

Are cysteinyl leukotrienes involved in allergic responses in human skin?

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Summary

Background Cysteinyl leukotrienes have been suggested to be involved in producing the symptoms of both the early and late phases of the allergic response in the lung and other tissues.

Objective To use scanning laser Doppler imaging, microdialysis and immunocytochemistry to explore the mediator and cellular mechanisms of the dermal allergic response.

Methods Thirteen atopic volunteers received intradermal injections into the forearm of grass pollen or *D. pteronyssinus* extract. Changes in dermal blood flow up to 8 h were monitored by scanning laser Doppler imaging. The release of histamine, PGD₂ and LTC₄/D₄/E₄ was assessed by dermal microdialysis. Skin biopsies were taken at 6 h to determine numbers of mast cells, eosinophils, basophils, Langerhans' cells, and monocytes/macrophages, and the expression of COX-1, COX-2, 5-LO and FLAP.

Results Allergen provocation produced an immediate weal and flare response followed by an erythematous induration peaking at 6 h. During the first hour, *c.* 84 pmoles of histamine and *c.* 0.3 pmoles of PGD₂ were recovered by microdialysis (both $P < 0.001$) but LTC₄/D₄/E₄ was undetectable. No histamine, PGD₂ or LTC₄/D₄/E₄ was detectable at later times. Immunocytochemical examination of biopsies taken at 8 h showed increased numbers of eosinophils and basophils and in COX-2, 5-LO and FLAP, but not COX-1. Expression of 5-LO and FLAP was associated primarily with eosinophils.

Conclusions These findings suggest that inflammatory cells recruited to the site of allergen injection are not activated to release detectable amounts of cysteinyl leukotrienes. Hence, it is unlikely that the late-phase erythematous induration is mediated by this autacoid.

Keywords 5-Lipoxygenase, allergen, cyclooxygenase-1, cyclooxygenase-2, EPR, FLAP, human, leukotriene C₄, LPR, prostaglandin D₂, skin

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Introduction

Immunological activation of human mast cells results in the generation of a plethora of pro-inflammatory mediators, including histamine, prostaglandin D₂ (PGD₂), leukotriene C₄ (LTC₄), proteases and proteoglycans. *In vivo*, the symptoms of the early-phase response (EPR) to allergen challenge largely depend upon the responsiveness of the constituent tissues of the organ in question to mast cell mediators. For example, a role for cysteinyl leukotrienes in allergen-induced bronchoconstriction is well established [1]. However, a role in mediating the weal and flare response in the skin is less obvious.

An early-phase release of histamine, prostaglandin D₂, cysteinyl leukotrienes and tryptase [2–4] from activated mast cells located in the upper dermis [5] has been demonstrated repeatedly in studies using skin chambers. Of these mediators, histamine is the major vasoactive agent, being both more efficacious [6, 7] and produced in greater quantities than the eicosanoids.

The relative molar concentrations generated by immunological challenge of human skin mast cells *in vitro* are 1000:25:2 for histamine, PGD₂ and LTC₄, respectively [8–10].

The LPR is seen macroscopically as a widespread erythematous and oedematous area, but the mechanisms underlying its development are unclear. Biopsies taken at the site of allergen challenge at the time of the LPR have shown increased numbers of neutrophils, eosinophils, basophils and lymphocytes [11]. Furthermore, studies using skin chambers have shown the generation of histamine and cysteinyl leukotrienes, but not PGD₂ or tryptase, at the time of the LPR [2, 3]. The observation that the rise in histamine is not accompanied by parallel rises in PGD₂ or tryptase [2, 3] suggests accumulating basophils are its source [11]. Similarly, the rise in cysteinyl leukotriene levels in the absence of accompanying PGD₂ and tryptase at this time [2] indicates that it is not mast cell derived. The identification of the leukotriene forming enzymes, 5-lipoxygenase (5-LO) and 5-lipoxygenase activating protein (FLAP), in both Langerhans' cells [12] and eosinophils [13, 14] make them possible sources of the cysteinyl leukotrienes. Thus, the temporal coincidence of histamine and cysteinyl leukotriene generation provides circumstantial evidence for their contributing to the symptoms of the dermal late response. In contrast, the failure of

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antihistamines [15] and tenidap sodium, a non-steroidal anti-inflammatory drug with weak lipoxygenase inhibitory activity [16], to modulate the late phase in the skin, provides some circumstantial evidence against the involvement of histamine and cysteinyl leukotrienes in its development.

