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Cetirizine and levocetirizine inhibit eotaxin-induced eosinophil transendothelial migration through human dermal or lung microvascular endothelial cells

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Summary

Background Several second-generation antihistamines have documented anti-inflammatory effects which appear independent of H_1-receptor blockade. We investigated the inhibitory effect of cetirizine and its active enantiomer levocetirizine on eosinophil transendothelial migration (TEM) through monolayers of normal human dermal microvascular endothelial cells (HMVEC-d) or human lung microvascular endothelial cells (HMVEC-l).

Methods HMVEC-d or HMVEC-l were grown to confluence on micropore filters in transwells inserted into a 24-well tissue culture dish. Eosinophils were isolated by density gradient centrifugation and negative immunomagnetic selection. Untreated eosinophils or eosinophils pre-incubated (30 min at 37 °C) with a concentration range of cetirizine or levocetirizine (10^{-5} to 10^{-9} m) were added to the upper chamber of the transwell which was incubated for 60 min at 37 °C. Both spontaneous eosinophil TEM and TEM to 100 ng/mL of human eotaxin in the lower chamber were assessed.

Results Between 8 and 10% of the eosinophils added to the upper chamber underwent spontaneous TEM through HMVEC-d or HMVEC-l. The addition of eotaxin to the lower chamber enhanced eosinophil TEM through HMVEC-d or HMVEC-l monolayers to over 20%, i.e. an enhanced TEM of approximately 100% in each case. Pre-incubation of eosinophils with cetirizine or levocetirizine dose-dependently inhibited eosinophil TEM to eotaxin through both HMVEC-d or HMVEC-l with total inhibition of eotaxin-induced TEM observed at 10^{-8} m for HMVEC-d and 10^{-7} m for HMVEC-l. Both drugs gave a reduced but significant inhibition of eosinophil TEM at lower concentrations. No concentration of cetirizine or levocetirizine had any significant effect on expression of CD11b, CD18 or CD49d by either resting or eotaxin-stimulated eosinophils. Furthermore, no effect on spontaneous eosinophil TEM, or eosinophil viability was seen with any concentration of cetirizine or levocetirizine.

Conclusion Levocetirizine inhibits eotaxin-induced eosinophil TEM through both dermal and lung microvascular endothelial cells suggesting that, like cetirizine, levocetirizine has potential anti-inflammatory effects.

Keywords cetirizine, eosinophil, eotaxin, levocetirizine, transendothelial migration

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Introduction

Therapeutic intervention in allergy has often focused on blocking the effects of histamine release from mast cells and basophils, a major contributor to the allergic response. The second-generation H_1 antagonists have been developed over the past 15 years and have major advantages over the earlier drugs, most notably their lack of significant CNS and anticholinergic side-effects and are considered to be important therapeutic tools in the treatment of atopic disease [1, 2]. More recently, a number of novel antihistamines have been developed which are either metabolites of active drugs or enantiomers. The aim was to develop antihistamines with improved potency, duration and onset of action together with predictability and safety. Unlike the majority of currently available antihistamines, cetirizine undergoes minimal hepatic metabolism and is composed of two enantiomers: levocetirizine and dextrocetirizine. Levocetirizine has twice the affinity for the H_1 receptor compared to cetirizine and is a potent antihistamine as demonstrated by inhibition of histamine-induced weal and flare reactions [3] and in clinical studies [4].

Allergic inflammation is characterized by an immediate IgE-dependent mast cell and basophil degranulation which is associated with the release of mediators and cytokines including histamine, platelet-activating factor (PAF), IL-3, IL-4 and TNF-α. These cytokines are particularly important in the subsequent late-phase reaction that results in the accumulation of leucocytes at the sites of inflammation. Though a minor constituent of circulating leucocytes, eosinophils are often

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prominent in leucocyte infiltration as a result of their selective recruitment to the tissues [5] where they are thought to make a major contribution to allergic inflammation [6]. It can be appreciated, therefore, that antagonizing the effects of histamine only targets one aspect of allergic inflammation. A more effective approach to the treatment of allergic disease might therefore require the inhibition of cell migration, mediator release, adhesion and adhesion molecule expression.

