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Cutting Edge: Serotonin Is a Chemotactic Factor for Eosinophils and Functions Additively with Eotaxin

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Elevated levels of serotonin (5-hydroxytryptamine, 5-HT) are observed in the serum of asthmatics. Herein, we demonstrate that 5-HT functions independently as an eosinophil chemoattractant that acts additively with eotaxin. 5-HT2A receptor antagonists (including MDL-100907 and cyproheptadine (CYP)) were found to inhibit 5-HT-induced, but not eotaxin-induced migration. Intravital microscopy studies revealed that eosinophils roll in response to 5-HT in venules under conditions of physiological shear stress, which could be blocked by pretreating eosinophils with CYP. OVA-induced pulmonary eosinophilia in wild-type mice was significantly inhibited using CYP alone and maximally in combination with a CCR3 receptor antagonist. Interestingly, OVA-induced pulmonary eosinophilia in eotaxin-knockout (Eot−/−) mice was inhibited by treatment with the 5-HT2A but not CCR3 receptor antagonist. These results suggest that 5-HT is a potent eosinophil-active chemoattractant that can function additively with eotaxin and a dual CCR3/5-HT2A receptor antagonist may be more effective in blocking allergen-induced eosinophil recruitment. The Journal of Immunology, 2004, 173: 3599–3603.

Eosinophils play a prominent proinflammatory role in airway allergic inflammation including the pathogenesis of asthma (1, 2, 3). This inflammatory role is mediated by lipid mediators, cytokines, and toxic granule proteins released by activated eosinophils (4). Several studies have demonstrated a critical role for eotaxin in the selective recruitment of eosinophils following allergen challenge (5–7). In experimental studies of allergic asthma, treatment of allergen-challenged mice with an anti-eotaxin Ab resulted in a 56% inhibition of eosinophil influx (7). Eot−/− mice demonstrated a 70% reduction of eosinophils in bronchoalveolar lavage (BAL) fluid 18 h postallergen challenge (5), while eotaxin receptor CCR3−/− mice exhibited an ∼60% decrease in pulmonary eosinophilia postchallenge (6). These studies demonstrate the importance of the eotaxin-CCR3 interaction in allergic asthma, but they also suggest that other eosinophil chemoattractants that function through different receptors are likely to be involved in selectively attracting eosinophils to respiratory tissue.

5-Hydroxytryptamine (5-HT, serotonin) is one of the most extensively studied neurotransmitters of the CNS that is also present in constituents of the immune system. It is an important inflammatory mediator that is released by mast cells upon IgE cross-linking and has recently been shown to play a role in the pathophysiology of asthma (8). Increased levels of free 5-HT are present in the plasma of symptomatic asthmatic patients compared with asymptomatic subjects (9), and recent studies have also demonstrated that 5-HT can induce lung fibroblasts to produce eotaxin (10). Although these studies suggest a role for 5-HT in allergic asthma, a direct effect of 5-HT in mediating eosinophil recruitment/chemotaxis relative to the function of eotaxin has not been determined. In the present study, we have investigated the role of 5-HT to function directly as an eosinophil-specific chemoattractant.

Materials and Methods

Eosinophil isolation

Eosinophils were purified from the peripheral blood of allergic donors (11). For the in vitro experiments, eosinophils were fluorescently labeled with carboxyfluorescein diacetate (CFDA, Invitrogen, Carlsbad, CA) (12).

Synthesis of the CCR3 antagonist N-(1(S)-4-(3,4-dichlorobenzyl)piperazin-1-yl)-1-{1-[2-(4-dimethylamino-2-methylpropyl)-4-methylbenzamide dihydrochloride salt (DPM)

DPM was synthesized following the protocol described in patent EP 0 903 349 A2. This antagonist has a Ki value of 62 nM for the human CCR3 receptor and >10 μM for the 5-HT2 receptors (data not shown).

