The Role of the Prostaglandin D$_2$ Receptor, DP, in Eosinophil Trafficking

Petra Schratl, Julia F. Royer, Evi Kostenis, Trond Ulven, Eva M. Sturm, Maria Waldhoer, Gerald Hoefler, Rufina Schuligoi, Irmgard Th. Lippe, Bernhard A. Peskar and Akos Heinemann

_J Immunol_ 2007; 179:4792-4799; doi: 10.4049/jimmunol.179.7.4792

http://www.jimmunol.org/content/179/7/4792

**References**

This article cites 51 articles, 25 of which you can access for free at:
http://www.jimmunol.org/content/179/7/4792.full#ref-list-1

**Subscription**

Information about subscribing to _The Journal of Immunology_ is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
The Role of the Prostaglandin D2 Receptor, DP, in Eosinophil Trafficking

Petra Schratl,* Julia F. Royer,* Evi Kostenis,† Trond Ulven,§ Eva M. Sturm,* Maria Waldhoer,* Gerald Hoeﬂer,† Rufina Schuligoi,* Irmgard Th. Lippe,* Bernhard A. Peskar,* and Akos Heinemann²*

Prostaglandin (PG) D₂ is a major mast cell product that acts via two receptors, the D-type prostanoid (DP) and the chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2) receptors. Whereas CRTH2 mediates the chemotaxis of eosinophils, basophils, and Th2 lymphocytes, the role of DP has remained unclear. We report in this study that, in addition to CRTH2, the DP receptor plays an important role in eosinophil trafficking. First, we investigated the release of eosinophils from bone marrow using the in situ perfused guinea pig hind limb preparation. PGD₂ induced the rapid release of eosinophils from bone marrow and this effect was inhibited by either the DP receptor antagonist BWA868c or the CRTH2 receptor antagonist ramatroban. In contrast, BWA868c did not inhibit the release of bone marrow eosinophils when this was induced by the CRTH2-selective agonist 13,14-dihydro-15-keto-PGD₂. In additional experiments, we isolated bone marrow eosinophils from the femoral cavity and found that these cells migrated toward PGD₂. We also observed that BWA868c inhibited this response to a similar extent as ramatroban. Finally, using immunohistochemistry we could demonstrate that eosinophils in human bone marrow specimens expressed DP and CRTH2 receptors at similar levels. Eosinophils isolated from human peripheral blood likewise expressed DP receptor protein but at lower levels than CRTH2. In agreement with this, the chemotaxis of human peripheral blood eosinophils was inhibited both by BWA868c and ramatroban. These findings suggest that DP receptors comediately with CRTH2 the mobilization of eosinophils from bone marrow and their chemotaxis, which might provide the rationale for DP antagonists in the treatment of allergic disease. The Journal of Immunology, 2007, 179: 4792–4799.

Received for publication January 9, 2007. Accepted for publication July 12, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by the Jubiläumsfonds of the Austrian National Bank (JubF) Grants 10287, 10934, and 11967; the Austrian Science Fund (FWF) Grants P16668-B05 and P19424-B05, and the Franz Lanzer Grant (2007) 288 and 315.

2 Address correspondence and reprint requests to Dr. Akos Heinemann, Department of Experimental and Clinical Pharmacology, Medical University Graz, Graz, Austria; Institute for Pharmaceutical Biology, Bonn, Germany; and Department of Chemistry, University of Southern Denmark, Odense M, Denmark.

The alternate PGD₂ receptor, DP, is expressed more widely, including platelets, several types of leukocytes, the vascular, the CNS, retina, nasal mucosa, lungs, and intestine (6, 20–23). Functionally, DP-mediated responses include inhibition of platelet aggregation, induction of vasorelaxation, mucin secretion, and lowering intraocular pressure (23–25). PG agonists have been suggested to inhibit neutrophil, basophil, and dendritic cell function (26–29). Eosinophils have been found to express the mRNA for the DP receptor and to show delayed apoptosis after treatment with supramaximal concentrations (1 μM) of the selective DP agonist BW245c (10). In vivo, a DP antagonist blocks Ag-induced rhinitis, conjunctivitis, and pulmonary inflammation in the guinea pig.
pig (30), and DP-deficient mice exhibit reduced pulmonary inflammation in response to allergen (31). These in vivo studies hence point to a proinflammatory role of DP but are difficult to explain based on the known functional responses of DP receptor activation.