To explore the mediator and cellular mechanisms of the early and late phases of the dermal response to allergen challenge, scanning laser Doppler imaging was used to monitor dermal blood flow [17], immunocytochemical staining of skin biopsies to determine cellular accumulation, and dermal microdialysis [18] to assess the release of histamine, PGD₂ and the cysteinyl leukotrienes (LTC₄/D₄/E₄).

Materials and methods

Study population

Thirteen atopic volunteers, eight male and five female, aged 21–44 years, took part in the study. Ethical approval was granted from Southampton and South-west Joint Research Ethics Committee and participants gave written informed consent. Exclusion criteria included subjects taking any drug with antihistaminic activity, non-steroidal anti-inflammatory drugs, corticosteroids or drugs that may affect the allergic response or the cardiovascular system. Subjects who suffered from dermatological or cardiovascular conditions were also excluded. Inclusion criteria included a positive skin test to extracts of either grass pollen or house dust mite (*Dermatophagoides pteronyssinus*).

Dermal provocation with allergen

An intradermal injection into the volar surface of the forearm of 20 µL containing 100 SQ units (equivalent to 30 biological units) of grass pollen or *D. pteronyssinus* extract (ALK, Hørsholm, Denmark) in phosphate buffered saline (PBS) was used to stimulate an allergic response in each subject. This dose was chosen following a preliminary study using intradermal injection of 3, 10, 30 and 100 SQ units that showed the highest dose to produce a consistently large area of indurated erythema at 8 and 24 h. PBS was used as a negative control. Injections were made 1 mm from and parallel to the microdialysis fibre (see later) using a 27 gauge needle and U-100 insulin syringe (Myjector, Terumo Europe NV, Leuven, Belgium).

Assessment of weal and flare responses

Changes in dermal blood flux were assessed using scanning laser Doppler imaging (SLDI, Moor LDI, Moor Instruments Ltd, Axminster, UK) as previously described [17]. Laser Doppler images were made by scanning an area of skin of 5 × 5 cm, giving *c.* 16 000 data points for analysis. The areas of the weal and flare responses were calculated from the stored images using the manufacturer's software. Our experience has shown that the changes in weal and flare areas may be measured to an accuracy of ± 0.05 cm² and changes in perfusion to ± 5% [17].

Assessment of mediator release

Two linear cutaneous microdialysis fibres of 2 kDa molecular mass cut-off, 216 µm diameter (Gambro model GFE 18, Gambro Dialysaten AG, Hechingen, Germany), were inserted, at least 10 cm apart, into the volar surface of one forearm of each

subject. They were inserted for a length of 20 mm at a depth of *c.* 0.7 mm under topical local anaesthesia (EMLA cream, 2.5%, prilocaine, 2.5% lignocaine, Astra Pharmaceuticals, Kings Langley, UK) as previously described [19]. A 2-h period was then allowed for recovery from local anaesthesia and trauma before the start of perfusion with sterile phosphate buffered saline (PBS) at a rate of 5 µL/min using a microinfusion pump (CMA/100, CMA/Microdialysis, Biotech, Luton, UK). To assess baseline mediator levels, dialysate was collected every 5 min for 30 min before the injection of allergen next to one fibre and PBS next to the other. To assess release of mediators during the EPR, dialysate collections were made every 5 min for 2 h after injection. To collect mediators released later, dialysis fibres were perfused for 30 min (3 × 10 min collections) at 2, 4, 6, 8, 10 and 12 h after challenge.

Histamine was measured using a spectrofluorimetric assay with a lower detection limit of approximately 25 nM [20]. PGD₂ was measured as its methoxime derivative using a commercial immunoassay with a detection limit of 3 pmol (Cayman Chemical, Ann Arbor, Michigan, USA). Total cysteinyl leukotrienes were measured using a commercial immunoassay that measures LTC₄ (100% efficiency), LTD₄ (100% efficiency) and LTE₄ (70% efficiency with a lower limit of sensitivity of 25 pM) (Amersham Biotrak, Amersham, UK).

