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Allergenic sensitization prevents upregulation of haemopoiesis by cyclo-oxygenase inhibitors in mice

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1 We evaluated whether immunization affects bone-marrow responses to indomethacin, because allergenic sensitization and challenge upregulate responses to haemopoietic cytokines (including IL-5-driven eosinopoiesis) in murine bone-marrow, while indomethacin upregulates haemopoiesis and protects bone-marrow from radiation damage.

2 Progenitor (semi-solid) and/or precursor (liquid) cultures were established from bone-marrow of: (a) normal mice; (b) ovalbumin-sensitized mice, with or without intranasal challenge. Cultures were established with GM-CSF (2 ng ml^{-1}) or IL-5 (1 ng ml^{-1}), respectively, alone or associated with indomethacin (10^{-7}–10^{-11} M) or aspirin (10^{-7}–10^{-8} M). Total myeloid colony numbers and numbers of eosinophil-peroxidase-positive cells were determined at day 7.

3 In naïve BALB/c mice, indomethacin (10^{-7}–10^{-9} M) increased GM-CSF-stimulated myeloid colony formation ($P=0.003$ and $P=0.009$, respectively). In contrast, it had no effect on bone-marrow of ovalbumin-sensitized and challenged mice. Indomethacin (10^{-7}–10^{-9} M) also increased eosinophil precursor responses to IL-5 in bone-marrow of naïve ($P<0.001$ and $P=0.002$ respectively), but not sensitized-challenged mice. Aspirin (10^{-7} M) had similar effects, equally abolished by sensitization. Enhancement of haemopoiesis by indomethacin required adherent cells from naïve bone-marrow. Nonadherent cells responded to IL-5 but not to indomethacin. Indomethacin was effective on bone-marrow from sham-sensitized, ovalbumin-challenged, but not from sensitized, saline-challenged mice. Plasma transfer from immune mice abolished eosinophil precursor responses to indomethacin in bone-marrow of naïve recipients. This was not prevented by previous removal of antibody from immune plasma.

4 COX inhibitors enhance haemopoiesis in naïve but not allergic mice. Responsiveness to indomethacin can be abolished either by active sensitization or by immune plasma transfer. Specific antibody is not involved.

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Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-5, interleukin-5; PG, prostaglandin

Introduction

Studies in asthmatic patients, as well as in animal models of asthma, have provided evidence for upregulation of responses to haemopoietic cytokines by airway exposure to allergens in sensitized subjects (Sehmi *et al*., 1997; Gaspar Elsas *et al*., 1997; Inman *et al*., 1996). More recently, we have shown that exogenous glucocorticoids enhance eosinopoiesis in bone-marrow culture from mice of different strains (Gaspar Elsas *et al*., 2000b), and have provided further evidence for a role of endogenous glucocorticoids in upregulating eosinopoiesis in a model of insoluble allergen-induced eosinophilia (Gaspar Elsas *et al*., 2000c). One possible mechanism by which exogenous and endogenous glucocorticoids might upregulate eosinopoiesis is through inhibition of prostanoid synthesis, one well-characterized effect of anti-inflammatory steroids (Giembycz & Lindsay, 1999). In support of this view, we have recently been able to show that prostaglandin E_2 (PGE_2) downmodulates eosinopoiesis in murine bone-marrow cultures (Gaspar Elsas *et al*., 2000a). Taken together, these findings raise the possibility that eosinopoiesis is regulated by mechanisms involving endogenous prostanoid production, and therefore can be modulated by cyclo-oxygenase (COX) inhibitors, such as indomethacin and aspirin.

There is extensive evidence for upregulation of haemopoiesis by COX inhibitors (Pelus, 1989; Campanile *et al*., 1993; Miller, 1992; Ogle *et al*., 1994; Kozubik *et al*., 1993; O'Reilly & Gamelli, 1990; Miller *et al*., 1989), even though opposite findings have been reported (Rossi *et al*., 1980; Snyder & Desforges, 1986; Giglio *et al*., 1984). None of these authors have addressed the effects of COX inhibitors in allergic versus nonallergic subjects, which are central to the understanding of the role of endogenous prostanoids in allergen-induced upregulation of bone-marrow. We have therefore evaluated whether endogenous prostanoid production regulates eosinopoiesis, and further evaluated whether the effects of cyclo-oxygenase inhibitors on eosinopoiesis are affected by allergen

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sensitization and challenge. Here we report that indomethacin and aspirin upregulate haemopoiesis in naïve, but not in allergic mice, through a mechanism involving circulating mediator(s) distinct from antibody.

Methods

Animals and animal procedures

For most of the study, male and female BALB/c mice bred at Fiocruz, Rio de Janeiro (Brazil), were used at 6–8 weeks of age. BALB/c mice bred at Elevage Janvier (Le Genest Saint-Isle, France) yielded comparable results. Mice were immunized with two s.c. 0.4 ml injections of 100 μg ovalbumin mixed with 4 mg ml^{-1} $Al(OH)_3$ in 0.9% NaCl, at 7 day intervals. BALB/c mice were intranasally challenged with 10 μg ovalbumin in 50 μl 0.9% NaCl, 1 week after the second injection, a procedure that increases responses to eosinopoietic cytokines (Gaspar Elsas *et al*., 1997). For selected experiments, mice were sensitized as above, and intranasally challenged with saline; alternatively, mice were sham-sensitized with alum-saline, and intranasally challenged with ovalbumin as above. The mice were sacrificed by cervical dislocation, 24 h after challenge. Animal handling followed the standard procedures adopted at the Institut Pasteur, Paris (France).

