Evidence that 13-14 di-hydro, 15-keto prostaglandin D_2-induced airway eosinophilia in guinea-pigs is independent of interleukin-5.

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Running title

DK-PGD_2 airway eosinophilia is independent of IL-5

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Abstract

Objective and design

Prostaglandin D_2 (PGD_2) has been shown to cause eosinophil, basophil and Th2 cell chemotaxis *in vitro* and *in vivo* through an action on the prostaglandin CRTH2 receptor. In the present study, the dependence of PGD_2-induced eosinophil accumulation *in vivo* on interleukin-5 (IL-5) blood eosinophilia was investigated.

Materials

Guinea-pigs were exposed to aerosols of 13,14di-hydro 15-keto PGD_2 (DK-PGD_2) or platelet activating factor (PAF) and the eosinophil content of the bronchoalveolar lavage fluid or blood determined. In some experiments, DK-PGD_2 was administered systemically and eosinophilia measured.

Results

Aerosols of DK-PGD_2 caused eosinophil accumulation in the lungs 24h after exposure. DK-PGD_2 ($10\mu g.ml^{-1}$)-induced airway eosinophilia was inhibited when animals were treated with the CRTH2 receptor antagonist ramatroban. 1-4h after exposure to DK-PGD_2 a significant decrease in blood eosinophil count was measured. The anti-IL-5 antibody TRFK-5 had no inhibitory effect of DK-PGD2-induced airway eosinophilia, but abolished airway eosinophilia induced by exposure of guinea-pigs to aerosols of PAF. Intracardiac injection of DK-PGD_2 induced a dose-related increase in blood eosinophil numbers.

Conclusions

It is concluded that, in the guinea-pig, DK-PGD_2-induced airway eosinophilia is mediated by an action on prostaglandin CRTH2 receptors and that this response appears to be independent of IL-5.

Introduction

Evidence has accumulated suggesting that prostaglandin D_2, released by mast cells, may be involved in allergic inflammation through an action on the CRTH2/DP2 receptor[1]. This G protein coupled receptor is expressed on eosinophils, lymphocytes and basophils [2] and, in common with other chemoattractant receptors, mediates cell activation through an action involving Gαi [1].

Prostaglandin D_2 (PGD_2) causes eosinophil and lymphocyte chemotaxis *in vitro* through an action on the CRTH2 receptor [3-5]. In addition, 13,14di-hydro 15-keto PGD_2 (DK-PGD_2), a PGD_2 metabolite, is a selective agonist at the CRTH2 receptor, has a markedly lower affinity for the DP1 receptor and is a potent activator of cells bearing these receptors [6].

Studies in laboratory animals have shown that PGD_2 administration augments antigen-induced airway inflammation [7] and induces eosinophil accumulation in the airways of rats primed with interleukin-5 [8]. Furthermore, CRTH2 knockout mice have a reduced inflammatory response to IgE induced inflammation of the skin [8]and to cedar pollen-induced dermatitis [9]. In addition, the prostaglandin CRTH2 receptor antagonist ramatroban inhibits eosinophil migration *in vitro* and *in vivo,* and is used in the treatment of allergic rhinitis in Japan [10]. Thus, it has been proposed that antagonists of the prostaglandin CRTH2 receptor may have therapeutic utility in the treatment of allergic disease [2].

However, other agents, including a range of chemokines, platelet activating factor (PAF) and histamine, also induce eosinophil recruitment [11-14] and antagonists of these agents may also have utility in the treatment of allergic inflammation.

Interleukin-5 (IL-5) causes blood eosinophilia [15] and may be central to eosinophil recruitment in disease. Thus, in order for eotaxin to induce eosinophil recruitment into the lung and skin a blood eosinophilia, induced by IL-5, is necessary [15]. Furthermore, in laboratory animals antigen- and PAF- induced airway eosinophilia was inhibited when animals are pre-treated with an antibody to IL-5 demonstrating the dependence of eosinophil accumulation on this cytokine [16-19].

In asthmatic patients, an antibody to IL-5, mepolizumab, caused a small reduction in blood eosinophil numbers and decreased antigen-induced eosinophil recruitment into the lungs but this treatment failed to inhibit antigen-induced late-phase bronchoconstriction. [20]. In a further analysis, mepolizumab caused a partial reduction in mucosal eosinophil numbers, suggesting that partial inhibition may be insufficient for a clinical effect [21]. One possible explanation for this observation is that, in man, mediators other than IL-5 contribute to the maintenance of eosinophilia. Such a factor could be PGD_2, which has been shown to increase blood eosinophil counts in laboratory animals through an action on CRTH2 and DP receptors [22, 23].

