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Prostaglandin E_2 Selectively Enhances the IgE-Mediated Production of IL-6 and Granulocyte-Macrophage Colony-Stimulating Factor by Mast Cells Through an EP_1/EP_3-Dependent Mechanism[1]

Kaede Gomi,* Fu-Gang Zhu,* and Jean S. Marshall[2]*†

PGE_2 is an endogenously synthesized inflammatory mediator that is over-produced in chronic inflammatory disorders such as allergic asthma. In this study, we investigated the regulatory effects of PGE_2 on mast cell degranulation and the production of cytokines relevant to allergic disease. Murine bone marrow-derived mast cells (BMMC) were treated with PGE_2 alone or in the context of IgE-mediated activation. PGE_2 treatment alone specifically enhanced IL-6 production, and neither induced nor inhibited degranulation and the release of other mast cell cytokines, including IL-4, IL-10, IFN-γ, and GM-CSF. IgE/Ag-mediated activation of BMMC induced the secretion of IL-4, IL-6, and GM-CSF, and concurrent PGE_2 stimulation synergistically increased mast cell degranulation and IL-6 and GM-CSF, but not IL-4, production. A similar potentiation of degranulation and IL-6 production by PGE_2, in the context of IgE-directed activation, was observed in the well-established IL-3-dependent murine mast cell line, MC/9. RT-PCR analysis of unstimulated MC/9 cells revealed the expression of EP_1, EP_3, and EP_4 PGE receptor subtypes, including a novel splice variant of the EP_1 receptor. Pharmacological studies using PGE receptor subtype-selective analogs showed that the potentiation of IgE/Ag-induced degranulation and IL-6 production by PGE_2 is mediated through EP_1 and/or EP_3 receptors. Our results suggest that PGE_2 may profoundly alter the nature of the mast cell degranulation and cytokine responses at sites of allergic inflammation through an EP_1/EP_3-dependent mechanism. ***The Journal of Immunology*****, 2000, 165: 6545–6552.**

Prostaglandin E_2, an arachidonic acid metabolite, is synthesized and secreted by diverse cell types in response to many physiologic and nonphysiologic stimuli, and is increasingly becoming recognized as a potent regulator of immune responses (1). PGE_2 differentially modulates type 1- and type 2-associated cytokine production (1, 2), strongly inhibiting the production of the type 1 cytokines IL-2 (3), IL-12 (4), and IFN-γ (5) and, depending on the stimulation conditions, either having no effect or enhancing production of the type 2-associated cytokines, such as IL-4 and IL-5 (2, 6). The general consensus is that PGE_2 acts to shift the immune response toward a type 2 cytokine profile. Moreover, this lipid mediator also up-regulates IgE production (1), and may consequently support the development of asthma and other type 2 cytokine-associated inflammatory disorders. However, there is evidence for a bronchoprotective role for PGE_2 in asthma (7–10).

Mast cells are critical effector cells of hypersensitivity reactions and allergy, and their expression of cell surface receptors for PGE_2 (11–13) combined with their close proximity to PGE_2-secreting cells, such as fibroblasts (14) and macrophages (15), make mast cells potential targets for immunoregulation by PGE_2. PGE_2 has been reported to be important for mast cell development from murine spleen cell precursors (16) and human umbilical cord endothelial cells (17). In addition, PGE_2 enhances IL-6 production by rat peritoneal mast cells (PMC)[3] (18) and potently inhibits TNF-α production by these cells (18, 19) and intestinal mucosal mast cells (19). Depending on the mast cell population and timing of prostanoid treatment, PGE_2 has been documented to either block the release of histamine and other inflammatory mediators from immunologically activated mast cells (19–21) or to potentiate such release (12, 18).

PGE_2 mediates many of its effects by binding to a specific group of seven-transmembrane domain, G protein-coupled receptors, of which there are four subtypes, designated EP_1, EP_2, EP_3, and EP_4 (22). EP_2 and EP_4 receptors activate adenylate cyclase and lead to increased levels in intracellular cAMP. Activation of EP_1 receptors is associated with increases in intracellular Ca^{2+}, and EP_3 generally couples to G_i and inhibits intracellular cAMP levels. To date, little work has been performed to characterize EP receptor expression on mast cells. The presence of EP_3 and EP_4 receptors has been reported for the murine mucosal type mast cell lines, BNu-2cl3 (12) and P815 (11), respectively. More recently, Chan et al. (13) provided evidence for possible EP receptor expression by rat PMC.

Here, we sought to investigate the effects of PGE_2 on mast cell cytokine responses in the context of IgE-mediated activation. As a model system, we have chosen the well-characterized murine bone marrow-derived mast cells (BMMC) (23) and an IL-3-dependent murine mast cell line, MC/9 (24). These cells have been demonstrated to share a number of characteristics with the mast cells resident in the airways and other mucosal sites of rodents and

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[3] Abbreviations used in this paper: PMC, peritoneal mast cell; BMMC, bone marrow-derived mast cell; HSA, human serum albumin.

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humans. We have focused on the effects of PGE_2 on three cytokines, IL-4, IL-6, and GM-CSF, which are produced in physiologically relevant quantities during allergic disease and are enhanced in symptomatic asthma (25, 26). IL-4 was selected for study in view of its critical role in the development of type 2 immune responses and IgE class switch (1); IL-6, for its role in inducing the acute phase response and down-regulating inflammatory processes (27); and GM-CSF, for its involvement in the pathogenesis of allergic inflammation largely through its role as a development and survival factor for eosinophils (28). EP receptor expression and usage by MC/9 cells was also examined in this study.

Materials and Methods

Mice

C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were housed in sterilized, filter-hooded cages and provided food and water ad libitum. All experiments were approved by the Animal Research Ethics Boards of McMaster University (Hamilton, Ontario, Canada) and Dalhousie University (Halifax, Nova Scotia, Canada).