Reagents

Heat-inactivated Foetal Calf Serum (FCS), L-glutamine and culture media were from Gibco (Life Technologies SARL, Cergy Pontoise, France), agar noble from Sigma (Sigma Chemical Co, St. Louis, U.S.A.), Diff-Quick from DADE Diagnostika GmbH (Unterschleisshei, Germany) and Harris' haematoxylin acidified from Shandon Inc. (Pittsburgh, PA, U.S.A.). Recombinant haemopoietic factors rmIL-5 and rmGM-CSF were from R&D (R&D Systems, Minneapolis, MN, U.S.A.). Ovalbumin (5 × crystallized) was from ICN Biomedicals, Inc. (Costa Mesa, CA, U.S.A.), $Al(OH)_3$ from Merck (Darmstadt, Germany). Indomethacin (I7378) and aspirin (A5376) were from Sigma.

Bone-marrow cell studies

Bone-marrow cell harvest, identification and separation Bone-marrow cells, collected by flushing the two femurs of mice with RPMI 1640 medium containing 1% FCS, were washed and counted. The frequency of cells stained for eosinophil peroxidase (EPO) following the protocol of Ten *et al*. (1989) was determined in cytocentrifuge smears (Gaspar Elsas *et al*., 1997). The cytochemical pattern of EPO+ cells in stained bone-marrow preparations was identical to that described by Horton *et al*. (1996).

Haemopoietic assays For bone-marrow culture, we used the conditions that were previously defined for BALB/c mice, on the basis of dose-response curves for GM-CSF and IL-5, for assaying colony growth (progenitor assay) and eosinophil differentiation in liquid culture (precursor assay), respectively. For IL-5, these dose-response relationships have been published (Gaspar Elsas *et al*., 2000b). For GM-CSF, dose-response relationships were established in preliminary experiments. In both cases, titration of novel stocks in the respective assays was carried out, covering at least a 100 fold range, and optimal concentration was defined as the lowest concentration that yielded plateau responses in the respective assays. Adding more cytokine did not increase the response significantly (nor decrease it). Decreasing cytokine concentrations to one-tenth or one-hundredth of this optimal level resulted in significantly lower responses. For IL-5, the optimal concentration defined as above was also the lowest concentration in which synergism with indomethacin was detectable (see Results). These conditions were designed to achieve full responses to the cytokine in the control cultures (i.e., those established in the absence of indomethacin or other agents), therefore ruling out low viability or reduced growth potential of bone-marrow cells as a cause of unresponsiveness.

Progenitor assays One ml semi-solid cultures were established in 35 mm culture dishes, from 2×10^5 cells, in a mixture of Iscove's modified Dulbecco's medium with 20% FCS and agar to 0.3% final concentration (Kurland *et al*., 1977; Gaspar Elsas *et al*., 1997), with or without rmGM-CSF (2 ng ml^{-1}), at least in triplicate. Colonies (progenitor-derived ensembles >50 cells; Bagby, 1994) were scored at day 7, and the frequency of eosinophil colonies was determined on agar layers dried (50°C), mounted on microscope slides, stained for EPO, and scored under high magnification (Gaspar Elsas *et al*., 1997). We have previously confirmed that these conditions were adequate for counting total myeloid colonies and for accurate differential counts of myeloid colony types on dried agar layers (Gaspar Elsas *et al*., 2000b).

Precursor assays Liquid bone-marrow cultures (10^6 cells in a 1 ml volume, in a 24-well cluster, Gaspar Elsas *et al*., 1997) were seeded in RPMI 1640 medium, with 10% FCS, 2 mM L-glutamine, and penicillin-streptomycin, at 37°C, 5% CO_2 and 95% air, for 7 days, at least in duplicate, in the absence or in the presence of rmIL-5 (1 ng ml^{-1}). The frequency of EPO+ cells was determined after 7 days of culture in cytocentrifuge smears. We have previously confirmed that these conditions are adequate for evaluating eosinophil differentiation in both naïve and sensitized and challenged murine bone-marrow, and that the increase in per cent of EPO+ cells, as directly determined on cytocentrifuge smears, was paralleled by increases in the total numbers of EPO+ cells in these cultures, as indirectly determined from cell counts (Gaspar Elsas *et al*., 2000b). In selected experiments, the supernatants of the liquid cultures were harvested and stored at −80°C for immunoassay quantitation of PGE_2.

Adherent cell depletion and addition Pooled bone-marrow cells from groups of five naive BALB/c mice were separated on discontinuous percoll gradients (Gaspar Elsas *et al*., 1997), for 45 min, at $g \times 270$, 20°C, after loading up to 2×10^7 cells/gradient. The low density mononuclear cells at the 40–60% percoll interface (Layer 2) were harvested, washed twice in RPMI/FCS, counted, and submitted to two cycles of adherence (30 min, 10^7 cells 100 mm^1 culture dish, at 37°C, with 95% air/5% CO_2). Non-adherent cells were collected, counted and used for liquid culture assays (10^6 cells well^{-1}). The effectiveness of the depletion procedure was assessed by

enumeration of contaminating mononuclear phagocytes in cytocentrifuge smears after panoptic staining. In selected experiments, aiming at defining the target of indomethacin modulation, bone-marrow cells from both naïve and immune animals were separated into adherent and nonadherent subpopulations, and used in reconstitution experiments, as described below. Unseparated bone-marrow cells were seeded in 24 well plates at 10^6 cells ml^{-1} in RPMI/FCS, 1 ml per well, and incubated for 8 h at 37°C. Nonadherent cells were aspirated with a Pasteur pipette, and the adherent cell layer was washed twice with RPMI/FCS at 37°C, to eliminate all recoverable nonadherent cells. During this adherence step, the remaining bone-marrow cells were used for purification of nonadherent cells as described above. Purified, 10^6 non-adherent cells in RPMI/FCS were then added to wells containing an adherent cell layer, to provide the following cell mixtures: (a) naïve nonadherent/immune adherent (N/I); (b) immune nonadherent/immune adherent (I/I); (c) immune adherent/naïve adherent (I/N). These reconstituted cell populations were cultured as described in the previous section.