In the present study the ability of the prostaglandin CRTH2 receptor agonist 13,14 di-hydro, 15-keto prostaglandin D_2 (DK-PGD2) [6, 22] to induce eosinophilia in guinea-pig lungs and the dependence of any eosinophilia on IL-5 was investigated.

Method

Materials

13,14 di-hydro, 15-keto prostaglandin D_2 (DK-PGD_2) and ramatroban were purchased from Cayman Chemical (Axxora (UK) Ltd., Nottingham, UK). Platelet activating Factor (PAF), heparin and dextran (MW 500,000) were purchased from Sigma-Aldrich, Poole, Dorset, UK. TRFK-5 was purchased from R&D systems Ltd., Abingdon, UK.

Eosinophilia in Guinea-pig airway and blood

Female Dunkin Hartley guinea-pigs (250-350 g) were purchased from Darley Oaks Farm, Newchurch, UK. Animals were housed and all experiments conducted under a project licence granted under the Animals (Scientific Procedures) Act 1986. Groups of guinea-pigs were placed in a plastic chamber and exposed to aerosols of PAF or DK-PGD_2, generated from a nebuliser (DeVilbiss) driven by a compressor (DeVilbiss Pulmostar), for 10 min. In experiments where guinea-pigs were treated with the anti-IL-5 antibody TRFK-5, this antibody was administered by intraperitoneal injection in PBS (1 $ml.kg^{-1}$) 30 min prior to exposure of animals to aerosols of PAF or DK-PGD_2. In these experiments control animals were given an intraperitoneal injection of PBS.

At intervals after exposure to aerosols (usually 24h) animals were killed by an overdose of pentobarbitone Na (Euthetal; Merial), the trachea was cannulated and the lungs lavaged twice with 5ml heparinised (10 $U.ml^{-1}$) PBS. A cytocentrifuge preparation (Thermo Shandon, cytospin 2) was prepared from an aliquot of the pooled bronchoalveolar lavage (BAL) fluid recovered and stained with May Grunwald Giemsa stain. A total leukocyte count was also made from the BAL fluid using a haemocytometer. The percentage eosinophils was determined from the cytocentrifuge preparation under oil immersion microscopy and the number of eosinophils/ml blood determined from this percentage and the total leukocyte count.

In experiments where the number of eosinophils in the blood was determined, animals were killed by an overdose of pentobarbitone Na and a blood sample (2 ml) was taken into heparin (0.05 ml) by cardiac puncture. 100µl of blood was mixed with 900µl Turk's solution and the total number of leukocytes determined with a

haemocytometer. 500 μl blood was mixed with 500 μl 4% Dextran (mol wt 500,000; Sigma) and the red cells allowed to sediment. A cytocentrifuge preparation was prepared from the leukocyte rich supernatant and stained with May Grunwald Giemsa stain (Sigma). The percentage eosinophils was determined from the cytocentrifuge preparation and the number of eosinophils/ml blood determined from this percentage and the total leukocyte count.

Data analysis

Each experiment was performed with groups of 2-4 animals and repeated to give the group sizes stated once data were pooled. Pooled data were analysed by ANOVA followed by Dunnett's post hoc test [24] using commercially available software (Prism; GraphPad, San Diego, California). A difference between groups was taken to be statistically significant when $P<0.05$.

Results

DK-PGD_2-induced airway eosinophilia

When the lungs of guinea-pigs were lavaged twice with 5 ml PBS, 7-8 ml of bronchoalveolar lavage fluid was recovered. The volume of BAL recovered was not affected by any of the treatments used. Bronchoalveolar lavage fluid from untreated guinea-pigs contained $8.76 \times 10^4 \pm 1.6 \times 10^4$ (n=9) eosinophils.ml^{-1} (Figure 1). Exposure of guinea-pigs to aerosols generated from solutions of DK-PGD_2 (0.1 to 100 $\mu g.ml^{-1}$) caused a small concentration-related accumulation of eosinophils in the airway, 24h after exposure to aerosols (Figure 1). Aerosols generated from solutions of DK-PGD_2 greater than 0.1 $\mu g.ml^{-1}$ caused a significant ($P<0.05$) eosinophil accumulation that appeared to be maximal when a concentration of 10 $\mu g.ml^{-1}$ was used (Figure 1). DK-PGD_2-induced eosinophil accumulation was also time-dependent. Following exposure to an aerosol of 10$\mu g.ml^{-1}$ DK-PGD_2 no significant ($P>0.05$) eosinophilia was detected 2 and 6h after exposure of animals whereas by 24h significant eosinophilia ($P<0.05$) was observed (Figure 2). While a small increase in eosinophil numbers in the recovered BAL was measured 18h after exposure to an aerosol of DK-PGD_2, this increase was not statistically significant ($P>0.05$).