To assess the absolute efficiencies for the dialysis of histamine, PGD₂ and total cysteinyl leukotrienes, *in vitro* studies were performed in which PBS was pumped at 5 µL/min through 20-mm lengths of dialysis fibre immersed in each mediator. Under these conditions, the absolute dialysis efficiencies for histamine, PGD₂ and total cysteinyl leukotrienes were approximately 50%, 20% and 20%, respectively.

Determination of cellular infiltration

Eight hours after challenge, the areas around the allergen and PBS injections were infiltrated with 2% w/v lignocaine local anaesthetic and a 3-mm punch biopsy taken (Steifel Laboratories, Woburn Green, UK). Each biopsy was divided into four, in order to obtain pieces small enough for the satisfactory infiltration of fixative, and fixed overnight in ice cold acetone containing the enzyme inhibitors iodoacetamide (20 mM) and phenyl methyl sulphonyl fluoride (PMSF, 2 mM). Specimens were then embedded in glycolmethacrylate (JB4 kit, Park Science Ltd, Northants, UK) as described previously [21] with the exception that the embedding time was extended to 7½ hours because of the dense nature of skin. Sections of 2-µm thickness were cut and stained immunocytochemically using biotinylated mouse anti-mouse F(ab)₂ (Dakopatts) as the secondary antibody as described previously [22]. The following antibodies were used for: mast cell tryptase, AA1 [23]; eosinophils, EG2 (Pharmacia & Upjohn, Uppsala, Sweden), basophils, antibody BB1 [24]; Langerhans' cells, antibody NA 1/34 (Dako, Ely, UK); CD68⁺ cells, antibody PG-M1 (Dako); COX-1, a mouse monoclonal IgG_{2b} antibody against purified ovine COX-1 protein that reacts with human COX-1 (Cayman Chemical); COX-2, a mouse monoclonal IgG₁ antibody raised against a KLH-conjugated synthetic peptide corresponding to amino acids 580–589 (NASSRSGLDDINPTVLLK) of human COX-2 (Cayman Chemical). For 5-LO and FLAP, rabbit polyclonal antibodies LO-32 and H4, respectively, were used (generous gifts from Dr Jilly Evans, Merck & Co., West Point, PA,

USA). Antibody LO-32, raised against purified human 5-LO, does not cross-react with 12-LO or 15-LO [25]. Antibody H4 is a rabbit IgG raised against a synthetic peptide corresponding to amino acids 41–52 of human FLAP [25, 26].

Cell counting was performed as previously described [22]. Briefly, immunocytochemically positive nucleated cells were counted in three high power fields and their numbers expressed as cells/mm², the area of dermis having been measured using semiautomated image analysis with Colorvision 1.7.4 software (Improvision, Coventry, UK). Co-localization studies were performed on adjacent sections using the camera lucida.

Statistics

Areas of the weal and flare responses and concentrations of mediators are expressed as mean $\pm$ standard error of the mean (SEM). The significance of differences was tested using Student's *t*-test for paired data. Cell numbers are expressed as medians (with 95% confidence limits) and the significance of differences tested using Wilcoxon's signed rank test.

Results

Dermal responses

Intradermal injection of allergen produced a dual response in 12 of the 13 individuals studied. Immediately following allergen

injection, a weal and flare reaction developed that reached a maximum at 10 min (Fig. 1) when the mean areas ($\pm$ SEM) of the weal and flare responses were 2.3 ± 0.8 cm² and 37.3 ± 4.0 cm², respectively (Fig. 2). Following a period during which the weal regressed and the area of erythema reduced in size, a late reaction developed, seen as an erythematous induration without a weal. This response reached a maximum at 6 h when its mean area was 29.4 ± 3.1 cm².

Mediator release

Histamine recovered by microdialysis was detected in all 13 subjects. The mean concentration of histamine in the dialysate rose from a baseline of 91 ± 22 nM to 665 ± 124 nM ($P < 0.001$) in the first 15 min sample collected following challenge (Fig. 3). Levels then fell slowly, so that by 40 min the concentration had returned to baseline levels. There was no detectable rise in histamine concentrations during the late-phase response.

