CRTH2 is a prominent effector in contact hypersensitivity-induced neutrophil inflammation

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Abstract

Chemoattractant receptor-homologous molecule expressed on Th2 lymphocytes, CRTH2, is a cognate receptor for prostaglandin (PG) D2 and, in humans, is suggested to play a functional role in Th2-dependent allergic inflammation. While peripheral blood leukocytes expressing high levels of surface CRTH2 have been detected in disease, little is known of the functional significance of CRTH2 in disease etiology. We have utilized a Th2-dependent murine model of FITC-induced contact hypersensitivity to assess the role, if any, CRTH2-PGD2 may play in the elicitation or maintenance of such pathobiology. Expression of both PGD2 and CRTH2 in lesional skin was paralleled by the release of the chemoattractants LTB4 and the chemokine KC, as well as a profuse dermal neutrophilic and eosinophilic infiltrate, closely paralleling the acute inflammatory pathology observed in human atopic dermatitis. A small molecule CRTH2 antagonist, but not a selective PGD2R (DP) receptor antagonist, was able to completely abrogate these responses. Inflammatory cascades mediated by CRTH2 ligation may therefore represent an important early step in the elicitation and maintenance of Th2-dependent skin inflammation.

Introduction

Prostaglandin D2 (PGD2), a product of the arachidonic acid/cyclooxygenase pathway, is generated at sites of inflammation and is believed to play an important regulatory role in inflammatory and immune responses. Being predominantly released by activated mast cells (1), PGD2 has a prominent role in allergic reactions (2–4) due to its ability to induce leukocyte extravasation and by acting as a chemoattractant for Th2 cells, eosinophils and basophils (5–8).

To date, two different G-protein-coupled receptors for PGD2 are known: the D prostanoid receptor, DP (9), and the more recently described chemoattractant receptor-homologous molecule expressed on Th2 lymphocytes, CRTH2 (5,10,11). In addition, PGD2 has been reported to interact with the thromboxane A2 receptor, TP (12). There is, in addition, the possibility that PGD2 can be non-enzymatically converted to other bioactive molecules such as 15-deoxy-

Δ12,14-PGJ2 (15d-PGJ2) and some effects of PGD2 might be explained indirectly by interaction of 15d-PGJ2 with nuclear receptors of the peroxisome-proliferator-activated receptor (PPAR) family (13,14). However, current evidence suggests that the levels of this metabolite found in vivo are substantially lower than the threshold levels required to activate the PPARs in vitro, raising the question of whether this pathway is relevant (15).

Through the activation of the DP receptor, PGD2 signals cAMP elevation and has been implicated in diverse physiological mechanisms such as sleep induction, apoptosis, mucus production, or ocular hypotension (16–19). In contrast, the biological functions of CRTH2 are much less defined. In certain human cells, activation of CRTH2 leads to a decrease in cAMP levels (19,20) and triggers a pertussis-sensitive Ca2+ signal (5,7), suggesting that CRTH2 couples to a G protein of the Goi/o class.

Although the tissue expression profiles seem to differ between mouse (11) and man (19), the high expression levels of CRTH2 in Th2 lymphocytes, basophils and eosinophils in both species imply a major role of CRTH2 in allergic diseases (7,8,10,21,22). Indeed, it has been shown that CRTH2 levels on circulating T cells correlate with the severity of atopic dermatitis (23,24), and recently it was demonstrated that...
CRTH2, but not DP or TP, is responsible for PGD2-induced chemotaxis of Th2 lymphocytes, eosinophils, basophils and monocytes (6–8,25). 13,14-dihydro-15-keto-PGD2 (DK-PGD2) (25) is a selective CRTH2 agonist without activating DP up to 30 μM (7), and has been useful in characterizing CRTH2 effects in cellular systems compounded by the presence of DP, although DK-PGD2 does weakly bind DP (26). Recent studies have illustrated that the biological activity of CRTH2 may differ between human and rodent leukocytes (26); however in vivo evidence for direct signal transduction through CRTH2 in mouse or rat leukocyte subpopulations has thus far been difficult to prove in our hands. We were therefore interested in a more detailed determination of the biological effects mediated by CRTH2 as a correlate to determinations of in vivo efficacy of small molecule, specific CRTH2 inhibitors in murine model systems. As an initial investigation we directly injected DK-PGD2 into mouse skin. The ensuing inflammation observed has been further characterized using an FITC-painted skin model for contact hypersensitivity, and the effects of the small molecule antagonist Ramatroban/BAY u3405 determined (8). Our studies indicate that CRTH2 activation may play a significant role in Th2-dependent inflammatory skin disorders, and that this effect is certainly inhibitable by specific antagonists.

