Cross-linking of the high-affinity IgE receptor induces the expression of cyclo-oxygenase 2 and attendant prostaglandin generation requiring interleukin 10 and interleukin 1β in mouse cultured mast cells

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When mouse bone marrow-derived mast cells (BMMC) developed in interleukin (IL)-3 were activated with IgE and antigen (IgE/antigen) in the presence of both IL-10 and IL-1β, two sequential phases of prostaglandin (PG)D_2 generation were elicited, in which the first phase occurred by 1 h and the second phase from 2 to 10 h. The delayed phase of PGD_2 generation was accompanied by a marked induction of cyclo-oxygenase (COX)-2 mRNA, which reached a peak at 1–2 h, followed by that of its protein from 2–10 h, with a peak at 5 h. The immediate phase of PGD_2 generation was completely abrogated by the irreversible inhibition of pre-exisiting COX-1 by aspirin pretreatment, whereas the delayed phase of PGD_2 generation was almost undetectable in the presence of the COX-2 inhibitor NS-398. A detailed analysis of the individual effects of IgE/antigen, IL-10 and IL-1β on COX-2 expression revealed that IgE/antigen and IL-10 each initiated and stabilized COX-2 mRNA expression, leading to an increase in the expression of its protein. Conversely, IL-1β stabilized the COX-2 protein without affecting its mRNA level. The induction of COX-2 by IgE/antigen with IL-10 and IL-1β preceded the induction of transcripts for endogenous cytokines such as IL-6, IL-1β and IL-10. The inhibition of PGD_2 generation by indomethacin did not affect the induction of COX-2 or these cytokines. Thus the two major delayed-phase responses of BMMC after IgE-dependent activation, namely COX-2-dependent PGD_2 generation and cytokine production, are regulated independently.

INTRODUCTION

The activation of mast cells through cross-linking of the high-affinity IgE receptor ($Fc_\epsilon RI$) by IgE and multivalent antigen (IgE/antigen) elicits three crucial responses that are thought to be involved in the allergic reaction. Two of these responses, which occur within a few minutes of cell activation, include the exocytosis of preformed mediators stored in the secretory granules, such as histamine, serotonin, proteoglycans and proteases, and the generation of lipid mediators such as leukotriene (LT)C_4 and prostaglandin (PG)D_2. The immediate responses are followed by the induction of the immediate–early genes and various cytokines, and this represents the delayed phase of mast cell activation [1–5].

Eicosanoids are synthesized by the oxidative metabolism of arachidonic acid, which is generally esterified at the *sn*-2 position of the major classes of glycerophospholipids. The first step of eicosanoid generation involves the liberation of free arachidonic acid from the phospholipid pools. This reaction is regulated mainly by two phospholipase A_2 (PLA_2) isoenzymes, namely the 85 kDa cytosolic PLA_2 ($cPLA_2$) and the 14 kDa secretory type II PLA_2 [6–8]. The second step leading to PG generation involves cyclo-oxygenases (COXs), which catalyse the conversion of arachidonic acid to PGH_2. There are two COX isoforms, COX-1 and COX-2, with similar molecular masses of approx. 70 kDa, and they have an approx. 60% amino acid identity with each other [9–11]. COX-1 is expressed constitutively with a modest change in expression in almost all tissues and cells, where it produces the PGs needed to regulate 'housekeeping activities'. COX-2 is induced markedly in response to a wide variety of stimuli. It produces PGs that function during specific stages of cell differentiation or replication, and has been implicated in PG production during inflammation.

To address the functional differences between the two COX isoforms it is important to use a single cell population that expresses both COX-1 and COX-2, each of which can exert a distinguishable function. The ability of mouse bone marrow-derived mast cells (BMMC) to respond to IgE/antigen and the stromal cytokine, c-*kit* ligand (KL), reveals the existence of biochemically separate responses over time [12–14]. The IgE-dependent activation of BMMC induces immediate PGD_2 generation (i.e. within a few minutes) that is absolutely dependent on COX-1 [13]. BMMC cultured with KL, when combined with particular accessory cytokines, interleukin (IL)-10 and IL-1β, elicits cytokine-initiated delayed PGD_2 generation, which depends entirely upon the induction of COX-2 *de novo*, over several hours [13]. Furthermore after more than 1 day of culture, KL primes increases in subsequent IgE-dependent immediate PGD_2 generation through increased expression of COX-1 but not COX-2 [14]. This probably reflects the maturation of immature BMMC towards a connective tissue mast cell-like phenotype [15,16]. Thus the segregated utilization of the two COX isoforms under different transmembrane stimuli, and the different kinetics within BMMC, together with other recent studies [17–20], imply that although COX-1 and -2 catalyse the same reaction *in vitro*, in intact cells each COX isoform utilizes endogenous arachidonic acid differently for subsequent PG generation in different phases of cell activation.

Here we show that the IgE-dependent activation of BMMC elicits a delayed response of PGD_2 generation when combined with IL-10 and IL-1β, as does KL-dependent activation [12,13]. The respective utilizations of COX-1 for immediate, and COX-

Abbreviations used: Ag, antigen; BMMC, bone marrow-derived mast cells; c, cytosolic; COX, cyclo-oxygenase; IL, interleukin; KL, c-*kit* ligand; LT, leukotriene; PG, prostaglandin; PLA_2, phospholipase A_2; RT–PCR, reverse transcriptase–PCR; TBS-T, Tris/HCl (pH 7.4) containing 150 mM NaCl and 0.1% Tween-20.

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2 for delayed, phases of PGD_2 generation have revealed that the biphasic eicosanoid generation over time requires different biosynthetic isoenzymes to provide the same product, after a particular stimulation related to the allergic reaction, $Fc_\epsilon RI$ cross-linking. We also provide evidence that delayed PGD_2 generation is independent of endogenous cytokine production.