Because of the larger volume of dialysate required for their assay, samples were pooled to give hourly estimates of PGD₂ in seven of the subjects and cysteinyl leukotriene levels in the remaining six. During the first hour after allergen challenge, the concentration of PGD₂ rose from a mean baseline level of 50 ± 10 pM to 1026 ± 279 pM (Fig. 4). After this, levels fell slowly to reach baseline again by 5 h. No rises in PGD₂ levels were seen at the time of the late response. At no time during the

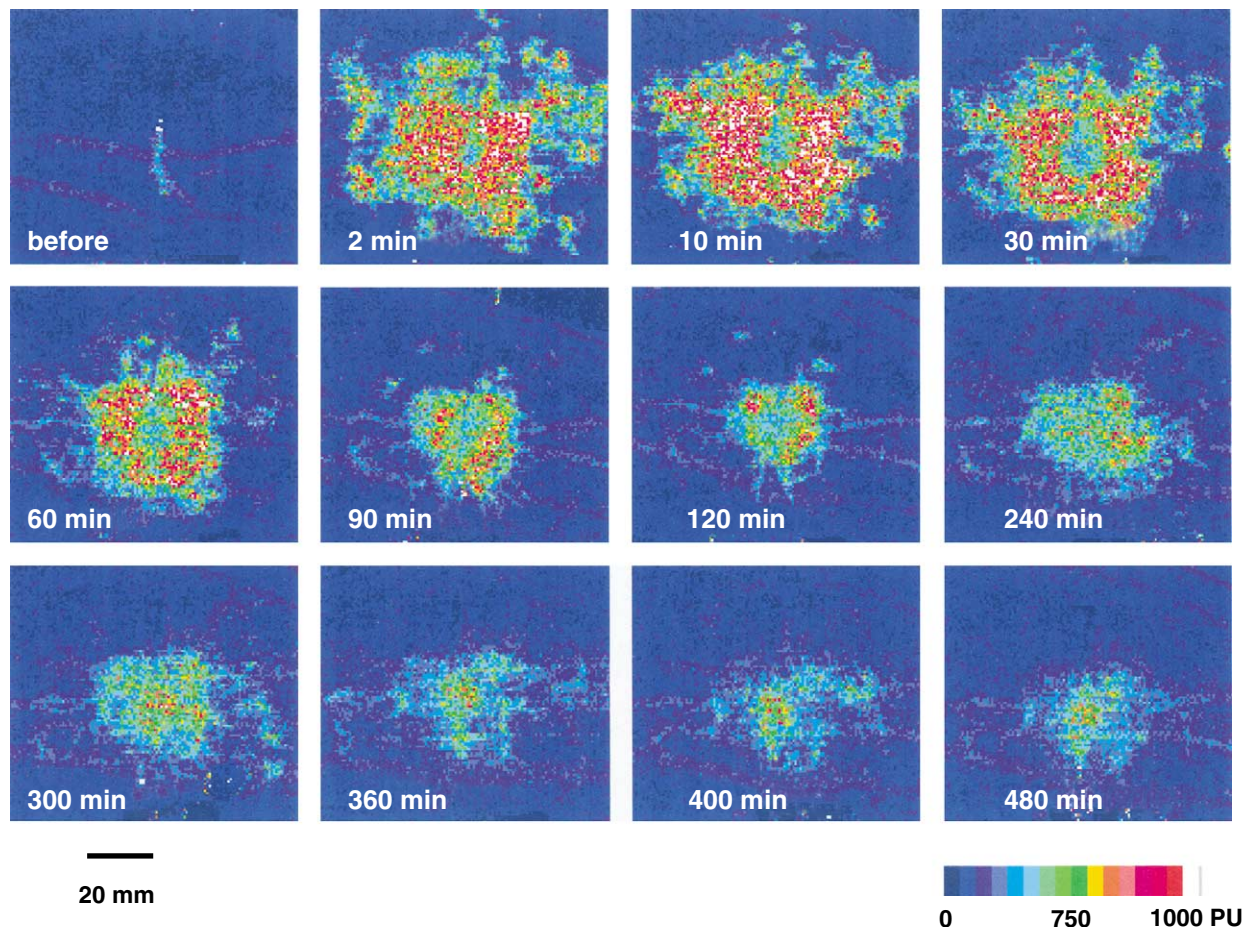


Fig. 1. Scanning laser Doppler images of the volar surface of the forearm before and up to 8 h after allergen challenge.