Mast cells

MC/9 cells (CRL 8306; American Type Culture Collection, Manassas, VA) were routinely grown in modified DMEM (Life Technologies, Burlington, Ontario, Canada) containing 36 mg/ml L-aspartate, 0.1 mM nonessential amino acids, 50 μM 2-ME, 10% FCS, and 3 ng/ml rmIL-3 (PeproTech, Rocky Hill, NJ) at 37°C, 10% CO_2. BMMC were generated from bone marrow of C57BL/6 mice. Briefly, mice were sacrificed, and intact femurs and tibias were removed. Sterile endotoxin-free medium was repeatedly flushed through the bone shaft using a needle and syringe, and the bone marrow cells were passed through a sterile wire screen to remove any bone fragments. The cell suspension was centrifuged at $320 \times g$ for 20 min at 4°C, and cultured at a concentration of $0.5–1 \times 10^6$ nucleated cells/ml in RPMI 1640 (Life Technologies) supplemented with 10% FCS (Sigma-Aldrich, Ontario, Canada), 10% v/v concentrated WEHI-3 conditioned medium as a source of IL-3, 1% penicillin/streptomycin (Life Technologies), and 50 μM 2-ME (BMMC medium). Nonadherent cells were transferred to fresh BMMC medium at least once a week. After 4–6 wk, mast cell purity of >95% was achieved as assessed by Alcian blue or Toluidine blue staining of fixed cytocentrifuge preparations.

Mast cell activation with various stimulating agents

Mast cells were resuspended in experimental medium consisting of RPMI 1640 (Canadian Life Technologies), 10% FCS (Sigma-Aldrich Canada), 1% penicillin/streptomycin (Life Technologies), 1% HEPES (Life Technologies), and 100 μg/ml soybean trypsin inhibitor (Sigma-Aldrich Canada). Mast cells were incubated at 1×10^6 cells/ml for up to 24 h at 37°C with the following reagents either alone or in combination: PGE_2 (Sigma-Aldrich Canada); PGE_1, PGE_1 alcohol, 17-phenyl-ω-trinor-PGE_2, sulprostone, and misoprostol (purchased from Cayman Chemicals, Ann Arbor, MI). In other studies, mast cells were also activated with the cAMP-elevating agents, pentoxifylline, forskolin, and β-isoproterenol (purchased from Sigma-Aldrich Canada). In our laboratory, each of these cAMP-elevating agents was observed to increase intracellular levels of cAMP in MC/9 cells by >2.5-fold (baseline levels were $\sim 1.7 \pm 0.4$ pmol/10^6 cells). All samples were stored at ≤ -20°C until assayed.

IgE-mediated mast cell activation

BMMC and MC/9 cells were incubated at 37°C for 18–30 h in their respective media with murine hybridoma supernatant containing anti-DNP IgE (gift from Dr. F. T. Lui, Scripps Institute, La Jolla, CA) or anti-TNP IgE (TIB-141; ATCC) as stated. Sensitized cells were washed three times by centrifugation to remove unbound IgE and used immediately in experiments. For activation, cells were incubated with DNP-human serum albumin (DNP-HSA; Sigma-Aldrich Canada) or TNP-BSA (Biosearch Technologies, Novato, CA) at a predetermined optimal concentration of 10 ng/ml for 20 min to assess β-hexosaminidase release or for up to 24 h to examine cytokine production in supernatant samples.

RT-PCR of murine EP receptors

MC/9 cells and BMMC were homogenized with Trizol Reagent (Life Technologies), and total RNA was isolated according to manufacturer's instructions. cDNAs were generated by reverse transcription using random primers. Primers used for PCR amplification of the EP receptor subtypes were purchased from Research Genetics (Huntsville, AL) and sequences were as follows (29): EP_1, 5′-CGCAGGGTTCACGCACACGA-3′ and 5′-CACTGTGCCGGGAACTACGC-3′ (336 bp); EP_2, 5′-AGGACTTC GATGGCAGAGGAGAC-3′ and 5′-CAGCCCCTTACACTTCTCCAA TG-3′ (401 bp); EP_3, 5′-CCGGGCACGTGGTGCTTCAT-3′ and 5′-TAGC AGCAGATAAACCCAGG-3′ (437 bp); and EP_4, 5′-TTCCGCTCGTG GTGCGAGTGTTC-3′ and 5′-GAGGTGGTGTCTGCTTGGGTCAG-3′ (423 bp). To further amplify resulting EP_1 PCR products, a second round of PCR was performed using the following "nesting" primers: 5′-TGGTGTCGT GCATCTGCTGG-3′ and 5′-TCCCAGGCACTCTTGGTTAG-3′ (249 bp). Splice variants exist for EP_3 ($EP_{3\alpha}$, $EP_{3\beta}$, and $EP_{3\gamma}$), and the primers used in this study recognized sequences shared by all three isoforms. PCR was performed in a 50-μl reaction mixture comprised of 1 μM of each forward and reverse oligonucleotide primer, 3 mM $MgCl_2$, 0.5 mM of the four deoxynucleotide triphosphates, 5 μl cDNA preparation, and 0.02 U/μl *Taq* DNA polymerase. PCR conditions were as follows: 3 min at 94°C followed by 40 cycles of 1 min at 94°C, 1 min at 55°C (EP_3) or 60°C (EP_1, EP_2, and EP_4), and 2 min at 72°C; followed by 7 min at 72°C. For DNase I treatment of MC/9 RNA, total RNA was incubated with DNase I (Life Technologies) for 15 min at room temperature, after which time DNase I activity was inactivated by the addition of 2 mM EDTA and heating between 60 and 65°C for 20 min.

B-9 bioassay

IL-6 bioactivity was measured by the B-9 hybridoma proliferation assay (30). B-9 cells were maintained in MEM or RPMI medium (Life Technologies) supplemented with 5% FCS, 1% penicillin/streptomycin, 50 μM 2-ME, and normal human lung fibroblast- or murine monocyte macrophage J774 cell line-conditioned medium supernatant as a source of IL-6. Briefly, serial dilutions of standards and samples were performed in triplicate in Nunc 96-well microtiter plates (Life Technologies). B-9 cells were washed, resuspended at 5×10^4 cells/ml in B-9 medium, and incubated with standards and samples for 3 days at 37°C. Then, 10 μl/well 0.5 mg/ml MTT (Sigma-Aldrich Canada) was added, and, 4 h later, 50 μl/well of 10% Triton-HCl was added and the plates were stored for 18–24 h in the dark. The optical densities of the resulting reaction product were determined at 570 nm. IL-6 concentrations were reported as U/ml of bioactivity, where 1 U equals ~0.45 pg of IL-6. The sensitivity of the B-9 assay has been determined to be 10 U/ml. None of the reagents used in this study, including PGE_2 at the highest concentration used in this study (1 μM), altered B-9 cell growth under these conditions. Moreover, other mast cell-derived cytokines, including TNF-α, GM-CSF, and IL-4, do not cause proliferation of B-9 cells under these conditions (18).