EXPERIMENTAL

Materials

Recombinant mouse IL-1β, IL-6 and tumour necrosis factor α were purchased from Genzyme (Boston, MA, U.S.A.). All other cytokines were obtained through their expression in baculovirus, as described previously [12–14]. Briefly, the cDNA of each cytokine was inserted into pVL1393 (Pharmingen, San Diego, CA, U.S.A.). The recombinant plasmids were co-transfected with Baculo Gold linearized baculovirus DNA (Pharmingen) into 3×10^6 Sf9 cells (Invitrogen, San Diego, CA, U.S.A.) with calcium phosphate. The cells were cultured at 27 °C in Grace's insect medium (Invitrogen) supplemented with lactalbumin hydrolysate, yeastolate and 10 % (v/v) fetal calf serum. After 7 days the recombinant virus in the supernatant was selected by plaque assay and amplified. Sf9 cells (10^6/ml) were infected with the recombinant virus and cultured for 7 days. Concentrations of cytokines were determined by [^{3}H]thymidine incorporation into BMMC cultured in the co-presence of IL-3, with corresponding authentic purified recombinant cytokines as standards.

Rabbit antiserum to human $cPLA_2$ [21] was provided by J. D. Clark (Genetics Institute, Cambridge, MA, U.S.A.). Rabbit antisera to mouse COX-1 and COX-2 were provided by W. L. Smith (Michigan State University, East Lansing, MI, U.S.A.). Mouse cDNA probe for COX-2 [22] and the COX-2 inhibitor NS-398 [17,23] were provided by J. Trzaskos (Merck DuPont, Wilmington, DE, U.S.A.). The cDNA probe for mouse β-actin was provided by J. P. Arm (Harvard Medical School, Boston, MA, U.S.A.). Aspirin and indomethacin were purchased from Sigma (St. Louis, MO, U.S.A.).

Preparation of BMMC

Bone marrow cells from male BALB/cJ mice were cultured for up to 10 weeks in 50 % enriched medium [RPMI 1640 containing 100 i.u./ml penicillin, 100 mg/ml streptomycin, 10 mg/ml gentamycin, 2 mM L-glutamine, 0.1 mM non-essential amino acids and 10 % (v/v) fetal calf serum] and 50 % WEHI-3 cell-conditioned medium as a source of IL-3. After 3 weeks, more than 98 % of the cells in the culture were BMMC, as assessed by staining with Toluidine Blue or Alcian Blue and Safranin [2].

Treatment of BMMC with cytokines and IgE/antigen

BMMC were washed once and suspended at a cell density of 10^7/ml in 50 % WEHI-conditioned medium, and were sensitized with 10 μg/ml monoclonal IgE anti-trinitrophenyl for 30 min at 37 °C. After washing twice with enriched medium, cells were suspended at 10^6/ml in enriched medium containing various cytokines, either alone or in combination, as required for the experiments. In most experiments the concentrations of cytokines used were 5 ng/ml IL-1β, 100 i.u./ml IL-3, 5 ng/ml IL-4, 100 i.u./ml IL-9, 100 i.u./ml IL-10 and 100 ng/ml KL. Some cells were stimulated with 20 ng/ml trinitrophenyl-conjugated BSA at 37 °C. After various periods, reactions were stopped by centrifugation at 120 ***g*** for 5 min at 4 °C. The supernatants were retained for assay of mediator release, and the cells for analysis of the expression of mRNA and protein. PGD_2 generation by BMMC was assayed with a PGD_2 assay kit (Amersham, Little Chalfont, Bucks. U.K.) with a modification of the manufacturer's instructions.

In another series of experiments, BMMC were adjusted to 2.5×10^5 cells/ml, activated with IgE/antigen in the presence of IL-3, and then cultured for up to 2 days. After being washed, the cells were incubated for 10 min with 100 ng/ml KL, and the PGD_2 released into the supernatant was quantified. Replicate cells were lysed and subjected to immunoblot analysis, as described below.

SDS/PAGE/immunoblot analysis

After activation with various cytokines and IgE/antigen, BMMC were washed once with PBS (10 mM phosphate buffer, pH 7.4, containing 150 mM NaCl), and lysed in PBS containing 0.1 % SDS and 10 mM 2-mercaptoethanol at 10^7 cells/ml. The lysate was subjected to SDS/PAGE [10 % (w/v) gel] (Novex, San Diego, CA, U.S.A.). The separated proteins were electroblotted on nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) with a semi-dry blotter (MilliBlot-SDE system, Millipore, Bedford, MA, U.S.A.) in accordance with the manufacturer's instructions. Membranes were then washed once with Tris/HCl, pH 7.4, containing 150 mM NaCl and 0.1 % Tween-20 (TBS-T), and then blocked for 1 h in TBS-T containing 3 % (w/v) skimmed milk. After the membranes had been washed with TBS-T, antibodies against $cPLA_2$, COX-1 or COX-2 were added at a dilution of 1:3500 in TBS-T and incubated for 2 h. After three washes with TBS-T, membranes were treated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (Zymed, South San Francisco, CA, U.S.A.) (diluted to 1:10000) in TBS-T. After six washes, the protein bands were revealed with an enhanced chemiluminesence Western blot analysis system (Amersham).