Plasma transfer and immunochemical procedures

BALB/c mice were sensitized/challenged as described in Methods. Twenty-four hours after allergen challenge, the mice were sacrificed and blood collected in heparinized tubes. Plasma was obtained by centrifugation (2000 r.p.m., 10 min). Three hundred μl immune plasma were transferred to each naïve BALB/c recipient by i.p. injection. In selected experiments, plasma was submitted to different treatments to deplete total antibody or to inactivate IgE before transfer. For antibody depletion, protein A sepharose (Sigma Ref. P-3391) was rehydrated in protein-free RPMI 1640 medium, under constant stirring, for 1 h 30 min at 4°C, washed three time and brought up to 10% until use. Absorption was carried out by mixing heparinized plasma (either naïve or immune) with pelleted protein A sepharose beads (10% beads final v v^{-1}), and incubating for 1 h at 4°C, with constant stirring. After centrifugation at 2500 r.p.m. for 5 min, the supernatant was submitted to two further cycles of absorption under identical conditions. To eliminate residual immunoglobulins (Ig) from classes that fail to bind directly to protein A sepharose, one final cycle of absorption was carried out after adding 50 μl of goat polyclonal antibody to mouse IgG with specificity for both heavy and light chains (H + L, Immunotech, Marseille, France, Ref. 0815) to each ml of the preabsorbed plasma. The mixture was incubated for 30 min at room temperature, and for 1 h more at 4°C, with stirring. The supernatant was collected and 300 μl were injected i.p. in each recipient mouse. The efficiency of these procedures in removing antibody was monitored as follows: (a) binding of Igs to the beads was monitored by SDS-polyacrylamide gel electrophoresis of the proteins that could be extracted from the protein A sepharose beads pelleted after each cycle, by boiling for 3 min in sample buffer; (b) complete absorption of IgG, including IgGl, was confirmed by immunoblotting (see below); (c) complete removal of specific antibody activity was directly demonstrated by ELISA (see below). For immunoblots, the following samples were separated by SDS–PAGE on a 7.5% minigel, for 1 h at 150 V: plasma from ovalbumin-sensitized and challenged mice (before and after absorption of Ig as described above); the eluate from the last cycle and the goat anti-mouse IgG (H + L) polyclonal antibody used for the last cycle (see above); and monoclonal mouse IgG (Ref. 0107–01, Southern Biotechnology Associates, Birmingham, AL, U.S.A.), used as a positive control for immune reactivity, as well as a molecular weight marker. The minigel was soaked for 30 min in transfer buffer (Tris 20 mM, glycine 100 mM), before transfer at 100 V 500 mA^{-1}, for 1 h. Complete transfer from the gel was demonstrated by Ponceau red staining of both the blot and the gel. The blot was washed in distilled water and quenching was carried out overnight with PBS–BSA 2%, at 4°C, before washing four times with PBS containing BSA 0.5% and tween 20 0.05%, for 15 min. Blots were incubated with anti-mouse IgG (Fab specific) peroxidase conjugate (Sigma, A-3682) at 1 : 4000, for 90 min washed four times (20 min each) as above and developed with diaminobenzidine solution (50 mg DAB, 100 μl de H_2O_2 30% in 100 ml PBS) for 5 min. Image processing was carried out with the help of the Kodak Digital Science 1 D Software, with inverted brightness-contrast for optimal visualization. For ELISA, ovalbumin (10 μg ml^{-1} in 0.1 M sodium phosphate buffer, pH 8), was used to coat 96-well plates (NUNC MaxiSorp™ Surface, 50 μl per well) overnight, at room temperature, with constant stirring, before washing with PBS-tween 20 (0.1%) and quenching with PBS–BSA 1% (200 μl per well), for 2 h. One hundred μl aliquots of immune plasma, naïve plasma or immune plasma absorbed on protein A sepharose, diluted in PBS–BSA, were added for 1 h, before washing and addition of goat anti-mouse IgG (γ-chain specific) alkaline phosphatase conjugate (Ref. A-1047, Sigma, 100 μl per well at 1 : 1000 dilution) for 2 h. Each well was washed and incubated with 100 μl p-nitrophenyl phosphate disodium (1 mg ml^{-1} in $MgCl_2$ 3 mM, Tris 100 mM) in the dark, for 15 min before reading OD_{405}. To control for the effects of the absorption procedure, as well as the effects of carryover of goat antimouse Ig antibody into the absorbed plasma samples, naïve plasma was treated under identical conditions and injected into naïve recipients (see Results). In order to selectively eliminate IgE, immune plasma was incubated for 30 min at 56°C before injecting 300 μl i.p. in each naïve recipient. In all cases, 24 h later, recipient mice were sacrificed and used as bone-marrow donors for liquid culture.

Statistical analysis

The data were analysed with the help of the Systat for Windows version 4 software, using factorial analysis of variance, and with the Tukey (HSD) correction for multiple comparisons between different treatments within each experimental group. For comparisons between two different experimental groups in identical conditions, the 2-tailed Student's *t*-test was used.

