British Journal of Pharmacology (1999) 128, 1149–1158
http://www.stockton-press.co.uk/bjp

'Outside-in' signalling mechanisms underlying CD11b/CD18-mediated NADPH oxidase activation in human adherent blood eosinophils

[1]Oonagh T. Lynch, [1]Mark A. Giembycz, [1]Peter J. Barnes, [2]Paul G. Hellewell & *,[1]Mark A. Lindsay

[1]Thoracic Medicine, Imperial College School of Medicine at the National Heart and Lung Institute, Dovehouse Street, London SW3 6LY and [2]Department of Vascular Biology, University of Sheffield, Sheffield

1 Incubation of human eosinophils in BSA-coated tissue culture plates resulted in time-dependent adhesion and attendant activation of the NADPH oxidase that were both inhibited (by >85%) by blocking antibodies raised against CD11b and CD18.

2 SB 203580, an inhibitor of p38 mitogen-activated protein (MAP) kinase, did not influence adhesion but inhibited superoxide anion generation ($pIC_{50} = -6.57$).

3 PP1, an inhibitor of the src-family of protein tyrosine kinases, inhibited adhesion and CD11b/CD18-mediated superoxide anion generation with similar potencies ($pEC_{50}s = -5.53$ and -5.99 respectively) suggesting that inhibition of the NADPH oxidase was a direct consequence of blocking adhesion.

4 The protein kinase C (PKC) inhibitors Ro-31 8220 (broad spectrum inhibitor), GF 109203X (inhibitor of conventional and novel isoforms) and Gö 6976 (inhibitor of conventional isoforms) suppressed adhesion-dependent NADPH oxidase activation ($pIC_{50}s = -6.61$, -6.05 and -4.89 respectively) without affecting adhesion. Based upon the selectivity of these drugs PKCδ and PKCε are implicated in the suppression of oxidant production.

5 Wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PtdIns 3-kinase), abolished superoxide anion production in adherent eosinophils ($pEC_{50} = -9.06$). Similarly, CD11b/CD18-dependent adhesion was suppressed with the same potency ($pEC_{50} = -9.29$) although the maximum effect did not exceed 50% implying that wortmannin also had an affect on those processes that govern adhesion-driven oxidase activation.

6 PD 098059 and piceatannol, inhibitors of MAP kinase kinase-1 and the syk tyrosine kinase respectively, had no effect on CD11b/CD18-mediated adhesion or NADPH oxidase activation.

7 The results of this study demonstrate that human eosinophils adhere to BSA-coated plastic by a CD11b/CD18-dependent mechanism, which is responsible for activation of the NADPH oxidase. Although the signalling pathway(s) utilized by CD11b/CD18 is still to be elucidated, the data presented herein implicate p38 MAP kinase, novel PKCs and PtdIns 3-kinase.

Keywords: Human adherent eosinophils; 'outside-in' signalling; protein kinase C; p38 MAP kinase; adhesion (CD11b/CD18)-dependent NADPH oxidase activation

Abbreviations: ERK, extracellular-regulated kinase; GM-CSF, granulocyte/macrophage colony-stimulating factor; H_2O_2, hydrogen peroxide; MAPKAPK-2, mitogen-activated protein kinase-activated protein kinase-2; MAP kinase, mitogen-activated protein kinase; PKC, protein kinase C; PtdIns 3-kinase, phosphatidylinositol 3-kinase; VLA4, very late antigen-4

Introduction

Eosinophils are actively motile, terminally differentiated leukocytes derived from the bone marrow and have been identified in many mammalian and non-mammalian species (Giembycz & Lindsay, 1999). In humans, eosinophils were originally thought to be primarily involved in immune defence against parasitic infection. However, it is now recognized that they are implicated in a number of allergic and non-allergic diseases such as hypereosinophilic syndromes, eosinophilic pneumonia, atopic dermatitis, asthma and rhinitis. In inflammatory disorders, such as asthma, eosinophils migrate into the airways where they become activated and secrete a host of cytotoxic species including granule proteins and lipid mediators together with the generation of highly toxic free radicals that are formed enzymatically from molecular oxygen by the NADPH oxidase complex (Giembycz & Lindsay, 1999). Activation of eosinophils in the airways is thought to cause localized tissue injury, contraction of airways smooth muscle and increased bronchial responsiveness to a diverse range of spasmogens (Barnes, 1996; Seminario & Gleich, 1994; Desreumaux & Capron, 1996).

The release of leukotriene C_4 (Anwar *et al*., 1994; Yoshida *et al*., 1995; Munoz *et al*., 1996), eosinophil peroxidase (Neeley *et al*., 1994), eosinophil-derived neurotoxin (Horie & Kita, 1994), interleukin-3, granulocyte/macropahge colony-stimulating factor (GM-CSF) (Anwar *et al*., 1993) and superoxide anions (Dri *et al*., 1991) is regulated by integrins upon their engagement by counter ligands expressed by extracellular matrix or plasma proteins. For example, platelet-activating factor and GM-CSF do not promote superoxide anion generation from eosinophils in suspension. However, a large and sustained activation of the NADPH oxidase occurs in the same eosinophils when they are adherent to albumin-coated polystyrene plates by a mechanism that is dependent upon the

*Author for correspondence; E-mail: m.lindsay@ic.ac.uk

β_2-integrin, CD18 (Horie & Kita, 1994). In another study, Laudanna *et al.* (1993) reported that coating plates with activating antibodies to β_1- and β_2-integrins triggers eosinophil spreading and oxidant generation. Similarly, an examination of the binding of eosinophils through the β_1-integrin, very late antigen-4 (VLA4; $\alpha_4\beta_1$; CD49d/CD29), revealed that adherence to vascular cell adhesion molecule-coated plates correlated with spontaneous production of superoxide and that this effect was increased by fMLP and inhibited by an antibody against CD18 (Nagata *et al.*, 1995).

Collectively, the aforementioned reports suggest a central role for β_1- and β_2-integrins in the mechanism of eosinophil adhesion and NADPH oxidase activation. However, little is known of the biochemical pathways underlying these two responses in eosinophils. Furthermore, these studies are complicated by the need to differentiate between the pathways that increase receptor-ligand binding ('inside-out' signalling) and those that mediate receptor-ligand-induced responses ('outside-in' signalling) such as cell spreading, and subsequent degranulation and activation of the NADPH oxidase (Nathan, 1987).

