Effect of *in vivo* desensitization to leukotriene B₄ on eosinophil infiltration in response to C5a in guinea-pig skin

¹E.R. Pettipher, E.D. Salter & H.J. Showell

Department of Immunology and Infectious Diseases, Central Research Division, Pfizer Inc., Groton, CT 06340, U.S.A.

1 The effect of *in vivo* desensitization to leukotriene B₄ (LTB₄) on eosinophil infiltration in response to recombinant C5a was examined in guinea-pig skin.

2 LTB₄ (10–300 ng) and C5a (1–10 µg) caused a dose-dependent increase in the levels of eosinophil peroxidase activity (a measure of eosinophil infiltration) 4 h after injection into guinea-pig skin. Leukotriene B₄ and C5a were approximately equipotent on a molar basis. Platelet activating factor (0.01–10 µg) also caused eosinophil accumulation but was much less active than LTB₄ or C5a.

3 20-Hydroxy-LTB₄ caused a dose-dependent desensitization of eosinophil responses to LTB₄ (ED₅₀ = 1.6 µg kg⁻¹, s.c.) and partially reduced responses to C5a. At a dose of 20-hydroxy-LTB₄ (10 µg) which inhibited responses to LTB₄ completely, responses to C5a were reduced by 56.5 ± 1.8% (n = 5). The structurally related metabolite of 20-hydroxy-LTB₄, 20-carboxy-LTB₄, which does not cause desensitization to the effects of LTB₄, did not inhibit eosinophil infiltration in response to C5a.

4 The LTB₄ receptor antagonist, SC-41,930 (10 mg kg⁻¹, p.o.), also inhibited eosinophil accumulation in response to C5a by 63.0 ± 3.9% (n = 5) at a dose which inhibited responses to LTB₄ by 86.5 ± 1.9% (n = 5).

5 These data indicate that eosinophil infiltration in response to C5a may, in part, be mediated by the generation of secondary chemotactic factors such as LTB₄.

Keywords: Leukotriene B₄; neutrophils; eosinophils; inflammation; asthma; C5a

Introduction

Eosinophils infiltrate the lungs of asthmatic patients and are thought to contribute to disease pathology by releasing granule constituents which are cytotoxic to bronchial epithelium (Gleich, 1990). While the mediators responsible for attracting eosinophils to the lungs of asthmatics have not been clearly identified, several mediators are capable of stimulating eosinophil chemotaxis *in vitro*. These include non-selective chemoattractants such as leukotriene B₄ (LTB₄), platelet activating factor (PAF) and C5a (Wardlaw *et al.*, 1986; Hakansson *et al.*, 1987; Sehmi *et al.*, 1992) which have activity on a wide variety of leukocyte types and have been demonstrated to cause eosinophil accumulation *in vivo* (Faccioli *et al.*, 1991). In contrast, RANTES, a β-chemokine, is chemotactic for eosinophils, monocytes and lymphocytes but does not affect neutrophils directly (Schall *et al.*, 1991; Kameyoshi *et al.*, 1992) while the α-chemokine, interleukin-8, is active on neutrophils but not eosinophils or monocytes (Baggiolini *et al.*, 1989).

Although the *in vitro* chemotactic effects of these mediators are due to direct actions on leukocytes, secondary mediators may be generated *in vivo* which contribute to the pattern of leukocyte infiltration observed in inflamed tissues. An example of this is the ability of interleukin-8, which lacks direct chemotactic activity for eosinophils, to stimulate eosinophil emigration *in vivo* through an indirect mechanism (Collins *et al.*, 1993). We have compared the ability of LTB₄, PAF and C5a to induce an eosinophilic infiltrate in guinea-pig skin *in vivo* over a 4 h period. As LTB₄ may be produced from resident macrophages and migrating leukocytes in response to other mediators, we have investigated the contribution of endogenously produced LTB₄ to eosinophil emigration induced by C5a by treating guinea-pigs with 20-hydroxy-LTB₄, an agent which causes specific desensitization to effects of LTB₄ *in vitro* and *in vivo* (Pettipher *et al.*, 1993). We have also used the selective LTB₄ receptor antagonist, SC-41930

(Fretland *et al.*, 1990), to investigate the role of LTB₄ receptor stimulation in eosinophil infiltration in these responses.