Results

Effect of COX inhibitors on bone-marrow of naïve and allergic mice

To define the effect of allergen sensitization and challenge on the ability of bone-marrow cells to respond to indomethacin,

we analysed colony formation by bone-marrow cells from naïve and from ovalbumin-sensitized and challenged BALB/c mice. Semi-solid bone-marrow cultures were established in the presence of GM-CSF, alone or with indomethacin. Conditions were chosen as detailed in Methods, to achieve plateau responses in the control cultures (those without indomethacin), as opposed to threshold or submaximal stimulation. As shown in Figure 1A, a significant increase in the total number of colonies formed in the presence of GM-CSF and indomethacin ($10^{-7}-10^{-9}$ M) ($P=0.003$ and $P=0.009$, respectively) was observed, relative to the control cultures with GM-CSF alone. At 10^{-11} M, the effect of indomethacin was not significant ($P=0.997$). No colony formation was observed in the absence of GM-CSF (not shown). Indomethacin by itself induced no colony formation (not shown). Over the entire series of experiments, ($n=8$) indomethacin at 10^{-7} M increased colony formation, on the average, by a factor of 2.01, and at 10^{-9} M by a factor of 1.68. In contrast, as shown in Figure 1B, indomethacin ($10^{-7}-10^{-11}$ M) had no effect on colony formation by bone-marrow cells from sensitized-challenged mice. This indicates that allergen exposure has a major effect on the ability of bone-marrow cells to respond to indomethacin in the presence of haemopoietic cytokines.

We have previously demonstrated that PGE_2 inhibits eosinophil precursor responses to IL-5, in addition to downmodulating myeloid colony formation by GM-CSF-stimulated progenitors (Gaspar Elsas *et al*., 2000a). To evaluate whether indomethacin, that inhibits production of endogenous prostanoids, would also modulate eosinophil precursor differentiation, liquid cultures were established from bone-marrow of naïve or ovalbumin-sensitized and challenged mice. Again, conditions yielding plateau responses in the control cultures (i.e., in the absence of indomethacin) were chosen. As shown in Figure 2A, a significant increase in the per cent of EPO+cells present at day 7 was observed in the presence of IL-5 and indomethacin ($10^{-7}-10^{-9}$ M), relative to the control cultures with IL-5 alone ($P<0.001$ for both concentrations). At 10^{-11} M, the effect of indomethacin was not significant ($P=0.123$). Indomethacin by itself induced no eosinophil differentiation (not shown). No eosinophil differentiation is observed in the absence of IL-5 (not shown). As shown in Figure 2B, response to IL-5 alone was increased in bone-marrow of sensitized and challenged mice, relative to the naïve controls shown in Figure 2A ($P=0.016$ for the difference between IL-5-stimulated cultures of naïve versus sensitized/challenged mice). However, as also shown in Figure 2B, indomethacin ($10^{-7}-10^{-11}$ M) had no effect on IL-5-induced eosinophil differentiation in bone-marrow cell culture from sensitized-challenged mice. This indicates that allergen exposure has two different effects on bone-marrow responses: (a) it upregulates responses to IL-5 alone, as previously described (Gaspar Elsas *et al*., 1997); (b) it interferes with the ability of eosinophil precursors to respond to indomethacin with increased proliferation and terminal differentiation. Taken together, these findings indicate that indomethacin upregulates myeloid progenitor and eosinophil precursor responses in naïve, but not allergic bone-marrow, and suggests different contributions of endogenous prostanoids to the regulation of haemopoiesis, depending on the immune status of the animal.

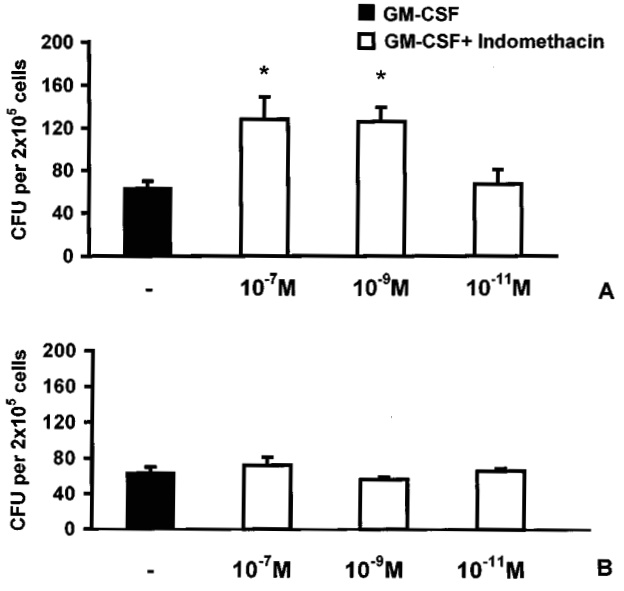

Figure 1 Effect of indomethacin on GM-CSF-induced colony formation. The data are means±s.e.mean of the number of myeloid colonies formed by bone-marrow from naïve (A) and from sensitized-challenged (B) BALB/c mice, in the presence of GM-CSF (2 ng ml^{-1}), alone, or in association with indomethacin, at the indicated concentrations. Data are derived from 3–9 experiments. *Indicates significant differences relative to the GM-CSF controls ($P=0.003$ and $P=0.009$, respectively to 10^{-7} M and 10^{-9} M indomethacin).