In human neutrophils, physiological stimuli including cytokines, complement fragments, immune complexes, bacterial products and chemotaxins are required to stimulate adhesion. The intracellular pathways implicated in this 'outside-in' signalling are under investigation and recent studies of β_2 integrin-mediated adhesion have implicated phosphatidylinositol 3-kinase (PI 3-kinase) (Jones *et al.*, 1998; Metzner *et al.*, 1997), p38 mitogen-activated protein (MAP) kinase (Detmers *et al.*, 1998), Rho GTPase and protein kinase C (PKC) ζ (Laudanna *et al.*, 1998). Following adhesion, signals generated from integrin-ligand interactions ('outside-in') are propagated *via* the short cytoplasmic tails of integrin molecules. These tails have no intrinsic enzymatic activity but appear to function by coupling to cytoplasmic proteins that nucleate the formation of large multicomponent complexes containing both cytoskeletal elements and signalling enzymes (Aplin *et al.*, 1998). Thus, neutrophil spreading and subsequent oxidant production is associated with the phosphorylation and/or activation of paxillin (Fuortes *et al.*, 1994), MAP kinases (Rafiee *et al.*, 1995), the src-related protein tyrosine kinases lyn, fgr and hck (Yan & Berton, 1996; Berton *et al.*, 1994; Lowell *et al.*, 1996), syk and focal adhesion kinases (Fernandez & Suchard, 1998; Yan *et al.*, 1997).

In this study, we describe the development of an *in vitro* model of integrin-mediated NADPH oxidase activation in human peripheral blood eosinophils and the role of MAP kinases, the src and syk family of cytosolic protein tyrosine kinases, the PKC family and PtdIns 3-kinase in this response.

Methods

Isolation of human eosinophils

Human eosinophils were purified according to the method of Hansel *et al.* (1989). Briefly, venous blood (50–100 ml) was collected into 10–20 ml of acid citrate dextrose anticoagulant. White blood cells were obtained by sedimentation with 3% hydroxyethyl starch, layered onto Ficoll-Paque and centrifuged at $1300 \times g$ for 30 min at 18°C. The mononuclear cell layer was discarded and the pellet, containing granulocyte and red blood cells, was washed in HBSS. Contaminating red blood cells were lysed by hypotonic lysis. The remaining granulocytes were washed, counted and resuspended in 300 μl RPMI 1640 containing 2% foetal calf serum and 5 mM EDTA (RPMI/FCS/EDTA). Eosinophils were purified from neutrophils using immunomagnetic anti-CD16 antibody-conjugated microbeads (1 μl of beads per 2×10^6 neutrophils). Following the addition of beads, cells were incubated at 4°C for 40 min before resuspension in 6 ml RPMI/FCS/EDTA. The mixture was loaded onto a separation column positioned within a magnetic field and eluted with 40 ml RPMI/FCS/EDTA. The CD16 negative cells (>99% eosinophils), which are not retained by the column, were collected, washed in buffer A (HBSS, 10 mM HEPES, pH 7.4, 0.1% (w v^{-1}) BSA), counted and resuspended at 10^7 cells ml^{-1}.

Measurement of superoxide anion generation and adhesion

Eosinophils (5×10^6 ml^{-1}) were incubated with 10 μM Calcein-AM in buffer A for 30 min at 37°C, washed three times and resuspended in the same buffer at 5×10^6 ml^{-1}. Aliquots (20 μl) of the cell suspension then were incubated in buffer A (+1 mM $CaCl_2$/1 mM $MgCl_2$), supplemented with 25 μM lucigenin and the relevant inhibitor/antibody in a total volume of 200 μl and seeded onto 96-well tisue culture plates coated with either BSA (0.1% w v^{-1}) or FCS. Superoxide anion generation was monitored by lucigenin-enhanced chemiluminescence (Gyllenhammar, 1987) with a plate reading luminometer (Lucy II, Labtech Ltd., Uckfield, U.K.). At the appropriate time points, the number of adherent cells was determined by measuring the fluorescence of cellular Calcein-AM. Briefly, total fluorescence was measured at the outset of the experiment (Reading 1) and then at pre-determined time intervals (see text; Reading 2) using a Biolite F1 plate reader ($\lambda_{excitation} = 485 \pm 20$ nm; $\lambda_{emission} = 530 \pm 25$ nm). Reading 2 was taken after non-adherent cells had been removed from the culture plates by gently washing in buffer A and the percentage of adherent eosinophils then was calculated by multiplying the ratio of fluorescence (Reading 2/Reading 1) by 100. Studies of the magnitude of the respiratory burst conducted in the presence and absence of eosinophils loaded with Calcein-AM showed that this fluorescent indicator had no adverse effects upon oxidant production or viability (data not shown).

Drugs and analytical reagents

PD 098059, SB 203580, piceatannol, wortmannin, Ro-31 8220, GF109203X and Gö 6976 were obtained from Calbiochem (Nottingham, U.K.). Flat clear-bottomed, white-walled 96-well tissue culture-treated plates, Ficoll-Paque and Calcein AM were purchased from Pharmacia (Uppsala, Sweden), Costar Ltd (Buckinghamshire, U.K.) and Molecular Probes (Eugene, Oregan, U.S.A.) respectively. Anti-CD16 microbeads and magnetic cell separation system were from Miltenyl Biotec (Surrey, U.K.). Blocking antibodies (6.5E, KIM225, MAX68P, MOPC21) to adhesion receptors and the inhibitor of the src-family of protein tyrosine kinases, PP1 (CP 118556), were kindly donated by Celltech Ltd (Slough, U.K.) and Pfizer (Groton, Connecticut, U.S.A.) respectively. All other reagents were purchased from Sigma (Poole, Dorset, U.K.).

Statistical analysis

Data points, bars and values in the text and Figure legends represent the mean ± s.e.mean of '*n*' independent determinations taken from different cell preparations. Concentration-response curves were analysed by least-squares non-linear iterative regression with the 'PRISM' curve fitting program

(GraphPad software, CA, U.S.A.) and pEC_{50}/pIC_{50} values were subsequently interpolated from curves of best-fit. Where appropriate, data were analysed non-parametrically using the Wilcoxon matched pairs test or Kruskal-Wallace one-way analysis of variance followed by Dunns multiple comparisons test. The null hypothesis was rejected when $P<0.05$.

