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Inhibition of eotaxin-mediated human eosinophil activation and migration by the selective cyclic nucleotide phosphodiesterase type 4 inhibitor rolipram

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1 The effect of the selective type 4 phosphodiesterase (PDE 4) inhibitor rolipram on human eosinophil activation and migration mediated by eotaxin was investigated.

2 Studies were performed with human freshly isolated eosinophils from peripheral blood of healthy donors by a magnetic cell separation (MACS) technique to a purity > 99%. To test the effect of rolipram, eosinophils were stimulated with recombinant human eotaxin and the cell surface activation markers CD11b and L-selectin were analysed by flow cytometry. Furthermore, eotaxin mediated eosinophil migration was measured in a transendothelial chemotaxis assay.

3 Our results indicate that rolipram inhibited eotaxin-induced CD11b up-regulation up to $60.6 \pm 7.6\%$ at the highest tested dose (10 μM), whereas transendothelial chemotaxis was partially inhibited reaching a plateau of approx. 30% at a rolipram concentration of 0.1 μM.

4 We conclude that the selective PDE 4 inhibitor rolipram decreases eotaxin mediated eosinophil activation, an observation that may contribute to elucidate the mechanism by which PDE 4 inhibitors reduce antigen-induced eosinophil infiltration in different animal models of allergic inflammation.

Keywords: Human eosinophil; eotaxin; PDE 4; phosphodiesterase inhibitor; rolipram

Introduction

Leukocyte infiltration into tissues is a multistep process regulated by adhesion and cell activation events (Springer, 1994). Chemokines are inflammatory mediators that specifically attract and activate different leukocyte populations. The chemokine family consists of 8–10 KDa secreted proteins that activate leukocytes by binding to serpentine receptors that require G proteins for signalling (Baggiolini *et al*., 1994). They are currently classified, based on the position of four cysteine residues in a conserved motif, into three subgroups (C-C, C-X-C and C) (Baggiolini *et al*., 1994).

Eosinophils are present in inflamed tissues and play an important role in asthma, dermatitis, inflammatory bowel disease and eosinophilic gastroenteritis (Garcia-Zepeda *et al*., 1996). The C-C chemokines RANTES (Rot *et al*., 1992), MCP-3 (Dahinden *et al*., 1994), MCP-4 (Uguccioni *et al*., 1996) and MIP-1α (Rot *et al*., 1992) have been described to activate, *in vitro*, or attract, *in vivo*, eosinophils. However, all these chemoattractans can also activate and attract other leukocyte subsets. In contrast, the C-C chemokine eotaxin preferentially attracts eosinophils. It was isolated, as an eosinophil chemoattractant factor, from bronchoalveolar lavages of a guinea-pig model of allergic lung inflammation (Griffiths-Johnson *et al*., 1993). Both human and mice homologous chemokines have been recently characterized (Rothenberg *et al*., 1995a; Ponath *et al*., 1996). Human eotaxin binds to the CCR3 receptor which is predominantly expressed by eosinophils (Daugherty *et al*., 1996) inducing Ca^{2+} influx and respiratory burst (Elsner *et al*., 1996). Tissue expression of eotaxin assessed by mRNA detection or immunohistochemical analysis reveals its presence in different tissues in resting or activating conditions. (Garcia-Zepeda *et al*., 1996) Interestingly, eotaxin parallels eosinophil accumulation into lung allergic inflammation and it is mainly produced by type I alveolar epithelial cells (Gonzalo *et al*., 1996). Hence, eotaxin modulation may be effective in dealing with eosinophil mediated diseases.

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Increases of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) have been shown to modulate inflammatory and immunological processes (Bourne *et al*., 1974; Moore & Willoughby, 1996). Rises in intracellular cyclic AMP are usually transient, since cyclic AMP is converted to adenosine 5′-monophosphate (AMP) by phosphodiesterases (PDEs). Seven PDE isoenzyme families have been currently identified (Nicholson & Shahid, 1994). The PDE isoenzyme 4 is the predominant form present in most inflammatory cells (Torphy & Undem, 1991). The present study provides evidence that the selective PDE 4 inhibitor, rolipram, inhibits eotaxin induced β2 integrin up-regulation and transendothelial chemotaxis of human eosinophils.