Methods

Chemicals and reagents

Ramatroban [(+]-(3R)-3-(4-fluorobenzenesulfonylamo)-1,2,3,4-tetra-hydrocarbazole-9-propanonic acid] was synthesized at Bayer Yakuhin Ltd. (Shiga, Japan), ridogrel [(E)-5-[[[(3-pyridinyl)(3-(trifluoromethyl)phenyl)methylene]amino]oxy] pentanoic acid] was prepared by Bayer AG (Wuppertal, Germany). Bayer Yakuhin Ltd. (Shiga, Japan), ridogrel [(E)-5-[[[(3-pyridinyl)(3-(trifluoromethyl)phenyl)methylene]amino]oxy] pentanoic acid] was prepared by Bayer AG (Wuppertal, Germany). BWA868C was synthesized by SOGO Pharmaceutical Co. Ltd (http://www.sogo-pharma.co.jp/index.html). PGD2 was from Sigma-Aldrich (St Louis, MO), DK-PGD2 was purchased from Cayman (Ann Arbor, MI). The following set of antibodies was used for staining histological skin sections: affinity purified anti-mouse Ly-6G (Gr-1) (clone: RB6-8C5, rat IgG2b, CA), purified anti-mouse Ly-6C (clone: A3-1, rat IgG2b, BMA Biochemicals AG, Augst, Switzerland), polyclonal goat anti CD3-e (M-20) (Santa Cruz Biotechnology, Santa Cruz, CA), purified anti-mouse F4/80 (clone: A3-1, rat IgG2b, BMA Biochemicals AG, Augst, Switzerland), anti-mouse Mac-3 (clone: M3/84, rat IgG1, K, BD Pharmingen, San Diego, CA), purified anti-mouse CD8a (Ly-2) (clone: 53–6.2, rat IgG2a, K, BD Pharmingen), purified anti-mouse CD45R/B220 (clone: RA3–6B2, rat IgG2a, K, BD Pharmingen), purified anti-human CD4 and CD8 (clone: L200, mouse IgG1, BD Pharmingen), purified anti-mouse CRTH2 mRNA, mouse skin was harvested after dorsal injection of PBS or DK-PGD2 and homogenized. For detection in leukocytes, spleen CD4+, CD8+ T and B lymphocytes were separated by using the mouse CD4, CD8 and B cell negative selection kit and MACS system according to the manufacturer’s instructions (Miltenyi, Bergisch Gladbach, Germany). Monocytes were separated by adhesion after incubation of splenocytes in RPMI 1640 supplemented with 10% FBS for 2 h. For isolation of neutrophils, anti-Gr-1 antibody (PharMingen) positive cells were separated from peripheral blood. The purity of each separated population of leukocytes was >95%. Total RNA was isolated from skin homogenates or leukocytes using RNAzol. The first strand cDNAs were synthesized with random hexamer primers and Superscript™ II RNase H-reverse transcriptase (Invitrogen, San Diego, CA) and the PCR reactions were carried out with the primers (synthesized by Nihon Gene Laboratories, Sendai, Japan) as follows: mouse CRTH2, 5'-CATGGCCAACGT-CACACTGAAGC-3' and 5'-GAG-GCTCAGCCCGCATGAC-3'; mouse b2 microglobulin as a standard, 5'-GAG-AATGGGAAGCCGAACA-3' and 5'-CTTTGTCGATGGTGGGTGTC-3'. For each PCR reaction, the samples were first denatured at 95°C and amplified by 35 cycles of PCR (95°C, 1 min: 65°C, 0.5 min; 72°C, 1 min).

Immunopharmacological studies

For Ab treatment, mice received an intravenous injection of 100 μg per mouse of anti-KC antibody plus Control Ig (rat

Mice

Female Balb/c mice were obtained from JCR (Charles River, Kanagawa, Japan), maintained under specific pathogen-free conditions, and used at 7–8 weeks of age. WB6F1-W/- mice and WB6F1+/+ mice were purchased from SLC (Shizuoka, Japan). Animals were kept under standard conditions in a 12 h day/night rhythm with free access to food and water ad libitum. All animals received humane care and the studies have been approved by the internal ethic committee in accordance with the guidelines recommended by JALAS (Japanese Association of Laboratory Animal Science).

Induction of contact hypersensitivity (CHS)

For FITC sensitization, 400 μl of 0.5% FITC dissolved in acetone: dibutyipthalate (1:1, DBP) were painted onto the shaved abdominal skin on days 0 and 1. Six days later (day 6), mice were challenged by applying 20 μl of 0.5% FITC onto both sides of the right ear.