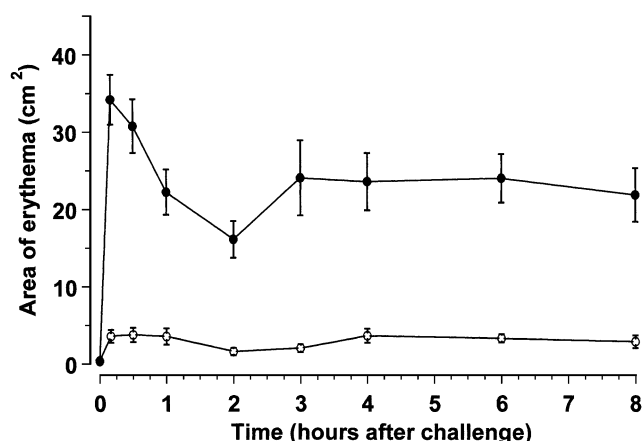


Fig. 2. The area of erythema estimated from scanning laser Doppler images of the volar surface of the forearm of 13 subjects before and up to 8 h after allergen challenge (solid circles) or saline (negative control, open circles).

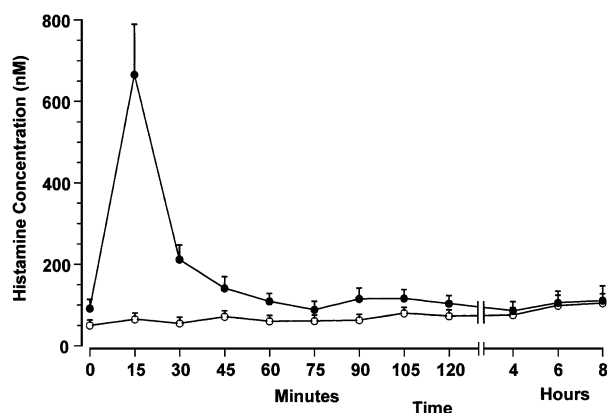


Fig. 3. Histamine levels in microdialysis fluid from 13 subjects before and up to 8 h after allergen challenge (solid circles) or saline (negative control, open circles). Dialysate was collected every 15 min for 2 h and then for three 10-min periods at 2, 4, 6 and 8 h after challenge.

8 h after allergen challenge did the levels of total cysteinyl leukotrienes rise above the lower limit of detection for the assay.

Biopsy studies

Biopsies were obtained from seven individuals at 8 h after challenge for analysis of cellular influx and the expression of eicosanoid forming enzymes. Comparison of saline control and allergen challenged sites showed that there were no significant changes in the numbers of mast cells, Langerhans' cells or CD68⁺ monocytes. The median numbers of these cells (with 95% confidence limits) at control and allergen sites were: mast cells 7 (3 to 35) and 11 (4 to 26) cells/mm² ($P=0.673$); Langerhans' cells 25 (5 to 49) and 18 (6 to 43) cells/mm² ($P=1.000$); and CD68⁺ monocytes, 32 (7 to 54) and 54 (20 to 85) cells/mm² ($P=0.353$). In contrast, the number of eosinophils rose eight-fold from 8 (0 to 19) to 68 (31 to 115) cells/mm² ($P=0.022$) and basophils 16-fold from 1 (0 to 2.3) to 16 (9 to 34) cells/mm² ($P=0.022$) (Fig. 5).

Figure 6 shows that there was no significant change in the number of cells immunocytochemically positive for COX-1, the medians in control and allergen challenged sites being 25 (5 to 44) and 20 (11 to 29) cells/mm², respectively ($P=0.993$). There

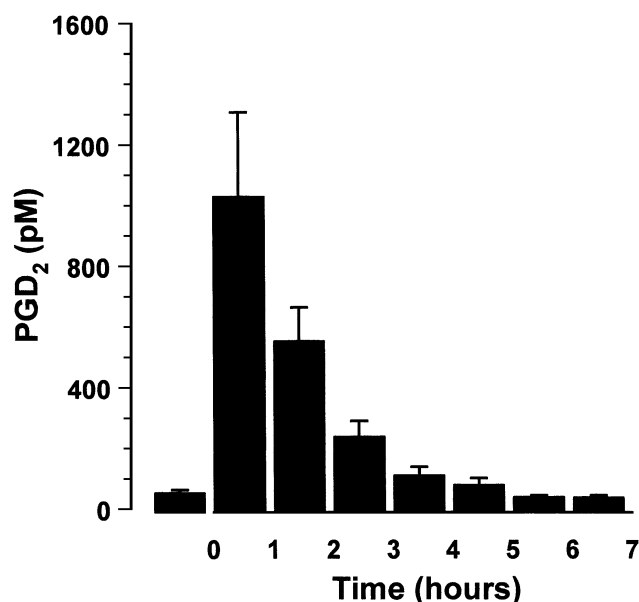


Fig. 4. PGD₂ levels in microdialysis fluid from seven subjects before and up to 8 h after allergen challenge. Dialysate was collected every 5 min for 2 h and then for three 10-min periods at 2, 4, 6 and 8 h after challenge. Samples were pooled for each hour to ensure that eicosanoid levels were above detectable limits.