Methods

Antibodies, cytokines, cell lines and reagents

The anti-CD9 and anti-L-selectin (CD62L) conjugated to fluorescein isothiocyanate (FITC) were from Immunotech (Marseille, Fr); anti-CD11b and IgG1 isotype matching FITC conjugated antibodies were from Sigma (St. Louis, U.S.A.). The anti-α-4 integrin (CD49d, VLA-4) antibody was kindly provided by C. R. Mackay (Institute for Immunology, Basel, Switzerland). Recombinant human eotaxin was bought from Preprotech (London, U.K.). The human endothelial cell line ECV-304 (Takahashi *et al*., 1990) was obtained from the European Collection of Animal Cell Cultures (ECACC) (Porton Down, U.K.). Only silylation grade dimethylsulphoxide (DMSO) from Pierce (Rockford, IL) was used to prepare stock solutions of rolipram (4-(3′-cyclopentyloxy-4′-methoxyphenyl)-2-pyrrolidone), that was synthesized at our chemical department. No effect was found in our assays of 0.005% DMSO, which was the highest amount used in the experiments.

Preparation of human eosinophils

Human eosinophils were isolated from EDTA-treated venous blood from healthy donors. Anticoagulated blood was diluted

(1:1, v:v) with PBS, and 35 ml aliquots were layered onto 15 ml isotonic Percoll solution 1.082 g ml^{-1} pH 7.4 (Pharmacia, Uppsala, Sweden) in a 50 ml graduated plastic tube. Erythrocytes were removed from the granulocyte suspension by dextran sedimentation and hypotonic lysis. Eosinophils were isolated by negative selection with anti-CD16 magnetic beads (Miltenyi, Bergisch Gladbach, Germany) (Hansel *et al*., 1991). Briefly, granulocytes were incubated with anti-CD16 microbeads for 1 h. Cells were passed through a MACS column (Miltenyi), and eosinophils were collected in the flow through. Eosinophil purity was >99% as determined by analysis of Diff-Quick-stained cytocentrifugation preparations by light microscopy and by flow cytometry with the CD9 antibody. Freshly isolated eosinophils were incubated in ice in Iscove's modified Dulbecco's medium (Life technologies, Paisley, U.K.) with 20% foetal calf serum (FCS; Life technologies) until used.

In vitro *stimulation of human eosinophils by eotaxin*

Freshly isolated eosinophils were preincubated with or without rolipram in culture medium, at the indicated concentrations, for 15 min at 37°C in a 96 well plate Nunclon (Roskilde, Denmark). Cells were then stimulated with 100 ng ml^{-1} eotaxin for 1 h at 37°C in the case of CD11b and VLA-4, or for 15 min at 37°C for L-selectin. Eosinophils were washed and stained for flow cytometry. The 100 ng ml^{-1} eotaxin dose was selected since this was the lowest amount giving the maximum CD11b up-regulation (data not shown).

Cell staining and flow cytometry

Four-parameter analysis was performed on an Coulter EPICS XL/XL-MCL flow cytometer (Coulter Corporation, Miami, FL). Typically, 10^5 cells were stained by directly coupled FITC antibodies. Stained cells were fixed in 1% paraformaldehyde in phosphate buffered saline (PBS) and stored at 4°C until they were analysed. Isotype control antibodies were used to verify specific staining.

Transendothelial chemotaxis assay

Eosinophil chemotaxis was based on a recent assay protocol (Qin *et al*., 1996). Endothelial cells were cultured on a 6.5-mm diameter Transwell culture inserts (Costar, Cambridge, MA, U.S.A.) with a 3.0 μm pore size. ECV-304 were cultured in RPMI-1640 medium (Life Technologies) supplemented with 10% foetal calf serum, 2 mM L-glutamine and 50 u ml^{-1} penicillin, all purchased from Life Technologies.