Chemotaxis assays

Boyden chamber assay. The ability of 5-HT to induce eosinophil migration was tested using the Boyden chamber assay (11). In some experiments, eosinophils were preincubated with cyproheptadine (CYP), ketanserin, pirenperone, or DPM for 15 min before the chemotaxis assay.

Transwell chamber assay. Eosinophils isolated from different allergic donors (n = 4) were preincubated with MDL-100907 (13, 14) (kindly provided by Dr. H. Y. Huang, Columbia University, New York, NY) at a

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3 Abbreviations used in this paper: BAL, bronchoalveolar lavage; %-HT, 5-hydroxytryptamine (serotonin); CFDA, carboxyfluorescein diacetate; DPM, N-[1(S)-4-(3,4-dichlorobenzyl)piperazin-1-yl)-1-{1-[2-(4-dimethylamino-2-methylpropyl)-4-methyl benzamide dihydrochloride salt; CYP, cyproheptadine; IVM, intravital microscopy; RF, rolling fraction; WT, wild type; hpf, high-power field.

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concentration of 20 μM for 20 min or with medium alone before addition (2 × 10^5/well) to Matrigel-coated (200 μg/ml) Transwell chambers. Culture medium alone or medium containing 5-HT (100 nM) or eotaxin (50 nM) was added to the lower chamber. In certain experiments, 5-HT was added to only upper or both upper and lower chambers and incubated at 37°C for 4 h, after which the chambers were removed and the number of migrated cells was quantitated and expressed as a percentage of total cells added to the well.

RT-PCR
Amplification of the gene for 5-HT2A was performed by RT-PCR of eosinophil RNA isolated from human allergic subjects. Eosinophil reverse transcriptase product underwent two rounds of PCR amplification using the following conditions: 15 s at 94°C, 30 s at 57°C, and 45 s at 72°C for 30 cycles. The sequence of the primers was as follows: 5′-TATATACTGTTAGCTTTTCAGG-3′ and internal antisense primer, 5′-GCTTCCACACACAGCTACC-3′. Two microtubes of the first PCR was used as a template for the second round. The internal nested primer sequences were as follows: 5-HT2A receptor internal nested sense primer, 5′-TATATACCGATCGTGCAGG-3′ and internal nested antisense primer, 5′-CCTACACACACACAGCTACC-3′. All primer pairs spanned an intron.

Western blot analysis
Western blot analysis was conducted using an anti-5-HT2A receptor Ab (BD Pharmingen, San Diego, CA). The bound Ab were detected using the chemiluminescent peroxidase substrate Super Signal Ultra (Pierce, Rockford, IL).

Animal preparation and superfusion of the vascular bed with 5-HT
The ability of 5-HT to alter the flux of human eosinophil rolling in postcapillary venules of New Zealand White rabbits was examined by intravital microscopy (IVM) (12). Briefly, between 6 and 10 h after IL-1β stimulation (i.p.), portions of the exposed rabbit mesentery were superfused with 5-HT (50 nM) or vehicle alone using a constant flow infusion syringe pump. CFDA-labeled human eosinophils (0.2–0.5 × 10^6 cells/ml) were administered into the cannulated side branch of the superior mesenteric artery after superfusion with 5-HT had ensued, and their ability to roll in postcapillary venules was determined. Data are expressed as rolling fraction (RF; percentage of rolling eosinophils in the total number of eosinophils passing through a segment of a given blood vessel per injection). The ability of CYP and DPM to alter the flux of 5-HT and eosinoid-induced eosinophil rolling was determined by incubating the eosinophils ex vivo with CYP or DPM (10 μM) for 15 min at room temperature before injecting into the rabbit mesentery.