RNA blot analysis

Total RNA was extracted in guanidinium thiocyanate with TRIzol-reagent (Gibco BRL, Grand Island, NY, U.S.A.) [24] in accordance with the manufacturer's instructions, and was quantified by measuring the absorbance at 260 nm. Approximately equal amounts (approx. 5 μg) of total RNA were applied to each lane of 1.2 % formaldehyde/agarose gels, subjected to electrophoresis and transferred to Immobilon-N (Millipore). The resulting blots were then sequentially probed with COX-2 and β-actin cDNA probes that had been labelled with [^{32}P]dCTP (Amersham) by random priming (TaKaRa, Ohtsu, Japan). All hybridizations were performed at 42 °C for 16 h in 50 % (v/v) formamide/0.75 M NaCl/75 mM sodium citrate/0.1 % SDS/1 mM EDTA/10 mM sodium phosphate (pH 6.8)/5 × Denhardt's solution (Sigma, St. Louis, MO, U.S.A.)/10 % dextran sulphate (Sigma)/100 μg/ml salmon sperm DNA (Sigma). The blots were washed three times at room temperature with 150 mM NaCl/15 mM sodium citrate/1 mM EDTA/0.1 % SDS/10 mM sodium phosphate (pH 6.8) for 5 min each, followed by two washes at 55 °C with 30 mM NaCl/3 mM sodium citrate/1 mM EDTA/0.1 % SDS/10 mM sodium phosphate (pH 6.8) for 15 min each. The blots were revealed by autoradiography with Kodak X-OMAT AR films and double-intensifying screens at −80 °C.

Reverse transcriptase–PCR (RT–PCR)

cDNA probes for mouse IL-1β and IL-6 were obtained by RT–PCR with the total RNA obtained from IgE/antigen-activated BMMC as a template for the reverse transcriptase

reaction. RT–PCR was performed with an RNA PCR kit (AMV) version 2 (TaKaRa), by using a modification of the manufacturer's instructions. Briefly, 1 μg of total RNA was subjected to the reverse transcriptase reaction with 2.5 pmol of $(dT)_{20}$ primer and 5 units of avian myeloblastosis virus reverse transcriptase in 10 mM Tris/HCl (pH 8.3)/50 mM KCl/5 mM $MgCl_2$/50 units/ml RNase inhibitor/1 mM dNTP mixture. The reaction was continued for 30 min at 55 °C, heated to 99 °C for 5 min and then chilled on ice. The reverse transcriptase product was subjected to PCR by using primer sets for each cytokine (Stratagene, La Jolla, CA, U.S.A.) as instructed by the manufacturer of the primers. After 30 cycles of amplification the DNA fragments were purified by 2.5% agarose gel electrophoresis, and eluted from the gel with a QIAEX II gel extraction kit (Qiagen, Germany). The purified fragments were then labelled with [^{32}P]dCTP by using a random primer kit, and used for RNA blotting as described above. The cDNA probe for mouse IL-10 was provided by K. Moore (DNAX) [25].

RESULTS

IgE-dependent biphasic PGD_2 generation

IgE-sensitized BMMC were cultured for 5 h with various cytokines either alone or in combination, in the presence of antigen. The PGD_2 released into the culture supernatant was then assayed (Table 1). In the presence of IL-10 and IL-1β together, IgE/antigen-activated BMMC generated 5.6 ng of $PGD_2/10^6$ cells, whereas IgE/antigen stimulation in the presence of other cytokine combinations elicited levels of PGD_2 generation that ranged from 1.0 to 1.4 ng/10^6 cells. In the absence of IgE/antigen stimulation, BMMC treated with IL-10 and IL-1β together produced only 0.37 ng of $PGD_2/10^6$ cells. PGD_2 generation by cells maintained in other cytokine combinations was negligible.

Time course experiments revealed that the IgE-dependent activation of BMMC in IL-10 with IL-1β elicited the first phase of PGD_2 generation by 1 h, when it reached 0.9 ng/10^6 cells (Figure 1A). This level of PGD_2 generation was maintained for up to 2 h, then reached a maximum of 3 ng of $PGD_2/10^6$ cells by 5 h, reaching a plateau up to 10 h. This represented a 3.3-fold increase over the PGD_2 generated by BMMC treated for 1 h with IL-10, IL-1β and IgE/antigen together. As has been reported previously [26], BMMC maintained in IL-3 and activated with IgE/antigen elicited only the immediate phase of PGD_2 generation, which was equivalent to that elicited by BMMC cultured for 1 h in IL-10, IL-1β and IgE/antigen together (Table 1).

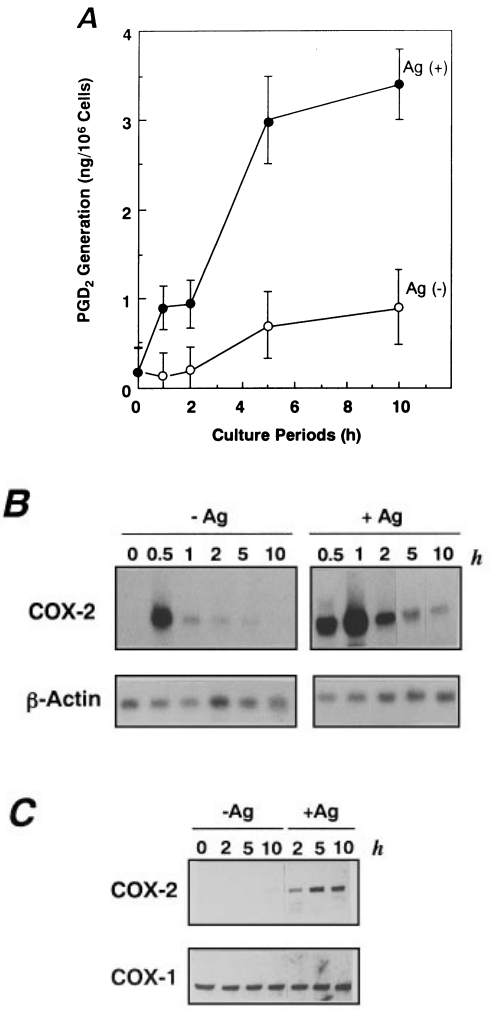

Figure 1 Time course of PGD_2 generation and COX expression in BMMC after stimulation with IL-10, IL-1β and IgE/antigen

IgE-sensitized BMMC were cultured for the indicated periods after stimulation with 100 i.u./ml IL-10 and 5 ng/ml IL-1β in the presence or absence of antigen (Ag). The PGD_2 released into the supernatants (**A**), the expression of transcripts for COX-2 and β-actin (**B**) and the expression of proteins for COX-1 and COX-2 (**C**) were assessed as described in the Experimental section. (**A**) Values are expressed as means ± S.E.M. for four independent experiments. (**B**, **C**) A representative result of three independent experiments.