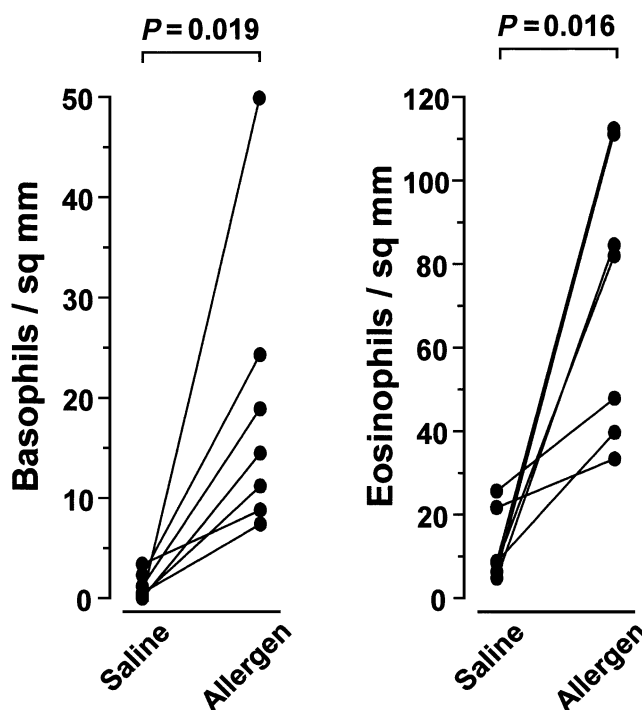


Fig. 5. Basophils and eosinophils in biopsies from the volar surface of the forearm of seven subjects 8 h after allergen challenge or saline injection (negative control). Basophils were detected using antibody BB1 and eosinophils detected using EG2. Significances were calculated using the Wilcoxon Signed Rank test.

were very few COX-2 positive cells in control biopsies, the median being 1.0 (0.5 to 2) cells/mm². Eight hours after allergen challenge, the median had risen eightfold to 8 (2 to 16) cells/mm² ($P=0.035$) (Fig. 6).

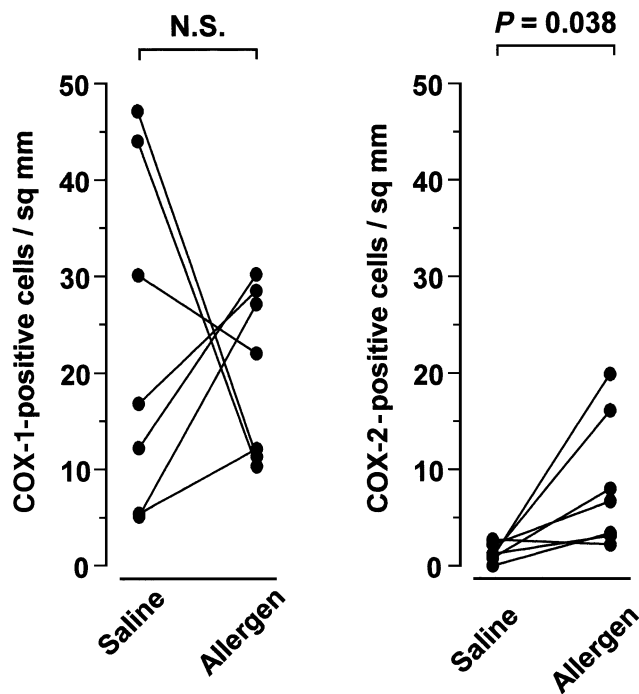


Fig. 6. COX-1 and COX-2 positive cells in biopsies from the volar surface of the forearm of six subjects 8 h after allergen challenge or saline injection (negative control). COX-1 and COX-2 positive cells were detected using specific monoclonal antibodies as described in the methods section. Significances were calculated using the Wilcoxon signed rank test.