Assay medium consisted of equal parts of RPMI 1640 and Iscove's medium with 0.5% BSA. ECV 304 cells (2×10^5) were plated onto each insert of a 24-well chemotaxis plate and incubated at 37°C for 24 h. Recombinant human eotaxin, diluted in assay medium with or without rolipram, was added to 24-well tissue culture plates in a final volume of 600 μl at a final concentration of 100 ng ml^{-1}. This was a submaximal dose but gives a clear chemotactic response. Endothelial cell-coated Transwells were inserted into each well and 3×10^5 eosinophils were added to the top chamber in a final volume of 100 μl. Previously eosinophils had been incubated for 15 min with rolipram or culture medium at 37°C. Rolipram was present in both chemotaxis chambers in all experiments in order to maintain a constant exposure of eosinophils to the drug.

Plates were incubated for 1 h at 37°C in 5% CO_2. Migrating cells into the bottom chamber were counted by flow cytometry. A 500 μl aliquot was taken from the lower chamber and relative cell counts were obtained in a 30 s acquisition period.

Data analysis

Results are expressed as mean ± s.e. mean. Percentage inhibition of CD11b up-regulation was calculated based on medium fluorescence intensity (MFI) as follows: (100-(MFI rolipram-MFI basal/MFI eotaxin-MFI basal) × 100). Assay conditions were run in duplicate for each independent condition. Data were analysed by Student's *t* test (one tail).

Results

Modulation of CD11b and L-selectin expression on human eosinophils by eotaxin

Freshly isolated eosinophils were incubated for 1 h with eotaxin (100 ng ml^{-1}) and stained with anti-CD11b antibody. Eotaxin induced an increase in CD11b cell surface expression of 20.1 ± 2.1% in 14 subjects studied. As shown in Figure 1a, the eotaxin-treated eosinophil population expressed higher surface intensity for the CD11b marker when compared to untreated cells or negative control. In contrast, preincubation of eosinophils for 15 min at 37°C with eotaxin (100 ng ml^{-1}) decreased basal levels of L-selectin expression. A decrease of 18.3 ± 5.4% was found for five subjects studied. As shown in Figure 1b, a significant L-selectin decrease can be observed in the flow cytometry histogram when compared with basal L-selectin expression levels. We also studied VLA-4 modulation by eotaxin but we did not find any effect (data not shown).

Inhibition of eotaxin induced CD11b up-regulation on eosinophils cell surface by rolipram

To evaluate the effect of a selective PDE 4 inhibitor on eotaxin-mediated eosinophil activation, we incubated eosinophils with rolipram at different concentrations for 15 min at