Table 1 Effect of various cytokines and IgE/Ag on PGD_2 generation by BMMC

BMMC were cultured for 5 h with the indicated stimuli, and the PGD_2 released into the supernatants was assessed as described in the Experimental section. Means ± S.E.M. for three independent experiments are shown. *$P < 0.05$ compared with no cytokine; †$P < 0.05$ compared with each group without IgE/antigen.

	PGD_2 generation (ng/10^6 cells)	
Cytokines	Without IgE/antigen	With IgE/antigen
No cytokine	< 0.1	1.03 ± 0.15†
IL-3	< 0.1	1.14 ± 0.11†
IL-10	< 0.1	1.27 ± 0.21†
IL-1β	< 0.1	1.02 ± 0.13†
IL-1β + IL-10	0.37 ± 0.15*	5.60 ± 1.18*†
IL-3 + IL-10	0.14 ± 0.08	1.40 ± 0.48†
IL-1β + IL-3	< 0.1	1.05 ± 0.18†

Expression of the two COX isoforms

BMMC were treated for 5 h with the cytokines, in either the presence or the absence of IgE/antigen stimulation, and COX-1 and COX-2 proteins expressed were revealed by immunoblotting. Although IL-10 with IL-1β induced a weak but significant expression of COX-2 protein relative to other cytokine treatments in which no expression of COX-2 was found, further activation of BMMC cultured in IL-10 and IL-1β together with IgE/antigen markedly enhanced its expression (Figure 2A). The

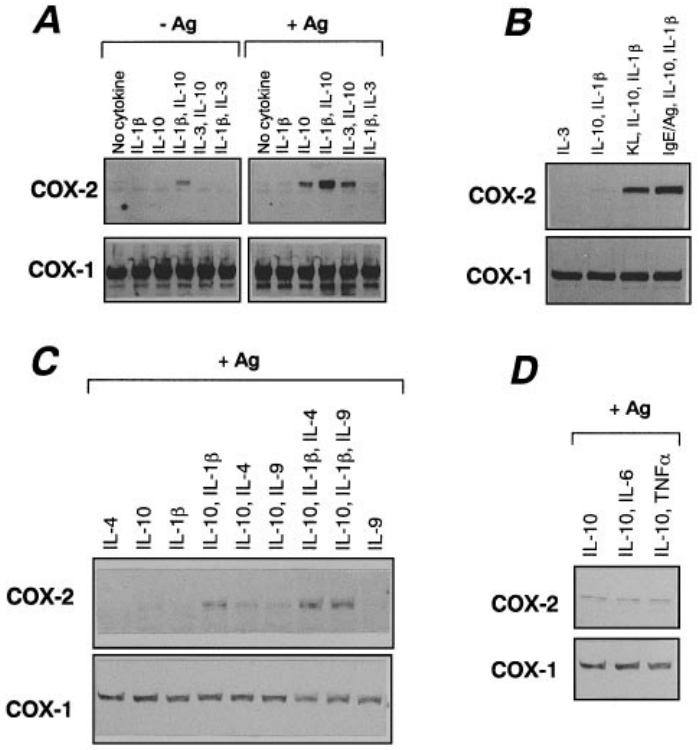

Figure 2 Effect of various cytokines and IgE/antigen on COX expression by BMMC

BMMC were cultured for 5 h with the indicated stimuli and the expression of COX-1 and COX-2 proteins (**A**–**D**) were assessed as described in the Experimental section. (**A**) A representative result of three independent experiments is shown. Abbreviation: Ag, antigen.

level of COX-2 protein expression was almost comparable to that induced by KL, IL-10 and IL-1β together [13] (Figure 2B). IL-10 alone also induced a weak expression of COX-2 protein when combined with IgE/antigen, whereas IL-3 had no effect on COX-2 protein expression (Figure 2A). Other cytokines tested, including IL-4 and IL-9 (Figure 2C), which are mast cell growth factors [27,28], and IL-6 and tumour necrosis factor α (Figure 2D), which are proinflammatory cytokines [29,30], exhibited no appreciable effect on COX-2 expression. None of these cytokines alone or in combination affected the constitutive level of the expression of COX-1 protein (Figure 2).

Time-dependent changes in the expression of COX-2 mRNA (Figure 1B) and protein (Figure 1C), after treatment with IL-10 and IL-1β together in the presence or absence of IgE/antigen stimulation, were examined. COX-2 mRNA was induced within 30 min, with little effect of IgE/antigen (Figure 1B). The COX-2 transcript, however, declined by 1 h in BMMC cultured without antigen, but it increased to a peak at 1 h, persisted up to 2 h, and gradually declined thereafter in BMMC cultured with antigen. In the presence of antigen, COX-2 protein was detectable at 2 h, and increased to reach a peak at 5–10 h. In the absence of antigen, COX-2 was induced only minimally at 10 h (Figure 1C). The expression of COX-1 protein did not change at any time point, in either the presence or absence of antigen (Figure 1C).