In a separate experiment, guinea-pigs were exposed to aerosols generated from solutions of DK-PGD_2 (10 $\mu g.ml^{-1}$) and blood samples taken at intervals after exposure, a significant ($P<0.05$) decrease in blood eosinophil count was seen 1-4h after exposure to aerosols of DK-PGD_2 (Figure 3). However, this eosinopenia recovered and 6h after exposure no significant ($P>0.05$) change in blood eosinophil count was seen (Figure 3).

Effect of ramatroban on DK-PGD_2-induced airway eosinophilia

When guinea-pigs were pretreated with ramatroban (0.1 - 100 $\mu g.kg^{-1}$ p.o.) 30 min prior to being exposed to an aerosol of DK-PGD_2 (10 $\mu g.ml^{-1}$), a dose-related inhibition of DK-PGD_2-induced eosinophil accumulation was seen (Figure 4). Doses of ramatroban greater than (1.0 $\mu g.kg^{-1}$ p.o.) caused a significant ($P<0.05$) inhibition of DK-PGD_2-induced eosinophilia. Furthermore 0.01 $mg.kg^{-1}$ ramatroban reduced

DK-PGD_2-induced BAL eosinophil count to a value that was not significantly different ($P>0.05$) from that seen in untreated animals (Figure 4).

Effect of TRFK-5 on DK-PGD_2-induced airway eosinophilia

When guinea-pigs were treated with the anti-IL-5 antibody TRFK-5 (100 $\mu g.kg^{-1}$ i.p.) 30 min prior to exposure to aerosols of DK-PGD_2 (10 $\mu g.ml^{-1}$), no significant ($P>0.05$) inhibition of DK-PGD_2-induced eosinophilia was seen (Figure 5A). In contrast, in a separate experiment, the same dose of TRFK-5 significantly inhibited airway eosinophilia induced by an aerosol of platelet activating factor (PAF; 100 $\mu g.ml^{-1}$)(Figure 5B).

Blood eosinophilia induced by systemic administration of DK-PGD_2

Following administration by intracardiac injection, DK-PGD_2 (1 – 100 μg) caused a dose-related blood eosinophilia (Figure 6A). Doses of DK-PGD_2 greater than 1.0 μg significantly ($P<0.05$) increased blood eosinophil count 1h after administration (Figure 6B). Blood eosinophilia induced by 10 μg DK-PGD_2 was evident 30 and 60 min after administration of DK-PGD_2 (Figure 6B).

Discussion

The experiments described above show that exposure of guinea-pigs to aerosols of the selective prostaglandin CRTH2 receptor agonist DK-PGD_2 caused eosinophil recruitment in the airways. This eosinophil accumulation appeared to be mediated through an action of DK-PGD_2 on the prostaglandin CRTH2 receptor since the effect was inhibited when guinea-pigs were treated with the prostaglandin CRTH2 receptor antagonist ramatroban prior to exposure to DK-PGD_2. The dose-range over which ramatroban inhibited DK-PGD2-induced eosinophil accumulation, in the present study, was slightly lower than that used by others to block the CRTH2 receptor in rats [23, 25], but this be because a relatively low dose of DK-PGD_2 was used. In the present study, a dose-effect curve for ramatroban was obtained and it was noted that higher doses produced total inhibition of eosinophilia similar to that obtained by others at the lowest dose tested [23, 25].

Ramatroban was originally developed as a thromboxane receptor antagonist and has affinity for both TP and CRTH2 receptors [10]. Thus, it is theoretically possible that DK-PGD_2-induced eosinophilia is mediated via an action on TP receptors. However, eosinophils do not express TP receptors [1] and eosinophilia-induced by DK-PGD_2 in the rat was unaffected by the specific TP receptor blocking drug SQ29548 but was blocked by ramatroban [25]. Thus, it seems unlikely that TP receptors contribute to DK-PGD_2-induced eosinophilia and its inhibition by ramatroban.