Results

Characteristics of eosinophil adhesion and superoxide anion generation

The addition of human eosinophils to 96 well tissue-culture plates coated in BSA (0.1% w v^{-1}) resulted in time-dependent adherence and the generation of superoxide anions as measured by lucigenin-enhanced chemiluminescence. A detectable and significant degree of adhesion was observed after a lag of approximately 20 min; thereafter, eosinophils adhered to the plastic rapidly over the duration of the experiment (Figure 1). Maximal adherence was achieved after approximately 100 min and the mean $t_{1/2}$ of this response was 43 min. The generation of superoxide anions increased in parallel with adherence, although the response was transient. Thus, after a lag of 20 min the rate of oxidant production increased rapidly, peaked at 40–60 min and then declined towards baseline (Figure 1). In contrast, relatively few eosinophils adhered to plates coated with FCS ($4.46\pm0.24\%$ at 60 min, $n=40$) and only a modest respiratory burst was detected under those conditions (0.102 ± 0.012 RLU at 60 min, $n=40$).

The nature of the adhesion of eosinophils to the tissue-culture plates and the requirement of that response for the activation of the NADPH oxidase was investigated using a panel of blocking antibodies raised against β_1 (CD29) and β_2 (CD18) integrins (Figure 2). Incubation of eosinophils with anti-CD18 (6.5E) and anti-CD11b (KIM225) antibodies suppressed adhesion by 63.2 ± 13.6 and $53.9\pm16\%$ respectively at 60 min. When both antibodies were used in combination a greater inhibitory effect ($75.2\pm7.2\%$) was produced than when either of them was used alone (Figure 2). In contrast, neither a blocking antibody (MAX68P) to the α_4 integrin, CD49d nor a non-specific, isotype-matched control antibody (MOPC21) affected eosinophil adhesion (Figure 2). Significantly, activation of the NADPH oxidase in eosinophils was dependent upon their prior adherence to the tissue-culture plates *via* a CD11b/CD18-dependent process. Thus, anti-CD18 and anti-CD11b antibodies inhibited superoxide anion generation by 82.8 ± 3.6 and $75.6\pm4\%$ respectively. When both antibodies were used in combination a greater inhibitory effect ($86.9\pm2.3\%$) was elicited when compared to the effect of either antibody in isolation (Figure 2). The anti-CD49d and the isotype-matched, control antibodies did not affect the respiratory burst (Figure 2).

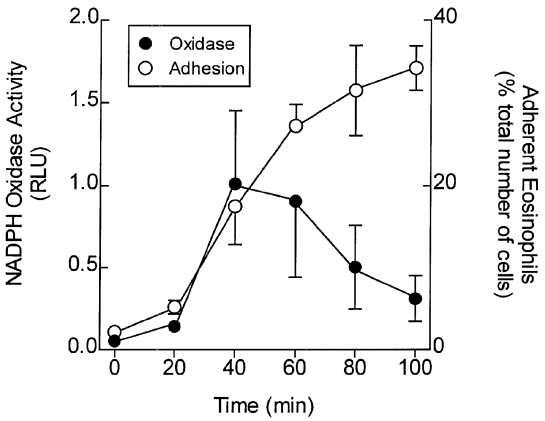

Figure 1 Time-course of eosinophil adhesion and NADPH oxidase activation. Cells (10^5) were placed in 96-well tissue culture treated plates and adhesion (○) and NADPH oxidase activity (●) was monitored over a period of 100 min. Each data point represents mean ± s.e.mean of five independent experiments using eosinophils purified from the blood of different donors. After 60 min, basal adhesion (per cent of total cells seeded) and respiratory burst, measured on FCS-coated plates, were $5.71\pm1.1\%$ and 0.012 ± 0.004 RLU respectively.

Effect of eosinophil number on superoxide anion generation and adherence

The magnitude of CD11b/CD18-induced superoxide anion generation was positively related to cell number with the maximum response achieved at approximately 0.8×10^5 to 10^5 eosinophils per well (Figure 3). Thus, at a seeding density of 0.2×10^5 eosinophils per well the respiratory burst amounted to 0.98 relative light units (RLU), which increased to 1.26, 2.65, 2.89 and 2.69 RLU at 0.4, 0.6, 0.8 and 1×10^5 cells per well respectively (Figure 3). In contrast, the percentage of eosinophils that adhered to the culture plate at each seeding density was invariant (range: 52.7–58.9%; Figure 3).

Comparison of CD11b/CD18-mediated superoxide anion generation with other stimuli

Using FCS-coated culture plates, which minimized the adhesion of eosinophil to less than 10%, the formylated tripeptide, fMLP (1 μM) failed to promote a respiratory burst (Figure 4a). However, in identical experiments using BSA-coated plates, to which approximately 40% of eosinophils adhered by a CD11b/CD18-dependent mechanism (Figure 4b),

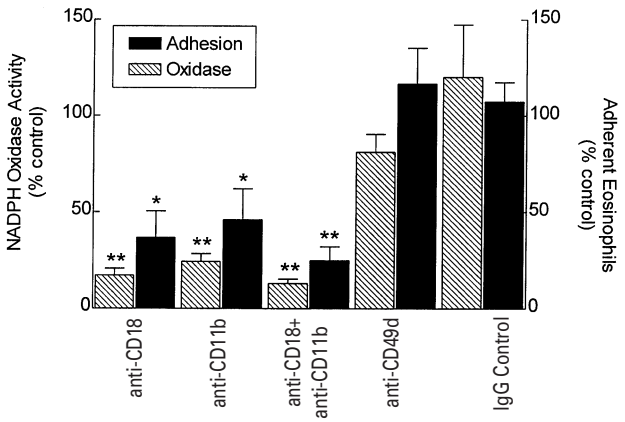

Figure 2 Effect of integrin blocking antibodies on the adhesion and NADPH oxidase activity of human eosinophils. Eosinophils (10^5) were incubated for 5 min at 37°C with blocking antibodies (10 μg ml^{-1}) to CD18 (6.5E), CD11b (KIM225), CD49d (MAX68P) or an isotype matched control (MOPC21) and then plated on to 96-well tissue culture plates and incubated for 60 min. Adherent cells (filled bars) and the maximal rate of NADPH oxidase activity (hatched bars) were determined and are expressed as a percentage of their respective responses obtained in untreated eosinophils. Each bar represents the mean ± s.e.mean of five independent experiments using eosinophils purified from the blood of different donors (*$P<0.05$ and **$P<0.001$ compared to untreated eosinophils). Basal adhesion (per cent of total cells seeded) and respiratory burst, measured on FCS-coated plates, were $3.43\pm0.8\%$ and 0.061 ± 0.007 RLU respectively.

fMLP significantly enhanced (~2 fold) NADPH oxidase activity over that produced by adhesion alone (Figure 4a). In contrast, the phorbol diester, PMA (100 nM) produced a robust (~5 fold) stimulation of the NADPH oxidase over that mediated by CD11b/CD18 in culture plates that were coated with FCS and BSA (Figure 4a). This was associated with an equivalent degree (37–51%) of adhesion to both surfaces (Figure 4b).