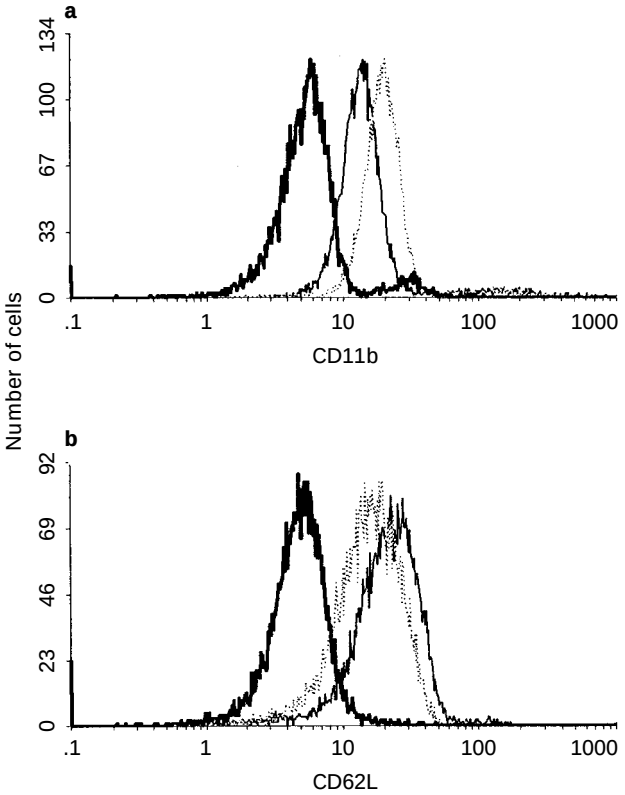

Figure 1 Eotaxin induced CD11b up-regulation and L-selectin shedding in human eosinophils. Freshly isolated human eosinophils were incubated with 100 ng ml^{-1} recombinant human eotaxin and stained for CD11b or L-selectin. Flow cytometry histograms show in (a) a significant CD11b upregulation in eosinophils cultured with eotaxin (. . . . .) compared to basal CD11b levels (———) or negative control antibody (———). In (b) eotaxin induced L-selectin shedding (. . . . .) compared to basal levels (———) or negative control antibody (———).

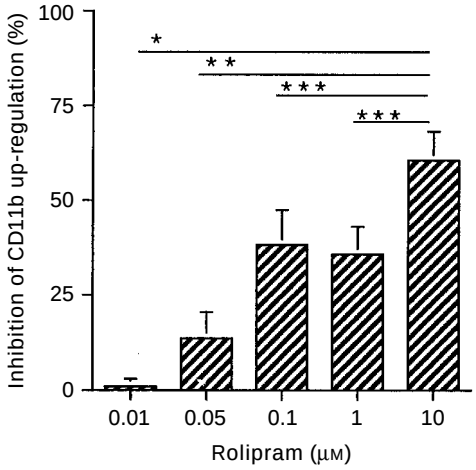

Figure 2 Rolipram inhibited eotaxin-mediated CD11b upregulation in human eosinophils. Freshly isolated human eosinophils were preincubated for 15 min with different amounts of rolipram before activation with 100 ng ml^{-1} eotaxin. Cells were incubated for 1 h at 37°C and stained for CD11b expression. Results are presented as percentage inhibition of CD11b upregulation. Data presented have been averaged from seven independent experiments ± s.e.mean. $*P<0.005$; $**P<0.01$; $***P<0.05$.

37°C before activation with eotaxin (100 ng ml^{-1}) for 1 h. Then, we stained cells with anti-CD11b antibody and analysed the samples by flow cytometry. In seven independent experiments run in duplicate, eotaxin-induced CD11b up-regulation was significantly inhibited in a dose-dependent way by rolipram, as shown in Figure 2. Inhibition was partial, amounting to 60.6 ± 7.6% at the highest tested dose ($P<0.05$). No effect of vehicle (DMSO) was found. Data are expressed as the percentage inhibition in CD11b up-regulation induced by eotaxin. A marginal inhibition in L-selectin shedding was present in some of the experiments (data not shown).

Effect of rolipram on the transendothelial chemotaxis induced by eotaxin

Since rolipram inhibited activation-dependent up-regulation of CD11b, we analysed its effect in a more functional assay such as transendothelial chemotaxis of human eosinophils. This chemotaxis method, where resting endothelial cells are used merely as a physiological barrier, has the advantage of having a high signal-to-noise ratio (Qin *et al*., 1996). In six independent assays with eosinophils from healthy donors we found a basal number of cells of 165 ± 29 which was increased to 3147 ± 829 in the presence of eotaxin (100 ng ml^{-1}). As shown in Figure 3, for a representative assay, the difference in the amount of attracted cells by medium alone (Figure 3a) or medium with eotaxin (Figure 3b) was clearly different. Based on such eotaxin-dependent transendothelial chemotaxis we studied the effect of rolipram.

As shown in Figure 3c, preincubation of eosinophils with different amounts of rolipram induced inhibition of the eotaxin-induced chemotaxis. This inhibition was partial, reaching a plateau of approx. 30% at a rolipram concentration of 0.1 μM ($P<0.05$).

Discussion

Eosinophils are preferentially present in tissues and especially in those with an epithelial surface (Weller, 1991). Besides their protective role against parasitic infections, eosinophils play an important role in inflammation. Eotaxin is a highly specific eosinophil chemoattractant with no effect on any other leukocyte type.