Methods

Induction of eosinophil infiltration in guinea-pig skin

Male Hartley guinea-pigs (400–450 g) were anaesthetized in an atmosphere of Metofane (methoxyflurane) and their backs shaved. LTB₄, PAF, human recombinant C5a were dissolved in saline containing 0.25% bovine serum albumin. Duplicate intradermal injections of saline or mediators were given in a volume of 0.1 ml such that there were a maximum of 6 sites per guinea-pig. Four hours after injection animals were killed, skin removed and sites punched out with a 13 mm gasket punch. Skin homogenates were prepared for assay as previously described (Pettipher *et al.*, 1993).

Animals were predosed with vehicle or 20-hydroxy-LTB₄ subcutaneously in 0.5 ml saline immediately prior to intradermal challenge. Animals were dosed orally with the LTB₄ antagonist, SC-41930 (7-[3-(4-acetyl-3-methoxy-2-propylphenoxy)-propoxy]-3, 4-dihydro-8-propyl-2H-1-benzopyran-2-carboxylic acid) in 0.5% carboxymethylcellulose vehicle (10 ml kg⁻¹ body weight) 1 h prior to intradermal challenge.

Assay of eosinophil peroxidase

Eosinophil peroxidase (EPO) was assayed in diluted skin homogenates as previously described (Strath *et al.*, 1985; Cheng *et al.*, 1993). Diluted sample (50 µl) was incubated with 0.6 mg ml⁻¹ o-phenylenediamine dihydrochloride in 50 mM Tris HCl (pH 8.0) containing 0.02% hydrogen peroxide for 5 min at room temperature and the reaction terminated by the addition of 4 M sulphuric acid (100 µl). The absorbance was read at 490 nm using a microtitre plate

¹ Author for correspondence.

reader (Molecular Devices, Menlo Park, CA, U.S.A.). Guinea-pig eosinophils were isolated on a Percoll gradient after harvest from the peritoneal cavities of animals which had been repeatedly injected with human serum. These preparations contained 100% eosinophils and $0.06 \times 10^3 - 3.0 \times 10^4$ cells per ml were included in each assay as a calibration curve. Neutrophils were harvested from the peritoneal cavities of guinea-pigs injected with casein and these preparations contained 92% neutrophils, 7% monocytes and 1% eosinophils.

Materials

Platelet activating factor (C-16), LTB_4 and 20-hydroxy- LTB_4 were purchased from Biomol, Plymouth Meeting, PA., U.S.A.; *o*-phenylenediamine dihydrochloride was from Eastman Kodak Co., Rochester, NY, U.S.A. SC-41930 was synthesized by Dr. Lawrence Reiter in the department of Medicinal Chemistry, Pfizer Inc., Groton, CT., U.S.A. All other reagents were purchased from the Sigma Chemical Company, St. Louis, MO, U.S.A.

Statistical analysis

The differences between drug-treated and control groups were analysed by ANOVA and considered significant when $P < 0.05$.

Results

Eosinophil peroxidase activities in guinea-pig eosinophils and neutrophils

The eosinophil peroxidase levels in guinea-pig eosinophils and neutrophils is shown in Figure 1. Guinea-pig eosinophils contain 34.8 times more eosinophil peroxidase activity than guinea-pig neutrophils. As the neutrophil preparation contained 1% contaminating eosinophils this is probably even a slight underestimate of the specificity of the enzyme for eosinophils versus neutrophils.

Effect of 3-amino-1,2,4-triazole on eosinophil peroxidase activity in guinea-pig skin sites injected with LTB_4

Eosinophil peroxidase activity was increased in guinea-pig skin sites injected with LTB_4 and this activity was reduced by 88.9% in the presence of 3-amino-1,2,4-triazole, a selective inhibitor of eosinophil peroxidase (Cramer *et al.*, 1984) (Figure 2).