We have previously shown that $\Delta^{10}$PGJ$_2$ and PGD$_2$ markedly prime eosinophils for chemotaxis toward other chemoattractants, such as the CCR3 ligand eotaxin, and that $\Delta^{12}$PGJ$_2$ is capable of mediating the rapid mobilization of eosinophils from the isolated hind limb of guinea pigs (8). These observations suggested novel mechanisms by which PGD$_2$ or its metabolites generated at sites of allergic inflammation could act as systemic signals for the supply of eosinophils from the bone marrow and enhance their extravasation to the tissue. Hence we set out to investigate the differential roles of CRTH2 and DP in the mobilization of eosinophils from the bone marrow and chemotaxis. Our data demonstrate that the DP receptor plays an important modulator role in the recruitment of eosinophils into the tissue. Thus, DP antagonists might be useful to treat conditions associated with eosinophilic inflammation such as asthma or other allergic diseases.

Materials and Methods

Animals

Adult Dunkin-Harley guinea pigs (either sex, 350–450 g body weight) were obtained from the Research Institute for Laboratory Animal Breeding (Medical University of Vienna, Himberg, Austria).

Chemicals

All laboratory reagents were obtained from Sigma-Aldrich, unless specified. Assay buffer as used in the shape change and chemotaxis experiments was made from Dulbecco’s modified PBS (with 0.9 mM Ca$^{2+}$ and 0.5 mM Mg$^{2+}$; Invitrogen Life Technologies), 0.1% BSA, 10 mM HEPES, and 10 mM glucose (pH 7.4). For the perfusion of the guinea pig hind limb, modified Krebs-Ringer bicarbonate buffer was prepared with 10 mM glucose (pH 7.4). For the perfusion of the guinea pig hind limb, modified Krebs-Ringer bicarbonate buffer was prepared with 10 mM glucose (pH 7.4). For the perfusion of the guinea pig hind limb, modified Krebs-Ringer bicarbonate buffer was prepared with 10 mM glucose (pH 7.4). For the perfusion of the guinea pig hind limb, modified Krebs-Ringer bicarbonate buffer was prepared with 10 mM glucose (pH 7.4). For the perfusion of the guinea pig hind limb, modified Krebs-Ringer bicarbonate buffer was prepared with 10 mM glucose (pH 7.4).

Chemotaxis of guinea pig bone marrow eosinophils

Competition binding experiments

HEK293 cells expressing guinea pig DP receptors were seeded into 96-well plates at a density of 30,000 cells/well. Competition binding experiments on whole cells were then performed 18–24 h later using $[^{3}H]$PGD$_2$, (PerkinElmer; 121 Ci/mmol) as a radiotracer in a binding buffer consisting of HBSS and 10 mM HEPES (pH 7.5); 0.6 nM $[^{3}H]$PGD$_2$ was used for guinea pig DP equilibrium competition binding. Competing ligands were diluted in DMSO that was kept constant at 1% (v/v) of the final incubation volume. Total and nonspecific binding were determined in the absence and presence of 10 $\mu$M PGD$_2$. Binding reactions were routinely conducted for 3 h at 4°C to exclude receptor internalization and terminated by two washes (100 µl each) with ice-cold binding buffer. Radioactivity was determined by liquid scintillation counting in a Topcount (Packard Instruments) following overnight incubation in Microscint 20.

Table I. Compounds used in this study and their receptor selectivity

<table>
<thead>
<tr>
<th>Name</th>
<th>Receptor of Selectivity and Rank Order of Affinity</th>
<th>Activity</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGD$_2$</td>
<td>DP = CRTH2 &gt; TP</td>
<td>Agonist</td>
<td>(6, 48)</td>
</tr>
<tr>
<td>DK-PGD$_2$</td>
<td>CRTH2</td>
<td>Agonist</td>
<td>(6)</td>
</tr>
<tr>
<td>BW245c</td>
<td>DP</td>
<td>Agonist</td>
<td>(49)</td>
</tr>
<tr>
<td>ZK110841</td>
<td>DP</td>
<td>Agonist</td>
<td>(50)</td>
</tr>
<tr>
<td>BWA868c</td>
<td>DP</td>
<td>Antagonist</td>
<td>(51)</td>
</tr>
<tr>
<td>SQ29548</td>
<td>TP</td>
<td>Antagonist</td>
<td>(52)</td>
</tr>
</tbody>
</table>