The ear thickness of all mice was measured by an engineer's micrometer (Mitutoyo, Japan). Animals were challenged on the right ear with FITC in DBP. DBP alone was applied to the left ear as a solvent control. Challenge-induced increases in ear thickness were measured at various time points thereafter. The CHS response was calculated as a function of challenge-induced increases in ear thickness according to the following formula: Increase in ear thickness = [(right ear thickness post challenge – left ear thickness post challenge) – (right ear thickness pre challenge – left ear thickness pre challenge)].
activity was developed using 3,3¢-biotin complex±HRP (DakoCytomation) for 30 min. Enzyme in methanol for 30 min, and then treated streptavidin±embedded in parafﬁn. The parafﬁn blocks were cut into 2 µm sections for hematoxylin and eosin staining, and cut into 4 µm sections for direct fast scarlet (DFS) stain and immunohistochemistry. To determine the presence of eosinophils, the sections were deparaffinized, rehydrated and treated with DFS solution (0.2% DFS, 0.8% Na2SO4 in 50% 2-propanol) for 5 min, and then were washed with tap water for 5 min. After overnight incubation in 50% 2-propanol to remove background staining, the samples were counterstained with Mayer’s hematoxylin. For immunohistochemistry, the sections were deparaffinized, rehydrated and treated with 1% BSA for 30 min at room temperature, and then diluted antibodies were applied. After overnight incubation at 4°C, biotinylated anti-mouse or anti-goat immunoglobulins (DakoCytomation Co. Ltd, Glostrup, Denmark) was applied to the samples for 30 min at room temperature, and then diluted antibodies were applied. After overnight incubation at 4°C, biotinylated antimouse or anti-goat immunoglobulins (DakoCytomation Co. Ltd, Glostrup, Denmark) was applied to the samples for 30 min at room temperature, and then diluted antibodies were applied. After overnight incubation at 4°C, biotinylated anti-mouse or anti-goat immunoglobulins (DakoCytomation Co. Ltd, Glostrup, Denmark) was applied to the samples for 30 min at room temperature, and then diluted antibodies were applied. After overnight incubation at 4°C, biotinylated anti-mouse or anti-goat immunoglobulins (DakoCytomation Co. Ltd, Glostrup, Denmark) was applied to the samples for 30 min at room temperature, and then diluted antibodies were applied.

**Histological examinations**

Ears and back skins were ﬁxed for 30 h in zinc ﬁxative (0.05% calcium acetate, 0.5% zinc acetate, 0.5% zinc chloride in 0.1 M Tris–HCl buffer pH7.4) at room temperature, and then embedded in parafﬁn. The parafﬁn blocks were cut into 2 µm sections for hematoxylin and eosin staining, and cut into 4 µm sections for direct fast scarlet (DFS) stain and immunohistochemistry. To determine the presence of eosinophils, the sections were deparaffinized, rehydrated and treated with DFS solution (0.2% DFS, 0.8% Na2SO4 in 50% 2-propanol) for 5 min, and then were washed with tap water for 5 min. After overnight incubation in 50% 2-propanol to remove background staining, the samples were counterstained with Mayer’s hematoxylin. For immunohistochemistry, the sections were deparaffinized, rehydrated and treated with 1% BSA for 30 min at room temperature, and then diluted antibodies were applied. After overnight incubation at 4°C, biotinylated anti-mouse or anti-goat immunoglobulins (DakoCytomation Co. Ltd, Glostrup, Denmark) was applied to the samples for 30 min at room temperature, and then diluted antibodies were applied. After overnight incubation at 4°C, biotinylated anti-mouse or anti-goat immunoglobulins (DakoCytomation Co. Ltd, Glostrup, Denmark) was applied to the samples for 30 min at room temperature, and then diluted antibodies were applied. After overnight incubation at 4°C, biotinylated anti-mouse or anti-goat immunoglobulins (DakoCytomation Co. Ltd, Glostrup, Denmark) was applied to the samples for 30 min at room temperature, and then diluted antibodies were applied. After overnight incubation at 4°C, biotinylated anti-mouse or anti-goat immunoglobulins (DakoCytomation Co. Ltd, Glostrup, Denmark) was applied to the samples for 30 min at room temperature, and then diluted antibodies were applied.

**Evan’s blue test**

Female mice (Balb/c mice 7 weeks) were injected at two locations intradermally on their shaved backs with increasing concentrations of 0.1–10 µg/site DK-PGD2 in PBS (±). Three minutes after injection of DK-PGD2, the mice were injected i.v. with 0.25 ml of saline containing 1.25 mg of Evan’s blue. Four hours after the injection, the mice were sacriﬁced and the skin of the back was removed. The severity of edema was assessed by quantifying the extravasated dye (punch diam-eter: 6 mm) with 0.3 ml of formamide. The extravasated dye density was measured at 620 nm.