The requirement of both IL-10 and IL-1β for delayed generation of PGD_2 (Figure 3A) and induction of COX-2 (Figure 3B) in IgE/antigen-stimulated BMMC was further demonstrated by dose–response experiments. When BMMC were cultured for 5 h with IL-10 and IL-1β in the presence of antigen, and where the concentration of one cytokine was varied while that of the other was held constant, PGD_2 generation and COX-2 expression increased in parallel, and in proportion to the incremental concentrations of each cytokine. A maximal effect occurred after the addition of 100 i.u./ml IL-10 and 5 ng/ml IL-1β. The addition of more cytokines did not enhance COX-2 expression further (results not shown). Thus PGD_2 generation induced by antigen alone was evident in BMMC in which only COX-1 was expressed, whereas when augmented by these cytokines, PGD_2 generation paralleled COX-2 expression.

Aspirin-pretreated BMMC, in which the immediate PGD_2 generation was completely abrogated through the irreversible inactivation of pre-existing COX-1 [26], produced 2.7 ng of $PGD_2/10^6$ cells 5 h after stimulation with IL-10, IL-1β and IgE/antigen together (Table 2). When aspirin-pretreated BMMC were activated with IL-10, IL-1β and IgE/antigen together for 5 h in the presence of 10 ng/ml NS-398, a COX-2-selective inhibitor [17,18,23], PGD_2 generation was undetectable. Aspirin pretreatment did not appreciably affect the induction of COX-2 expression (results not shown). These results imply that COX-2 is the dominant isoenzyme regulating the delayed phase of PGD_2 generation in BMMC, irrespective of the presence of COX-1.

Differential regulation of COX-2 expression by IL-10, IL-1β and IgE/antigen

The effect of each member of the triad, IgE/antigen, IL-10 and IL-1β, on the induction of COX-2 was assessed by holding two of the components fixed, with or without the third. As shown in

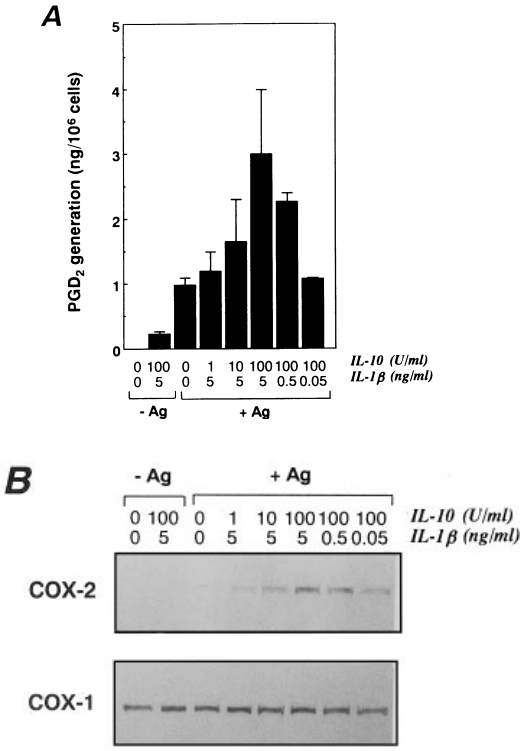

Figure 3 Dependence of PGD_2 generation and COX protein expression on IL-10 and IL-1β concentrations

BMMC were cultured for 5 h with various concentrations of IL-10 and IL-1β either with or without IgE/antigen stimulation. PGD_2 generation (**A**) and expression of COX-1 and COX-2 proteins (**B**) were assessed as described in the Experimental section. Means $\pm$ S.E.M. (**A**) and a representative result (**B**) of three independent experiments are shown. Abbreviation: Ag, antigen.

Table 2 Inhibition of the delayed phase of PGD_2 generation by NS-398

BMMC, pretreated for 5 h with or without 1 μg/ml aspirin, were sensitized with IgE and activated with antigen in the presence of IL-10 and IL-1β, either with or without 10 ng/ml NS-398. Immediate and delayed PGD_2 generation was assessed at 1 and 5 h respectively. Values are expressed as the means $\pm$ S.D. for three independent experiments. *$P < 0.05$ compared with PGD_2 generation at 1 h after the same treatment; †$P < 0.01$ compared with PGD_2 generation at 1 h without aspirin pretreatment; ‡$P < 0.05$ compared with PGD_2 generation after pretreatment with aspirin, and activation in the absence of NS-398.

Aspirin pretreatment	NS-398 treatment	Incubation time (h) ...	PGD_2 generation ($ng/10^6$ cells) 1	5
−	−		1.1 ± 0.2	3.5 ± 0.8*
−	+		1.0 ± 0.3	0.9 ± 0.3
+	−		< 0.1†	2.7 ± 1.1*
+	+		< 0.1†	< 0.1‡

Figure 1, COX-2 mRNA increased markedly after 1–2 h of IgE/antigen stimulation relative to its absence in BMMC cultured in IL-10 with IL-1β (Figure 1B), and was accompanied by a marked enhancement of COX-2 protein expression by IgE/antigen after 2–10 h of culture (Figure 1C).

The effect of IL-10 was assessed by culturing BMMC with IL-1β and IgE/antigen together in the presence or absence of IL-10

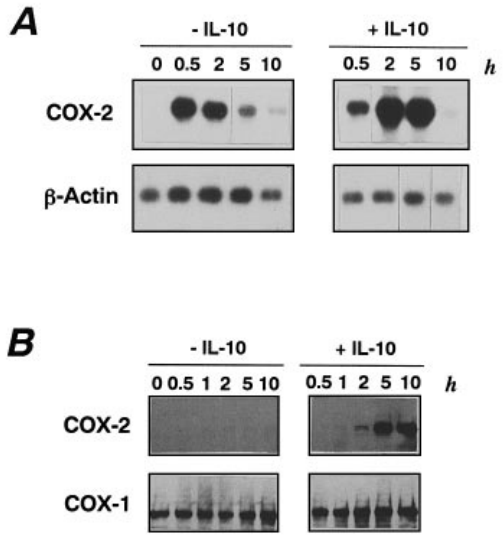

Figure 4 Effect of IL-10 on COX-2 expression

BMMC were activated for the indicated periods with IgE/antigen and IL-1β, in either the presence or the absence of IL-10, and the expression of transcripts for COX-2 and β-actin (**A**) and proteins for COX-1 and COX-2 (**B**) was assessed as described in the Experimental section. A representative result of three independent experiments is shown.