cAMP accumulation assays

Preparations of polymorphonuclear leukocytes (PMNL) were prepared by dextran sedimentation and Histopaque gradients, and eosinophils were further purified by negative magnetic selection using an Ab mixture directed against CD2, CD14, CD16, CD19, CD56, glycophorin A from StemCell Technologies. The purity of the eosinophil preparations was usually above 97%, the contaminating cells being mononuclear cells. The viability of the cells was >98%. Thirty microliter aliquots of citrated whole blood were stimulated with 10 $\mu$l of agonists for 4 min at 37°C. The samples were then transferred to ice and fixed with 250 $\mu$l of fixative solution followed by NH$_4$Cl-induced lysis of RBC (33). Cells were then washed and resuspended in 250 $\mu$l of fixative solution. Samples were immediately analyzed on a FACSCalibur flow cytometer (BD Biosciences). Eosinophils were distinguished from other cells by means of their autofluorescence in the FL-1 and FL-2 fluorescence channels (8). Shape change was determined as the increase of forward scatter compared with vehicle stimulation. The forward scatter values are shown as dimensionless arbitrary units.

Chemotaxis of human eosinophils

Preparations of polymorphonuclear leukocytes (PMNL) were prepared by dextran sedimentation and Histopaque gradients, and eosinophils were further purified by negative magnetic selection using an Ab mixture directed against CD2, CD14, CD16, CD19, CD56, glycophorin A from StemCell Technologies. The purity of the eosinophil preparations was usually above 97%, the contaminating cells being mononuclear cells. The viability of the cells was >98%. Thirty microliter aliquots of citrated whole blood were stimulated with 10 $\mu$l of agonists for 4 min at 37°C. The samples were then transferred to ice and fixed with 250 $\mu$l of fixative solution followed by NH$_4$Cl-induced lysis of RBC (33). Cells were then washed and resuspended in 250 $\mu$l of fixative solution. Samples were immediately analyzed on a FACSCalibur flow cytometer (BD Biosciences). Eosinophils were distinguished from other cells by means of their autofluorescence in the FL-1 and FL-2 fluorescence channels (8). Shape change was determined as the increase of forward scatter compared with vehicle stimulation. The forward scatter values are shown as dimensionless arbitrary units.

Chemotaxis of guinea pig bone marrow eosinophils

The femoral bone cavity was flushed, erythrocytes were lysed by hypotonic shock, and PMNLs were prepared by Histopaque gradients. The cells were resuspended in RPMI 1640 (1% FCS) at 10 $\times$10$^6$/ml and 100-$\mu$l aliquots of the suspension were placed into Transwell inserts with 5-$\mu$m pore size polycylinpolygromidolene-free polycarbonate filters (Corning). The cells were allowed to migrate toward 600 $\mu$l of assay buffer or agonists in the bottom wells of 24-well tissue culture plates for 1 h at 37°C in a humidified CO$_2$ incubator for 1 h and the membrane was carefully removed. Cells that had migrated to the bottom wells were enumerated by flow cytometry (FACSCalibur, BD Biosciences), and contaminating cells were gated out by side scatter and autofluorescence as previously described (8).
**In situ perfusion of the guinea pig hind limb**

The guinea pig hind limb was perfused as previously described (8, 34). The external iliac artery and vein were exposed and the caudal abdominal artery, superficial iliac circumflex artery, and pudendoepigastric trunk along with their satellite veins were ligated. Polyethylene cannulas (0.8 mm outside diameter) were inserted into the external iliac artery and vein. Modified Krebs-Ringer bicarbonate buffer (gassed with 95% O₂ and 5% CO₂, 37°C) was infused at 4 ml/min via the arterial cannula and removed from the venous cannula using a peristaltic pump. After an equilibration period of 20 min, the perfusate fractions were collected every 10 min and centrifuged at 300 × g for 10 min. The cell pellet was resuspended in Kimura’s stain and nucleated leukocytes and Kimura-positive eosinophils were counted in a Neubauer hemacytometer.