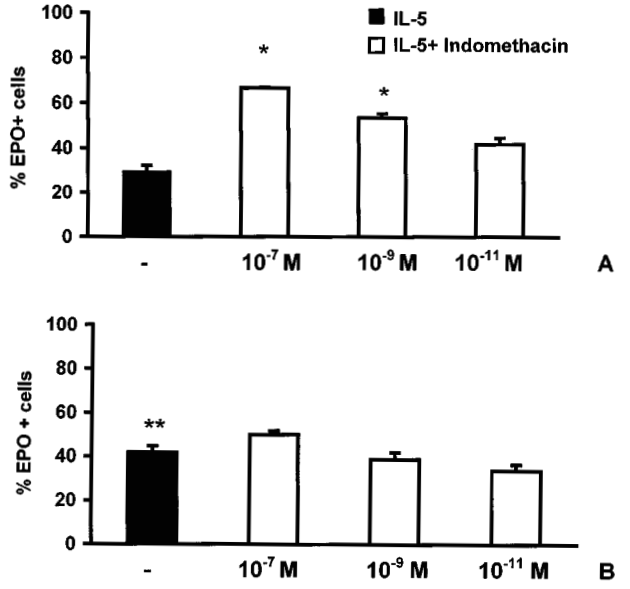

Figure 2 Effect of indomethacin on IL-5-driven eosinophil differentiation. The data are means±s.e.mean of the per cent of EPO+cells present at day 7 in liquid cultures with bone-marrow cells from naïve (A) and from sensitized-challenged (B) BALB/c mice, in the presence of IL-5 (1 ng ml^{-1}), alone, or in association with indomethacin, at the indicated concentrations. Data are derived from 3–22 experiments. *Indicates significant differences relative to the IL-5 controls ($P<0.001$ for both concentrations). **Indicates significant difference relative to the IL-5 control of naïve mice (A) ($P=0.016$). In B, no significant differences were observed between indomethacin-treated and control cultures.

In a separate series of experiments, we evaluated whether synergism between indomethacin and IL-5 was dependent on IL-5 concentration. Indomethacin (10^{-7} and 10^{-9} M) significantly increased responses of naive bone-marrow cells to IL-5 when the latter was used at either 1 or 2 ng ml^{-1} ($P<0.01$ for all comparisons between indomethacin-treated and control cultures, data derived from four experiments; indomethacin at 10^{-11} M had no effect in these conditions). In contrast, indomethacin, from 10^{-7} to 10^{-11} M, had no effect on responses to IL-5 when stimulus was provided at 0.01 or 0.1 ng ml^{-1}. Hence, synergism depended on the stimulus strength, and was not detected at submaximally effective concentrations, while it was present at maximally and supramaximally effective concentrations.

In order to define whether the effects of allergen sensitization and challenge were also detectable with other COX inhibitors, we evaluated responses of bone-marrow cells to aspirin. Semi-solid cultures were established with bone-marrow from naïve or from ovalbumin sensitized mice, in the absence or in the presence of aspirin (10^{-7}–10^{-8} M). As shown in Table 1, there was a significant increase ($P=0.046$) in the number of colonies formed in the presence of GM-CSF and aspirin (10^{-7} M), relative to control cultures stimulated by GM-CSF alone. Aspirin by itself did not induce colony formation (not shown). At 10^{-8} M, the effect of aspirin was not significant ($P=0.999$). As also shown in Table 1, when bone-marrow from ovalbumin sensitized/challenged mice was used, colony numbers were not increased.

Because colony-formation assays and eosinophil differentiation assays yielded comparable results, and because the latter made it possible to evaluate the role of cell subpopulations in immune modulation of responses to indomethacin, subsequent experiments were carried out, unless otherwise indicated, in the liquid culture system.

Cellular targets of cyclo-oxygenase inhibitors in bone-marrow

In order to define the cellular source of endogenous prostanoids in bone-marrow culture, naïve bone-marrow cells were submitted to depletion of mature, adherent cells by sequential passage on percoll gradients and two cycles of adherence. As shown in Figure 3A, nonadherent bone-marrow cells presented responses to IL-5 that were very close to those of unseparated bone-marrow. However, indomethacin did not enhance responses to IL-5 in cultures of nonadherent cells, in contrast to its strong enhancing effect in cultures of unseparated bone-marrow. This points to adherent cells (most likely macrophages) as the mediators of the modulatory effects of indomethacin, which are indirect, in contrast to the direct effect of IL-5. To evaluate whether differences in their adherent cell populations account for the differences between naïve and immune bone-marrow cells in response to indomethacin, adherent and nonadherent bone-marrow cells were isolated from both sets of donors and used in reconstitution experiments. As shown in Figure 3B, adherent cells from immune bone-marrow did not reconstitute the response to indomethacin in purified nonadherent cells from naïve donors ($P=0.599$). Accordingly, they had no effect when used to reconstitute purified nonadherent cells from immune mice ($P=0.643$). In contrast, adherent cells from naïve bone-marrow conferred the ability to respond to indomethacin when added to nonadherent cells from immune bone-marrow ($P=0.038$). This indicates that enhancement of haemopoiesis by indomethacin requires adherent cells from naïve mice, and that adherent cells from immune bone-marrow are unable to duplicate this effect.

Role of allergen sensitization and challenge

We evaluated the relative contributions of sensitization, on the one hand, and intranasal allergen exposure, on the other hand, to the change in responsiveness to indomethacin

Table 1 Effect of asprin on GM-CSF-induced colony formation

	Colonies per 2×10^5 cells		
		Aspirin	
	GM-CSF	*10^{-7} M*	*10^{-8} M*
Naïve mice	48.66±4.16	68.59±6.73*	55.30±5.72
Immune, ovalbumin-challenged mice	56.13±5.52	64.15±3.61	56.38±1.88

The data are means±s.e.mean of the number of myeloid colonies formed by bone-marrow from naïve and from sensitized-challenge BALB/c mice, in the presence of GM-CSF (2 ng ml^{-1}), alone, or in association with aspirin, at the indicated concentrations. Data are derived from 4–7 experiments. *Indicates significant differences relative to the GM-CSF controls ($P=0.046$ to aspirin 10^{-7} M).