Role of MAP kinases in CD11b/CD18-mediated adhesion and superoxide anion generation

The MAP kinases are a group of proline-directed, serine/threonine-specific kinases that become activated following their dual phosphorylation at conserved threonine and tyrosine residues. Currently, three MAP kinase families have been biochemically classified: extracellular-regulated kinase-1 and 2 (ERK-1/2), c-*jun* N terminal kinase 46 and 54 and the p38 MAP kinase. To examine the role of ERK-1/2 and p38 MAP kinase in CD11b/CD18-dependent adhesion and superoxide generation of human eosinophils a pharmacological approach was adopted using selective inhibitors of these enzyme families.

PD 098059, which inhibits ERK-1 and ERK-2 indirectly by binding to the inactive form of MAP kinase kinase-1 thereby preventing its phosphorylation and activation by c-Raf (Alessi *et al*., 1995; Dudley *et al*., 1995), did not affect eosinophil adhesion or respiratory burst activity (Figure 5a) over a concentration range (300 pM – 10 μM) that we have previously shown to inhibit ERK-1 and ERK-2 phosphorylation in eosinophils (Kankaanranta *et al*., 1999). In contrast, SB 203580 (100 pM – 10 μM), an ATP binding site inhibitor of the α and β isoforms of p38 MAP kinase (Young *et al*., 1997; Tong *et al*., 1997) that has been shown to abolish MAPKAPK-2 phosphorylation in leukocytes (Dean *et al*., 1999; Lal *et al*., 1999), had no significant effect on CD11b/CD18-dependent eosinophil adhesion to the tissue-culture plates (Figure 3b) but inhibited the activation of the NADPH oxidase in a concentration-dependent manner ($pIC_{50} = -6.57 \pm 0.14$). At the highest concentration (10 μM) of SB 203580 examined, the generation of superoxide anions was suppressed by $69 \pm 5\%$ (Figure 5b).

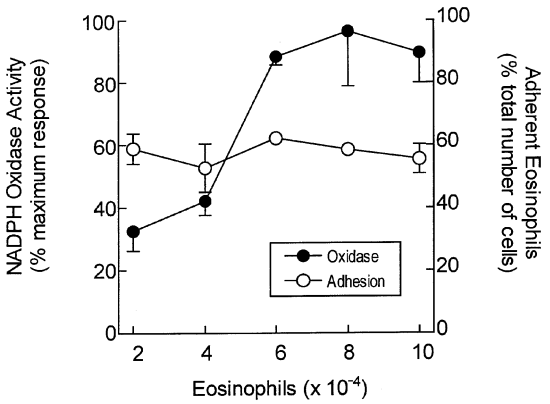

Figure 3 Effect of eosinophil seeding density on eosinophil adhesion and NADPH oxidase activity. Cells ($2 \times 10^4 - 10^5$) were placed in BSA-coated 96-well tissue culture treated plates and the maximum rate of NADPH oxidase activity (●) and the percentage of adherent eosinophils at 60 min (○) were measured. Each data point represents mean ± s.e.mean of four independent experiment using eosinophils purified from the blood of different donors. Basal adhesion (per cent of total cells seeded) and respiratory burst, measured on FCS-coated plates, were 4.42 ± 1.7% and 0.034 ± 0.008 RLU respectively.

Role of non-receptor-linked tyrosine kinases in CD11b/CD18-mediated adhesion and superoxide anion generation

The potential role of the src and syk families of cytosolic, or non-receptor-linked, protein tyrosine kinases in CD11b/CD18-mediated adhesion and superoxide generation was evaluated using the potent and selective membrane-permeable inhibitors, PP1 (Hanke *et al*., 1996) and piceatannol (Geahlen & McLaughlin, 1989), respectively.

PP1 (30 nM – 100 μM) inhibited CD11b/CD18-mediated superoxide anion generation in a concentration-dependent manner with a pEC_{50} of -5.99 ± 0.11; complete inhibition was achieved at 10 μM PP1 (Figure 6a). In contrast to the results obtained with SB 203580, PP1 also suppressed the adhesion of eosinophils to tissue-culture plates with a similar potency ($pEC_{50} = -5.53 \pm 0.11$) suggesting that inhibition of the NADPH oxidase was a direct consequence of blocking adhesion (Figure 6a). However, only partial inhibition (~70%) of adhesion was effected by PP1 implying that additional PP1-insensitive mechanisms are involved. In these experiments there

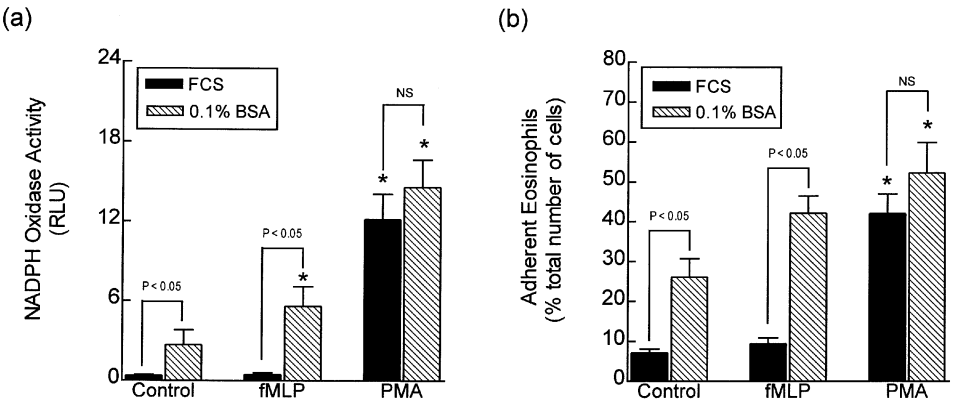

Figure 4 Comparative effects of CD11b/CD18, fMLP (1 μM) and PMA (100 nM) on eosinophil adhesion and NADPH oxidase activity. Cells (10^5) were placed in BSA- and FCS-coated 96-well tissue culture treated plates and the maximum rate of NADPH oxidase activity (a) and the percentage of adherent cells at 60 min (b) were measured. Each bar represents the mean ± s.e.mean of seven independent experiments using eosinophils purified from the blood of different donors. *$P < 0.05$, adherence and respiratory burst significantly greater than respective controls.

