Anti-Inflammatory Drug Effects on Apoptosis of Eosinophil Granulocytes Derived from Murine Bone-Marrow: Cellular Mechanisms as Related to Lineage, Developmental Stage and Hemopoietic Environment

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Abstract: The effects of a variety of widely used anti-inflammatory agents (dexamethasone, indomethacin, and montelukast) as well as ubiquitous mediators of inflammation (prostaglandin E₂ and nitric oxide) on the development of murine eosinophils ex vivo and in vivo have been studied over the last decade. The results indicate that developing eosinophils differ markedly in their responses to these agents from the mature forms of the same lineage, studied either in allergic human subjects or experimental animal models of allergic disease. Most strikingly, glucocorticoids strongly enhance eosinophil development, both in vitro and in vivo. The enhancing effects are also observed during stress reactions and are strictly dependent on stress-induced glucocorticoid hormone production from the adrenal glands. Some, but not all, of the developmental effects of glucocorticoids on eosinophils could be accounted for their ability to prevent generation of nitric oxide through inducible NO synthase, which leads to apoptosis through the CD95-CD95L pathway. A novel mechanism for the effects of indomethacin in upregulating the development of eosinophils has also been documented. Evidence that lineage-specific as well as stage-specific cellular response programmes determine these different outcomes is discussed, along with the perspectives for future research.

PURPOSE AND SCOPE OF THIS REVIEW

This review intends to examine some emerging issues concerning the mechanisms of action of a number of well-known anti-inflammatory drugs, including dexamethasone, indomethacin and montelukast, with a focus on their ability to modulate the development of eosinophils from bone-marrow. The issue of interest will be the relationship between the targets of these pharmacological agents and the molecules that determine whether developing eosinophils will undergo programmed cell death, or become resistant to it. In addition, we have tried to relate our findings to those of other laboratories which examined similar mechanisms in the circulating (fully mature) forms of two hemopoietic cell lineages, eosinophils and neutrophils, because even though these are closely related at their origin in bone-marrow, they diverge during development to take up different functions in host defense and allergic reactions, and in their mature stages seem to present very distinct patterns of response to a series of apoptosis-inducing stimuli, as well as to the anti-inflammatory drugs that affect this response [1-4].

Using this comparative approach, we will review, in the following sections, the available evidence indicating that the way eosinophils respond to both apoptosis inducing stimuli and anti-inflammatory drugs is strongly influenced by their developmental stage, as well as by the environment in which they complete maturation.

Eosinophils and neutrophils are central effectors of allergic reactions and antimicrobial immunity, respectively. The agents whose actions are reviewed here are currently used for controlling the undesired consequences of the inflammatory response. Therefore, the following discussion is likely to be of interest to those concerned with the effects of anti-inflammatory agents at the cellular level, as well as with the development of novel drugs combining the known properties of distinct agents available today (as, for instance, molecules that combine the ability to release nitric oxide with the properties of nonsteroideal anti-inflammatory agents) [5-6]. In addition, due to the great current interest in strategies that employ apoptosis-promoting stimuli to terminate chronic inflammatory reactions and to promote the clearance of dead infiltrating leukocytes [2-3], we feel it is important to appreciate that some of these stimuli will act differently in inflammatory sites and in the bone-marrow, which represent environments compatible with the opposing processes of leukocyte consumption and replenishment, respectively.

Regulation of Murine Eosinopoiesis by Allergen, Inflammatory Mediators, and Anti-Inflammatory Drugs

Over the last decade, our own group has concentrated on the production of eosinophils in the bone-marrow, and its modulation by allergen, anti-inflammatory drugs and chemical mediators of inflammation [7]. As detailed below, some of our initial observations failed to conform to commonly accepted views of anti-inflammatory drug action, and prompted us to explore further the cellular basis of the ef-
fects of these drugs on developing eosinophils. Our results have pointed to the control of apoptosis during hemopoiesis as an important mechanism of action for both mediators of inflammation and anti-inflammatory drugs; furthermore, they have raised the issue of whether the patterns of responsiveness previously described for the eosinophil and the neutrophil lineage represent stable, fundamental characteristics of these lineages, or, alternatively, the behaviour of specific developmental stages in a specific environment.