ELISAs

Murine IL-4 and IL-10 were assayed using ELISA kits purchased from R&D Systems (Minneapolis, MN). IL-5 and IL-12 ELISA kits were obtained from Amersham Life Science (ON, Canada) and Genzyme Diagnostics (Cambridge, MA), respectively. GM-CSF was assayed using ELISA kits purchased from both R&D Systems and Amersham Life Science. Cyclic AMP was measured by enzyme immunoassay purchased from Amersham Pharmacia Biotech (Quebec, Canada).

Murine IFN-γ was measured by an "in-house" sandwich ELISA with all incubations performed at room temperature. Briefly, Maxisorp ELISA plates (Nunc/Inter Med, ON, Canada) were coated for 18–24 h at 4°C with 50 μl/well of 2 μg/ml anti-mouse IFN-γ capture Ab (BD PharMingen, ON, Canada) diluted in either borate-buffered saline (pH 8.3) or freshly prepared 0.1 M bicarbonate solution (in distilled water). The wells were aspirated, and incubated for 1 h with 100 μl/well blocking solution (10 mg BSA/ml PBS, pH 7.4). The blocking solution was decanted, and the wells were washed four times with TBS (pH 7.4) containing 0.05% Tween 20. Wells were aspirated after the final wash to ensure complete removal of liquid. Standards and samples were added to the plate at 50 μl/well and incubated between 1.5 and 2 h. The wells were washed as described above, and secondary biotinylated anti-mouse IFN-γ Ab (BD PharMingen) at 0.5 μg/ml in blocking solution was added at 50 μl/well. After 1 h, the wells were washed and 50 μl/well of streptavidin-alkaline phosphatase (Canadian Life Technologies) prepared in blocking solution was added to the plates for 1 h. The wells were washed, and bound labeled IFN-γ was detected with the Life Technologies ELISA Amplification System (Canadian Life Technologies). The colored product was read at 492 nm.

β-Hexosaminidase release assay

Briefly, 1×10^6 BMMC or MC/9 cells per ml were incubated for 15 min at 37°C in HEPES-Tyrodes buffer (137 mM Na, 5.6 mM glucose, 2.7 mM KCl, 0.5 mM NaH_2PO_4, 1 mM $CaCl_2$, 10 mM HEPES, 0.1% BSA, pH 7.3,

~300 mOsm/kg) in the presence of various stimulating agents. β-Hexosaminidase release was stopped by pelleting the cells at 140 × *g* for 10 min at 4°C. Supernatants were collected and the pellets were resuspended in the original volume of HEPES-Tyrode's buffer lacking the stimulating agents. β-Hexosaminidase content in the supernatant and pellet samples was determined using a previously reported method (31). Briefly, 50 μl of samples were transferred to individual wells of a 96-well plate and incubated with 50 μl of 1 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Sigma-Aldrich Canada) dissolved in 0.1 M citrate buffer, pH 5, for 1 h at 37°C. The reaction was stopped by the addition of 200 μl/well of 0.1 M carbonate buffer, pH 10.5. The resulting yellow reaction product was read at 405 nm in an ELISA reader, and net percent β-hexosaminidase release was calculated as follows: β-hexosaminidase in supernatant/(β-hexosaminidase in supernatant + β-hexosaminidase in pellet) × 100%.

Statistical analysis

All data are expressed as the mean ± SEM. Statistical analysis was performed by one-way ANOVA. The effects of different treatments were compared using the Student-Newman-Keuls post test for comparison of individual groups and controls with the exception of IL-6 production data, which, in view of the data distribution, were compared using the Bonferroni multiple comparisons test.

Results

PGE_2 induces enhancement of IL-6 production by BMMC

To assess the effects of PGE_2 activation alone on mast cell cytokine production, BMMC were activated with different doses of PGE_2 for up to 24 h, and supernatants were assayed for cytokines of interest. As previously demonstrated in rat PMCs (18), IL-6 production in BMMC was enhanced by PGE_2 in a dose-dependent manner (baseline IL-6 production of 21.6 ± 5.5 U/ml was increased to 595 ± 72 U/ml ($p < 0.001$) and 280 ± 77 U/ml ($p < 0.01$) following stimulation with PGE_2 at 10^{-6} M and 10^{-8} M, respectively ($n = 8$)). In contrast, PGE_2 lacked any significant effect on the production of IL-4, IL-5, IL-10, IFN-γ, and GM-CSF (data not shown), whereas BMMC were capable of producing each of these cytokines in response to FcεRI cross-linking alone (IL-4, IL-5, GM-CSF) (32–34), IL-3 treatment (IL-10) (35), or IL-12 treatment (IFN-γ) (36).

PGE_2 synergistically increases IL-6 and GM-CSF responses in the context of IgE-mediated activation

Mast cells are known to be activated via cross-linking of their surface FcεRI by specific allergen. To examine the regulatory effects of PGE_2 in the context of IgE-mediated activation, BMMC were passively sensitized with anti-DNP IgE or anti-TNP IgE for 18–30 h, and subsequently incubated with respective Ag, DNP-HSA, or TNP-BSA (at 10 ng/ml), in the presence or absence of PGE_2. IgE-mediated activation increased the BMMC production of IL-6, GM-CSF, and IL-4 over that of media-treated controls (Fig. 1, *A–C*). Costimulation of IgE-sensitized mast cells with DNP-HSA and PGE_2 resulted in increased IL-6 and GM-CSF production over IgE-mediated activation alone ($p < 0.01$ for IL-6; $p < 0.001$ for GM-CSF) (Fig. 1, *A* and *B*). IgE-mediated IL-4 production, in contrast, was not enhanced by PGE_2, and at higher concentrations, PGE_2 (≥10 nM) had suppressive effects on IL-4 production ($p < 0.01$) (Fig. 1*C*). We also investigated the modulation of IL-6 production by PGE_2 in an IL-3-dependent murine mast cell line, MC/9. IL-6 production by MC/9 cells was also potentiated by PGE_2 in the context of IgE-mediated activation; however, PGE_2 alone failed to consistently induce IL-6 production by a range of PGE_2 doses (10^{-8}, 10^{-7}, 10^{-6} M) (data not shown).