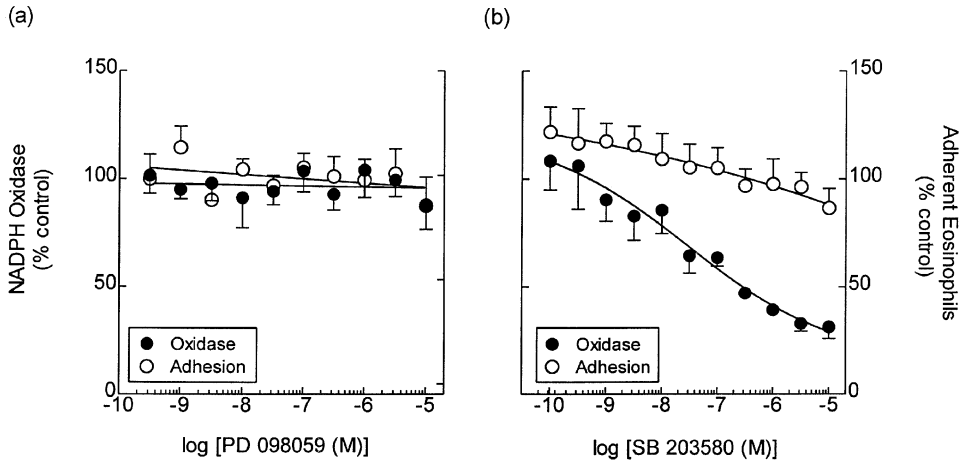

Figure 5 Effect of PD 098059 and SB 203580 on eosinophil adhesion and NADPH oxidase activity. Eosinophils (10^5) were seeded in BSA-coated 96-well tissue culture treated plates at 37°C containing PD 098059 (a), SB 203580 (b) or their respective vehicles. The number of adherent eosinophils after 60 min (○) and the maximal rate of NADPH oxidase activity (●) were measured and are expressed as a percentage of their respective responses obtained in untreated eosinophils. Each data point represents the mean ± s.e.mean of five independent experiments using eosinophils purified from the blood of different donors. Basal adhesion (per cent of total cells seeded) and respiratory burst, measured on FCS-coated plates, were 3.56 ± 0.66% and 0.12 ± 0.024 RLU respectively.

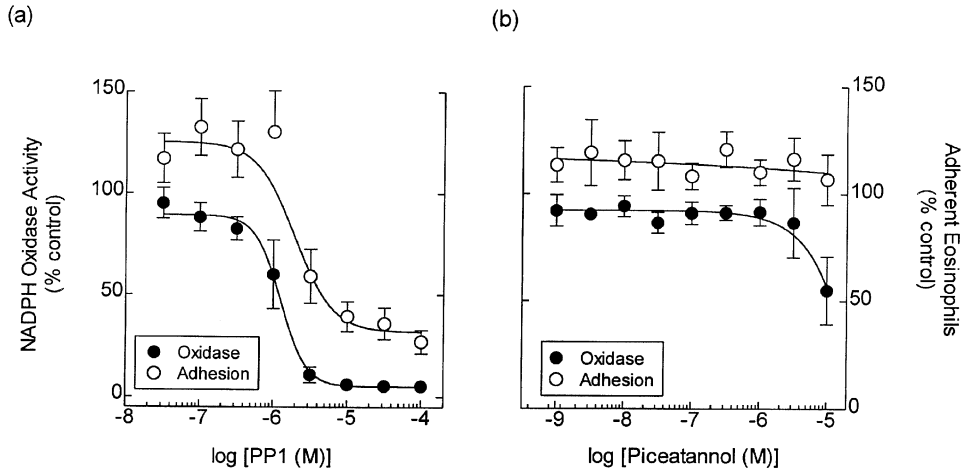

Figure 6 Effect of PP1 and piceatannol on eosinophil adhesion and NADPH oxidase activity. Eosinophils (10^5) were seeded in BSA-coated 96-well tissue culture treated plates at 37°C containing PP1 (a), piceatannol (b) or their respective vehicles. The number of adherent eosinophils after 60 min (○) and the maximal rate of NADPH oxidase activity (●) were measured and are expressed as a percentage of their respective responses obtained in untreated eosinophils. Each data point represents the mean ± s.e.mean of five independent experiments using eosinophils purified from the blood of different donors. Basal adhesion (per cent of total cells seeded) and respiratory burst, measured on FCS-coated plates, were and 4.09 ± 0.37% and 0.103 ± 0.022 RLU respectively.

was a tendency of PP1 to enhance adhesion at low concentrations although this failed to reach statistical significance (Figure 6a).

Piceatannol (1 nM – 10 μM) had no significant affect upon eosinophil adhesion or on the activity of the NADPH oxidase accept at the highest concentration (10 μM) studied, where it reduced superoxide anion generation by 44 ± 16% (Figure 6b).

Role of protein kinase C isoenzymes in CD11b/CD18-mediated adhesion and superoxide anion generation

Three PKC inhibitors (Ro-31 8220, GF109203X and Gö 6976) were used in an attempt to dissect which isoenzymes might play a role in CD11b/CD18-mediated adhesion and superoxide anion generation. These drugs were selected on the basis of the PKC isoenzymes they selectively inhibit. Thus, Ro-31 8220 is a broad-spectrum PKC inhibitor (Davis *et al.*, 1989) while GF109203X shows selectivity for the conventional and novel isoforms (cPKC and nPKC families respectively) and Gö 6976 is reported to selectively block the activity of cPKC family members (Martiny-Baron *et al.*, 1993).

As shown in Figure 7, Ro-31 8220 (30 nM – 10 μM) and GF 109203X (30 nM – 100 μM) inhibited CD11b/CD18-mediated superoxide anion generation in a concentration-dependent manner ($pIC_{50}s = -6.61 \pm 0.11$ and -6.05 ± 0.2 respectively). However, concentrations of Ro-31 8220 (3 μM) and GF 109203X (10 μM) that abolished the respiratory burst, failed to significantly affect the adhesion of eosinophils to the tissue culture plates. In fact, there was a tendency of the former inhibitor to enhance this response (Figure 7a,b). Gö 6976 also suppressed superoxide anion generation in a concentration-dependent

manner in the absence of a detectable affect on adhesion. However, the pIC_{50} of Gö 6976 (-4.89 ± 0.56) was low relative to the potency of Ro-31 8220 and GF 109203X (Figure 7c).

Role of PtdIns 3-kinase in CD11b/CD18-mediated adhesion and superoxide anion generation

Wortmannin (Arcaro & Wymann, 1993) was used to investigate the role of PtdIns 3-kinases in CD11b/CD18-mediated adhesion of, and superoxide generation by, human eosinophils. As illustrated in Figure 8, wortmannin (10 pM–1 μM) elicited a concentration-dependent inhibition both of NADPH oxidase activity and adhesion. For both responses the pEC_{50} values were not significantly different (-9.06 ± 0.23 and -9.27 ± 0.29 respectively) although the maximum inhibition of CD11b/CD18-mediated adhesion effected by wortmannin amounted to $50.4 \pm 7.7\%$ under conditions where superoxide anion production was abolished.