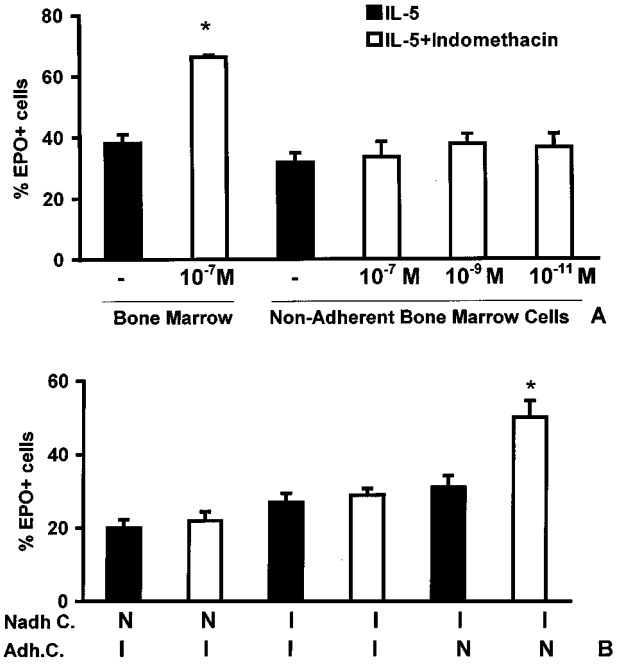

Figure 3 Effect of adherent cell depletion on bone-marrow response to indomethacin. Data are means±s.e.mean of the per cent of EPO+ cells present at day 7 in liquid cultures with bone-marrow cells from naïve BALB/c mice, in the presence of IL-5 (1 ng.ml^{-1}) alone, or in association with indomethacin at 10^{-7}–10^{-11} M (A) or at 10^{-7} M (B). In A, total (unseparated) or non-adherent bone-marrow cells were cultured. In B, cultures were established with a mixture of nonadherent cells (NAdh C) and adherent cells (Adh C) purified from naïve (N) or immune (I) donors. Data for A are derived from 3–8 experiments, and for B from four experiments. *Indicates significant differences to the IL-5 control ($P<0.038$).

observed in sensitized-challenged mice. In colony formation assays with bone-marrow cells from ovalbumin-sensitized, saline-challenged and from sham-sensitized, ovalbumin-challenged BALB/c mice, indomethacin (10^{-7}–10^{-11} M) had no effect when cells from ovalbumin-sensitized, saline-challenged animals were used. In contrast, indomethacin (10^{-9} M) significantly enhanced (from 55.1 ± 1.04 to 68.9 ± 2.03 colonies per 2×10^5 cells plated, means ± s.e.mean, $P < 0.001$, data from four experiments) colony formation by bone-marrow from sham-sensitized, ovalbumin-challenged mice. Indomethacin at 10^{-11} M was ineffective in these conditions. As shown in Figure 4A, indomethacin (10^{-7}–10^{-11} M) had no effect on eosinophil differentiation induced by IL-5 in bone-marrow culture from ovalbumin-sensitized, saline-challenged animals. In contrast, as shown in Figure 4B, indomethacin (10^{-7}–10^{-9} M) significantly enhanced the responses to IL-5 in bone-marrow from sham-sensitized, ovalbumin-challenged controls ($P < 0.001$, for both concentrations). Again, indomethacin at 10^{-11} M was ineffective on the latter. Taken together, these findings indicate that sensitization alone is sufficient to interfere with the modulatory activity of indomethacin on haemopoiesis, and that airway allergen exposure has no effect by itself. They also rule out any nonspecific effects of the adjuvant used in the sensitization procedure, which was similarly present in both groups, unlike antigen, which was only injected into one group.

To define whether the effects of allergen sensitization could be passively transferred from immune to naïve animals, plasma from sensitized BALB/c mice was injected in naïve recipients, 24 h before the recipients' bone-marrow was cultured. In additional experiments, recipient mice were injected with plasma from naïve or from immune donors, after the removal of Igs by immunoaffinity chromatography, or after heat-inactivation of IgE. To ensure that removal of Ig was complete, specific antibodies and total Ig were detected before and after absorption. As shown in Figure 5A, specific IgG was thoroughly removed by the absorption procedure: while immune plasma had a high titer of anti-ovalbumin antibody, absorbed immune plasma had antibody levels comparable to those of nonimmune plasma. As shown in Figure 5B,C, all immunoreactive IgG was removed from immune plasma by absorption. While most IgG was removed by the three initial cycles of absorption, as assessed by SDS–PAGE of proteins bound to the protein A-sepharose (not shown), the residual IgG_1 was absorbed by the last cycle, with the help of goat-antimouse IgG antibody (which is not reactive in the immunoblot). Taken together, these findings document complete removal of IgG from the immune plasma used for transfer.

As shown in Figure 6, transfer of naïve plasma did not downmodulate responses to COX inhibitors in bone-marrow of naïve recipients, which remained fully responsive to indomethacin ($P = 0.005$) as well as aspirin (not shown). In

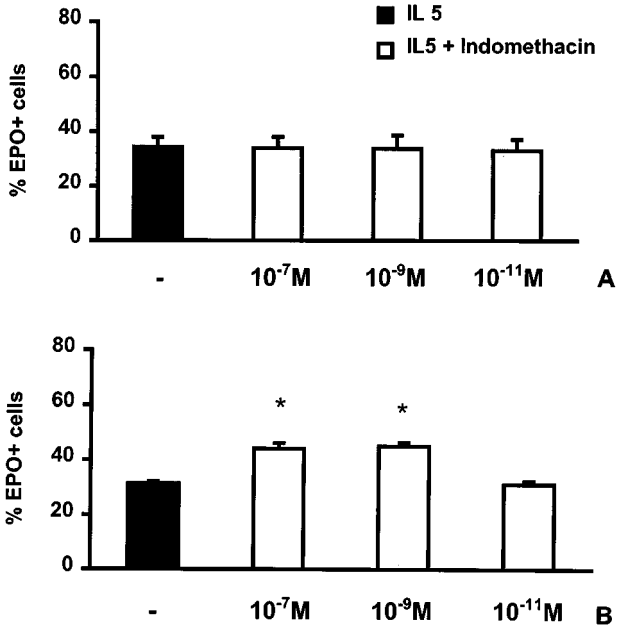

Figure 4 Effect of indomethacin on IL-5-driven eosinophil differentiation. The data are means ± s.e.mean of the per cent EPO+ cells present at day 7 in liquid cultures with bone-marrow cells from sensitized/sham-challenged (A) and from sham-sensitized/challenged (B) BALB/c mice, in the presence of IL-5 (1 ng ml^{-1}), alone, or in association with indomethacin, at the indicated concentrations. Data are derived from five experiments. *Indicates significant differences relative to the IL-5 controls ($P < 0.001$ for both concentrations of indomethacin).