**Eosinophil peroxidase activity**

Skin sections were homogenized in 1 ml ice-cold buffer (0.05 M Tris–HCl pH 8.0 containing 0.1% Triton X-100) using a tissue homogenizer. The samples were then centrifuged at 10 000 g for 20 min at 4°C and the supernatants collected for EPO measurements. The substrate solution (100 µl) consisting of 10 mM O-phenylenediamine in 0.05 M Tris–HCl and 4 mM H2O2 was added to 20-fold diluted homogenate in buffer (100 µl) in a 96-well microwell plate. The reaction mixture was incubated at room temperature for 60 min before the reaction was stopped by the addition of 100 µl of 2M sulfuric acid. The absorbance at 490 nm was measured in a microplate reader (Labsystems Multiskan Multisoft, Osaka, Japan).

**Statistics**

Unless otherwise stated, data are expressed as means ± SE of at least three independent experiments. Statistical signiﬁcance was determined using the unpaired Student’s t-test if applicable, or with the Dunnett’s or Bonferroni test if variances were non-homogeneous using commercially available statistic software (GraphPad Software, San Diego, CA).

**Results**

**Local reaction to DK-PGD2 injections into mouse skin**

Injection of the CRTH2 agonist, DK-PGD2, into the epidermis of mice led to a dose-dependent local inﬂammation characterized by edema formation and signiﬁcant leukocyte invasion as assessed by the Evan’s blue method and histological evaluation, respectively. The maximal response was reached at a DK-PGD2 dose of 3 µg/injection site within 1–4 h after challenge, and no sign of local inﬂammation was observed at the control injection site where the corresponding volume of solvent was applied instead of DK-PGD2 (Fig. 1A–C). Dermal inﬁltrates were observed in the DK-injected mice, the dermal inﬁltrates tendency to be focused closer to the dermal–epidermal interface, including what appear to be spongiform lesions dispersed throughout the general area of inﬁltration.

To prove the speciﬁcity of the reactions induced by DK-PGD2, we pretreated mice with ramatroban, a small molecule CRTH2 antagonist (8), prior to DK-PGD2 administration. As expected, the pronounced leucocyte inﬁltrate in skin sections was completely abrogated (compare Fig. 2A, B and D) as was edema formation (Fig. 2E). In contrast, pretreatment of mice with dexamethasone had no effect (Fig. 2C).

In line with these observations, mRNA for CRTH2 was detectable in skin specimens obtained from DK-PGD2-challenged but not from vehicle-treated control sites (Fig. 3A). CRTH2 mRNA expression was not detectable in DK-PGD2-challenged skin of mice pretreated with ramatroban (R) or dexamethasone (D) (Fig. 3A). In addition, numerous immune cells isolated and puriﬁed from naive mice expressed high mRNA levels for CRTH2 (Fig. 3B).

In an attempt to identify the cell population being recruited into the dermal and epidermal layers by DK-PGD2, we stained the skin sections with a set of antibodies speciﬁc for various mouse leukocyte populations: surprisingly, the presence of B cells (B220+), T cells (CD3+, CD4+, CD8+), Langerhans cells (LC), monocytes and macrophages (stained with F4/80), did not signiﬁcantly differ between sections taken from DK-PGD2-treated and control animals, i.e. only a few cells stained positive (Fig. 4A, B and data not shown). In addition, HE staining did not reveal any evidence for signiﬁcantly high numbers of eosinophils being recruited following DK-PGD2 injection into the epidermis (data not shown). However, almost all cells in the skin sections from DK-PGD2-challenged mice stained positive when exposed to the neutrophil-speciﬁc Ab Ly-6G (Fig. 4C and D) which is in line with the highest mRNA signal for CRTH2 among all murine cell types investigated (c.f. Fig. 3B and data not shown). In addition, a signiﬁcantly increased number of activated macrophages as assessed by the antibody directed against the activation marker Mac-3...
was observed in samples from DK-PGD$_2$-treated animals (Fig. 4E–H).

Thus, intradermal activation of CRTH2 primarily leads to a local inflammatory reaction characterized by infiltrating neutrophils, activated macrophages and edema formation. DK-PGD$_2$ does not directly stimulate mouse neutrophil migration or signal transduction in vitro

As a correlate to the in vivo neutrophil infiltration of injection sites, we assessed the effect of DK-PGD$_2$ on neutrophil migration and signaling in vitro. Contrary to expectations, no induced migration or activation was observed in response to DK-PGD$_2$ over a wide concentration range. Surprisingly, the basal migration and chemoattractant-induced (LTB$_4$, KC) migration of isolated peripheral blood neutrophils from mice was slightly, yet consistently, inhibited in the presence of DK-PGD$_2$ (data not shown). Since these leukocytes express DP1 as well as CRTH2, and it was known that DK-PGD$_2$ has an effect, albeit at lower potency, on DP1, we postulated that there may have been preferential activation of DP1 and inhibition of migration via the cAMP signaling pathway. Contrary to expectation however, there was no effect of the DP antagonist BWA868C in the presence of DK-PGD$_2$ and no induction of cAMP as measured by ELISA, in the DK-PGD$_2$-stimulated neutrophils (not shown). In addition to these studies, investigations of calcium mobilization (in whole populations or at the single cell level) and ERK phosphorylation all failed to reveal any activity, suggesting a total lack of direct signaling by DK-PGD$_2$ compared with standard controls (not shown).