Murine model of allergic airway inflammation
Pulmonary eosinophilia was induced in wild-type (WT) and Eot−/− mice, bred on the SVEV background (kindly provided by Dr. M. Rothenberg, Children’s Hospital Medical Center, Cincinnati, OH) (5, 15). In brief, mice were sensitized by two i.p. injections of 50 μg OVA in 200 μl of alum (Pierce). Nonsensitized mice received 200 μl of alum alone. Ten days after the second injection, CYP (0.2 mg/mouse), DPM (2 mg/mouse), or both were administered (i.p.) to different groups of mice (n = 3 mice/group). Fifteen minutes after injection of the inhibitor, mice were exposed to three inhalations (30 min each) of aerosolized OVA (10 mg/ml in 0.9% sterile saline) at 1-h intervals. Nonsensitized mice received an i.p. injection of vehicle and aerosol challenge of saline only. One hour after the last allergen-challenge, mice were sacrificed and processed for BAL fluid collection to determine eosinophil counts (15).

Results
Eosinophils respond functionally to 5-HT
5-HT alone was found to induce migration of human eosinophils in a dose-dependent manner, which was maximal at 10−6 M (Fig. 1A). Furthermore, 5-HT, at various concentrations, had an additive effect on eosinophil chemotaxis when tested in combination with 50 nM eotaxin. As a positive control, 50 nM eotaxin alone also induced eosinophil migration, consistent with our previous studies (11). The migration induced by 5-HT was chemotactic and not chemokinetic (Fig. 1B). 5-HT (100 nM) was found to be selective for eosinophils and did not induce migration of neutrophils in Transwell chamber chemotaxis assays (18 ± 5% [control] vs 20 ± 4% (5-HT) of total cells added) while, C5a (10−7 M), which was used as a positive control, was found to induce migration of both eosinophils and neutrophils (data not shown).

5-HT2A receptor antagonists inhibit 5-HT-induced eosinophil chemotaxis in vitro
We tested the ability of the 5-HT2A receptor antagonists CYP, ketanserin, and pirenperone as well as a CCR3 receptor antagonist (DPM) to block human eosinophil migration in response to 5-HT or eotaxin stimulation. CYP, ketanserin, and pirenperone blocked eosinophil chemotaxis in response to 5-HT (p = 0.05, 0.05, 0.09, respectively; Fig. 1C). However, ketanserin and pirenperone, but not CYP, were found to antagonize the CCR3 receptor in radioligand binding assays, suggesting that CYP can interact with 5-HT2A and not CCR3 (data not shown).

FIGURE 1. Eosinophils respond functionally to 5-HT. A, Migration of eosinophils in response to various concentrations of 5-HT alone or in combination with 50 nM eotaxin was evaluated. The dashed line indicates the background migration with medium alone, while the dotted line indicates migration in response to 50 nM eotaxin. Five high-power fields (hpf) were counted per well (10 hpf/condition) and the average number of cells per hpf ± SEM is shown. This experiment was repeated with three different donors independently and a representative experiment is shown. B, 5-HT is chemotactic but not chemokinetic for eosinophils. Eosinophil migration (n = 3 donors) across Transwell membranes was performed with 5-HT only in the upper wells or in the upper and lower wells as indicated. Results are expressed as percent migration of eosinophils ± SE. C, 5-HT2A receptor antagonists inhibit 5-HT-induced eosinophil chemotaxis in vitro. Eosinophil migration in response to 50 nM eotaxin (left panels) or 5-HT2A M 5-HT (right panels) were examined following incubation with CCR3-specific antagonist DPM (300 nM) and the 5-HT2A receptor antagonists CYP, ketanserin, and pirenperone (each at 50 nM). Results are expressed as mean ± SEM of a representative experiment of four separate experiments using different allergic donors.
shown). Eotaxin-mediated eosinophil chemotaxis was not inhibited by CYP (Fig. 1C), but was blocked by DPM ($p = 0.01$). However, DPM did not inhibit 5-HT-mediated eosinophil chemotaxis (Fig. 1C).