The finding that DK-PGD_2 induced airway eosinophilia in guinea-pigs after aerosol administration is consistent with data reported by others in rats [26]. However, in some studies in the rat [25] DK-PGD2-induced airway eosinophilia only after a blood eosinophilia was induced by intravenous administration of IL-5. In contrast to laboratory rats, guinea-pigs often have a higher resting blood eosinophil count. Thus, one explanation for the finding that in the guinea-pig DK-PGD_2-induced airway eosinophil accumulation without the need for a treatment to induce blood eosinophilia is that the animals had a pre-existing blood eosinophilia that is not present in rats.

The anti IL-5 antibody, TRFK-5, has been widely used to show a role for IL-5 in allergen induced airway inflammation in experimental animals, including guinea-pigs [16-18, 27]. This antibody has also been used to show that eosinophil accumulation

in the airways of guinea-pigs induced by PAF is IL-5 dependent [19]. In the present study, TRFK-5 inhibited PAF-induced eosinophilia confirming earlier findings [19] but TRFK-5 failed to inhibit eosinophilia induced by DK-PGD_2. These findings suggest that, in guinea-pigs, eosinophilia induced by agents that act on the CRTH2 receptor is independent of IL-5.

It has been proposed that IL-5 recruits eosinophils from the bone marrow to the blood and has a "priming" action on these cells such that they can migrate into a lesion under the influence of other agents, such as the chemokine eotaxin [15]. In the present study, exposure of guinea-pigs to an aerosol of DK-PGD_2 caused a fall in blood eosinophil numbers shortly after treatment that recovered by 6h after treatment. These findings support the findings that DK-PGD_2-induced eosinophilia is independent of IL-5 and suggest that following inhalation of DK-PGD_2, eosinophils are directly reruited into the lung from the blood and this results in a decrease in blood eosinophils. Subsequently, eosinophils are released from the bone marrow into the blood to restore the blood eosinophil count. In the present study, blood eosinopenia was rapid yet eosinophil accumulation in the BAL was only detected 18-24h after exposure to aerosols of DK-PGD_2. It is possible that the reason for the delay in detecting airway eosinophilia is because eosinophils have to migrate into the airway wall before entering the lumen of the airway where they can be recovered by BAL. However, further experiments measuring eosinophilia in the lung tissue would be necessary in order to resolve this discrepancy.

The findings described in the present study contrast with those of Shichijo *et al*. who reported that intravenous administration of PGD_2 and DK-PGD_2 caused a rapid increase in blood eosinophil counts in rats, mice and guinea-pigs through an action on CRTH2 receptors[22]. These authors concluded that DK-PGD_2 caused the release of eosinophils from the bone marrow, a conclusion confirmed in the guinea-pig by the demonstration that PGD_2 and DK-PGD_2 release eosinophils from the bone marrow of perfused guinea-pig hind quarters [23]. Furthermore, in guinea-pigs PGD_2-induced eosinophilia was mediated by both DP and CRTH2 receptors [23]. However, the doses of PGD_2 and DK-PGD_2 used in these experiments [22] are higher than those likely to reach the blood in the present study. Recent studies [23] in guinea-pig perfused hind quarters used a low concentration of DK-PGD_2 to release eosinophils

from the bone marrow. In these experiments the tissue was perfused with physiological salt solution and it is not clear whether or not higher concentrations would be necessary to achieve the same response if blood were used as a perfusion fluid. Furthermore, the supposition that insufficient DK-PGD2 reaches the blood in the present study is supported by the observation that intravenous administration of doses of DK-PGD_2 equal to the concentration in the nebuliser used in the present study was necessary to induce blood eosinophilia in the experiments described by others [22]. Similar findings have been reported by others in rats where intratracheal administration of DK-PGD_2 induced airway eosinophil accumulation without a blood eosinophilia [25].

The finding that DK-PGD2-induced airway eosinophilia is not dependent on IL-5 may be of therapeutic significance. Eosinophils are thought to be key effector cells in the pathology of allergic inflammation [28, 29] and eosinophil chemoattractants, such as eotaxin, act in concert with IL-5 to cause eosinophil recruitment [15, 30]. Thus, a model has been proposed where IL-5 acts on the bone marrow to cause a blood eosinophilia and to prime these cells while agents such as eotaxin recruit IL-5 primed eosinophila from the blood [15]. If such a mechanism were pivotal to the pathology of allergic inflammatory diseases then an inhibitor of IL-5 should inhibit eosinophil recruitment and alleviate the disorder.