Time course of PGE_2 effects on cytokine production

Kinetic studies were performed investigating IL-6 and GM-CSF release in response to PGE_2 and IgE-mediated activation of BMMC. IgE-mediated IL-6 and GM-CSF production, which was minimal or absent at 1 h, was readily detected by 6 h, and cytokine levels were maintained up to the 24-h time point (Table I). PGE_2-mediated potentiation of IL-6 and GM-CSF production in IgE/Ag-activated cells was readily apparent by 6 h poststimulation. IgE-mediated activation also induced significant IL-4 release by 6 h (98.7 ± 11.4 pg/ml; $p < 0.001$ with respect to the media control value of 11.3 ± 4.7 pg/ml; $n = 2$), and such secretion was not modulated at this time point by PGE_2 (102 ± 4 pg/ml for concurrent IgE/Ag and PGE_2 treatment; $n = 3$).

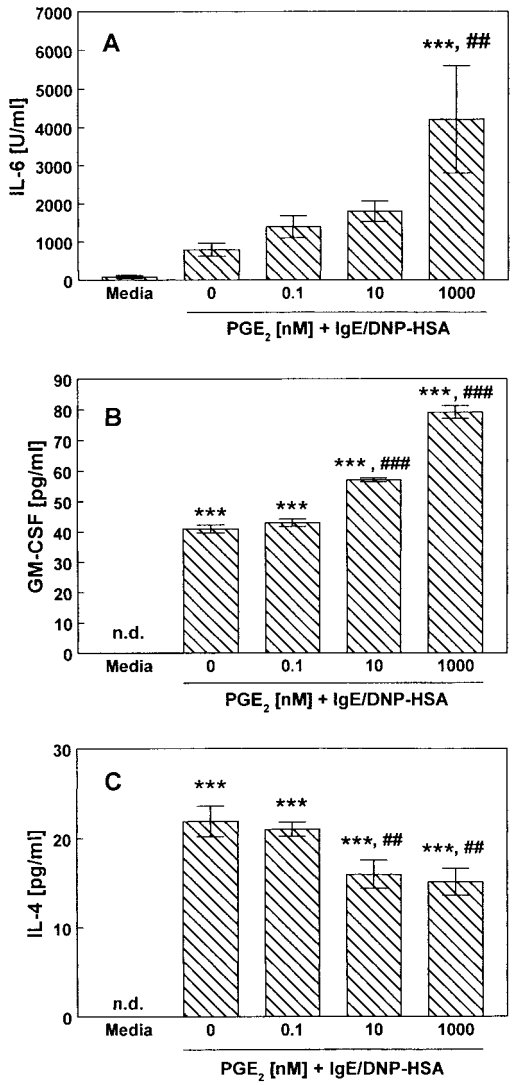

FIGURE 1. PGE_2 effects on IgE- activated mast cells. BMMC passively sensitized with anti-DNP IgE were incubated with DNP-HSA alone or in the presence of various doses of PGE_2. BMMC incubated with medium alone were used as controls. Following 24 h of incubation, supernatants were harvested and assayed for IL-6 (*A*), GM-CSF (*B*), and IL-4 (*C*) content. Bars represent mean values ± SEM. ***, $p < 0.001$ compared with media controls. ##, $p < 0.01$; ###, $p < 0.001$ compared with IgE-mediated activation alone. n.d., not detected in the assay (limit of detection for the GM-CSF and IL-4 ELISAs were 1 and 2 pg/ml, respectively).

PGE_2 induces potentiation of mast cell degranulation

To examine the effects of PGE_2 on mast cell degranulation, BMMC and MC/9 cells were activated for 20 min with PGE_2 alone or in combination with IgE/Ag-activation, and the degree of β-hexosaminidase release was measured as a marker of degranulation. PGE_2 activation alone did not induce β-hexosaminidase

Table I. *Kinetics of cytokine production by BMMC in response to IgE/Ag and PGE_2*

	IL-6 (U/ml)			GM-CSF (pg/ml)		
	1 h	6 h	24 h	1 h	6 h	24 h
Media	12.7 ± 1.3[a]	78.0 ± 15.5	116 ± 14	ND	ND	1.1 ± 0.1
PGE_2	10.0 ± 0.0	63.3 ± 9.3	263 ± 38	ND	ND	1.5 ± 0.2
IgE/DNP	12.7 ± 1.3	643 ± 20*	393 ± 87	1.1 ± 0.1	29.0 ± 1.5***	25.3 ± 2.2***
IgE/DNP + PGE_2	19.0 ± 2.5	1110 ± 283***	1190 ± 307***,##	ND	55.3 ± 1.3***,###	54.0 ± 2.1***,###

[a] Cells were passively sensitized with anti-DNP IgE, and incubated with DNP-HSA (10 ng/ml) in the presence or absence of PGE_2 (1 μM). Supernatants were harvested at different times and assayed for IL-6 and GM-CSF. Figures represent mean values ± SEM. *, Denotes $p < 0.05$; ***, denotes $p < 0.001$ compared with media controls; ##, denotes $p < 0.01$; ###, denotes $p < 0.001$ compared with IgE-mediated activation alone. ND, Not detectable in the assay (limit of detection for the GM-CSF ELISA was 1 pg/ml).

release by BMMC (Fig. 2*A*) or MC/9 cells (Fig. 2*B*). IgE-mediated activation induced significant β-hexosaminidase release by both mast cell populations, and concurrent stimulation with PGE_2 consistently enhanced this release by at least 30% (Fig. 2).

Effects of PGE_2 on mast cell degranulation and IL-6 production are unlikely to be mediated by the second messenger, cAMP

Our findings of enhanced degranulation induced by PGE_2 in the context of IgE-mediated activation are in contrast to the inhibitory effects of this prostanoid on mast cell degranulation reported when mast cells were preincubated with PGE_2 before addition of other mast cell stimuli (19–21). In the latter studies, intracellular cAMP was implicated as the second messenger mediating the inhibitory effects. To investigate whether cAMP played a critical role in PGE_2 -mediated enhancement of degranulation and IL-6 production in IgE/Ag-activated mast cells, BMMC and MC/9 were stimulated with cAMP-elevating agents. In contrast to the stimulatory effects observed with PGE_2, forskolin, a direct activator of adenylate cyclase, inhibited IgE-mediated β-hexosaminidase release in both BMMC and MC/9 cells (Fig. 2), and failed to potentiate IL-6 production in IgE/Ag-activated MC/9 cells (Fig. 3). Two additional cAMP-elevating agents, β-isoproterenol and the phosphodiesterase inhibitor, pentoxifylline, also failed to potentiate IL-6 production in the context of IgE-mediated activation (Fig. 3).