Although ketanserin, pirenperone, and CYP bind to 5-HT2A, they are also known to bind to the 5-HT2B and 5-HT2C receptors at a much lower affinity (16, 17). To further confirm the receptor involved in 5-HT-mediated eosinophil migration, the effect of MDL-100907, a highly selective antagonist of the 5-HT2A receptor (14), on 5-HT-mediated eosinophil chemotaxis was investigated using Transwell chambers (Fig. 2A). MDL-100907 significantly inhibited 5-HT-induced, but not eotaxin-induced chemotaxis of human eosinophils. Next, eosinophils from allergic subjects were observed to express the 5-HT2A receptor when analyzed by RT-PCR (Fig. 2B, lane 2 and 3) as well as Western blot analysis (Fig. 2C). Stimulation with 50 nM eotaxin or 20 ng/ml IL-5 did not further enhance eosinophil 5-HT2A receptor expression (Fig. 2C, lanes 2 and 3, respectively).

5-HT2A antagonist inhibits eosinophil rolling in vivo

We next determined the effect of 5-HT and its receptor antagonist on the rolling of CFDA-labeled human eosinophils in inflamed blood vessels of the rabbit mesentry by IVM (Fig. 3). Superfusion of the mesentry with 50 nM 5-HT resulted in a 3.5-fold increase in the flux of rolling eosinophils (RF: 16 ± 3% (control) vs 56 ± 8% (5-HT); $p = 0.004$). Pretreatment of eosinophils with CYP (10 μM) resulted in near complete inhibition of rolling ($p < 0.003$). The rolling of vehicle-treated eosinophils was not affected (data not shown). As a control, superfusion of the mesentry with eotaxin (50 nM) resulted in a 3-fold increase in eosinophil rolling (RF: 33 ± 5% (eotaxin) vs 11 ± 2% (control) $p = 0.01$). This eotaxin-induced eosinophil rolling was completely inhibited by DPM ($p = 0.002$). These results suggest that a functional 5-HT2A receptor is required for multiple steps during 5-HT-induced migration of eosinophils.

5-HT2A antagonist inhibits allergen-induced pulmonary eosinophilia in Eot$^{-/-}$ mice and functions additively with a CCR3 antagonist in WT mice

To determine whether treatment with the 5-HT2A receptor antagonist CYP had any effect on a pathological eosinophil influx in a murine model of allergic asthma, WT as well as Eot$^{-/-}$ mice were sensitized with OVA and then treated with CYP, DPM, or saline (control) before allergen challenge (Fig. 4). OVA sensitization followed by aerosolized allergen challenge induced significant BAL eosinophilia in Eot$^{-/-}$ as well as WT mice. There was a 68% decrease in the total number of eosinophils recovered in the BAL fluid from Eot$^{-/-}$ mice compared with that of WT mice, consistent with previous findings (5). In the WT mice, i.p. administration of CYP or DPM before OVA challenge significantly inhibited (>80%, $p = 0.01$ for both inhibitors) pulmonary eosinophilia compared with vehicle-treated OVA-challenged mice. Furthermore, a greater inhibition of the eosinophil influx was observed when both antagonists were used (94% reduction, $p = 0.01$). Interestingly, in the Eot$^{-/-}$ mice, CYP was effective in significantly reducing pulmonary eosinophilia (80%, $p = 0.04$) while the administration of DPM had no effect in reducing early stage eosinophilia under these experimental conditions. When both antagonists...
were administered in combination, there was no further reduction in pulmonary eosinophilia compared with administration of CYP alone (78%, $p > 0.01$). These results clearly demonstrate a role for 5-HT as a chemoattractant for eosinophils in allergen-challenged WT as well as Eot$^{−/−}$ mice. Overall, our studies demonstrate that there is an additive effect in preventing pulmonary eosinophilia when both 5-HT2A and CCR3 antagonists are used in WT mice and that 5-HT independently plays a significant role in the eosinophilia observed in the murine model of allergic inflammation as demonstrated by the studies with Eot$^{−/−}$ mice.