**Immunofluorescence microscopy**

The expression of DP and CRTH2 receptors were visualized on human peripheral blood eosinophils by immunofluorescence following a double antibody staining protocol. For visualization of the CRTH2 receptor, a suspension of unfixed 1 × 10⁷ PMNL in PBS was blocked with human IgG (Sigma-Aldrich) for 30 min at 4°C. Cells were washed once with PBS by centrifugation at 400 × g for 5 min and then incubated with rat mAb against CRTH2 (clone BM16; BD Biosciences) diluted 1:25, or the isotype control (BD Biosciences) in PBS for 30 min at 4°C. Following a washing step, cells were incubated for 30 min in the dark at 4°C with the secondary Ab (Cy3-conjugated goat anti-rabbit secondary Ab; Chemicon; dilution 1:250). PMNL were washed twice with PBS, and cells were cyto-spun on cover slips. Subsequently, PMNL were fixed with 3.7% formaldehyde in PBS at 4°C overnight. After washing, the cell pellets were blocked with powdered milk and human IgG (Sigma-Aldrich) in PBS for 60 min at room temperature. Cells were washed twice with PBS and then incubated with rabbit polyclonal Ab against the DP receptor (Sigma-Aldrich) diluted 1:125 or rabbit IgG (Sigma-Aldrich) as negative control in PBS with powdered milk. After incubation with primary Ab, PMNL were washed six times with PBS and then incubated for 30 min in the dark at room temperature with secondary Ab (Alkaline Phosphor 488 goat anti-rabbit IgG; Invitrogen Life Technologies; dilution 1:500). Finally, after six washes with PBS, the nuclei were stained with 4’,6’-diamidino-2-phenylindole (DAPI; Invitrogen Life Technologies). The fluorescent signal was recorded with an Olympus IX70 fluorescence microscope and an Olympus UPlanApo - 60x/1.20 lens, using Olympus DP50-CU digital camera and Olympus CellP® software. Images were further processed also with CellP® for contrast and brightness adjustments.

**Immunohistochemistry**

The expression of DP and CRTH2 receptors were investigated in human bone marrow. Sections (3 μm) were prepared from EDTA decalcified material, deparaffinized by xylene, rehydrated in graded alcohols, and submitted to microwave epitope retrieval (10 min at 750 W using pH 9.0 Ag material, deparaffinized by xylene, rehydrated in graded alcohols, and sub-

**Calculations and data analysis**

IC₅₀ and EC₅₀ values were determined by nonlinear regression analysis using the Prism 3.0 software (GraphPad Software). Antagonistic potencies in functional assays are given as pIC₅₀ values that were obtained by competing with increasing concentrations of inhibitor compound for an agonist concentration required to elicit ~75–80% of the maximal agonist efficacy. IC₅₀ values generated by this procedure are very closely matching the true affinity of antagonists determined by the analysis according to Anrunkahana and Schuld (35).

Data are shown as the mean ± SEM except where otherwise stated. Statistical comparisons of groups were performed using two-way ANOVA for repeated measurements or the Mann-Whitney U test. Probability values of p < 0.05 were considered statistically significant.

**Results**

**Pharmacological characterization of BWA868c**

While BWA868c has been investigated before with respect to its affinity and selectivity toward human DP and CRTH2 receptors (36), guinea pig DP has been characterized so far only in functional assays. Thus we expressed the guinea pig DP receptor in HEK293 and 1321N1 cells for binding experiments and assay of cAMP accumulation, respectively. As expected, BWA868c and the DP-selective agonist BW245c exhibited high affinity for the guinea pig DP receptor, with log Kᵢ values of −7.95 ± 0.08 and −8.95 ± 0.06, respectively, and displaced PGD₂, from the receptor (Fig. 1A). In assays of cAMP accumulation, BW245c was a full agonist of DP comparable with PGD₂, with pEC₅₀ values of −8.89 ± 0.11 and −8.48 ± 0.09, respectively. In contrast, BWA868c caused a minute degree of cAMP accumulation at higher concentrations (Fig. 1B), which was compatible with partial agonism already reported for human DP (20).

The selectivity of BWA868c against guinea pig CRTH2 was investigated in eosinophils, which naturally express CRTH2, using the shape change assay. Stimulation with chemotactractants results in immediate responses of granulocytes including reorganization of the cytoskeleton and shape change, which can be detected by flow cytometry (8). PGD₂ was a potent and highly effective inducer of eosinophil shape change of guinea pig eosinophils, although with a 3- to 10-fold lower potency than in human whole blood (Fig. 2). Ramatroban inhibited the PGD₂-induced shape change of human and guinea pig eosinophils (Fig. 2), but had no effect on eotaxin-induced shape change (data not shown, n = 4). In contrast, BWA868c had no effect on shape change responses of guinea pig and human eosinophils, irrespective of the stimulant used (PGD₂, Fig. 2; eotaxin, data not shown, n = 4). Since the DP agonist BW245c did not cause shape change (data not shown, n = 4), it seems that eosinophil shape change responses to PGD₂ are exclusively mediated by CRTH2.