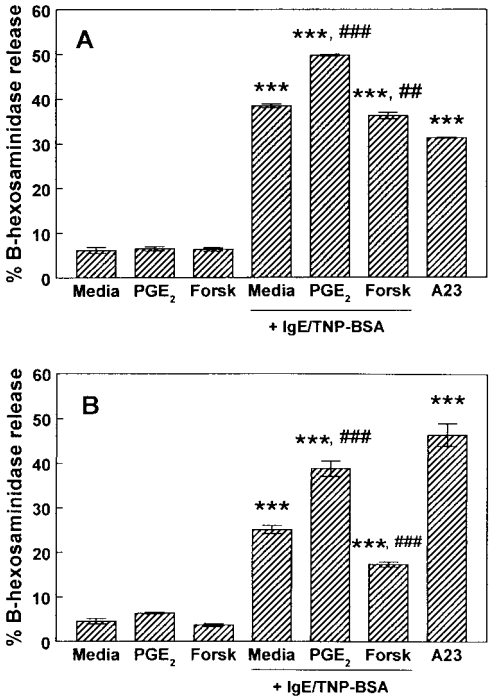

FIGURE 2. β-Hexosaminidase release by BMMC and MC/9 in response to PGE_2 and IgE-mediated activation. BMMC (*A*) and MC/9 (*B*) were previously sensitized with anti-TNP IgE and incubated with TNP-BSA (10 ng/ml) in the presence or absence of PGE_2 (1 μM) for 15 min at 37°C. BMMC incubated in buffer alone served as a control for spontaneous β-hexosaminidase release. Bars represent mean values ± SEM. ***, $p <$ 0.001 compared with media controls. ###, $p < 0.001$ compared with IgE-mediated activation alone. A23, A23187 (calcium ionophore; 1 μM); Forsk, Forskolin (10 μM). Data shown are representative of at least three independent experiments.

Involvement of EP_1/EP_3 receptors in the potentiation of β-hexosaminidase and IL-6 production in IgE/Ag-activated mast cells

PGE_2 acts by interacting with one of four receptor subtypes designated EP_1, EP_2, EP_3, and EP_4 (22). To examine whether PGE_2 receptor agonists could modulate IgE/Ag-induced β-hexosaminidase release and IL-6 production, MC/9 cells were stimulated with a panel of synthetic agonists that demonstrate preferential binding of one or more EP subtypes. The EP_1 agonist, 17-phenyl-ω-trinor-PGE_2, and the EP_1/EP_3 selective agonist, sulprostone, potentiated β-hexosaminidase release (Fig. 4) and IL-6 production (Fig. 5 and Table II) by IgE/Ag-activated mast cells. PGE_1, a PGE_2 homologue which binds with comparable affinity as PGE_2 to EP_2, EP_3, and EP_4 yet more weakly to EP_1, strongly potentiated β-hexosaminidase release by MC/9 cells. However, PGE_1 induced IL-6 production to a substantially lower degree than PGE_2 (Fig. 5 and Table II). The EP_2/EP_4-selective agonist, PGE_1 alcohol, failed to enhance β-hexosaminidase (Fig. 4) and IL-6 production (Fig. 5 and Table II) above IgE-mediated activation alone. These data implicate the involvement of the EP_1 and/or EP_3 receptors in β-hexosaminidase release and IL-6 production. Involvement of EP_3 in mediating β-hexosaminidase release was further suggested

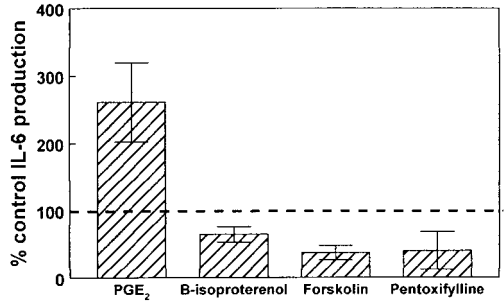

FIGURE 3. Effect of cAMP-elevating agents on IL-6 production by IgE/Ag-activated mast cells. MC/9 cells were passively sensitized with anti-TNP IgE and subsequently incubated for 24 h with TNP-BSA (10 ng/ml) alone or in the presence of β-isoproterenol (10 μM), forskolin (10 μM), or pentoxifylline (1 mg/ml). MC/9 cells incubated with PGE_2 (1 μM) and TNP-BSA (10 ng/ml) served as controls. Bars represent mean % change (±SEM) in IL-6 response with respect to IgE-mediated activation from at least two independent experiments.

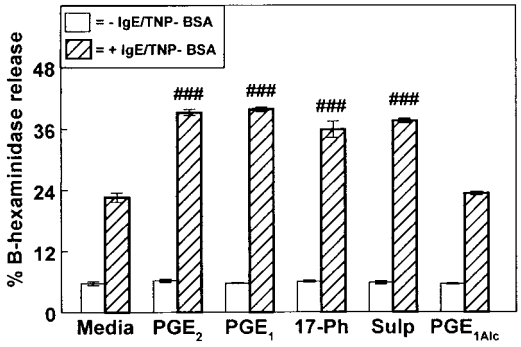

FIGURE 4. Effect of EP-selective agonists on β-hexosaminidase release by IgE/Ag-activated MC/9 cells. MC/9 cells were passively sensitized with anti-TNP IgE and incubated with TNP-BSA (10 ng/ml) in the presence or absence of PGE_1, PGE_2, EP_1-selective agonist 17-phenyl-ω-trinor-PGE_2 (17-Ph), EP_1/EP_3-selective agonist sulprostone (Sulp), or EP_2/EP_4-selective agonist PGE_1 alcohol (PGE_1 Alc). PGE_1, PGE_2 and the EP analogs were used at a concentration of 1 μM. Following a 15-min incubation, cell supernatants and pellets were harvested and assayed for β-hexosaminidase. Bars represent mean % change ± SEM in β-hexosaminidase release with respect to IgE-mediated activation alone from three independent experiments.

by the observation that β-hexosaminidase release by IgE/Ag-activated mast cells was potentiated by the $EP_2/EP_3/EP_4$ selective analog, misoprostol (15.4 ± 1.5% release (IgE/Ag-activation alone) vs 24.8 ± 2.0% release (concurrent IgE/Ag and misoprostol treatment); $p < 0.001$; comparison of means of three independent experiments); whereas, such potentiation was not observed with the EP_2/EP_4-selective agonist, PGE_1 alcohol as mentioned above.