**Discussion**

In this study, we demonstrate that 5-HT by itself is a potent eosinophil chemoattractant that can act additively with eotaxin. Furthermore, we demonstrate that pharmacological antagonism of the 5-HT2A receptor by multiple receptor antagonists (CYP, ketanserin, pitiperone, and MDL-109007) blocks 5-HT-mediated chemotaxis in highly controlled in vitro chemotaxis experiments. In addition, CYP was also observed to block 5-HT-mediated rolling of eosinophils under conditions of physiologic blood flow in postcapillary venules and to inhibit allergen-induced pulmonary eosinophilia in mice. It is conceivable that 5-HT provides a gradient to circulating eosinophils and, through interaction with the 5-HT2A receptor expressed by eosinophils, induces a series of signaling events resulting in rolling (the earliest step of the adhesion cascade) and subsequent transmigration of the cells toward the source of the chemoattractant as observed in the case of other eosinophil active chemoattractants (11, 18, 19). Overall, these data strongly suggest that the 5-HT2A receptor is responsible for mediating the chemoattractant effects of 5-HT.

These observations are critical for understanding the role of various chemoattractants in orchestrating eosinophil migration. Several studies have clearly demonstrated the importance of CCR3 and eotaxin in regulating eosinophil migration using murine models of allergic inflammation (5–7). However, although these studies demonstrate the importance of the eotaxin-CCR3 interaction in allergic inflammation, they also illustrate that other eosinophil chemoattractants that function through different receptors are also likely to be involved in attracting eosinophils to lung tissue since blocking CCR3 and/or eotaxin resulted only in partial inhibition of pulmonary eosinophilia. This study confirms previous findings demonstrating the role eotaxin plays in early-stage eosinophil recruitment to the airway tissue (5) as there was a 67% decrease of eosinophils in the BAL fluid isolated from OVA-challenged Eot$^{−/−}$ mice compared with that of WT mice. However, in studies examining WT mice, the 5-HT2A antagonist CYP inhibited eosinophilia to a similar extent as the CCR3 antagonist DPM. Since 5-HT has been shown to induce lung fibroblasts to produce eotaxin (10), this raises the question of whether CYP acts by blocking eotaxin production by lung fibroblasts or whether it acts in a more direct manner to inhibit eosinophil recruitment by 5-HT. Examination of the Eot$^{−/−}$ mice challenged with aerosolized OVA and treated with CYP showed a profound decrease in pulmonary eosinophilia, suggesting that 5-HT can play an integral role in allergic eosinophil recruitment independent of the involvement of eotaxin. This is underscored by the lack of effect elicited by DPM treatment in the Eot$^{−/−}$ mice. Furthermore, treatment of OVA-challenged WT mice with CYP and DPM had a greater inhibitory effect than either antagonist alone.

A recent study demonstrated that airway hyperresponsiveness and eosinophilia can be modulated by histamine and 5-HT2 receptor antagonists in a mouse model of allergic inflammation (20). However, the mechanism by which the effects of 5-HT were mediated is difficult to interpret in these studies as ketanserin, the 5-HT2A antagonist used in the study, also antagonizes the CCR3 receptor. Additionally, another study investigating bronchospasm in asthmatic humans showed that ketanserin had a protective effect on adenosine-induced bronchoconstriction (21). However, the mechanism by which ketanserin elicited these protective effects was not determined. The current study using multiple 5-HT2A receptor antagonists in in vitro chemotaxis and in vivo rolling studies as well as in a murine model of allergic inflammation with WT and Eot$^{−/−}$ mice clearly shows that the effects of 5-HT are mediated by this receptor. Although CYP has been shown to have some antagonist effect on the histamine H2 receptor, CYP can block 5-HT-mediated eosinophil chemotaxis in vitro as well as 5-HT-induced eosinophil rolling in rabbit postcapillary venules in the absence of histamine stimulation. Thus, the effects of CYP observed in the allergen-challenged WT and Eot$^{−/−}$ mice are most likely due to antagonism of the 5-HT2A receptor.

In summary, we demonstrate that 5-HT independently acts as a chemoattractant for eosinophils and this effect appears to be mediated by the 5-HT2A receptor. Additionally, 5-HT has an additive effect with eotaxin, inducing eosinophil chemotaxis and pulmonary eosinophilia.

**References**