Eosinophil peroxidase activity in guinea-pig skin sites after injection of LTB_4 , C5a and PAF

LTB_4 (10–300 ng) and recombinant C5a (1–10 μ g) caused a dose-dependent increase in the levels of eosinophil peroxidase activity in skin sites after injection into guinea-pig skin (Figure 3). LTB_4 and C5a were approximately equipotent on a molar basis and both induced an approximately 50 fold increase in the levels of eosinophil peroxidase over the saline control at the highest doses tested. In contrast, PAF caused a relatively low level of eosinophil infiltration compared to LTB_4 and C5a ($5.7 \times$ saline response at the highest dose tested, Figure 3).

Effects of 20-hydroxy- LTB_4 and SC41930 on eosinophil infiltration in response to LTB_4 and C5a

For inhibition studies with 20-hydroxy- LTB_4 , doses of LTB_4 (100 ng) and C5a (2 μ g) were chosen. Systemic treatment of guinea-pigs with 20-hydroxy LTB_4 inhibited eosinophil emigration in response to LTB_4 (100 ng) with an ED_{50} of 1.6μ g kg^{-1} (Figure 4). 20-Hydroxy- LTB_4 also dose-depen-

Figure 1 Eosinophil peroxidase activity in guinea-pig eosinophils (●) and neutrophils (○).

Figure 2 Effect of 3-amino-1,2,4-triazole on eosinophil peroxidase activity in guinea-pig skin sites. Skin sites were removed 4 h after injection of LTB_4 (100 ng) or saline and homogenized in 8 ml phosphate-buffered saline (pH 7.2) containing 0.5% hexadecyltrimethylammonium bromide and assayed for eosinophil peroxidase as described in the Methods. Activity in untreated samples is shown by open columns and activity in the presence of 3-amino-1,2,4-triazole (10 mM) is shown by hatched columns. Data are presented as mean $\pm$ s.e. mean ($n = 5$).

Figure 3 Eosinophil infiltration (as measured by eosinophil peroxidase activity) 4 h after intradermal injection of LTB_4 (●), human recombinant C5a (■) or PAF (▲) into guinea-pig skin. Each point represents the mean $\pm$ s.e. mean of data derived from 5–10 animals (* $P < 0.05$ vs saline control).

Figure 4 Effect of systemic treatment with 20-hydroxy-LTB₄ (given subcutaneously in 0.5 ml saline containing 0.25% bovine serum albumin) on eosinophil infiltration in response to LTB₄ (100 ng) (●) or human recombinant C5a (2 µg) (▲) at 4 h after intradermal injection. Results are expressed as a % of the untreated control responses which in absolute numbers were $412.0 \pm 65.8 \times 10^{-3}$ ($n = 10$) eosinophil equivalents for LTB₄ and $154.9 \pm 25.8 \times 10^{-3}$ ($n = 10$) eosinophil equivalents for C5a. Saline-injected sites contained $10.9 \pm 7.3 \times 10^{-3}$ ($n = 10$) eosinophil equivalents. Each point is the mean $\pm$ s.e. mean of data derived from 5–10 animals (* $P < 0.05$ versus vehicle-treated control animals).

Figure 5 Effect of systemic treatment with 20-carboxy-LTB₄ (10 µg s.c.) on eosinophil infiltration measured 4 h after intradermal injection of human recombinant C5a (2 µg). Data are the mean $\pm$ s.e. mean of values from 5 animals. Responses to saline are depicted by open columns and responses to LTB₄ are shown by hatched columns (* $P < 0.05$ versus control).

dently inhibited eosinophil responses to C5a (Figure 4). At a dose of 20-hydroxy-LTB₄ which completely inhibited responses to 100 ng LTB₄, responses to C5a were reduced by $56.5 \pm 1.8\%$ ($n = 5$). 20-Carboxy LTB₄ did not affect eosinophil responses to C5a (Figure 5).

The LTB₄ receptor antagonist, SC-41930, also reduced eosinophil infiltration in response to C5a (Figure 6). At the highest dose tested (10 mg kg⁻¹, p.o.) which caused $86.5 \pm 1.9\%$ inhibition of responses to 100 ng LTB₄, eosinophil infiltration in response to C5a was inhibited by $63.0 \pm 3.9\%$ ($n = 5$).