The role of CRTH2 in CHS in the mouse

In light of the prominent recruitment of neutrophils into the skin in response to DK-PGD$_2$ which may be counter-intuitive to a role of CRTH2 in a chronic Th2-mediated allergic inflammatory state (5,7), we were interested in determining the presence and role of CRTH2 in a more pathologically relevant animal model of skin inflammation. The FITC-painted skin CHS model was chosen, as this displays considerable Th2-dependency (27,28). Following a regimen of sensitization and ensuing challenge of the ear by FITC (see Methods for details), a CHS reaction as assessed and quantified by the measurement of ear thickness upon local FITC challenge was reproducibly obtained in Balb/c mice. Preliminary examinations revealed high enzymatic activity of eosinophil peroxidase in the lesions, but not in skin samples taken from the control ears, indicating the infiltration, and potentially activation, of eosinophils in response to FITC. High power photomicrographs of the lesional skin revealed a significant presence of eosinophils in the dermal layer in addition to leukocyte-filled micro-abscesses (Fig. 5A). The edematous reaction peaked at 24 h after challenge and lasted for more than 3 days (data not shown).

We first determined whether CRTH2 and its endogenous ligand, PGD$_2$, would be detectable in skin lesions. Indeed, significant CRTH2 mRNA signals were seen in ear samples 24 h after the FITC challenge but not in control ears. CRTH2 was not detectable at earlier time points, indicating that FITC was most likely inducing recruitment of CRTH2-expressing cells following antigen presentation in the draining afferent lymph nodes. In line with this interpretation, the kinetics of PGD$_2$ release upon FITC challenge preceded...
the induction of CRTH2 message as shown in Fig. 5(B) and (C), respectively. Together, these findings present evidence for PGD2/CRTH2 in elicited CHS and are suggestive of a potential causal role in the establishment of inflammatory infiltrates.

**Ramatroban, a CRTH2 antagonist, protects mice from Th2-dependent CHS**

We then tested ramatroban for its inhibitory potential in this CHS model. A dose-dependent, highly significant reduction of the FITC-induced ear thickness was observed in mice pretreated with ramatroban, and the EPO activity was completely reduced to control levels. Importantly, the TP antagonist ridogrel was without any effect on all parameters, suggesting that ramatroban’s efficacy was most likely due to its CRTH2 receptor blockade (Fig. 6A and B). Further studies utilizing orally-administered CRTH2-selective small molecule antagonists have also shown similar efficacy in reducing the inflammation (data not shown).

Histological examinations of the challenged mouse ears confirmed those findings: profuse leukocyte infiltrates were seen 24 h after FITC challenge compared to PBS-treated controls, with both dermal and epidermal infiltration. Foci of inflammatory aggregates and microabscesses were observed in the epidermis, reminiscent of subcorneal pustules and neutrophilic spongiosis typically observed in acute and subacute dermatitis. Indeed, the microabscesses contained significant proportions of intact and degranulated eosinophils, as well as lymphocytes, neutrophils and monocyte/macrophages (Fig. 7A). Overall, the model correlates well with the acute manifestations of the IgE-dependent cutaneous late-phase allergic reaction often found in atopic dermatitis patients (29). Again, ramatroban, but not ridogrel (TP antagonist) or BWA868C (DP antagonist), abrogated the FITC-induced cell influx as shown in Fig. 7(B–D). Importantly, the subcorneal abscesses were completely absent following ramatroban treatment, indicating a crucial role for CRTH2 in the formation of these pathological structures.

Surprisingly—but in accordance with what we have found upon DK-PGD2 injection (c.f. Fig. 4)—leukocyte typing revealed that the majority of cells were neutrophils, although there were eosinophils, macrophages and lymphocytes visibly present. Whether the neutrophils were activated is difficult to say since measurements of neutrophil elastase or myeloperoxidase have not been undertaken. Ramatroban clearly inhibited neutrophilia, while the DP antagonist had a moderate effect and ridogrel completely lacked efficacy (Fig. 8A–E). Though obvious, the number of cells staining positively for the eosinophil dye DFS was small compared to the neutrophil numbers (Fig. 8F and G), however, considerably greater than are found in normal (unchallenged) skin, and, more surpris-
ingly, this infiltrate was not significantly affected by any receptor antagonist (data not shown).