Expression of EP receptor subtypes by MC/9 cells

We used RT-PCR to determine which PGE receptor subtypes were expressed by MC/9 cells. Quiescent MC/9 cells expressed EP_1, EP_3, and EP_4 receptors (Fig. 6*A*). However, MC/9 cells failed to express mRNA encoding EP_2 in three independent RNA preparations, whereas a signal for EP_2 of the expected size (401 bp) was observed in murine uterus (data not shown). For the EP_1 receptor, in addition to a weak signal for the expected PCR product (336 bp), a more intense band corresponding to a larger amplicon at ~750 bp was observed (Fig. 6*A*), and the latter PCR product may represent a splice variant similar to that described in the rat (37). To rule out the possibility of genomic contamination, RNA preparations were treated with DNase I to degrade any contaminating genomic DNA, and then subjected to PCR with or without prior reverse transcription. No PCR products were obtained for any of the EP receptors including EP_1 when reverse transcription was not performed (Fig. 6*A*). Nesting primers were employed to amplify the EP_1 receptor signal, and two PCR products of expected sizes (249 bp and 668 bp) were obtained (Fig. 6*B*). Subsequent sequence analysis indicated that the putative EP_1-variant receptor contained an intron positioned within the sixth transmembrane domain (data not shown), and, hence, as in the rat, the EP_1-variant receptor arose from the failure to use a splice site located within this domain (37).

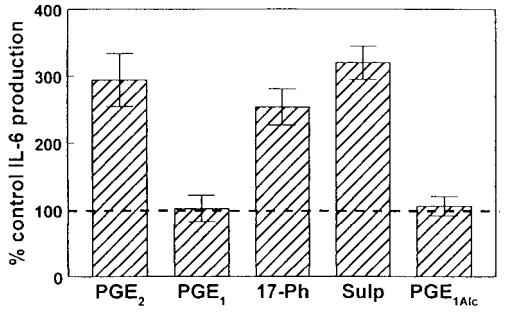

FIGURE 5. Effect of EP-selective agonists on the IL-6 response by IgE/Ag-activated MC/9 cells. MC/9 cells were passively sensitized with anti-TNP IgE and incubated with TNP-BSA (10 ng/ml) in the presence or absence of PGE_1, PGE_2, EP_1-selective agonist 17-phenyl-ω-trinor-PGE_2 (17-Ph), EP_1/EP_3-selective agonist sulprostone (Sulp), or EP_2/EP_4-selective agonist PGE_1 alcohol (PGE_1 Alc). PGE_1, PGE_2, and the EP agonists were used at a concentration of 1 μM. Following a 24-h incubation, supernatants were harvested and assayed for IL-6 by B9 bioassay. Bars represent mean % change ± SEM in IL-6 response with respect to IgE-mediated activation alone from three independent experiments.

Table II. *Effects of EP-selective agonists on the IL-6 response by IgE/Ag-activated MC/9 cells*

	Percentage of Change in IL-6 Production by IgE/TNP-Activated MC/9 Cells Following Treatment with EP Receptor Agonists		
	0.01 μM	0.1 μM	1 μM
PGE_2	133 ± 5[a]	232 ± 27	294 ± 40
PGE_1	139 ± 6	176 ± 39	102 ± 20
17-Phenyl-ω-trinor-PGE_2	115 ± 24	163 ± 23	254 ± 27
Sulprostone	163 ± 3	257 ± 38	320 ± 25
PGE_1 alcohol	96.2 ± 8.4	102 ± 18	106 ± 14

[a] Figures represent mean percent change ± SEM in IL-6 response compared with IgE-mediated responses alone of three experiments for PGE_2 and two experiments for PGE_1 and the EP agonists. MC/9 cells were passively sensitized with anti-TNP IgE and concurrently incubated with TNP-BSA (10 ng/ml) and various doses of PGE_2, PGE_1, EP_1 agonist 17-phenyl-trinor-PGE_2, $EP_{1/3}$-selective agonist sulprostone, or $EP_{2/4}$-selective agonist PGE_1 alcohol. IL-6 production was determined in cell-free supernatants following 24-h incubation. IL-6 production in response to IgE/TNP-activation alone was 480 ± 88 U/ml (shown as mean ± SEM of three independent experiments).

Discussion

Elevated numbers of mast cells and evidence of mast cell activation are observed in a variety of inflammatory disorders, including asthma (38), rheumatoid arthritis (39), and inflammatory bowel disease (40). However, the full role of mast cells in the pathogenesis of such inflammatory disorders is largely unexplored. Mast cells are storehouses of preformed mediators including histamine

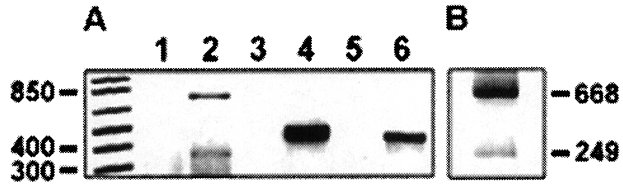

FIGURE 6. Resting MC/9 cells express mRNA for EP_1, EP_3, and EP_4 receptors. Total RNA was isolated from MC/9 cells and DNase I treated to remove genomic DNA contamination. *A*, RT-PCR was performed with primers specific for mouse EP receptor subtypes. In *lanes 2, 4*, and *6*, are EP_1, EP_3, and EP_4 PCR products, respectively. *Lanes 1, 3*, and *5* are PCR where RNA samples were not reverse transcribed (as controls for genomic DNA contamination) for EP_1, EP_3, and EP_4, respectively. The 1 Kb Plus DNA Ladder (Life Technologies) was used for sizing the PCR products. *B*, Ethidium bromide-stained gel electrophoresis of RT-PCR showing amplification of EP_1 PCR products shown in *A* using "nesting" primers as described in *Materials and Methods*. Molecular sizes are indicated in base pairs. Results are representative of three independent MC/9 RNA preparations.

and proteases, and are potent sources of a number of proinflammatory cytokines and chemokines. Levels of the lipid mediator, PGE_2, are also elevated in the context of many inflammatory conditions (41), and PGE_2 has been demonstrated to possess potent immunomodulatory actions and to shift the immune response toward a type 2 response through inhibition of type 1 cytokine production and either enhancing or having no effect on the production type 2 cytokines (1). Consequently, PGE_2 may support the induction and chronicity of certain types of inflammation.