**Mobilization of eosinophils from the guinea pig femoral bone marrow**

Using BWA868c and ramatroban as potent and selective antagonists for DP and CRTH2, respectively, we investigated which of the PGD₂ receptors were mediating the mobilization of mature eosinophils from bone marrow in the in situ perfused guinea pig hind limb preparation. PGD₂ caused an immediate increase of eosinophils in the perfusate when added during the period of 20–40 min.
after the start of the experiments, whereas the vehicle of the prostanoid alone had no effect (Fig. 3A); 10 nM of PGD2 caused a similar degree of eosinophil release as 1 nM eotaxin. The release of noneosinophilic cells from the bone marrow was not increased by PGD2 or eotaxin, whereas LTB4 nonselectively induced the release of both eosinophils and other nucleated cells (Fig. 3A).

To determine the role of CRTH2, DP, and TP receptors in eosinophil mobilization from bone marrow, PGD2 antagonists or their vehicle were infused throughout the experiment, and PGD2 was added to the perfusate for the period of 20–40 min after the start of the perfusion. The ability of PGD2 to increase the release of eosinophils from bone marrow was not altered by the TP-selective antagonist SQ29548 (100 nM), but it was inhibited by the CRTH2 antagonist ramatroban (1/9262 M) by 70–80%, and also by the DP antagonist BWA868c (100 nM) to a similar extent (Fig. 3B). In contrast, the DP antagonist BWA868c did not inhibit the response to DK-PGD2 (Fig. 3B). Therefore, these data show that PGD2-induced release of eosinophils is mediated both by CRTH2 and DP receptors, while in agreement with its known selectivity for CRTH2, DK-PGD2 mobilized eosinophils solely via CRTH2. Unexpectedly, the DP agonists BW245c or ZK110841 did not induce eosinophil release by themselves and the addition of BW245c to the infusion of DK-PGD2 did not enhance the response of bone marrow eosinophils when compared with DK-PGD2 alone (Fig. 4A). Hence, these findings raised the question whether BW245c was inactive on DP receptors in this preparation or might even behave as a DP antagonist. When infused throughout the experiment, BW245c (100 nM) in fact reduced the ability of PGD2 to release eosinophils from the bone marrow, whereas the response to DK-PGD2 was not affected (Fig. 4B). From these observations it may be inferred that BW245c behaves as an antagonist of DP receptors in guinea pig bone marrow.

**Chemotaxis of eosinophils**

To further investigate the mechanisms by which CRTH2 and DP activation induce the release of eosinophils from bone marrow, we harvested the leukocytes from the cavity of the guinea pig femoral bone to determine their migration. Bone marrow eosinophils showed a chemotactic response to PGD2 (3–300 nM) similar to
human peripheral blood eosinophils, whereas neutrophil chemotaxis was not stimulated by PGD$_2$ (Fig. 5A). Migration of bone marrow eosinophils was significantly inhibited by the CRTH2 antagonist ramatroban and, to a larger extent, also by the DP antagonist BWA868c (Fig. 5B). Human eosinophils purified from peripheral blood likewise migrated toward PGD$_2$, and this response was inhibited by ramatroban but not SQ29548 (Fig. 6A). BWA868c (100 nM) also attenuated the migration of human eosinophils by causing a 3- to 6-fold shift of the PGD$_2$ concentration-response curve to the right (Fig. 6A). The inhibitory effect of BWA868c was unrelated to nonspecific attenuation of eosinophil responsiveness, because BWA868c pretreatment did not alter the migration toward eotaxin (Fig. 6A) or LTB$_4$ (data not shown). The DP-selective agonists BW245c (3–300 nM) or ZK110841 (10–30 nM) did not stimulate the chemotaxis of eosinophils, neither alone (Fig. 6B) nor in combination with DP-PGD$_2$ or eotaxin (data not shown). However, pretreatment of eosinophils with BW245c (100 nM) significantly attenuated the migratory response toward PGD$_2$ (Fig. 6C). This suggests again that, in contrast to PGD$_2$, conventional DP agonist do not activate chemotactic pathways in eosinophils, but might even act as antagonist at DP-mediated eosinophil responses.