The presence of neutrophil chemoattractants LTB₄, KC and MIP-2 may contribute to the neutrophil inflammation. The leukocyte infiltration provoked during the CHS reaction induced by FITC in Balb/c mice is most likely in part mediated by the products of activated mast cells (K. Takeshita, T. Yamasaki, S. Akira, F. Gantner and K. B. Bacon, submitted for publication). Measurements of three well-characterized murine chemoattractants likely synthesized or induced following mast cell activation, LTB₄ and the chemokines KC and MIP-2 (30), revealed a time-dependent increase in these mediators over the duration of the CHS reaction. LTB₄ kinetics of release showed an early peak (1 h) followed by a more sustained release over 8 h prior to returning to baseline (Fig. 9). The calculated concentration of ~0.1 μM would suggest sufficiently high levels to induce both neutrophil and lymphocyte migration (31,32). KC however showed a distinct kinetic with maximal concentrations being achieved at 8–12 h after

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![Image](https://academic.oup.com/intimm/article-abstract/16/7/947/839149)

**Fig. 4.** Immunohistological determination of infiltrating leukocytes. Mice were treated with DK-PGD₂ (3 μg per site), then back skin removed by punch biopsy as detailed in Methods. Staining for specific leukocyte markers was performed as described on at least three mice per treatment and five high power fields counted. Following staining, sections were photographed under high power (×40). Arrows indicate specifically-stained activated macrophages (Mac-3 antibody).
FITC challenge (Fig. 9). The calculated maximal concentration (making no assumptions in terms of protein or GAG binding) of ~50 pM while low, would still promote significant neutrophil migration, if assessed in in vitro assays (33). In contrast, however, MIP-2 showed a biphasic kinetic peaking at 30 min and then at 4–8 h post-challenge (Fig. 9). Similarly, the apparent maximal concentration of 300 pM would be well within the range of effective concentrations, and close to the optimal concentration of MIP-2 for promoting migration of neutrophils.

Using the FITC model to assess this possibility, antibodies specific to murine KC and MIP-2 were injected (i.v.) into mice 30 min prior to FITC challenge and the ears assessed for edema from T = 0 to T = 24, and histologically after 24 h (to parallel the kinetic). As shown in Fig. 10(A), there was a significant decrease in edema as early as the 4 h period when either antibody was used independently and >50% inhibition in swelling when the antibodies were combined. In contrast, no inhibition was observed in mice treated with anti-MCP-1 antibody (Fig. 10C). In addition, that this response was secondary to release of mediators from mast cells, was suggested using the WWv mouse (Fig. 10B). These mice, being deficient in functional mast cells showed no edematous response to FITC, suggesting the requirement for mast-cell derived mediators in elicitation of the response.

Upon histological analysis of the FITC-challenged ears it was clear that the neutrophil infiltrates were significantly reduced (Fig. 10D); however, this appeared to be restricted to the KC-treated animals. In contrast to the effects of ramatroban, an inflammatory infiltrate still appeared to be present. On closer examination however (H&E stained sections), it was clear that while the migration of the majority of neutrophils had been inhibited, there was a substantial eosinophil presence, in addition to what appeared to be mononuclear cells (possibly activated macrophages). This appeared to be most obvious in the dermis where an almost complete inhibition of neutrophilia was observed, leaving an almost exclusive eosinophilic infiltrate.
Discussion

Results generated from the present experiments further detail the propensity for DK-PGD2, a selective CRTH2 agonist, to promote murine leukocyte infiltration in an inflammatory setting (26). The profuse infiltrates, reminiscent of the acute phase Th2-mediated allergic response in part corroborates biological effects predicted from the in vitro human cell activity (5–7). What is difficult to reconcile is the lack of obvious in vitro effects in numerous validated bioassays for murine leukocytes. Indeed, in our hands to date, only the activation (phosphorylation) of p42/p44 ERK has been unequivocally proven, in murine splenocytes and CD4+ T cells derived from mouse spleen (H. Sugimoto, M. Shichijo and K. B. Bacon, unpublished data). Analyses of cell activation in vivo suggest a direct effect on rat leukocytes (26), however, there has to date been little if any evidence in the literature for defined functional responses to DK-PGD2/CRTH2 activation.