In order for eosinophils to move from the circulation to the tissues, they must first adhere to the vascular endothelium by the binding of cell surface adhesion molecules followed by transmigration into the tissues under the control of inflammatory cytokines, e.g. IL-1, TNF-α, IL-4 [7–10]. Endothelial cells synthesize chemokines that activate and attract eosinophils. One such chemokine is eotaxin, a potent and specific eosinophil chemoattractant [11]. Although very little is known about the molecular mechanisms involved, eotaxin has been shown to up-regulate adhesion molecule expression on human nasal microvascular endothelial cells [12] and is known to stimulate eosinophil adhesion to human lung microvascular endothelial cells [13]. Many *in vitro* studies have used large vein-derived endothelial cells, i.e. human umbilical vein endothelial cells (HUVEC), to mimic the inflammatory adhesion and transendothelial migration (TEM) processes that occur in the capillaries [7–10]. The relevance of using HUVEC has been questioned because not only do macro- and microvascular endothelial cells show heterogeneity, there are also differences in microvascular endothelial cells of different origins [14].

Cetirizine has well-documented anti-inflammatory effects both *in vitro* and *in vivo* [15, 16], including the inhibition of PAF-dependent eosinophil chemotaxis and adhesion of eosinophils to HUVEC [17]. We therefore investigated the potential inhibitory effects of levocetirizine and cetirizine on spontaneous and eotaxin-induced eosinophil TEM through human microvascular endothelial cells of dermal and pulmonary origin.

Methods

Reagents

Normal human dermal microvascular endothelial cells (HMVEC-d) and human lung microvascular endothelial cells (HMVEC-l) and their recommended growth media (microvascular endothelial cell growth medium, EGM-2-MV), produced by Clonetics Inc. (Walkersville, MD, USA), were purchased from Biowhittaker (Berkshire, UK) as cryopreserved 3° or 4° passage cultures, respectively. Falcon 8-μm micropore cell culture inserts were obtained from Fred Baker Scientific (Runcorn, UK). Percoll was supplied by Pharmacia (Milton Keynes, UK) and the CD-16 immunomagnetic beads purchased from Miltenyi Biotec (Surrey, UK). Isotype-matched control, CD11b and CD18 mAbs were from Dako Ltd (Cambridgeshire, UK) and VLA4 mAb from Serotec Ltd (Kidlington, Oxon, UK). All were mouse IgG1 isotype. Human eotaxin was purchased from R & D Systems (Abingdon, UK). Levocetirizine and cetirizine were provided by UCB Pharma (Brussels, Belgium).

Eosinophil isolation

Blood (80 mL) was obtained from normal donors or individuals with a history of mild allergic disease with an eosinophilia not greater than 0.5×10^6 eosinophils/mL who were not taking any medication at the time of venesection and who gave informed consent. Eosinophils were purified using dextran sedimentation and centrifugation on Percoll gradients followed by CD16-dependent negative immunomagnetic selection as described [18]. Isolated eosinophils were > 99% pure with > 98% viability as judged by trypan blue exclusion.

Endothelial cell culture

Normal HMVEC-d or HMVEC-l were grown to confluence on 8-μm micropore filters in transwells inserted into the wells of a 24-well tissue culture dish. HMVEC-d and HMVEC-l, used at passage 5–6 and 6–7, respectively, became confluent within 10 days. The cells were fed every 2 days with EGM-2-MV and maintained in an atmosphere of 5% CO_2 at 37 °C.