Expression of CRTH2 and DP in human eosinophils
To further substantiate our findings of DP being involved in eosinophil release from bone marrow and eosinophil chemotaxis, we attempted to visualize the expression of DP and CRTH2 in human peripheral blood eosinophils and eosinophils in bone marrow biopsy specimens. Using immunohistochemistry, we identified DP and CRTH2 in normal bone marrow specimens of three different individuals (data not shown) and one patient suffering from hypereosinophilia of the bone marrow. In these samples, DP was located in mature eosinophils with typical bi-lobed nuclei that also stained positive for eosinophil peroxidase (Fig. 7A). CRTH2 could be observed in mature eosinophils and also in cells with larger, nonsegmented nuclei consistent with eosinophil precursors (Fig. 7B). Using double-staining immunofluorescence microscopy, we observed that eosinophils from peripheral blood expressed both DP and CRTH2, whereas neutrophils expressed DP only, although at higher levels than eosinophils (Fig. 8). In contrast, cells incubated with the respective isotype-matched primary control Abs followed by the secondary Abs did not exhibit positive staining.

FIGURE 5. PGD$_2$ selectively induces the migration of guinea pig bone marrow eosinophils (A), and the DP antagonist BWA868c and the CRTH2 antagonist ramatroban inhibit this response (B). Eosinophils and neutrophils were isolated from the femoral bone cavity. Cells were placed on Transwell filter inserts and allowed to migrate toward PGD$_2$. In A, responses are expressed as the chemotactic index (i.e., relative to migration toward vehicle). In B, data are shown as percentage of the maximal control response (i.e., 100 nM PGD$_2$). Data are shown as mean ± SEM. *, $p < 0.05$ versus vehicle, $n = 5–7$.

FIGURE 6. The PGD$_2$-induced migration of human peripheral blood eosinophils is inhibited by the DP antagonist BWA868c and the CRTH2 antagonist ramatroban but not the TP antagonist SQ29548. In A, purified eosinophils were pretreated with the antagonists, were then loaded into the top wells of a microBoyden chamber and allowed to migrate toward PGD$_2$ or eotaxin. In B, the migration of eosinophils toward the DP agonists ZK110841 and BW245c was determined. In C, eosinophils were pretreated with BW245c and were then allowed to migrate toward PGD$_2$. Cells that had migrated into the bottom wells were enumerated by flow cytometry and the counts of eosinophil events gathered for 30 s are shown as mean ± SEM. *, $p < 0.05$ versus vehicle, $n = 4–6$.

FIGURE 7. Human bone marrow eosinophils express DP and CRTH2 receptors. In A, eosinophil peroxidase-positive cells are stained brown by immunohistochemistry and DP-positive eosinophils (arrows) appear in red. In B, cells positive for eosinophil peroxidase are stained red, CRTH2-positive eosinophils and eosinophil precursors appear in brown (arrows).
A demonstrates the DP selectivity of this antagonist. The mobilization of bone marrow, but BWA868c did not inhibit this response, which might be the blockade of DP receptors on endothelial cells, the femoral cavity. One possible mechanism underlying the inhibitory effect of BWA868c, but not the TP antagonist SQ29548. However, the DP antagonist BWA868c did not inhibit the chemotaxis of human eosinophils purified from peripheral blood, whereas the TP-selective antagonist SQ29548 had no effect. However, the DP antagonist BWA868c also significantly reduced the migration toward PGD2 by inducing a 6-fold rightward shift of the concentration response curve to PGD2 without altering the maximal response.

Our data suggested for the first time that, in addition to CRTH2, the alternate PGD2 receptor DP might also be involved in eosinophil chemotaxis and mobilization of eosinophils from the bone marrow. This conclusion was based on the observation that BWA868c inhibited the chemotaxis and mobilization from bone marrow of eosinophils that had been stimulated with PGD2. BWA868c has previously been characterized as a highly selective antagonist at human DP receptors with virtually no affinity for human CRTH2 (36). The same seems to hold true for the guinea pig, since BWA868c potently inhibited the binding of PGD2 to recombinant guinea pig DP, but it did not inhibit the PGD2-induced shape change of guinea pig eosinophils, a response solely mediated by CRTH2. Moreover, BWA868c did not inhibit the chemotaxis of human eosinophils toward eosin and LTB4. Finally, the selectivity of the DP antagonist was also demonstrated by its lack of effect when eosinophil mobilization from bone marrow was induced by the selective CRTH2 agonist, DK-PGD2.