Analysis of the leukotriene forming enzymes showed that there were significant rises in the expression of 5-LO and FLAP, the median number of cells showing immunoreactivity for these enzymes in control and allergen challenged biopsies being 30 (3 to 57) and 78 (58 to 115) cells/mm² ($P = 0.035$) for 5-LO and 39 (6 to 72) and 87 (59 to 113) cells/mm² ($P = 0.036$) for FLAP (Fig. 7).

Co-localization using the camera lucida (Fig. 8) showed that in control biopsies 5-LO and FLAP immunoreactivity was associated with mast cells, eosinophils, monocytes and Langerhans' cells. Following allergen challenge, the association of the enzymes with mast cells and Langerhans' cells fell marginally, while the number of eosinophils expressing them rose 10-fold.

Discussion

Allergen provocation of the skin produced an immediate weal and flare response that was accompanied by the rapid release of histamine and a more prolonged generation of PGD₂. Levels of cysteinyl leukotrienes remained below detectable limits. A late response, seen as a large area of erythematous induration, developed more slowly and reached a maximum at 6 h. Histamine, PGD₂ and cysteinyl leukotrienes were undetectable at this time. Immunocytochemical examination of biopsies taken at 8 h showed statistically significant increases in the number of eosinophils and basophils and in the number of cells staining positive for COX-2, 5-LO and FLAP, but not COX-1. Co-localization studies showed 5-LO and FLAP to be associated

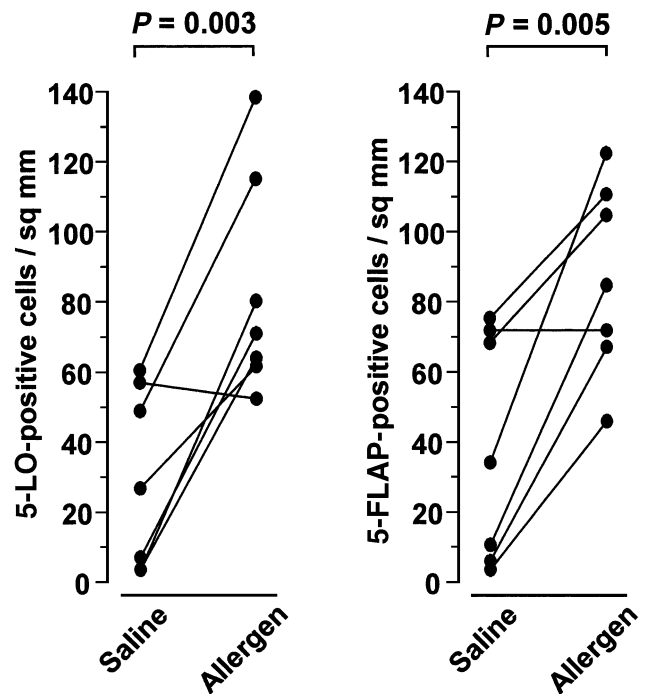


Fig. 7. 5-Lipoxygenase and FLAP positive cells in biopsies from the volar surface of the forearm of six subjects 8 h after allergen challenge or saline injection (negative control). 5-LO and FLAP positive cells were detected using rabbit polyclonal antibodies as described in the methods section. Significances were calculated using the Wilcoxon signed rank test.

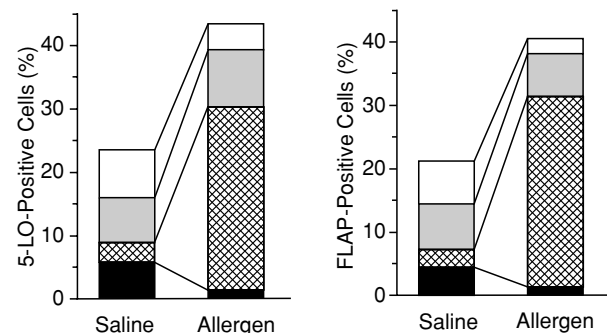


Fig. 8. Co-localization of 5-lipoxygenase and FLAP to Langerhans' cells, monocytes/macrophages, eosinophils and mast cells in biopsies from the volar surface of the forearm of six subjects 8 h after allergen challenge or saline injection (negative control).

primarily with eosinophils recruited as a consequence of the allergic response.