(Figure 4A). In the absence of IL-10 there was a marked induction of COX-2 mRNA from 0.5 h that declined gradually by 5–10 h. In the presence of IL-10, COX-2 mRNA was induced markedly by 30 min, continued to increase to reach a maximum by 2–5 h, and declined by 10 h. COX-2 protein in BMMC treated with IL-10, IL-1β and IgE/antigen was induced as early as 2 h and reached a maximum by 5–10 h. There was only minimal expression at any time point when replicate cells were cultured in the absence of IL-10 (Figure 4B). These results suggest that IL-10 and IgE/antigen each not only initiate and enhance the expression of COX-2 mRNA, but also prolong its half-life, leading to an enhanced expression of the COX-2 protein.

To determine the effect of IL-1β on COX-2 mRNA expression in BMMC, IgE-sensitized cells were cultured in IL-10 and antigen together, either with or without IL-1β for various periods, and the expression of COX-2 mRNA was examined. IL-1β had no effect on the expression of COX-2 mRNA (Figure 5A), whereas the expression of COX-2 protein in BMMC that was induced by IL-10 with IgE/antigen was augmented markedly by IL-1β after 2–10 h (Figure 5B). To assess the effect of IL-1β on the stability of COX-2 protein, half of the BMMC that had been activated for 5 h with IL-10, IL-1β and IgE/antigen together were washed and resuspended in medium containing IL-10 and antigen without IL-1β, and replicate cells were cultured in the continued presence of IL-10, IL-1β and antigen for up to 24 h (Figure 5C). By 5 h after the withdrawal of IL-1β, the level of COX-2 protein decreased markedly (Figure 5C, lane e) compared with its level in cells maintained in the continued presence of IL-1β (Figure 5C, lane c). COX-2 was undetectable 20 h after the cells were resuspended in IL-1β-free medium (Figure 5C, lane f), whereas a trace amount of COX-2 was still detected at this time point in cells maintained in IL-10, IL-1β and antigen (Figure 5C, lane d). These results demonstrate that the addition of IL-1β to IL-10

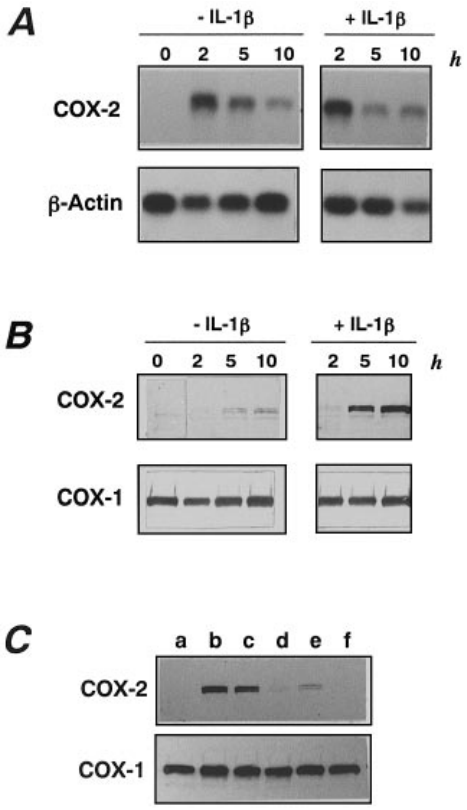

Figure 5 Effect of IL-1β on COX-2 expression

BMMC were activated for the indicated periods with IgE/antigen and IL-10 and in the presence or absence of IL-1β. The expression of transcripts for COX-2 and β-actin (**A**) and of proteins for COX-1 and COX-2 (**B**) were assessed as described in the Experimental section. (**C**) BMMC, maintained in IL-3 (lane a), were cultured first for 5 h with IL-10 + IL-1β + IgE/antigen (lane b), and for a further 5 h (lanes c and e) or 14 h (lanes d and f) with antigen + IL-10 in either the continued presence (lanes c and d) or absence (lanes e and f) of IL-1β. The expression of COX-1 and COX-2 proteins was assessed at each time point. A representative result of three independent experiments is shown.

plus IgE/antigen increased the expression of COX-2 protein in BMMC by its stabilization, without affecting its mRNA.

Relation of delayed PGD_2 generation to cytokine production

To assess whether endogenous cytokine production affects the COX-2-dependent delayed PGD_2 generation, and vice versa, we compared the kinetics of the induction of the transcript for COX-2 with those for several cytokines, such as IL-1β, IL-10 and IL-6, that are known to be induced in IgE/antigen-activated BMMC [4,5,31]. The COX-2 transcript reached maximal expression 2 h after activation with IgE/antigen, IL-10 and IL-1β together, and this was followed after only 5 h of culture by a marked increase in the expression of IL-1β and the induction of IL-10 *de novo* (Figure 6). IL-6 expression was induced markedly by 2 h, and increased further by 5 h. The expression of these cytokines occurring later than that of COX-2 suggests that endogenous cytokines are not required for COX-2 expression. Furthermore the expression of COX-2, IL-1β, IL-10 and IL-6 was not affected significantly, relative to β-actin, by treatment with indomethacin (Figure 6) at a concentration at which PGD_2 generation was completely suppressed (results not shown). This indicates that prostanoid generation is not necessary for the induction of transcripts for COX-2 and cytokines.