In an analysis of neutrophil activation in vitro, the only noticeable, yet insignificant, inhibition of basal and chemo-attractant-induced migration. While counter-intuitive, such a finding may have significant relevance to the pathology observed in the DK-induced and CHS models. We have seen that the release of PGD2 occurs over the entire period of 24 h, peaking at around 4 h. In the DK-PGD2 skin injection model, peak infiltrates were seen at 4 h, while in the FITC CHS model, significant infiltration was observed at 1 h post-challenge, with peak infiltrates being observed at 8 h. In the DK-PGD2 skin injection model, it is arguable that pre-formed/ stored chemoattractants are released from activated cells in the dermal/epidermal layers that induce the neutrophil influx and DK-PGD2 retains the cells by virtue of its inhibitory activity on migration. Similarly, in the more complex setting of FITC-induced CHS, preformed or locally generated chemo-
attractants over the course of the sensitization and challenge period prime or induce the migration of cells, and as the concentration of PGD2 rises, the cells are retained at the challenge site. It is clear from these hypotheses that only a more comprehensive analysis of the kinetics of chemokine generation will satisfactorily clarify what role CRTH2 activation on leukocytes serves. However, our preliminary investigations into these phenomena suggest that chemokines (LTB4, MIP-2 and KC) are synthesized and released in sufficient concentrations and with a kinetic parallel that of PGD2 and the leukocyte infiltrates, thus may fulfill this role at least for neutrophils. One surprising finding, however, was the apparent lack of efficacy of the anti-MIP-2 antibody in reducing the neutrophilia, although it appeared efficacious in reducing the edema. It will be interesting to further define the mechanistic specificity of this apparent discrepancy between two ligands that supposedly interact at the same receptor, CXCR2.

In the current experiments, it was intriguing to find that DK-PGD2 failed to promote neutrophil migration in vitro on the one hand, while ramatroban was capable of completely eliminating all infiltrates following DK-PGD2 injection. Further, DK-PGD2 was not capable of priming cells to respond to other chemokines; rather it desensitized the reaction. An explanation for such a discrepancy may center on the kinetics of antagonism by ramatroban. In the direct injection scenario, DK-PGD2 may have been acting on an as yet uncharacterized expressor of CRTH2 in the mouse (mast cells, endothelium, fibroblast, Langerhans cells), inducing the release of secondary chemokines. Alternatively, DK-PGD2 may simply induce chemokinetic priming of cells and not the directed, haptotactic migration. In addition, or alternatively, DK-PGD2 may increase certain adhesion receptors that can promote the migration—a phenomenon unlikely to become evident in the artificial environment of the transwell chemotaxis assay. However, using murine cells, we have not observed any change in surface expression of more than 20 surface receptors associated with adhesion and cell migration/activation, including CD11a-c, CD54, CD44, CD28, CD45R, CD23, Gr-1 and CD49d, in response to DK-PGD2 (H. Sugimoto, M. Shichijo and K. B. Bacon, data not shown). That ramatroban can inhibit the response may thus lie in selective down-regulation of these entities, and/or inhibition of chemokinetic priming of leukocytes in the circulation; hence their migration out of the circulation.

Given the profound effects of CRTH2 antagonists on the overall pathology of the FITC CHS model, it will be important to further dissect the potential to inhibit the individual components responsible for the gross pathology. Understanding such regulation may further contribute to our

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**Fig. 9.** Measurement of release of chemokines, LTB4, KC and MIP-2. Following FITC treatment in the 1 week model, ear tissue was homogenized (see Methods) and the concentration of the chemokines, LTB4, KC and MIP-2 measured by specific DuoSet ELISA development kits. ELISA. Each point represents the mean ± SEM concentration from five mouse ears, with significance being determined by Student’s t-test; *P < 0.05 and **P < 0.01.
understanding of how PGD₂ and CRTH2 are effecting such pathology. For example, it is well-established that the hapten contact sensitizers promote differential T lymphocyte responses (27,28,34). A fundamental characteristic of this acquired immune response is the T lymphocyte and its ability to provide the necessary signals for antibody generation in addition to secreting IL-5 and promoting eosinophil infiltration. In our analyses, there was no significant effect on the dermal eosinophil infiltrate as assessed by histological examination of the sections. In addition, there is no noticeable effect on T-cell

Fig. 10. Effect of antibodies to murine KC and MIP-2 on the FITC-induced neutrophil infiltrates. Polyclonal anti-mouse KC and MIP-2 antibodies (see Methods) were administered i.v. at 100 μg/mouse. Thirty minutes later, the mice were challenged with FITC as described. After 24 h (the optimal time for neutrophil infiltrates), the mice were sacrificed and the ear thickness measured using a micrometer screw-gauge (A), after which the ears were sectioned for histological analysis using H&E and anti-Ly-6G antibody (D). At least 10 mice per challenge and time point were used. Mast cell deficient mice (B) and antibody to MCP-1 (C) were used as negative controls in the experiment.
numbers (although a thorough statistical analysis has not been performed). Two important caveats must be detailed here; (i) there was an almost complete reduction in the EPO measured from the injection sites; and (ii) we did not perform differential staining using specific Th1 vs Th2 markers for the T lymphocyte populations. If the disease model is a suitable correlate for human AD, measurements of EPO could reflect the degree of dermal eosinophil cytology, especially if morphologically intact eosinophils are still identifiable (35). Since we have not observed marked changes in dermal eosinophils in histology sections, it is unlikely that the EPO measurements represent degranulated cells. However, the complete absence of the microabscesses following ramatroban treatment is the likely reason for dramatically reduced EPO.