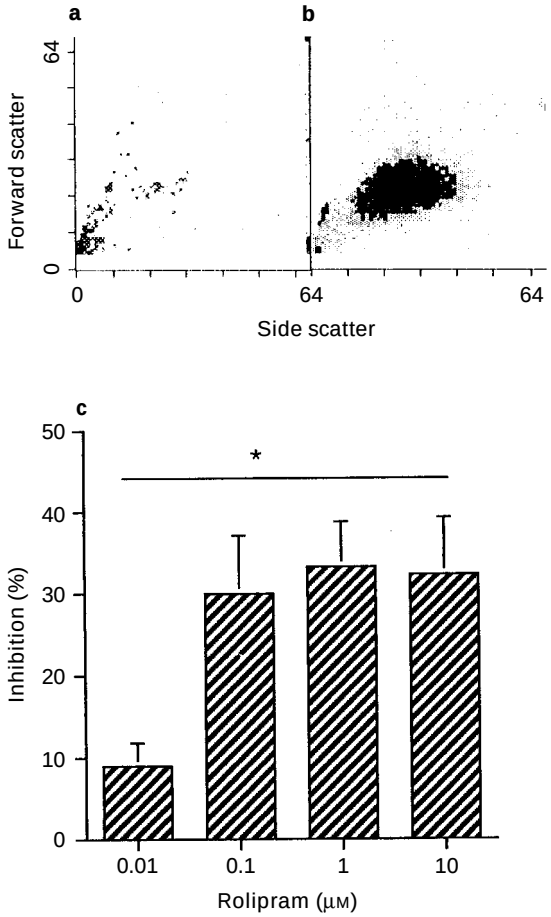

Figure 3 Rolipram inhibited eotaxin-induced transendothelial chemotaxis of human eosinophils. Eotaxin chemotaxis of human eosinophils was measured with Transwell culture inserts coated with resting human endothelial cells ECV-304. (a) and (b) Show a representative experiment performed in the presence of medium (a) and 100 ng ml^{-1} eotaxin (b). The inhibitory effect of rolipram on eotaxin-induced transendothelial eosinophil migration is shown in (c). Data presented have been averaged from six independent experiments ± s.e.mean ($*P<0.05$).

Intracellular increases of cyclic AMP have been shown to mediate a variety of anti-inflammatory effects (Bourne *et al*., 1974; Kammer, 1988). Compounds inhibiting PDEs, the cyclic AMP degrading enzymes (Beavo, 1995) have been suggested as potential anti-inflammatory agents (Hall, 1993), especially those inhibiting the type 4 PDE, which is preferentially found in inflammatory cells (Giembycz, 1992; Palfreyman, 1995). Several groups have previously shown that human eosinophils, as well as those from other species, exclusively express the type 4 PDE isoenzyme (Dent *et al*., 1994; Hatzelmann *et al*., 1995; Souness *et al*., 1995). PDE 4 inhibitors have been shown to mediate an inhibitory effect on eosinophil activation induced by different stimuli such as formyl-methionyl-leucyl-phenylalanine (FMLP), platelet activating factor (PAF) and leukotriene B_4 (LTB_4) at doses corresponding with their potencies as inhibitors of the isolated PDE 4 isoenzyme (Torphy *et al*., 1994; Hatzelmann *et al*., 1995; Souness *et al*., 1995; Lagente *et al*., 1995). These functional effects correlate with the increases in cyclic AMP observed in eosinophils when treated with these drugs (Dent *et al*., 1994; Souness *et al*., 1995; Hallsworth *et al*., 1996).

Because most chemotactic factors modulate cell surface adhesion molecules expression (Kishimoto *et al*., 1989), we studied the effect of eotaxin on CD11b and L-selectin expression on human eosinophils. Our data indicate that eotaxin induces up-regulation of CD11b in parallel to a shedding of L-selectin. Since CD11b increase is a marker of leukocyte che-