While eosinophilia (the presence of increased eosinophil numbers in blood or tissues) has been evaluated in a large number of studies of allergy in humans and mice, very few studies of allergy actually have evaluated eosinopoiesis (the actual production of eosinophils from more immature hemopoietic cells), a difference that is not always appreciated. The study of eosinopoiesis demands specific assays, which are usually carried out with bone-marrow cells, although they can be adapted to other cell populations, in which the ability to carry out extramedullary hemopoiesis has either been demonstrated or suspected. Such assays include both clonal growth in semisolid media (agar, methylcellulose) and precursor differentiation from lineage-committed precursors in liquid culture. A third important parameter that can be studied is the eosinophilia of bone-marrow, where the numbers of eosinophils present are believed to reflect the balance between their production (and possibly their destruction before reaching maturity), on the one hand, and their emigration from bone-marrow, on the other.

These ways of looking at eosinophil production may present important differences, which should be kept in mind when comparing the outcomes of independent studies. Bone-marrow eosinophilia is probably the most physiologically relevant parameter, because it reflects an actual change that has taken place in vivo. However, because it represents the net result from the competing processes of production and export of eosinophils, it does not provide straightforward estimates of the rate of eosinopoiesis. For that, clonal assays and eosinophil differentiation assays are more informative. These, however, must be carried out in tissue culture, because the entire sequence of events takes several days and cannot be easily followed inside the bone-marrow of normal animals (where all cell lineages are simultaneously present and often resemble each other in their more immature stages). This is especially true in the case of a minority population such as eosinophils. Hemopoietic cultures of either type often require addition of exogenous cytokines as hemopoietic growth and differentiation factors (IL-5, the eosinophil-selective growth factor, is active in both types of assays, and is required for maintenance of bone-marrow eosinophilia in vivo) in order to support continued viability, growth and differentiation of the hemopoietic cells. For this reason, they readily provide information on the potential for responding to these exogenous factors (i.e. the size and responsiveness of the responsive population), but are not directly informative about the extent of actual responses to their endogenous counterparts taking place in vivo. When exogenous cytokines are not purposely added to the cultures, any of a number of endogenous stimuli is usually included, to drive the endogenous cytokine production without which hemopoiesis cannot go on. Again, this does not inform us about how hemopoiesis proceeds in the absence of these inducers of cytokine production.