Discussion

A number of studies have demonstrated that the NADPH oxidase in human eosinophils is activated following ligation of the β_2-integrin, CD11b/CD18. However, little is known of the intracellular mechanisms that mediate this physiological/pathophysiological response. In the experiments described herein, we report the development of an *in vitro* model to directly examine signalling by CD11b/CD18 and demonstrate, using a pharmacological approach, a role for nPKCs, p38 MAP kinase and PtdIns 3-kinase in CD11b/CD18-mediated superoxide anion generation.

Using a panel of blocking antibodies to β_1- and β_2-integrins, we initially set out to characterize the interaction of human eosinophils with tissue culture plates and how this effect relates to the production of a respiratory burst. The demonstration that 6.5E and KIM225 (anti-CD18 and anti-CD11b antibodies respectively) markedly suppressed eosinophil adhesion strongly implicates the β_2 integrin CD11b/CD18 in this response. The observation that neither MAX68P, an antibody against CD49d, nor an isotype-matched control antibody (MOPC21), affected the process of eosinophil adhesion excludes a role for VLA-4 and a non-specific antibody effect. These data also support the idea that the binding of human eosinophils to tissue culture plates reflects a specific interaction involving the β_2 integrin. Furthermore, the additional finding that superoxide anion generation followed a similar time-course to adhesion, together with the ability of 6.5E and KIM225 also to attenuate this response demonstrates that activation of the NADPH oxidase is dependent upon ligation of CD11b/CD18.

A consistent finding in these experiments was that the percentage of eosinophils that adhered to BSA-coated 96-well tissue culture plates varied significantly (30–60%) between donors and that on no occasion was 100% adherence observed. The inability of all eosinophils to stick to BSA-coated plastic is consistent with the results of similar experiments using other substrates (see Walsh *et al.*, 1993) although why this occurs is unclear. One simple explanation is that too many eosinophils were seeded per well such that only a proportion of those gained access to the BSA. However, no

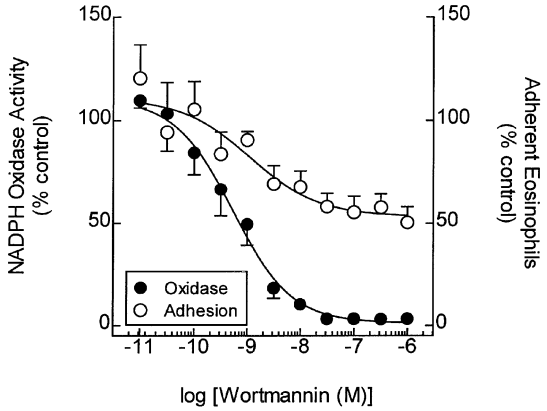

Figure 8 Effect of wortmannin on eosinophil adhesion and NADPH oxidase activity. Eosinophils (10^5) were seeded in BSA-coated 96-well tissue culture treated plates at 37°C containing wortmannin or its vehicle. The number of adherent eosinophils after 60 min (○) and the maximal rate of NADPH oxidase activity (●) were measured and are expressed as a percentage of their respective responses obtained in untreated eosinophils. Each data point represents the mean ± s.e.mean of five independent experiments using eosinophils purified from the blood of different donors. Basal adhesion (per cent of total cells seeded) and respiratory burst, measured on FCS-coated plates, were $3.83 \pm 0.49\%$ and 0.16 ± 0.029 RLU respectively.

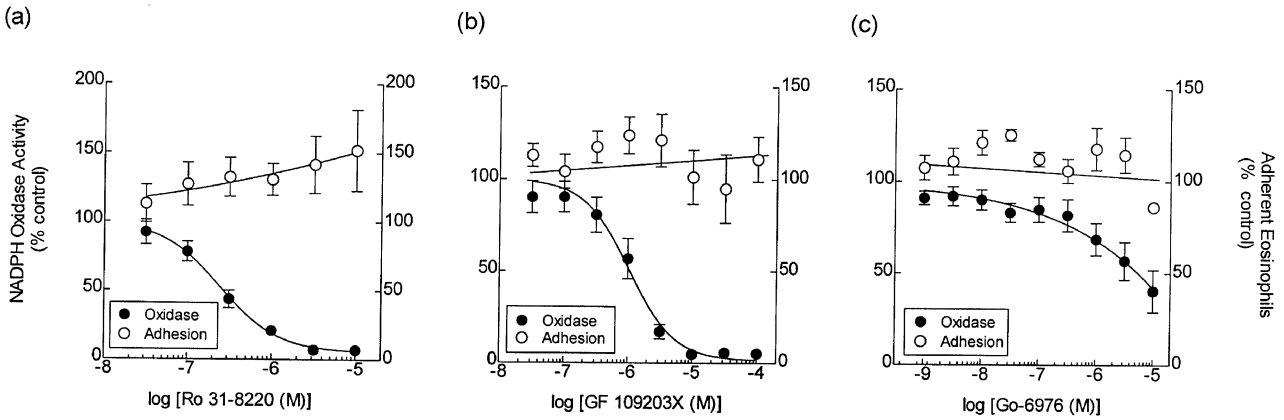

Figure 7 Effect of Ro-31 8220, GF 109203X and Gö 6976 on eosinophil adhesion and NADPH oxidase activity. Eosinophils (10^5) were seeded in BSA-coated 96-well tissue culture treated plates at 37°C containing Ro-31 8220 (a), GF 109203X (b), Gö 6976 (c) or their respective vehicles. The number of adherent eosinophils after 60 min (○) and the maximal rate of NADPH oxidase activity (●) were measured and are expressed as a percentage of their respective responses obtained in untreated eosinophils. Each data point represents the mean ± s.e.mean of five independent experiments using eosinophils purified from the blood of different donors. Basal adhesion (per cent of total cells seeded) and respiratory burst, measured on FCS-coated plates, were $5.36 \pm 0.51\%$ and 0.077 ± 0.017 RLU respectively.