In man a humanised anti-IL-5 antibody, mepolizumab, caused a partial reduction of blood eosinophilia in asthmatic patients but did not significantly improve the symptoms of asthma [20, 31] suggesting that factors other than IL-5 may contribute to the eosinophilia seen in the lungs of asthmatic patients. One such factor could be PGD_2 released by the mast cell acting on the CRTH2 receptor. The CRTH2 receptor promotes eosinophil, basophil and Th2 lymphocyte chemotaxis [2] and has recently been shown to activate Th2 cells to release many of the cytokines found in asthma including IL-5 [32]. Furthermore, ramatroban has therapeutic utility in allergic rhinitis suggesting a role for CRTH2 receptors in the pathology of this disorder in man. If eosinophil recruitment mediated by CRTH2 receptor activation in man is also independent of IL-5 then the existence of such mechanisms could explain the lack of clinical efficacy of preparations such as mepolizumab in the clinic.

In conclusion, the experiments described in the present study show that the CRTH2 receptor agonist DK-PGD_2 induces airway eosinophil accumulation in guinea-pig lungs after aerosol administration. This lung eosinophilia is mediated through an action on the CRTH2 receptor and appears to be independent of IL-5. Because the CRTH2 receptor antagonist ramatroban has clinical utility in the treatment of allergic inflammation it is suggested that this IL-5 independent pathway may offer an explanation for the failure of anti-IL-5 antibodies to produce a clinically beneficial effect in the treatment of allergic inflammation in man.

Figure 1. Eosinophil accumulation in the airways of guinea-pigs 24h after exposure to aerosols of DK-PGD_2. Guinea-pigs were placed in a plastic chamber and exposed to aerosols of DK-PGD_2 for 10 min. Bars represent the mean ± s.e.mean of the number of eosinophils.ml^{-1} in the BAL recovered from 6 guinea-pigs.

Figure 2. Time course for DK-PGD_2-induced airway eosinophilia in guinea-pigs. Guinea-pigs were exposed to an aerosol generated from a solution of DK-PGD_2 (10μg.ml^{-1}) and the lungs lavaged at the time indicated. Bars represent the mean ± s.e.mean of the number of eosinophils.ml^{-1} in the BAL recovered from 4 guinea-pigs.

Figure 3. Blood eosinopenia induced by aerosols of DK-PGD_2 (10μg.ml^{-1}) in the guinea-pig. Guinea-pigs were exposed to aerosols of DK-PGD_2 for 10 min and a blood sample taken at the time indicated. Bars represent the mean ± s.e.mean of the number of eosinophils.ml^{-1} in blood samples from 6 guinea-pigs.

Figure 4. Inhibition of DK-PGD_2-induced airway eosinophilia in the guinea-pig by ramatroban. Guinea-pigs were dosed with ramatroban orally 30 min prior to exposure to an aerosol of DK-PGD_2 (10μg.ml^{-1}). 24h after exposure to DK-PGD_2 the eosinophil content of the recovered BAL was determined. Bars represent the mean ± s.e.mean of the number of eosinophils.ml^{-1} in the BAL recovered from 6 guinea-pigs.

Figure 5. The effect of the anti-IL-5 antibody TRFK-5 on (A) DK-PGD_2-, or (B) PAF-induced eosinophil accumulation in guinea-pig lungs. Guinea-pigs were treated with TRFK-5 30 min prior to exposure to an aerosol of DK-PGD_2 (10μg.ml^{-1}) or PAF (100μg.ml^{-1}). The number of eosinophils in the recovered BAL was determined 24h after exposure to aerosols. Bars represent the mean ± s.e.mean of the number of eosinophils.ml^{-1} in the BAL recovered from 6 guinea-pigs.

Figure 6. Blood eosinophilia induced by intracardiac administration of DK-PGD_2 to guinea-pigs. (A) Anaesthetised guinea-pigs received an intracardiac injection of DK-PGD_2 (1-100 μg) and the blood eosinophil count determined 60 min later. (B) Anaesthetised guinea-pigs received an intracardiac injection of DK-PGD_2 (10mg) and the blood eosinophil count determined at the time indicated. Bars represent the mean ± s.e.mean of the number of eosinophils.ml^{-1} of blood from 6 guinea-pigs.

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