Our current data show that PGE_2 alone selectively modulates cytokine production by murine mast cells, BMMC and MC/9, both of which are considered models of mucosal mast cells. In otherwise unactivated BMMC, PGE_2 enhanced IL-6 production and failed to alter the production of many other cytokines, including IL-4, IL-5, IL-10, and GM-CSF, that are known to be produced by mast cells under alternate stimulation conditions. However, PGE_2 displayed a broader range of potent effects on cytokine production when used in conjunction with IgE/Ag stimulation. IgE-mediated activation alone induced significant release of IL-4, IL-6, and GM-CSF, and further addition of PGE_2 led to a synergistic increase in the production of both IL-6 and GM-CSF, but not IL-4, suggesting selectivity in the ability of PGE_2 to interact with FcεRI-mediated cytokine induction.

The potentiation of IL-6 release by PGE_2 in the context of IgE-mediated activation was unlikely to be the result of increased secretion of stored cytokines as detectable levels of IL-6 were not observed in the cell pellets of unstimulated BMMC or PGE_2-stimulated BMMC; moreover, in IgE/Ag-activated BMMC, where low levels of IL-6 were recovered from cell pellets, concurrent PGE_2 treatment slightly increased these levels rather than decreasing them as one would expect if PGE_2 was acting by facilitating the release of stored cytokine (data not shown).

Originally described as a proinflammatory cytokine, there is growing evidence that IL-6 exerts important anti-inflammatory actions both in vivo and in vitro (27). For instance, endotoxemia-induced circulating levels of proinflammatory cytokines TNF-α, MIP-2, IFN-γ, and GM-CSF were higher in IL-6 gene knockout mice than in wild-type littermates (42), and, in humans, recombinant IL-6 administration up-regulated production of antagonists for the proinflammatory cytokines, IL-1 and TNF-α (43). Moreover, PGE_2 was recently reported to induce production of the anti-inflammatory agent, α_1-acid glycoprotein, in rat alveolar macrophages costimulated with dexamethasone (44). This acute phase protein possesses anticomplement activities and inhibits neutrophil activation, among other anti-inflammatory effects that serve to reduce existing inflammation. In light of these data, the observed potentiation of IL-6 production by PGE_2 during IgE-mediated activation of mast cells may have in vivo significance by potentially facilitating the resolution of inflammation induced by earlier release of histamine and other proinflammatory mast cell-derived mediators.

GM-CSF is a potent growth factor for granulocytes and macrophages, and induces the differentiation of neutrophils, eosinophils, and macrophages from myeloid progenitor cells (28). GM-CSF also maintains the viability and enhances the activity of mature eosinophils and neutrophils. Our data indicate that GM-CSF production by mast cells is increased in the presence of PGE_2 and IgE-mediated activation, and such increased levels of secreted GM-CSF may partly explain the selective retention of granulocytes observed at sites of mast cell activation and PGE_2 production in chronic inflammation.

Previous studies examining the effects of PGE_2 on mast cell degranulation have led to conflicting findings. Several groups have reported an inhibitory effect of PGE_2 and PGE_1 on histamine release. Kaliner and Austen (20) demonstrated that PGE_1 (1 μM) inhibited histamine release by rat mast cells in response to FcεRI cross-linking, and a similar inhibitory effect on degranulation was observed in human lung mast cells preincubated with PGE_2 (>1 μM) for 5 min before FcεRI cross-linking with anti-IgE (21). Hogaboam et al. (19) reported that PGE_2 treatment inhibited histamine release in rat PMCs activated with calcium ionophore, A23187; however, PGE_2 was without effect on IgE-mediated histamine release by rat PMCs under the experimental conditions employed by this group. In contrast, PGE_2 has also been shown to potentiate histamine release by mast cells. Nishigaki et al. (12) reported that PGE_2 potentiated ionomycin-mediated degranulation in the murine mast cell line, BNu-2cl3, and our group has previously demonstrated that although PGE_2 alone neither induced nor inhibited spontaneous histamine release by rat PMCs, PGE_2 enhanced such release from mast cells concurrently activated with anti-IgE (18). Here, we further demonstrate PGE_2-mediated potentiation of degranulation in two different mast cell populations, BMMC and the IL-3-dependent mast cell line, MC/9. As observed in rat PMCs, PGE_2 treatment alone did not induce degranulation in either mast cell population yet strongly enhanced β-hexosaminidase release induced by IgE/Ag-activation.

The opposing stimulatory and inhibitory actions described for PGE_2 in the context of mast cell degranulation may reflect differences in the timing of PGE_2 treatment relative to the administration of other stimuli, and to possible differences in EP receptor subtype expression by the mast cell populations. In studies describing an inhibitory effect for PGE_2 on histamine release, mast cells were preincubated with PGE_2 for $\geq$5 min before the addition of the other stimuli (19, 21); whereas, in experiments where PGE_2 potentiated mast cell degranulation, concurrent activation with PGE_2 and the secretagogue was employed (12, 18, and this study). Cyclic AMP has been implicated as the second messenger mediating PGE_2-directed inhibition of degranulation (21, 45, 46). Conversely, increased Ca^{2+} rather than cAMP was implicated in a study where degranulation was potentiated by PGE_2 (12), and these observations are not surprising considering the absolute requirement for increased intracellular Ca^{2+} in the induction of mast cell degranulation (47). The role of cAMP in mediating degranulation is less clear. Biphasic increases in cAMP are observed in IgE-mediated degranulation; however a causal link between increased cAMP and histamine release has not been established. Here, we have shown that cAMP-elevating agents, forskolin, pentoxifylline, and β-isoproterenol, fail to reproduce the enhancing effects of PGE_2 on both β-hexosaminidase release and IL-6 production, suggesting that the observed effects of PGE_2 are mediated by a cAMP-independent mechanism.