Whether eosinophils express the corresponding DP receptor protein has not been investigated before. Using immunohistochemistry we found that eosinophils in human bone marrow and in peripheral blood express both CRTH2 and DP receptors, which further emphasizes the importance of DP in eosinophil trafficking. BW245c and ZK110841 have previously been described as selective DP agonists, which can potently mimic those PGD2 effects that are mediated by the DP receptor, such as inhibition of platelet aggregation, vasodilatation and ocular hypotension (24, 37, 38). BW245c has been reported to delay eosinophil apoptosis at supramaximal (1 μM) concentration (10). In this study, we found that BW245c and ZK110841 were not chemotactic for eosinophils, and none of the DP agonists induced the mobilization of eosinophils from in the in situ perfused guinea pig hind limb preparation. On the contrary, BW245c actually inhibited the responses to PGD2, i.e., the chemotaxis of peripheral blood eosinophils and eosinophil release from bone marrow. However, eosinophil mobilization induced by DK-PGD2 was not attenuated by BW245c.

These observations suggest that the DP receptor, that is involved in eosinophil locomotion, behaves differently than the DP receptors in the vasculature or platelets: although BWA868c is a DP antagonist in all these cell types (38, 39), BW245c is a DP agonist in platelets and vascular smooth muscle (39, 40), but it does not activate chemotactic pathways in eosinophils and even might act as a DP antagonist in eosinophil responses. Similar disparities have been observed in the conjunctiva, where PGD2 increased conjunctival microvascular permeability in a BWA868C-sensitive manner, despite the fact that BW245c failed to evoke a plasma exudation response (25). These findings might be explained by unique coupling of the DP receptor in eosinophils: previous studies by others and our own data suggest that activation of CRTH2 in eosinophils leads to shape change through Gq G-proteins, phospholipase C, Ca2+ mobilization from intracellular stores, MAP kinases, and

Discussion

In the current study, we show for the first time that the DP receptor plays an important role in eosinophil trafficking, i.e., chemotaxis and mobilization of eosinophils from the bone marrow. Hence, our data provide a rationale for the use of DP receptor antagonists or mixed CRTH2/DP antagonists in the treatment of allergic disease. In this study, we observed that PGD2 induced the release of mature eosinophils from the bone marrow and that this effect was inhibited by either the CRTH2 antagonist ramatroban or the DP antagonist BWA868c, but not the TP antagonist SQ29548. The CRTH2-selective agonist DK-PGD2 also mobilized eosinophils from the bone marrow, but BWA868c did not inhibit this response, which demonstrates the DP selectivity of this antagonist. The mobilization of eosinophils from the bone marrow was apparently linked with the chemotactic activity of PGD2, as the prostanoid induced the in vitro migration of eosinophils isolated from the guinea pig femoral cavity. One possible mechanism underlying the inhibitory effect of BWA868c on eosinophil mobilization from bone marrow might be the blockade of DP receptors on endothelial cells, the activation of which by PGD2 may support the release of bone marrow cells to the blood stream when the eosinophils are stimulated through CRTH2. However, both DP and CRTH2 seem to be involved in the migration of bone marrow eosinophils at the cellular level, because either BWA868c or ramatroban inhibited the in vitro chemotaxis of isolated bone marrow eosinophils. This was unexpected because the DP receptor has not been implicated in the chemotactic response of eosinophils to PGD2 before. Therefore we further investigated the role of DP in the migration of human eosinophils purified from peripheral blood. As expected, the CRTH2 antagonist markedly attenuated the PGD2-induced chemotaxis of human eosinophils purified from peripheral blood, whereas the TP-selective antagonist SQ29548 had no effect. However, the DP antagonist BWA868c also significantly reduced the migration toward PGD2, by inducing a 6-fold rightward shift of the concentration response curve to PGD2 without altering the maximal response.