Secondly, given that no Th1/Th2 differential staining was performed, it is difficult for us to conclude that one population of CRTH2-expressing T cells has been affected over another. One cell type that has not been analyzed in these studies is the Langerhans cell (LC). Given that this is the first line of defence in hapten recognition and presentation to the T cells in the afferent lymph nodes (27), it is critical to investigate the effects of DK-PGD₂ and ramatroban on these cells. It has been published that LC express both DP and CRTH2 (25,36). However, the major biological responses to PGD₂ seen in in vitro and in vivo models analyzing LC and DC maturation, migration and antigen presentation suggest that the major receptor activated is DP, and that the majority of responses are inhibitory/anti-inflammatory, in direct contrast to what one observes with DK-PGD₂ injections and the CHS model. In our hands, murine monocyte-derived DC also did not show robust signaling (calcium mobilization and ERK phosphorylation) in response to DK-PGD₂, and failed to augment Th2-derived IL-4 release in a co-culture assay in vitro (K. Nakashima, F. Gantner, M. Shichijo and K. B. Bacon, unpublished data). It will therefore be critical to determine the contributions of DP and CRTH2 under a variety of stimulatory conditions in naive T lymphocytes, the specific skin-homing T cells and dermal DC and LC.

The finding that ramatroban (and other selective small molecule CRTH2 antagonists developed in-house, data not shown) can completely abrogate the experimentally induced contact dermatitis (as measured by ear swelling) is a particularly encouraging signal for the development of effective therapeutics for dermatoses characterized by such clinical manifestations. Of particular interest was the finding that the significant levels of EPO were measurably reduced. It is thus likely that the high EPO activity (c.f. Fig. 6B) is an indicator of the ability of a single ligand±receptor system to control the homeostasis and inflammation by leukotrienes and other mast cell-dependent compounds. Since elevated PGD₂ levels are found in allergen-challenged skin from AD patients, as well as in samples from atopic donors (38,39), and an increased number of peripheral blood CRTH2+ T lymphocytes are detected in the CLA+ skin-homing lymphocyte population from AD patients (40), it is likely that the PGD₂/CRTH2 system is playing a role in the inflammatory dermal infiltrate in this disease. Even though numerous chemoattractants capable of attracting the specific leukocyte populations are elevated in AD skin (41,42), the ability of a single ligand–receptor system to control the recruitment of all the relevant populations is thus far unknown. Eotaxin, an eosinophil and Th2 cell chemoattractant, has not been shown to promote human neutrophil or monocyte migration. In our hands, human T lymphocytes with either a Th1 or Th2 cytokine profile have been shown to express CCR3 and migrate in vitro in response to eotaxin (H. Sugimoto and K. B. Bacon, unpublished data). Similarly, IL-8, a well-known neutrophil chemoattractant, has much weaker pro-migratory effects on lymphocytes (43). Leukotriene B₄, for many years known to be a neutrophil and T lymphocyte chemoattractant was recently shown to promote effector CD4 and CD8, Th1 and Th2 lymphocyte migration in a model of allergic inflammation (44). While this may contribute significantly to the pro-inflammatory cascade in AD (41), the weak effects of LTB₄ on eosinophils argues against a prominent role in the disease etiology.

We have identified a potential role for CRTH2 and PGD₂ in the elicitation of inflammation in a dermatological context. An initiating or maintenance role for CRTH2 is difficult to prove categorically and final proof of the fundamental role of this system will come only from the use of specific antagonists to CRTH2 in a clinical setting.

**Abbreviations**

CHS    contact hypersensitivity  
DFS    direct fast scarlet  
DK-PGD₂    13,14-dihydro-15-keto-PGD₂  
15α-PGJ₂    15-deoxy-Δ12, 14-PGJ₂  
LC    Langerhan cell  
PGD₂    prostaglandin D₂  
PPAR    peroxisome-proliferator-activated receptor

**References**

CRTH2 is involved in contact hypersensitivity


Seuter, F., Perzbach, E., Rosentreu, U., Boshagen, H. and Fiedler, V. B. 1989. Inhibition of platelet aggregation in vitro and ex vivo by the new thromboxane antagonist (3R)-3-(4-fluorophenylsulfonylamo)-1,2,3,4-tetrahydro-9-carbazolepropanoic acid, Azimil 98-128.


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