Discussion

We have confirmed the observations of Cheng *et al.* (1993) that eosinophil peroxidase is a selective marker for the

Figure 6 Effect of oral administration of the LTB₄ receptor antagonist, SC-41930, on eosinophil infiltration in response to LTB₄ (100 ng) (●) or C5a (2 µg) (■) in guinea-pig skin. Each point is the mean $\pm$ s.e. mean of data from 5 animals (* $P < 0.05$ versus responses in vehicle-treated animals).

presence of eosinophils in inflamed tissues and using this enzyme marker, we have compared the ability of various chemotactic factors to induce eosinophil infiltration after intradermal injection in guinea-pigs. The inflammatory mediators, LTB₄, C5a and PAF all elicited an increase in eosinophil peroxidase activity after intradermal injection in guinea-pigs. The peroxidase activity detected in this assay is likely to be predominantly due to the presence of eosinophils as reflected by the ability of a selective inhibitor of eosinophil peroxidase, 3-amino-1,2,4-triazole (Cramer *et al.*, 1984), to reduce peroxidase activity in skin sites by approximately 90%. However, based on data presented in this paper, neutrophils may contain low levels of eosinophil peroxidase-like activity and since these cells accumulate in skin sites in much higher numbers than eosinophils, a small contribution of neutrophil-derived peroxidase to the activity detected in skin sites cannot be excluded. LTB₄ and C5a were approximately equipotent on a molar basis, while PAF was much less active in this assay, confirming previous observations with these mediators in guinea-pig skin (Czarnetzki & Mertensmeier, 1985; Faccioli *et al.*, 1991) which suggests that the oedema producing activity of PAF may predominate over its effects on leukocyte activation at sites of inflammation. Invading neutrophils in inflamed tissues are a rich source of LTB₄ (Simmons *et al.*, 1983) which, in addition to its effects on neutrophils, is chemotactic for eosinophils and may therefore amplify neutrophil and eosinophil recruitment into skin sites injected with other chemotactic factors, particularly at late time points. In order to investigate the contribution of LTB₄ to eosinophil infiltration in response to C5a we have treated guinea-pigs with 20-hydroxy-LTB₄ which causes *in vivo* desensitization to the effects of LTB₄ (Pettipher *et al.*, 1993).

20-Hydroxy-LTB₄ reduced eosinophil infiltration in response to LTB₄ with a potency similar to its effect on neutrophil accumulation (Pettipher *et al.*, 1993). 20-Hydroxy-LTB₄ also caused a dose-related inhibition of eosinophil accumulation measured 4 h after injection of C5a. Doses of 20-hydroxy-LTB₄ which cause maximal desensitization to LTB₄ inhibited responses to C5a by over 50%. These data indicate that endogenously generated LTB₄ may contribute to eosinophil accumulation after injection of C5a, although other mediators which interact with the LTB₄ receptor such as 12(R)-HETE may be responsible for the observed effects. In terms of specificity controls, we have previously shown that 20-hydroxy-LTB₄ does not affect neutrophil infiltration in response to C5a (Pettipher *et al.*, 1993) and the structurally related metabolite of 20-hydroxy-LTB₄, 20-carboxy-LTB₄, did not inhibit eosinophil infiltration in response to

C5a. Furthermore, the LTB₄ receptor antagonist, SC-41930, also inhibited eosinophil infiltration in response to C5a and to much the same extent as 20-hydroxy-LTB₄. Taken together these data suggest that the observed effects of 20-hydroxy-LTB₄ can be interpreted as an effect involving LTB₄ receptors and is not due to general toxicity or some other nonspecific effect. The fact that 20-hydroxy-LTB₄ does not significantly affect the levels of myeloperoxidase in skin sites injected with C5a (Pettipher *et al.*, 1993) suggests neutrophils do not contribute significantly to the eosinophil peroxidase activity present in these inflamed skin sites.

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