Transendothelial migration assay

Eosinophils were either untreated or pre-incubated at 37 °C for 30 min with a concentration range of levocetirizine or cetirizine (10^{-5} to 10^{-9} M). Following a wash to remove either the levocetirizine or cetirizine, the eosinophils (2×10^5 per insert), were added to the upper chamber of the transwell. The lower chamber contained either medium or 100 ng/mL eotaxin. Plates were incubated at 37 °C for 60 min. After the 60-min incubation, 50 μL of 1 M EDTA were added to the upper chamber to prevent further transmigration. An additional 50 μL were used to rinse the underside of the transwell and the plates lightly tapped to dislodge any transmigrated eosinophils that had adhered to the underside. Transmigrated eosinophils were recovered from the wells, combined with a phosphate-buffered saline rinse of each well and the cells centrifuged at 300 ***g*** for 5 min. The eosinophils were then resuspended in 50 μL of 0.1% paraformaldehyde and incubated at 4 °C for at least 10 min. The fixed cells were then counted using a haemocytometer by an investigator blinded to the protocol. Percentage TEM was calculated as:

$$\frac{\text{Number of eosinophils recovered from the lower chamber}}{\text{Number of eosinophils added initially to the upper chamber}} \times 100$$

Effects of levocetirizine or cetirizine on eosinophil adhesion molecule receptor expression

Resting or eotaxin (100 ng/mL) stimulated eosinophils were pre-treated with increasing concentrations of levocetirizine or cetirizine. Immunostaining and flow cytometry were performed as previously described [19]. Briefly, treated and control eosinophils (2×10^5) were immunostained with the relevant antibodies or isotype-matched control at saturating concentrations for 40 min at 4 °C. Following a wash and staining with an appropriate fluorescein isothiocyanate (FITC)-conjugated secondary mAb, the cells were analysed on a flow cytometer (FACScan; Becton Dickinson, Franklin Lakes, NJ, USA) with eosinophils cells gated on the basis of their forward and side scatter profile with any cell debris excluded from analysis.

Effects of levocetirizine or cetirizine on eosinophil viability

Viability of untreated eosinophils and eosinophils treated with 10^{-5} M cetirizine or levocetirizine was determined by trypan blue exclusion and averaged 98% and 97%, respectively.

Furthermore, neither cetirizine or levocetirizine accelerated eosinophil apoptosis or necrosis as judged by annexin V binding or propidium iodide exclusion, respectively, quantified as described [20].

Statistical analysis

All data are presented as mean ± SEM and where *n* is given this represents the number of experiments each performed in duplicate. Statistical analysis was by the unpaired two-tailed Student's *t*-test where a *P*-value of < 0.05 was considered significant.

Results

Between 8 and 10% of the freshly isolated untreated eosinophils added to the upper chamber underwent spontaneous TEM through HMVEC-d or HMVEC-l. The addition of eotaxin to the lower chamber significantly enhanced eosinophil TEM through HMVEC-d or HMVEC-l monolayers to over 20%, respectively, i.e. approximately 100% in each case. Preliminary experiments demonstrated that both spontaneous and eotaxin-induced transmigration were optimal at 60 min of incubation. The effect plateaued at this time-point and greater TEM was not associated with longer incubation periods (data not shown). Eotaxin-induced dose-dependently increased eosinophil TEM that was maximal at an eotaxin concentration of 100 ng/mL (data not shown).

Pre-incubation of eosinophils with cetirizine or levocetirizine dose-dependently inhibited eosinophil TEM to eotaxin through both HMVEC-d or HMVEC-l. Cetirizine had its maximal effect on eotaxin-induced eosinophil TEM through HMVEC-d at a concentration of 10^{-8} M (Fig. 1) while, in contrast, eosinophil TEM through HMVEC-l was only completely inhibited at a drug concentration of 10^{-7} M although partial but significant ($P < 0.005$) inhibition was observed at 10^{-8} M (Fig. 2). Total inhibition of eotaxin-induced TEM was observed at a levocetirizine concentration of 10^{-8} M for HMVEC-d (Fig. 3) and 10^{-7} M for eosinophil TEM through HMVEC-l (Fig. 4). Partial but significant inhibition of eosinophil TEM through HMVEC-d by levocetirizine was seen at a concentration of 10^{-9} M