Our data suggested for the first time that, in addition to CRTH2, the alternate PGD2 receptor DP might also be involved in eosinophil chemotaxis and mobilization of eosinophils from the bone marrow. This conclusion was based on the observation that BWA868c inhibited the chemotaxis and mobilization from bone marrow of eosinophils that had been stimulated with PGD2. BWA868c has previously been characterized as a highly selective antagonist at human DP receptors with virtually no affinity for human CRTH2 (36). The same seems to hold true for the guinea pig, since BWA868c potently inhibited the binding of PGD2 to recombinant guinea pig DP, but it did not inhibit the PGD2-induced shape change of guinea pig eosinophils, a response solely mediated by CRTH2. Moreover, BWA868c did not inhibit the chemotaxis of human eosinophils toward eosin and LTB4. Finally, the selectivity of the DP antagonist was also demonstrated by its lack of effect when eosinophil mobilization from bone marrow was induced by the selective CRTH2 agonist, DK-PGD2.

Whether eosinophils express the corresponding DP receptor protein has not been investigated before. Using immunohistochemistry we found that eosinophils in human bone marrow and in peripheral blood express both CRTH2 and DP receptors, which further emphasizes the importance of DP in eosinophil trafficking. BW245c and ZK110841 have previously been described as selective DP agonists, which can potently mimic those PGD2 effects that are mediated by the DP receptor, such as inhibition of platelet aggregation, vasodilatation and ocular hypotension (24, 37, 38). BW245c has been reported to delay eosinophil apoptosis at supramaximal (1 μM) concentration (10). In this study, we found that BW245c and ZK110841 were not chemotactic for eosinophils, and none of the DP agonists induced the mobilization of eosinophils from in the in situ perfused guinea pig hind limb preparation. On the contrary, BW245c actually inhibited the responses to PGD2, i.e., the chemotaxis of peripheral blood eosinophils and eosinophil release from bone marrow. However, eosinophil mobilization induced by DK-PGD2 was not attenuated by BW245c.

These observations suggest that the DP receptor, that is involved in eosinophil locomotion, behaves differently than the DP receptors in the vasculature or platelets: although BWA868c is a DP antagonist in all these cell types (38, 39), BW245c is a DP agonist in platelets and vascular smooth muscle (39, 40), but it does not activate chemotactic pathways in eosinophils and even might act as a DP antagonist in eosinophil responses. Similar disparities have been observed in the conjunctiva, where PGD2 increased conjunctival microvascular permeability in a BWA868C-sensitive manner, despite the fact that BW245c failed to evoke a plasma exudation response (25). These findings might be explained by unique coupling of the DP receptor in eosinophils: previous studies by others and our own data suggest that activation of CRTH2 in eosinophils leads to shape change through Gq G-proteins, phospholipase C, Ca2+ mobilization from intracellular stores, MAP kinases, and

FIGURE 8. Human peripheral blood eosinophils express DP and CRTH2 receptors. In D, double-staining immunofluorescence shows an eosinophil (Eo) and a neutrophil (N) stained positive for DP (green), whereas B demonstrates that the same eosinophil also expresses CRTH2 (red). A and C are images taken with the respective control Abs. E and F show overlay-images of A and C, and B and D, respectively.
actin polymerization (17, 41), while the CRTH2-mediated chemotaxis of eosinophils depends on G<sub>q</sub> (36). By contrast, DP is coupled to G<sub>q</sub> and activation of adenylyl cyclase (3, 41). From our data it appears that the events leading to PGD<sub>2</sub>-stimulated shape change are solely mediated by CRTH2 without an involvement of DP. Monneret et al. (41) have shown that both PGD<sub>2</sub> and BW245c stimulate cAMP formation in eosinophils in a BWA868c-sensitive manner. However, an increase of cAMP is likely to attenuate eosinophil locomotion and decrease the magnitude of eosinophil infiltration at inflammatory sites is determined by the balance between CRTH2 and DP antagonists (31). However, the pharmacological mechanisms that underlie these observations have remained unclear. Here we have demonstrated for the first time that eosinophils in the bone marrow and peripheral blood express the DP receptor and that DP is substantially involved in eosinophil recruitment. This notion applies both to eosinophil release from the bone marrow and chemotaxis. Our current data provide novel insights into the roles of PGD<sub>2</sub> in eosinophil function and hence add further evidence for the potential usefulness of DP antagonists in the treatment of allergic disease.

**Disclosures**

The authors have no financial conflict of interest.

**References**