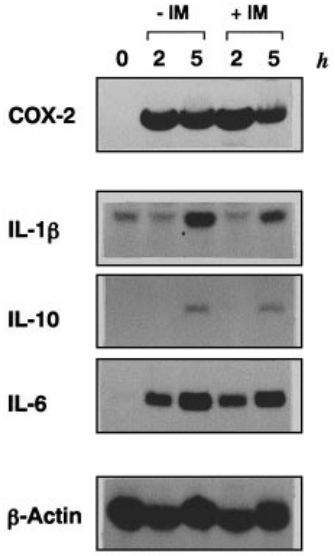

Figure 6 Changes in the expression of transcripts for COX-2, IL-1β, IL-10, IL-6 and β-actin after activation of BMMC with IgE/antigen + IL-10 + IL-1β, either with or without 100 ng/ml indomethacin (IM)

A representative result of four independent experiments is shown.

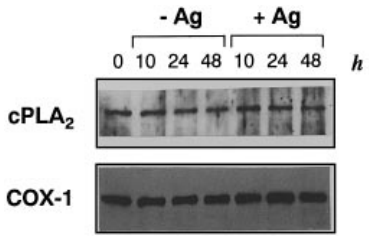

Figure 7 Effect of IgE/antigen on the expression of $cPLA_2$ and COX-1 in BMMC after long-term culture

IgE-sensitized BMMC were cultured for the indicated periods, either with or without antigen (Ag), in the presence of IL-3. The expression of $cPLA_2$ and COX-1 proteins was assessed by using immunoblotting as described in the Experimental section. A representative result of three independent experiments is shown.

Effect of IgE/antigen on subsequent KL-initiated immediate PGD_2 generation

KL mediates the increased expression of $cPLA_2$ and COX-1, after more than 24 h of culture, that is associated with priming for the increased PGD_2 generation after subsequent IgE-dependent [14] and A23187-initiated [32] immediate activation. Because KL and IgE/antigen both elicit COX-2-dependent delayed PGD_2 generation in the presence of the same accessory cytokine combination, we examined whether IgE/antigen mediated increased $cPLA_2$ and COX-1 expression during a 48 h culture, with attendant priming for secondary stimulus-initiated PGD_2 generation. When BMMC were cultured for up to 48 h with IgE/antigen in the presence of IL-3 (added to maintain their viability during this culture period), there was no appreciable change in the expression of $cPLA_2$ and COX-1 proteins (Figure 7). When these BMMC were stimulated further with 100 ng/ml KL for 10 min, KL-initiated PGD_2 generation by BMMC

pretreated with or without IgE/antigen (means ± S.E.M.) was 0.82 ± 0.25 and 1.00 ± 0.35 ($n = 3$; $P > 0.05$) respectively.

DISCUSSION

Whereas various chemical mediators released from mast cells can cause acute allergic or anaphylactic reactions, they do not completely account for the pathology of the chronic states of allergy that are of more clinical and therapeutic importance in bronchial asthma or atopic dermatitis. The finding that mast cells produce a variety of cytokines and eicosanoids has provided an insight into the participation of this particular effector cell population in prolonged inflammatory responses. In the study reported here we have shown the IgE-dependent regulation of the later phases of arachidonate metabolism in BMMC. Reports showing elevated levels of PGD_2, a potent bronchoconstrictor and vasodilator, in patients with chronic asthma compared with normal individuals [33], and the suppressive effect of non-steroidal anti-inflammatory drugs on the delayed phase of allergen-induced bronchoconstriction [34] might provide support for a correlation between delayed PGD_2 generation by allergen-exposed mast cells and some phases of chronic allergic responses.

When BMMC were activated with IgE/antigen, immediate PGD_2 generation, which occurred within 1 h, was followed by the second phase of PGD_2 generation, extending over 2–10 h. This latter phase was greatly augmented by the co-presence of IL-10 and IL-1β (Figure 1A). Cytokine specificity (Table 1 and Figure 2), kinetics (Figure 1) and dose-dependence (Figure 3) studies on the expression of the two COX proteins and transcripts, as well as studies on the pharmacological inhibition of each COX isoform (Table 2), have revealed that constitutive COX-1 and inducible COX-2 were the absolute requirements for the immediate and delayed phases of PGD_2 generation respectively. That the delayed, but not the immediate, phase was augmented by IL-10 and IL-1β further implies that different biochemical events occur in these two phases. Of particular importance, despite the presence of both COX isoforms 5–10 h after stimulation with IgE/antigen, IL-10 and IL-1β together, only COX-2 was utilized for delayed PGD_2 generation. The utilization of COX-2, but not COX-1, has also been demonstrated in endotoxin- or proinflammatory-cytokine-stimulated PGE_2 generation over a number of hours by monocytes/macrophages and fibroblasts, by means of a COX-2-specific inhibitor [17,18], COX-2 anti-sense [19] and COX-2 knockout mice [20]. To account for preferential utilization of COX-2 in the delayed phase, the following possibilities have been proposed. First, COX-1 and COX-2 exhibit different intracellular localizations [35], suggesting the existence of certain compartments responsible for particular phases of prostanoid biosynthesis. Secondly, COX-2 can utilize a lower concentration of substrate than can COX-1, implying the involvement of COX-1 in the burst of events after immediate activation, and of COX-2 in the downstream events that last for hours [36]. Thirdly, each COX is coupled to different types of downstream terminal prostanoid synthases [37]. Finally, arachidonic acid is supplied to each COX from different phospholipid pools by discrete PLA_2 isoforms [38,39]. With regard to the last point, we have recently obtained a preliminary result that different PLA_2 isoforms are activated in immediate and delayed phases in IgE/antigen/IL-10/IL-1β-activated BMMC (M. Ashraf, M. Murakami and I. Kudo, unpublished work).