evidence was found to support this idea. Indeed, the percentage of eosinophils that adhered to BSA remained invariant over a range of seeding densities in which the number of eosinophils was reduced from 10^5 to 0.2×10^5 cells per well. An alternative explanation is eosinophil heterogeneity. In this scenario only a certain proportion of eosinophils within each preparation express the necessary adhesion molecules to bind to BSA. It has been known for sometime that human blood eosinophils are physically, morphologically and functionally heterogeneous and that the expression of many cell surface epitopes varies between phenotypes (see Giembycz & Lindsay, 1999). In this respect, it is significant that cell surface CD18 is markedly ($\sim$50%) lower in eosinophils of low buoyant density (Hartnell *et al*., 1990), which are considered to represent an activated phenotype associated with eosinophilia, when compared to their autologous normodense counterparts. Moreover, it has been proposed that low density eosinophils in the circulation have undergone down-regulation of their capacity to adhere to the vascular endothelium (Hartnell *et al*., 1990). Thus, given that the method of eosinophil purification used in the present study does not distinguish between eosinophil phenotypes, hypodense cells will be present in all preparations, and their number will vary significantly depending on whether or not donors have eosinophil-associated disease. Clearly, this could have an impact on the percentage of eosinophils able to adhere to BSA-coated plastic *via* a CD18-dependent mechanism.

fMLP failed to stimulate the NADPH oxidase in eosinophils seeded in FCS-coated plates but effectively primed CD11b/CD18-induced oxidative metabolism and adherence over the basal levels. These results are consistent with previous studies where fMLP is reported to increase the expression of CD11b/CD18 on human eosinophils and enhance adhesion to human umbilical vein endothelial cells (Neeley *et al*., 1993; Kimani *et al*., 1988). The phorbol ester, PMA, also activated the NADPH oxidase and effected adhesion of eosinophils but these effects differed from fMLP-induced responses. In particular, PMA evoked a respiratory burst in eosinophils regardless of whether FCS or BSA was used as substrate. One interpretation of this result is that PMA is active on non-adherent cells. However, PMA also augmented the adhesion of eosinophils to FCS-coated plates to a level that was not significantly different from that achieved with BSA. Thus, the role of adhesion molecules in PMA-induced oxidase activation in human eosinophils is equivocal and requires formal investigation.

It is clear from the above discussion that eosinophil adhesion and subsequent oxidant generation did not require the addition of an exogenous stimulus (so-called 'inside-out' signalling) and so permitted a pharmacological examination of the mechanism of CD11b/CD18-mediated 'outside-in' signalling. The identification of putative MAP kinase phosphorylation sites on $p47^{phox}$, a cytosolic component of the NADPH oxidase (el Benna *et al*., 1994; 1996), prompted an examination of the role of these kinases in adhesion and/or adhesion-dependent superoxide generation. SB 203580, an inhibitor of the α- and β-isoforms of the p38 MAP kinase family, inhibited superoxide anion generation in a concentration-dependent manner but had no significant effect on CD11b/CD18-dependent adhesion. These results clearly demonstrate that SB 203580 suppresses activation of the NADPH oxidase directly and implicates p38 MAP kinase in CD11b/CD18-mediated 'outside-in' signalling. Indeed, the ability of SB 203580 to suppress superoxide anion generation in human eosinophils occurred over a concentration range similar to a variety of p38 MAP kinase-driven responses including CD11b/CD18-dependent adhesion and adhesion-dependent oxidative burst of human neutrophils in response to lipopolysacchide and tumour necrosis factor-α (Detmers *et al*., 1998), fMLP-induced NADPH oxidase activation in non-adherent human neutrophils (Nick *et al*., 1997), interleukin-6 generation from interleukin-1β-stimulated human fibroblast-like synoviocytes (Miyazawa *et al*., 1998), the phosphorylation, in intact cells, of mitogen-activated protein kinase-activated protein kinase-2 (MAPKAPK-2; Miyazawa *et al*., 1998), a down-stream substrate of p38 MAP kinase, and the phosphorylation of heat-shock protein-27, a down-stream substrate of MAPKAPK-2 (Ridley *et al*., 1997). However, it is noteworthy that in the study by Detmers *et al*. (1998), the ability of SB 203580 to prevent the activation of the NADPH oxidase was secondary to the inhibition of adhesion suggesting that different mechanisms regulate superoxide anion generation between adherent granulocytes. As illustrated in Figure 5b, SB 203580 only partially suppressed CD11b/CD18-mediated superoxide anion generation even at high concentrations (10 μM) where selectivity for p38 MAP kinase is presumably lost, implying that other mechanisms also contribute to this response. The possible role of MEK-1 was excluded based on the consistent finding that PD 098059 had no affect on oxidant production even at concentrations (10 μM) that we have shown previously to inhibit the dual phosphorylation of ERK-1 and ERK-2 in eosinophils (Lindsay *et al*., 1998; Kankaanranta *et al*., 1999). This conclusion also is consistent with results obtained with fMLP-induced NADPH oxidase activation in non-adherent human neutrophils where PD 098059 similarly is inactive (Kuroki & O'Flaherty, 1997).

Previous studies have found that the binding of eosinophils through VLA4 (Nagata *et al*., 1995) and CD11b/CD18 (Kato *et al*., 1998) results in protein tyrosine phosphorylation. A number of candidate kinases could account for this effect including members of the syk and/or src-families of cytosolic protein tyrosine kinases. Indeed, on the basis of studies with selective inhibitors, evidence has been published implicating the syk and src families in 'outside-in' signalling in a host of cells (Aplin *et al*., 1998) including neutrophils (Yan & Berton, 1996; Berton *et al*., 1994; Lowell *et al*., 1996; Fernandez & Suchard, 1998). To assess if tyrosine phosphorylation could be involved in CD11b/CD18-mediated superoxide anion generation in human adherent eosinophils, a similar pharmacological approach was adopted. However, no evidence was found to implicate either enzyme family. Thus, although PP1, a selective inhibitor of the src-related family of protein tyrosine kinases (Hanke *et al*., 1996), inhibited NADPH oxidase activation, it also prevented adhesion with equal potency suggesting a causal relationship between these responses. This conclusion is consistent with the results obtained with neutrophils isolated from $hck^{-/-}$ $fgr^{-/-}$ double-knockout mice that were unable to generate oxidants when plated on to surfaces coated with extracellular matrix proteins or murine ICAM-1 in the presence of TNF and fMLP (Lowell *et al*., 1996). Similarly, superoxide anion generation was not observed in neutrophils from the same double mutant animals following cross-linking of β_2-subunits with surface-bound monoclonal antibodies, when compared to the wide-type mice (Lowell *et al*., 1996). In both cases, photomicroscopy revealed that impaired NADPH oxidase activity was due to defective spreading and tight adhesion (Lowell *et al*., 1996). A similar conclusion was reached with a naturally occurring inhibitor of syk, piceatannol (Geahlen & McLaughlin, 1989), which had no effect upon eosinophil adhesion or superoxide anion generation (accept at a high concentration). This was somewhat unexpected given that piceatannol is reported to attenuate syk

activity, cell spreading and H_2O_2 production in fMLP-stimulated human neutrophils with an IC_{50} of approximately 1 μM (Fernandez & Suchard, 1998). However, this apparent discrepancy might relate simply to differences in activating stimulus, given that fMLP acts through a G-protein-coupled receptor (unlike CD11b/CD18). Alternatively, these data might be explained by a more fundamental difference between eosinophils and neutrophils in the mechanism of NADPH oxidase activation.