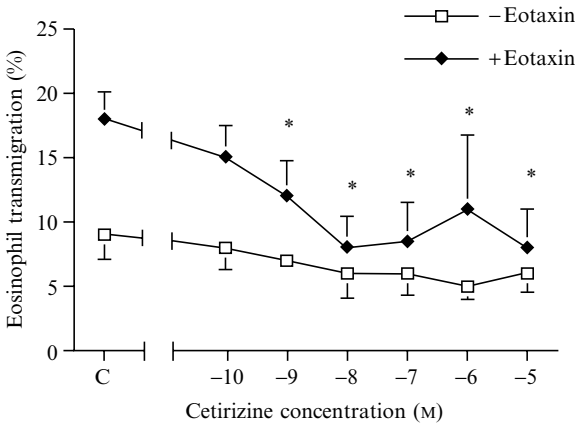

Fig. 1. The effect of increasing concentrations of cetirizine on spontaneous (□) and eotaxin-induced (♦) eosinophil TEM through monolayers of cultured HMVEC-d. Each point represents the mean ± SEM of at least four experiments (*$P < 0.05$).

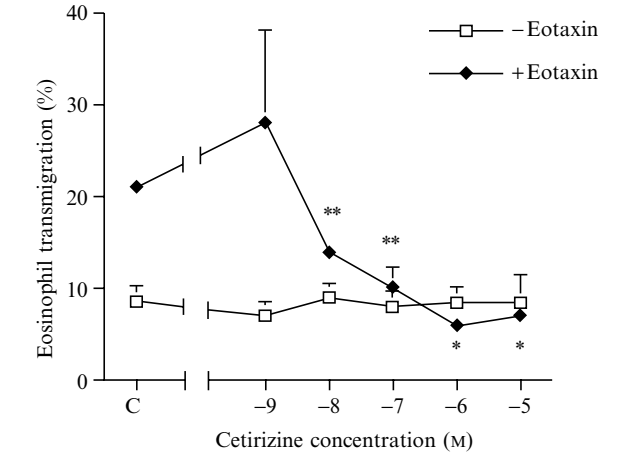

Fig. 2. The effect of increasing concentrations of cetirizine on spontaneous (□) and eotaxin-induced (♦) eosinophil TEM through monolayers of cultured HMVEC-1. Each point represents the mean ± SEM of at least four experiments (*$P < 0.05$, *$P < 0.005$).

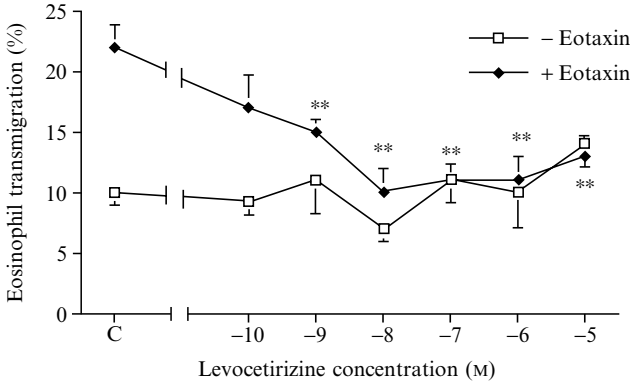

Fig. 3. The effect of increasing concentrations of levocetirizine on spontaneous (□) and eotaxin-induced (♦) eosinophil TEM through monolayers of cultured HMVEC-d. Each point represents the mean ± SEM of at least four experiments (**$P < 0.005$).

