.G.R. (1986). The late allergic reaction in bronchial asthma. *Thesis, Groningen.*

POZZAN, T., LEW, D.P., WOLLHEIM, C.B. & TSIEN, R.Y. (1983). Is cytosolic ionized calcium regulating neutrophil activation? *Science*, **221**, 1413–1415.

SHAW, J.O., PINCKARD, R.N., FERRIGNI, K.S., MCMANUS, L.M. & HANAHAN, D.J. (1981). Activation of human neutrophils with 1-O-hexadecyl/octadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine (platelet activating factor). *J. Immunol.*, **127**, 1250–1255.

SKEDINGER, M.C., AUGUSTINE, N.H., MORRIS, E.Z., NIELSON, D.W., ZIMMERMAN, G.A. & HILL, H.R. (1987). Effect of disodium cromoglycate on neutrophil movement and intracellular calcium mobilization. *J. Allergy Clin. Immunol.*, **80**, 573–577.

TAKASUGI, M. (1971). An improved fluorochromatic cytotoxic test. *Transplantation*, **12**, 148–151.

VERHAGEN, J. & BRUIJNZEEL, P.L.B. (1985). Leukotrienes and their possible significance for the pathogenesis of asthma. *Allergol. Immunopathol.*, **13**, 531–537.

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