PGE_2 exerts its effects on target cells by interacting with specific G protein-coupled receptors, of which there are four subtypes (EP_1, EP_2, EP_3, and EP_4). EP_1 coupling elevates intracellular Ca^{2+} levels; signaling through EP_2 and EP_4 results in the activation of adenylate cyclase and subsequent increases in intracellular cAMP; and signaling through EP_3 is generally associated with diminished levels of intracellular cAMP although a number of splice variants of this receptor coupled to different G proteins have been described (22). Using RT-PCR, we have demonstrated that MC/9 cells express EP_1, EP_3, and EP_4, but not EP_2, receptors. The presence of EP_3 and EP_4 receptors has been reported for the mucosal type mast cells BNu-2cl3 (12) and P815 (11), respectively. EP_3 and EP_4 receptors are ubiquitously expressed in tissues (51) and have been identified on murine macrophage-like cell line, RAW 264.7 cells (29), primary and transformed murine B lymphocytes (48, 49), and human HSB.2 early T cells (50). EP_1 expression is somewhat more limited, and is most abundantly expressed in the kidney (51) where

it is restricted to the collecting duct and regulates natriuretic actions of PGE_2 (52). Using primers specific for EP_1, we observed two bands, a minor band of 336 bp corresponding to the expected PCR product and a stronger band of ~750 bp. Thus far, a splice variant for EP_1 receptors (EP_1-v) has only been described in the rat and arises from failure to use a potential splice site located in the sixth transmembrane domain (37). In contrast to the EP_1 receptor, EP_1-v is devoid of a carboxyl terminus and lacks signaling capacity. Experiments where CHO cells were cotransfected with EP_1 and EP_1-v showed that although the variant receptor alone was not coupled to Ca^{2+} mobilization, it inhibited Ca^{2+} mobilization mediated by EP_1 (37) and hence, may serve as a sink for the EP_1 receptor (53). The larger EP_1 PCR product observed in this study is of the predicted size for a splice variant analogous to that observed in the rat, and did not arise from genomic DNA contamination in RNA samples. Sequence analysis confirmed that it contained the second intron as would be expected in the absence of splicing events occurring in the sixth transmembrane domain during processing of primary RNA transcripts.

To identify the EP receptors mediating PGE_2-directed potentiation of degranulation and IL-6 production, MC/9 cells were stimulated with EP subtype-selective agonists in the presence of IgE/Ag-activation. Both the EP_1 agonist, 17-phenyl-ω-trinor-PGE_2, and the EP_1/EP_3 selective agonist, sulprostone, potentiated β-hexosaminidase release and IL-6 production in IgE/Ag-activated mast cells. Misoprostol, an EP_2/EP_3/EP_4 -selective agonist also enhanced IgE-mediated degranulation. Such potentiation of degranulation or IL-6 production was not observed with the EP_2/EP_4 -selective agonist, PGE_1 alcohol. PGE_1, a structural homologue of PGE_2 that binds EP_1 with weaker affinity than PGE_2 and binds with comparable affinity to EP_2, EP_3, and EP_4, enhanced IgE-mediated degranulation to a similar degree as PGE_2 but did not potentiate IL-6 production. Taken together, these findings strongly suggest the involvement of both EP_1 and/or EP_3 receptors in PGE_2-directed potentiation of degranulation and IL-6 production by IgE/Ag-activated mast cells.

The importance of EP_1 and/or EP_3 receptors in regulating mast cell function is intriguing in view of the fact that EP_2 and EP_4 receptors have generally been associated with immunological modulation. For instance, TNF-α inhibition in human blood monocytes (54), B cell differentiation to IgE-secreting plasma cells (48), and IL-8 production by human colonic epithelial cells (55) have all been reported to be mediated by PGE_2 via EP_2 and/or EP_4 receptors. Moreover, in the human HSB.2 early T cell line, PGE_2 induced IL-6 production via EP_2/EP_4 receptors, and costimulation with Con A further enhanced IL-6 levels by up-regulating EP_4 receptor expression and down-regulating that of EP_2 and EP_3 (50). Interestingly, a study by Kozawa and colleagues (56) investigating PGE_2-induced IL-6 synthesis in the murine osteoblast-like cell line, MC3T3, reported that both EP_1 and EP_2 receptors contributed to the production of IL-6. These data implicate the involvement of second messengers, Ca^{2+} and cAMP in IL-6 induction by osteoblasts, and a similar role for these two second messengers may be involved in IL-6 production by mast cells as rat PMC IL-6 production is both highly calcium dependent and is induced by the cAMP-elevating agent, cholera toxin (47). In this study, EP_1 and/or EP_3 appear to play a substantial role in mast cell IL-6 production. Although activation of EP_3 receptors is generally associated with diminished intracellular cAMP levels, an isoform in the mouse has been shown, at higher agonist concentrations, to stimulate adenylate cyclase and increase intracellular cAMP levels (22). Coupling through EP_3 has also been linked with elevated Ca^{2+} in the murine mast cell line, Bnu-2cl3 (12). Hence, stimulation of mast cells with PGE_2 alone may, through coupling to EP_1/EP_3 receptors, elevate intracellular Ca^{2+} and/or cAMP to levels exceeding the threshold required for IL-6 production, and it is possible that concurrent activation with PGE_2 and IgE/Ag results in synergism of such initial responses leading to potentiation of IL-6 production.

Taken overall, our results suggest a more complex role for PGE_2 in the modulation of allergic inflammation and disease than has been previously recognized. We have demonstrated that PGE_2 modulates IL-6 production in otherwise unstimulated BMMCs with no change in the production of many other cytokines or in the induction of mast cell degranulation. However, in the context of IgE-mediated activation, PGE_2 enhances preformed mediator release and selectively up-regulates the production of IL-6 and GM-CSF, and these effects likely occur through coupling to EP_1 and/or EP_3 receptors. The residence of mast cells in the skin and mucosal linings positions them among our first line of defense against environmental insults, irritants, and pathogens. Mast cell mediators induce PGE_2 production by neighboring tissue cells (57, 58), and newly secreted PGE_2 may act to modulate cytokine production by mast cells and alter localized inflammatory reactions in an autocrine and paracrine manner. Understanding the mechanisms by which PGE_2 modulates cytokine production will undoubtedly be of prime importance if we are to harness the beneficial effects of prostanoids and related molecules in the treatment of inflammatory disease.

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