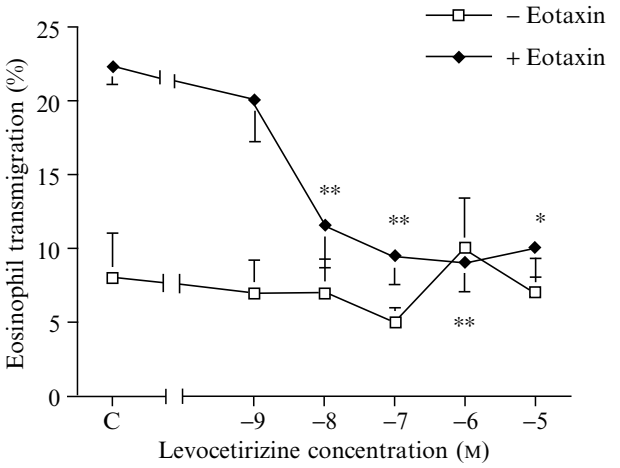

Fig. 4. The effect of increasing concentrations of levocetirizine on spontaneous (□) and eotaxin-induced (♦) eosinophil TEM through monolayers of cultured HMVEC-1. Each point represents the mean ± SEM of at least four experiments (*$P < 0.05$, **$P < 0.005$).

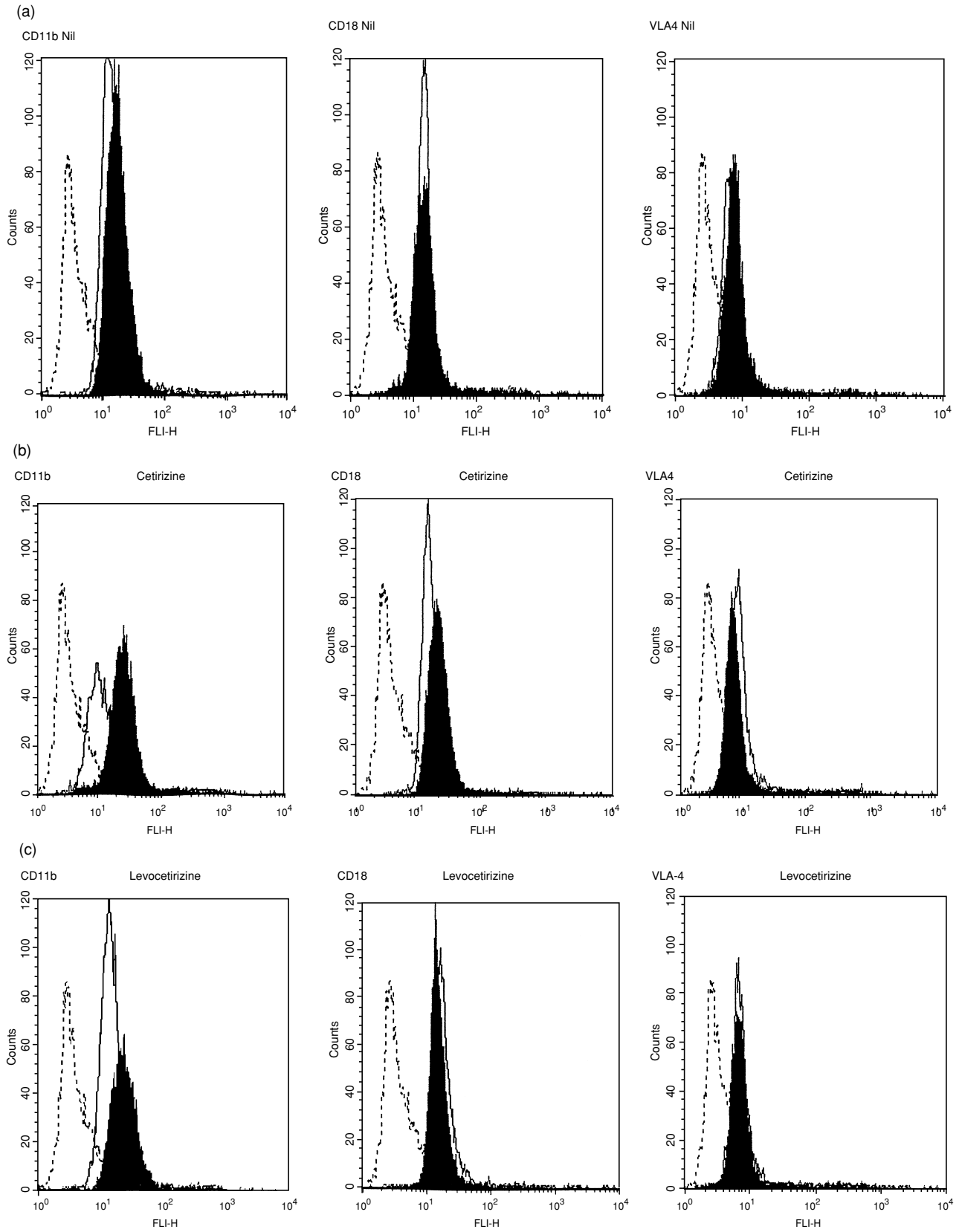

Fig. 5. Representative flow cytometry experiment showing expression of CD11b, CD18 and VLA-4 by resting (solid line) and eotaxin-stimulated (filled histogram) eosinophils (a). The dotted line represents the isotype-negative control mAb in each case. Neither cetirizine (b) nor levocetirizine (c) had any inhibitory effect on the expression of CD11b, CD18 or VLA-4. For simplicity, a single concentration of each drug (10^{-8} M) is shown. However, comparable data were obtained with a concentration range (10^{-5} to 10^{-10} M) of cetirizine or levocetirizine in three separate experiments.

($P < 0.005$, Fig. 3). The effect of cetirizine and levocetirizine on eotaxin-induced eosinophil TEM did not appear to be a consequence of an inhibitory effect on eosinophil adhesion molecule expression. We confirmed a previous report [21] that eotaxin stimulation (100 ng/mL) resulted in a modest increase in eosinophil expression of CD11b. Eotaxin had no effect on expression of CD18 or VLA-4. No concentration of cetirizine or levocetirizine had any significant inhibitory effect on expression of CD11b, CD18 or CD49d by either resting or eotaxin-stimulated (100 ng/mL) eosinophils (Fig. 5). Furthermore, we observed no significant differences in the inhibitory effects of either cetirizine