More detailed analyses of the role of each factor in the optimal expression of COX-2 in BMMC have demonstrated that IgE/antigen and IL-10 each induced COX-2 transcript accumulation, whereas IL-1β enhanced COX-2 protein expression through its stabilization, with little effect on its mRNA level (Figures 2, 4 and 5). Although it is not surprising that $Fc_\epsilon RI$ aggregation, which leads to activation of the *ras*/*raf*-1/MAP kinase pathway [40], induces COX-2 mRNA expression, because COX-2 induction has been observed generally after stimulation with many growth factors that activate this pathway [41], the effects of the two accessory cytokines IL-10 and IL-1β on COX-2 expression in BMMC are not readily understood. Although IL-10, whose receptor belongs to the type II cytokine receptor superfamily to which receptors for interferons α/β and γ also belong [42], is reported to stimulate the JAK/STAT pathway [43], there has so far been no demonstration of the presence of a STAT recognition consensus sequence in the 5′-upstream sequence of the COX-2 gene [44–46]. Furthermore, in contrast with its stimulatory effect on mast cells (Figure 4), IL-10 is a potent inactivator of other inflammatory cells, including macrophages, Th1 cells and neutrophils [47], and in human monocytes the COX-2 expression that was induced by proinflammatory stimuli was counteracted by IL-10 [48]. Therefore we hypothesize the presence of mast cell-specific intracellular machinery for the expression of COX-2 by IL-10. IL-1β has been reported to stimulate COX-2 mRNA transcription and stabilization in many types of cell [49,50], in contrast with its effect on COX-2 expression, which occurs only at the post-transcriptional level in BMMC (Figure 5). As the NF-κB recognition site, which exists in the promoter region of the COX-2 gene, was shown to be insufficient for the optimal induction of COX-2 mRNA [45,46], the additional signalling pathway required for COX-2 mRNA expression, which is activated by IL-1β in other cell types, might not be present in BMMC.

The ability of BMMC to display delayed PGD_2 generation after stimulation with IgE/antigen instead of KL, with a requirement for the same combination of accessory cytokines, namely IL-1β and IL-10 [13], not only reveals a KL-independent route for the induction of COX-2 but also implies the presence of a signalling pathway that is shared by $Fc_\epsilon RI$ and c-*kit*, leading to the induction of the onset of the same genes. Similarities between IgE/antigen and KL are not limited to the delayed phase of PGD_2 generation but are also observed in a number of mast cell responses, such as immediate exocytosis, immediate LTC_4 and PGD_2 generation, cell adhesion, chemotaxis and induction of immediate–early genes [12,51–55]. These similarities can be accounted for, at least in part, by the facts that although stimulations via $Fc_\epsilon RI$ and c-*kit* activate different tyrosine kinases in the initiation of the transmembrane signalling, in which *syk* and c-*kit* tyrosine kinases respectively play crucial roles [56,57], downstream signals through both receptors converge on the *ras*/*raf*-1/MAP kinase pathway [40,41,58,59] and that Ca^{2+} signalling occurs through the activation of phospholipase C-γ [60–62]. Furthermore stimulations by IgE/antigen and KL synergize each other to enhance the immediate [63,64] and delayed [26] responses when they are added to mast cells together. It seems likely that IgE-dependent responses are rather restricted to allergic inflammation, whether depending upon synergy with KL or with other haematopoietic cytokines, whereas KL-dependent mast cell activation is more relevant to innate immunity or the maintenance of tissue homeostasis.

Prolonged culture of BMMC with KL changes the mast cell phenotype towards more mature, connective tissue mast cell-like cells [15,16] that produce more PGD_2 on IgE-dependent [14] and A23187-dependent [32] activation, and this effect is explained by the increased expression by KL of $cPLA_2$, COX-1 and PGD_2 synthase. To address whether IgE/antigen exhibits a similar effect, BMMC were cultured for up to 2 days after stimulation with IgE/antigen and were subsequently activated with another

mast cell stimulator, KL (Figure 7) or A23187 (results not shown). However, the constitutive levels of the expression of $cPLA_2$ and COX-1 were unchanged and the cells were not primed for increased KL- or A23187-dependent PGD_2 generation. These results indicate that whereas both $Fc_\epsilon RI$ and c-*kit* produce signals that are linked to inflammatory responses over hours, only c-*kit* can transduce specific signals that lead to mast cell differentiation and maturation extending over a longer period.

In either situation, the optimal delayed PGD_2 generation depends on elevated levels of IL-1β and IL-10, one of the main sources of which is activated macrophages [46,65]. Because activated mast cells release various cytokines that can activate macrophages [4,5], the cytokine network between these two cell populations might reflect a further amplification of local inflammatory responses to the surrounding microenvironment. In contrast, the utilization of endogenously derived cytokines for the enhancement of delayed PGD_2 generation by BMMC is less likely because COX-2 expression preceded the induction of IL-1β, IL-10 and IL-6. In addition, the suppression of PGD_2 generation by indomethacin did not affect the induction of these cytokines (Figure 6). These results, together with the previous observations that cytokine induction was observed even under conditions without degranulation [66] and that eicosanoid generation was independent of exocytosis [67,68] in mast cells, suggest that there is no significant cross-talk between the three major reactions after mast cell activation: exocytosis, eicosanoid generation and cytokine induction. We cannot rule out the possibility, however, that the expression of other cytokines that we have not yet examined is affected by granule-associated preformed mediators or eicosanoids.

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