moattractant activation (Detmers *et al*., 1990; Walker *et al*., 1993), we analysed whether rolipram may interfere with the eotaxin-mediated eosinophil activation by analysing CD11b expression by flow cytometry. We found that eotaxin-mediated CD11b up-regulation was inhibited by rolipram, in a dose-dependent way, compatible with the effect of the drug on isolated PDE 4 and on PDE 4-mediated cell responses (Souness *et al*., 1995). On the other hand, eotaxin-mediated L-selectin shedding was only marginally affected by rolipram (data not shown). We also studied the modulation of cell surface VLA-4, but we did not find any significant change after eotaxin activation. However, we cannot discard such a possibility because we did not use monoclonal antibodies identifying the high affinity state of β1 integrins (Dransfield *et al*., 1990). Moreover, it has recently been shown that chemoattractants can differentially regulate β1 and β2 integrins expressed on the same eosinophil (Weber *et al*., 1996).

Eosinophil extravasation is a process involving L-selectin, β1 and β2 integrins (Sriramarao *et al*., 1994). Eosinophils present in human broncho-alveolar lavages show increased expression of CD11b (Walker *et al*., 1993) which is a counterreceptor for ICAM-1 (Diamond *et al*., 1990). Interestingly, human eotaxin mediated eosinophil accumulation in rat skin is dependent on both VCAM-1 and ICAM-1 (Sanz *et al*., 1996). To characterize further the effect of rolipram on eosinophil migration we selected a model of transendothelial chemotaxis. Our data indicate that rolipram not only decreases CD11b up-regulation but also inhibits eotaxin-mediated transendothelial chemotaxis. The fact that eotaxin-induced eosinophil chemotaxis was only partially inhibited by rolipram indicates that this process is likely to involve other mechanisms, apart from CD11b up-regulation, that are not modulated by the expected drug effect of cyclic AMP elevation. One of these factors may be L-selectin shedding or VLA-4 up-regulation.

Many animal models for asthma have demonstrated a potential benefit of PDE 4 inhibitors, including rolipram, because they can inhibit eosinophil recruitment into the respiratory tract (Banner & Page, 1995). Interestingly, several *in vivo* studies suggest that eotaxin may have an important role in such models. Thus, eotaxin is induced by allergen challenge in the guinea-pig lung (Rothenberg *et al*., 1995b) and mouse eotaxin expression, which was mainly produced by type I alveolar epithelial cells, parallels eosinophil accumulation during lung inflammation in the mice (Gonzalo *et al*., 1996). Moreover, a strong correlation has been shown between eosinophil accumulation and eotaxin expression on human polyp nasal mucosa (Ponath *et al*., 1996). Our *in vitro* data support the idea that at least part of the inhibitory effect of rolipram on eosinophil infiltration in asthma models could be due to inhibition of eotaxin-mediated activation. Eosinophil migration into tissues involves different mechanisms (Teixeira *et al*., 1995). Hence, it has been shown that rolipram and other PDE 4 inhibitors are able to block interleukin-5 (IL-5) induced eosinophilia (Hughes *et al*., 1996). The fact that IL-5 and eotaxin cooperate to induce eosinophil accumulation *in vivo* (Collins *et al*., 1995) may suggest that PDE 4 inhibitors could be acting at different levels. This may be a reason why we only found a partial inhibition of rolipram on eotaxin-mediated eosinophil extravasation, in contrast to the *in vivo* situation, where more PDE 4 susceptible mechanisms are acting and, possibly, produce a greater inhibitory effect on eosinophil extravasation.

Additionally, eosinophils are also implicated in the pathology of allergic skin diseases (Leiferman, 1994). The role of eotaxin in cutaneous eosinophil infiltration is supported by several *in vivo* experiments. Thus, intradermal injection of human eotaxin induces eosinophil recruitment in rhesus monkey (Ponath *et al*., 1996) and in the guinea-pig (Jose *et al*., 1994), where PDE 4 inhibitors are able to reduce the inflammation (Teixeira *et al*., 1994). Recently it has been shown that the PDE 4 inhibitor CP80,633 has clinical anti-inflammatory effects in atopic dermatitis (Hanifin *et al*., 1996), where eosinophils may play an active role (Leiferman, 1994). In summary, our results may be relevant to the understanding of the mechanism by which rolipram and related drugs reduce eosinophil infiltration into tissues like lung and skin.

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