or levocetirizine on TEM of eosinophils from either allergic or non-allergic donors. Neither cetirizine nor levocetizine had any significant effect on spontaneous eosinophil TEM through HMVEC-l or HMVEC-d. Moreover, even high non-physiological concentrations of either cetirizine or levocetirizine had no effect on eosinophil viability or apoptosis induction (data not shown).

Discussion

It has been recognized for some years that, in addition to being potent antihistamines, a number of the second-generation drugs appear to possess several antiallergic effects which cannot be explained by antagonism of the H_1 receptor [22, 23]. Thus, they may play an enhanced role in treating allergic disease if, in addition to antagonizing the effects of histamine, they also inhibit the influx and/or activation of pro-inflammatory cells. In general terms, these studies can be divided into *in vitro* effects [22] on the function of isolated pro-inflammatory cells or *in vivo* effects [24] where the impact of a given drug on inflammatory parameters is assessed in addition to its effects on symptom relief. The question of whether these additional attributes enhance the therapeutic effect of an antihistamine has been the subject of considerable debate [15, 16, 22–24]. It is difficult to give a definitive answer owing to the problems inherent in isolating the many anti-inflammatory effects from the H_1-blocking effect of a given drug. Notwithstanding this limitation, demonstrable suppressive effects on allergic inflammation, additional to potent H_1 blockade, must be seen as a positive attribute for a drug of this class.