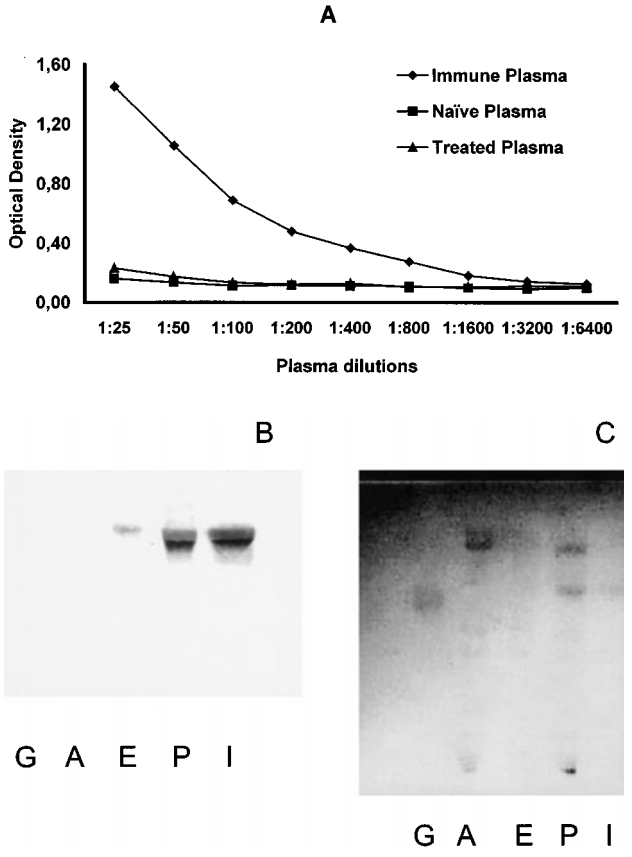

Figure 5 Depletion of antibody from immune plasma. (A) Data are OD_{405} readings from the indicated dilutions of immune plasma, naïve plasma and Protein A-Sepharose-absorbed immune plasma, assayed in ELISA for ovalbumin-specific IgG antibodies, as described in Methods. (B), immuno-blot and (C), Ponceau Red-stained blot of 'P' plasma from ovalbumin-sensitized and challenged mice; 'A' absorbed immune plasma; 'E' eluate from the last absorption cycle; shown as controls, 'G' goat antimouse IgG (H+L) polyclonal antibody and 'I' monoclonal mouse IgG.

contrast, the response to indomethacin was abolished by transfer of immune plasma to naïve recipients ($P=0.999$ for the difference between indomethacin-treated and control cultures). As also shown in Figure 6, depletion of Igs did not eliminate the ability of immune plasma to downmodulate responses to indomethacin ($P=0.960$ for the difference between indomethacin-treated and control cultures). No effect of the Ig depletion procedure by itself could be observed in mice receiving similarly treated naïve plasma (not shown). In another series of experiments, heat-inactivation did not eliminate the ability of immune plasma to downmodulate responses to indomethacin in bone-marrow of naïve recipients (Table 2). This rules out any role for IgE in downmodulating responsiveness to indomethacin. Taken together, these findings indicate that immune plasma modulates bone-marrow responses by antibody-independent mechanisms.

Discussion

The findings reported here support the view that endogenous prostanoids regulate eosinopoiesis in bone-marrow cultures. This is, to our knowledge, the first report of enhancement of eosinophil production in the bone-marrow by COX inhibitors. Since exogenously added PGE_2 suppresses eosinopoiesis (Gaspar Elsas *et al*., 2000a), this suggests a role for endogenous prostanoid production in regulating the responsiveness of the eosinophil lineage to haemopoietic cytokines. This also provides one possible mechanism for the enhancing effect of exogenous (Gaspar Elsas *et al*., 2000b) and endogenous (Gaspar Elsas *et al*., 2000c) glucocorticoids on eosinopoiesis. However, it also raises questions regarding the relationship between allergen exposure and prostanoid production in regulation of eosinopoiesis. Because most, if not all, reports addressing the haemopoietic effects of indomethacin concern nonsensitized animals, we evaluated whether the enhancing effects of COX inhibitors were modified by allergen exposure.

Our findings document, for the first time, a major effect of allergen exposure on responsiveness to COX inhibitors. Bone-marrow from ovalbumin-sensitized and challenged mice did not respond to indomethacin and aspirin, in contrast to naïve bone-marrow. Sensitization by itself was sufficient to interfere with responsiveness to indomethacin, while airway allergen exposure was insufficient. Furthermore, circulating immune mediators were sufficient to reproduce the effects of active sensitization, in passive plasma transfer protocols.