Visual examination of the skin over the 6 h following allergen challenge showed two distinct dermal responses. The first, the weal and flare response, was seen as a central area of oedema surrounded by a neurogenic flare [18]. This response was maximum at 10 min and resolved slowly after that time. The second, and visually quite different, response was an area of erythematous induration that developed more slowly and reached a maximum at about 6 h. The different appearance and time course of these responses suggest that, although both are initiated by allergen, their development may be independent.

During the first hour after challenge, *c.* 84 pmoles of histamine and *c.* 0.3 pmoles of PGD₂ were recovered by dermal microdialysis, giving a ratio of approximately 270:1. This

may be compared with a histamine:PGD₂ ratio of approximately 40:1 in the supernatant of human skin mast cells, dispersed from healthy foreskin, following challenge with anti-IgE *in vitro* [9]. That the ratio *in vivo* is higher than that observed *in vitro* may be explained in part by the 2.5-fold lower efficiency of PGD₂ dialysis compared with that of histamine and partly by the protein binding and rapid metabolism of PGD₂ in the *in vivo* environment [27].

Levels of cysteinyl leukotrienes recovered by microdialysis were below detectable limits during the early-phase response. This may not be surprising considering that studies *in vitro* have shown that the amount of LTC₄ measured in the supernatant following anti-IgE challenge of human skin mast cells is 10 to 20 times lower than that of PGD₂ [9]. That the failure to detect cysteinyl leukotrienes was not purely a technical problem is supported by our observation that, using an identical allergen challenge protocol in subjects with atopic dermatitis, relatively large amounts of LTC₄/D₄/E₄ were recovered by microdialysis during the early-phase response [28].

Microscopical examination of biopsies showed sevenfold and 17-fold increases in the numbers of eosinophils and basophils, respectively, at the site of allergen injection 8 h after challenge. These observations confirm previous studies [11, 29]. The observation that the increases in 5-LO and FLAP immunoreactivity in the late response were associated predominantly with eosinophils matches closely changes observed in bronchial mucosal biopsies of atopic asthmatics following exposure to environmental allergen, which were linked to deterioration in lung function [30]. Nasal and bronchial biopsy studies have also demonstrated modest increases in COX-2, but not COX-1, after allergic and viral stimuli [31].

An association between inflammatory mediators, particularly histamine and cysteinyl leukotrienes, and the LPR stems from the plethora of studies utilizing allergen challenge of mucosal surfaces of the eye [32], nose [33] and lung [34], in which inflammatory cells migrate onto the tissue surface where they become activated to release their mediators. Similar elevations of histamine have been found using skin chambers or windows, which mimic mucosal membranes in that inflammatory cells may migrate on to the surface of the denuded dermis where they are activated by the allergen in the chamber [4, 11, 35]. However, this study utilized the focal injection into the dermis of a small volume (20 µL) of allergen that can diffuse laterally for a maximum distance of 2 to 3 mm from the site of injection [18]. While this was sufficient to attract increased numbers of basophils and eosinophils and therefore an increased potential for the generation of these mediators into the extracellular environment, microdialysis fluid levels of histamine remained at baseline and cysteinyl leukotrienes below the limit of detection throughout the period of the LPR. Interestingly, even in atopic dermatitis, where relatively large amounts of cysteinyl leukotrienes are detected during the early phase response, none were detected during the late phase [28]. Further, the ability of the immunoassay to detect LTE₄ with 70% of the efficiency with which it detects LTC₄ and LTD₄, means that leukotriene metabolism cannot be used as an explanation for our inability to detect them. This finding strongly suggests that, although inflammatory cells are being recruited to the site of the initial allergen injection, they are not becoming activated to release their mediators, possibly because the allergen has been cleared from the tissue by this time.

In conclusion, as relatively large concentrations of cysteinyl leukotrienes are required to induce weal and flare responses in the skin [36, 37] it is unlikely that the mediators play a role in the early-phase allergic response in asymptomatic atopic individuals. Furthermore, it is unlikely that the erythematous induration, which characterizes the macroscopical appearance of the late-phase allergic response in the skin, is caused by the classical mast cell, basophil or eosinophil derived autocrine mediators, including cysteinyl leukotrienes. Alternative mechanisms need to be considered.

Acknowledgements

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