Protein kinase C is a generic term that represents an increasingly diverse superfamily of cell signalling enzymes (Nishizuka, 1992; 1995) that are implicated in eosinophil adhesion (Dobrina *et al*., 1991), degranulation (Kroegel *et al*., 1989) and NADPH oxidase activation (Sedgwick *et al*., 1990). Currently, at least eleven isoforms of PKC have been identified that, on the basis of molecular structure and biochemical properties, have been categorized into three groups: cPKCs (α, β_I, β_{II} and γ), which are Ca^{2+}- and phospholipid-dependent; nPKCs (δ, ε, η and θ), which lack the Ca^{2+}-binding region and are, therefore, Ca^{2+}-independent; and atypical PKCs (aPKCs; ι, μ and ζ), which lack both the Ca^{2+}- and diacylglycerol/phorbol ester binding-site (Nishizuka, 1992; 1995). We have reported previously that human eosinophils express representatives of the conventional (α, β_1, β_{II}), novel (δ, ε) and atypical (ι, μ, ζ) PKC subtypes (Evans *et al*., 1999), and other studies have established that these three broad classes of enzymes can be differentiated by the use of the family-selective inhibitors, Ro-31 8220 (broad spectrum), GF109203X (cPKC and nPKC-selective) and Gö 6976 (cPKC-selective) (Davis *et al*., 1989; Martiny-Baron *et al*., 1993).

The results obtained with the aforementioned PKC inhibitors provide persuasive evidence that specific PKC isoforms are involved in the mechanism of CD11b/CD18-mediated NADPH oxidase activation in human eosinophils. Thus, while all three inhibitors blocked superoxide anion generation, none of them suppressed adhesion. The isoenzyme family responsible for this effect was inferred from the IC_{50} values obtained for each inhibitor. Thus, Ro-31 8220 ($IC_{50} \sim 0.25$ μM) and GF 109203X ($IC_{50} \sim 1$ μM) effectively suppressed oxidant production with potencies similar to those found for inhibition of fMLP-induced activation of the NADPH oxidase in human neutrophils (respective IC_{50}'s = 0.2 and 0.85 nM; Lindsay *et al*., 1996; Wenzel-Seifert *et al*., 1994); in contrast, Gö 6976 was a relatively weak inhibitor ($IC_{50} \sim 13$ μM). Since Ro 31-8220, GF 109203X and Gö 6976 all are bisindolylmaleimide derivatives with similar physical characteristics, these data suggest that the α, β_1 and β_{II} isoforms (cPKCs) in the eosinophils are unlikely to be involved in the adhesion-dependent activation of the NADPH oxidase. Additional support for this proposal comes from studies with macrophages where Gö 6976 abolished PMA-induced arachidonic acid release at a concentration (1 μM) that had only a modest inhibitory effect ($\sim$30%) on CD11b/CD18-dependent superoxide anion production reported herein (Lin & Chen, 1998). Thus, it is suggested that CD11b/CD18-mediated NADPH oxidase activation requires the participation of nPKC family members of which the δ- and ε-subtypes have been identified in human eosinophils (Evans *et al*., 1999). In this respect, Kato *et al*. (1998) have reported that the adherence of IL-5-treated human eosinophils to protein-coated tissue culture plates *via* β_2-integrins is associated with inositol phosphate accumulation. Clearly, this effect would provide the necessary diglyceride for the activation of nPKCs although the extent to which this accounts for CD11b/CD18-dependent signalling in the present study requires formal investigation. Again, differences in cell type and/or stimulus apparently dictate the subtype or complement of PKCs involved in a specific response given that fMLP, immune complex and PMA activate the NADPH oxidase, but not adhesion, in HL60 cells, by a PKCβ-dependent mechanism (Korchak *et al*., 1998).

Previous studies, using wortmannin, have implicated PtdIns 3-kinase in CD11b expression (Powell *et al*., 1999), chemotaxis (Palframan *et al*., 1998) and NADPH oxidase activation in human non-adherent eosinophils in response to agonists that act through G-protein-coupled receptors (Elsner *et al*., 1996). The experiments described in the present report extend those studies by demonstrating that wortmannin abolished superoxide anion production in adherent eosinophils with a potency ($IC_{50} \sim 0.9$ nM) comparable to its ability to inhibit purified PtdIns 3-kinase (IC_{50}'s = 2–5 nM) (Powis *et al*., 1994). This is an important observation since it reduces the possibility that wortmannin is working non-specifically on other enzymes such as myosin light chain kinase (Nakanishi *et al*., 1992) and PtdIns 4-kinase (Nakanishi *et al*., 1995) that are sensitive only to high nanomolar concentrations of the drug. It is possible that part of the inhibitory effect of wortmannin on superoxide anion generation is due to the inhibition of eosinophil adhesion since the EC_{50} values for both responses were almost identical (0.9 and 0.5 nM respectively). However, the finding that CD11b/CD18-dependent adhesion was maximally suppressed by only 50% implies that either a threshold level of adhesion is required to mount an oxidase response or that wortmannin also has an affect on those processes that govern 'outside-in' (CD11b/CD18-driven) oxidase activation.

In conclusion, the experiments described herein demonstrate that human eosinophils adhere to BSA-coated tissue culture plastic by a CD11b/CD18-dependent mechanism that is responsible for the subsequent activation of the NADPH oxidase. Furthermore, although the precise 'outside-in' signalling pathway(s) responsible for this effect still is to be elucidated, the results obtained with the selective inhibitors used in this study implicate p38 MAP kinase, nPKCs and PtdIns 3-kinase.

The authors gratefully acknowledge the British Lung Foundation [BLF], the Wellcome Trust (Grant #056814) and GlaxoWellcome for financial support, and Dr Martyn Robinson (Celltech Therapeutics Ltd, Slough) for the generous gift of the antibodies used in these studies.

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(Received February 9, 1999
Revised July 26, 1999
Accepted August 24, 1999)