The cellular target for COX inhibitors, in this study, is an adherent bone-marrow cell population, most likely macrophages, which are well-known prostanoid producers. This was directly shown by reconstitution experiments, in which adherent cells from naïve bone-marrow conferred the ability to respond to indomethacin. At this point, there is no evidence for the involvement of COX-2 in this production, because bone-marrow from normal mice was cultured, in the absence of any known inflammatory stimulus, and because adjuvant played no role in modulation. On the other hand, it has been shown that ovalbumin sensitization results in increased COX-2 and 5-lipoxygenase activating protein expression in murine peritoneal macrophages, associated with a decrease in cytosolic phospholipase A_2 (PLA_2) expression (Escoubet *et al*., 2000). As a result, basal arachidonic acid (AA) metabolism was reduced, even though this could be rescued by several nonphysiological stimuli. It is unclear how this relates to our findings, because larger antigen and adjuvant loads were repeatedly administered in the study by Escoubet *et al*. (2000), and because peritoneal macrophages may differ in this respect from those in bone-marrow. However, the loss of sensitivity to COX inhibitors in allergic mice in our study is probably due to a decrease in prostanoid production, rather than to decreased sensitivity of haemopoietic cells to prostanoids, because bone-marrow from normal and allergic mice is equally sensitive to inhibition by PGE_2 (Gaspar Elsas *et al*., 2000a). Hence, it might reflect decreased cytosolic PLA_2 activity in immune bone-marrow

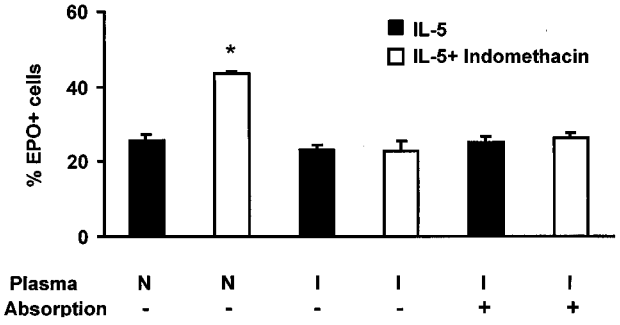

Figure 6 Effect of plasma transfer on bone-marrow response to indomethacin. The data are means ± s.e.mean of the per cent of EPO + cells present at day 7 in liquid cultures with total bone-marrow cells from naïve BALB/c mice, in the presence of IL-5 (1 ng ml^{-1}) alone, or in association with indomethacin (10^{-7} M). Mice were injected with plasma from naïve (N) or immune (I) donors. Plasma was absorbed as described in Methods to eliminate antibodies (+) or left untreated (−). Data are derived from 3–8 experiments. *Indicates significant differences to the IL-5 control ($P=0.005$).

Table 2 Downregulation of responses to indomethacin by heat-unactivated immune plasma

	Indomethacin concentration			
In vivo Treatment	*0*	*10^{-7} M*	*10^{-9} M*	*10^{-11} M*
Naïve plasma	25.50 ± 1.85	43.43 ± 0.72*	39.23 ± 5.90*	26.25 ± 1.11
Immune plasma	22.92 ± 1.33	22.71 ± 2.65	21.90 ± 1.01	19.92 ± 2.30
Immune plasma, heat-inactivated	23.00 ± 1.46	26.06 ± 1.91	20.40 ± 0.80	21.89 ± 0.98

The data are means ± s.e.mean of the per cent of EPO + cells present at day 7 in liquid cultures with total bone-marrow cells from naïve BALB/c mice, in the presence of IL-5 (1 ng.ml^{-1}) alone, or in association with indomethacin at the indicated concentrations. Mice were injected with plasma from naïve or immune donors. Plasma was heat-inactivated as described in Methods to eliminate antibodies of the IgE class. Data derived from 3–8 experiments. *Indicates significant differences to the IL-5 control ($P=0.005$)

macrophages, as described by Escoubet *et al*. (2000) for those in the peritoneal cavity.

Interestingly, in reconstitution experiments, naïve adherent cells did not suppress haemopoietic cells from immune mice, in the absence of COX inhibitors. In contrast, addition of indomethacin resulted in a very significant enhancement. This argues against the constitutive production of suppressive AA mediators by naïve adherent cells. It is, however, consistent with deviation of basal AA metabolism from the COX to the lipoxygenase pathway, due to COX inhibition by indomethacin, with the resulting generation of a product that enhances eosinopoiesis. We are currently investigating the possible role of lipoxygenase products in this enhancement.

The ability of immune plasma transfer to modulate the pattern of response to indomethacin in the bone-marrow made it possible to evaluate the role of specific antibody. Unexpectedly, depletion of virtually all circulating antibody did not reduce the ability of immune plasma to exert its regulatory effects. In addition, no change was observed after selective elimination of IgE. This indicates that antibody is not required for the modulatory activity of immune plasma on AA metabolism in the bone-marrow, and raises questions about the nature of the mediator(s) involved. Even though specific antibody is the most obvious factor that differs between immune and naïve plasma, other immunomodulating substances may behave similarly. In humans, neopterin, a metabolite of guanosine triphosphate with pleiotropic regulatory activities, is found in the circulation after immunization, and its levels closely correlate with cellular immune activation (Baier-Bitterlich *et al*., 1997). Interestingly, neopterin has marked enhancing effects on murine haemopoiesis, which depend on a stromal (adherent) cell population in the bone-marrow (Aizawa *et al*., 1998). Even though mice are poor producers of neopterin (Amadori *et al*., 2001), murine endothelial cells release large amounts of a closely related product, tetrahydrobiopterin, which is an essential regulator of nitric oxide synthase (Walter *et al*., 1994). It remains to be established whether neopterin (or a related regulatory substance) plays a role in modulating responsiveness to COX inhibitors in the bone-marrow.

These observations highlight the relevance of the immune status of the host for the effectiveness of anti-inflammatory drugs. Although it remains to be determined whether this applies to tissues other than the bone-marrow, this should be of interest to those studying haemopoietic recovery in irradiated animals, as well as to those approaching the effects of cyclo-oxygenase inhibitors on leukocyte accumulation in inflammatory sites.

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