Here we have demonstrated that cetirizine, together with its active enantiomer levocetirizine, exhibit potent *in vitro* inhibitory effects on eotaxin-induced eosinophil transmigration through monolayers of both lung and dermal microvascular endothelial cells. We observed somewhat greater inhibition by both cetirizine and levocetirizine of eotaxin-induced eosinophil TEM across HMVEC-d compared with HMVEC-l. These findings are interesting in the light of the fact that high concentrations of cetirizine have been reported in the skin of patients treated with the drug [25]. It is therefore tempting to speculate that cetirizine and levocetirizine have differential effects on eosinophil TEM across microvascular endothelial cells from different tissue sites. Furthermore, several studies demonstrated that cetirizine inhibited eosinophil accumulation at skin sites challenged with pollen or PAF [26–29]. These observations taken together with the *in vitro* findings presented here raises the possibility that cetirizine and levocetizine might inhibit eosinophil accumulation *in vivo* through an effect on transmigration at post-capillary venues.

We observed total inhibition of eotaxin-induced eosinophil TEM across both HMVEC-d and HMVEC-l at low and physiologically relevant concentrations of both cetirizine and levocetirizine. These findings support earlier *in vitro* observations showing that PAF-induced eosinophil hyperadherence to cultured HUVEC was significantly inhibited by cetirizine at concentrations as low as 10^{-9} M [17], while a separate study demonstrated that other adhesion-dependent eosinophil functions were also inhibited by pre-treatment of the cells with physiologically relevant concentrations of cetirizine [30]. These inhibitory effects at low drug concentrations are important findings, as many reported *in vitro* anti-inflammatory effects for many second-generation drugs were only achieved at concentrations several order of magnitude higher than could be achieved *in vivo* [22, 23]. Indeed, it could be argued that, in some *in vitro* studies which utilized very high antihistamine concentrations, the reported inhibitory effects were a consequence of drug toxicity. In this respect the present study demonstrated that high concentrations of either cetirizine or levocetirizine had no effect on the viability of the isolated eosinophils.

The precise point(s) at which cetirizine or levocetirizine inhibits the intracellular signalling pathways controlling eosinophil TEM through microvascular endothelial cells remain to be elucidated. The inhibitory effects exerted by each drug on eosinophil transmigration could not be attributed to a direct antagonistic effect on eotaxin-induced TEM as neither cetirizine nor levocetirizine are CCR3 antagonists (UCB Pharma, Brussels, Belgium, data on file). Furthermore, neither cetirizine nor levocetirizine appeared to exert their inhibitory effect via a direct effect of eosinophil adhesion molecule expression as no concentration of either drug had any significant effect on expression of CD11b, CD18 or CD49d by either resting or eotaxin-stimulated eosinophils. However, conformational changes in adhesion receptors are known to be important in activated leucocyte integrin-dependent interaction with endothelial cells [31]. Thus it cannot be ruled out that cetirizine or levocetirizine exert their effects via an inhibition of eotaxin-induced conformational changes in the integrins involved in eosinophil TEM. Furthermore, one study has demonstrated that low concentrations of cetirizine (1 μg/mL) induced a significant increase in the lipid order in the exterior part of the membrane, reduced eosinophil membrane heterogeneity and also blocked the PAF-induced changes in membrane fluidity in eosinophils [32]. Moreover, activation of the transcription factor nuclear factor kappa B (NF-κB) is a requirement for adhesion molecule expression, and low concentrations of cetirizine down-regulate NF-κB expression in a bronchial epithelial cell line [33]. Whether these effects contribute to the inhibitory effects of cetirizine or levocetirizine on eosinophil TEM remain to be elucidated.

In conclusion, we have demonstrated that levocetirizine inhibits eotaxin-induced eosinophil TEM through both dermal and lung microvascular endothelial cells at physiologically relevant concentrations suggesting that, like cetirizine, levocetirizine has potential anti-inflammatory effects. These extra anti-inflammatory effects may enhance the therapeutic effects of these drugs in the treatment of allergic disease.

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