Clonal assays for growth of hemopoietic cells in semisolid media (agar, methylcellulose) have made it possible to identify the first hemopoietic growth factors by their ability to support the growth of colonies from seeded bone-marrow cells (reviewed in [8]). Even after sophisticated assays for the long-term repopulating ability of hemopoietic cells in vivo became available, colony formation assays remained useful, for they evaluate a target that is able to respond to a short-term demand, but not to repopulate irradiated animals for prolonged periods. It is now clear that the ability to reconstitute hemopoietic tissues permanently is a property of hemopoietic stem cells, which are self-renewing, while the ability to sustain the production of specific hemopoietic lineages over short periods characterizes hemopoietic progenitors, which are not, and therefore ultimately depend on de novo generation from stem cells to persist. Because most progenitors give origin to colonies which can be easily identified on morphological and cytochemical criteria, it is generally thought that progenitors are restricted in their developmental potential, or committed to a specific lineage. However, some cells give rise to colonies containing more than one lineage (mixed), and in some cases it is possible to show that cells of a given lineage can be found both in pure and in some subtypes of mixed colonies [9]. The current explanation for this is a hierarchical model of progressive commitment, in which progenitors common to several hemopoietic lineages give rise to progenitors of progressively more restricted potential. Even so, eosinophil formation has been described in different studies as arising in pure eosinophil colonies [10] as well as in colonies which also contain neutrophils [11], or neutrophils and monocytes [10], or basophils [12]. From recent studies with monitoring of gene expression for eosinophil-specific gene products as well as GATA-1 transcription factor activity at a single cell level [9], it seems that eosinophil colonies can be derived from two types of cells: one that also gives rise to monocyte and neutrophil colonies, and therefore qualifies as a mixed progenitor, and one that only gives rise to eosinophil colonies. The same studies provided evidence against a shared origin between eosinophils and basophils [9], and supported the notion that expression of IL-5 receptors is the result, and not the cause, of eosinophil lineage commitment. If confirmed, this would be consistent with the evidence from our own studies [10]. It is not clear at present whether the different colony patterns described by other laboratories can be reconciled into the same hierarchical model. It is probably safe to conclude that the exact types of colonies that will be detected depend on the exact experimental conditions. If so, we cannot rule out, at present, a common origin for eosinophils and basophils [12] or for eosinophils and neutrophils with the exclusion of macrophages [11], in other specific experimental conditions. As a result, depending on the conditions used in each laboratory, eosinophils will be considered to be very close relatives of neutrophils (sharing an immediate ancestor that gives rise to committed progenitors for both lineages), or, alternatively, as a little more distant relatives (not sharing an immediate common ancestor, but a common origin two levels above, at a common mixed progenitor).
By definition, clonal assays depend on the ability of cells in a specific developmental stage (the progenitor) to initiate growth in semisolid medium. Because colonies are scored as such when they have reached a size compatible with 40-50 cells, depending on the study, they are the outcome of 5-6 rounds of cell division from one colony forming cell (the progenitor). It is clear that not every immature myeloid cell in bone-marrow has the ability to initiate colony growth, even in very favorable conditions; indeed, at the beginning of the culture one usually sees a relatively dense cell population but no colonies, and as the latter develop the cells between colonies – which are much more numerous – tend to disappear, leaving a relatively clear picture. Nevertheless, very little is known about the cellular properties that enable a progenitor to give rise to a colony. Picking up cells from inside a colony and replating them, in several studies, has led to growth of novel colonies, indicating that at least some of the cells inside a growing colony remain in a progenitor stage [8]. Nevertheless, many of the cells in colonies look like progressively mature stages of cell lineages which are morphologically and cytochemically identifiable, but not necessarily capable of proliferation. It is, therefore, generally believed that the proliferative potential of cells inside colonies from most lineages (neutrophils and eosinophils included) decreases as they mature.

On the other hand, the emergence of progressively mature cells of these lineages can be followed in liquid cultures of bone-marrow cells that originally lack them, provided the appropriate cytokines are present. This forms the basis of differentiation assays, in which colony growth is not required (even though, on occasion, tightly associated clusters of developing eosinophils, for instance, can be found in liquid cultures, indicating that cluster emergence is not necessarily a result of mechanical limitations on cell movement imposed by the semisolid medium). The entire sequence is assumed to be the result of proliferation and terminal differentiation, from cells that lack the markers of interest but are nevertheless committed to one specific lineage (the so-called precursors), to their completely differentiated progeny, resembling the mature cells that are ready to leave the bone-marrow. The extent of proliferation taking place in these conditions is probably lesser than the 5-6 rounds of cell division involved in colony formation, but this has not been directly established, due to the lack of an unambiguous marker for the precursors. This should be borne in mind when discussing the responses of progenitors and precursors to different agents, because the underlying assumption is that precursors arise from progenitors (that is, a colony should contain precursors as a transient component, just as it contains progenitors, both eventually disappearing to leave only their terminally differentiated progeny), even though precursors seem to have lost the ability to initiate colony growth.

In the case of eosinophils, one concern with these assays is the extent to which survival of mature eosinophils present in the bone-marrow inoculum contributes to the numbers observed at the end of the culture. This concern is important because immunological mediators released following challenge, such as IL-5 itself, may increase mature eosinophil survival, and because mature eosinophils are to be found in the inocula from sensitized and challenged donors than in those of controls. However, prolonged survival does not mean indefinite survival; while it might contribute to eosinophil counts at day 3, it did not seem to account for results observed at 7 days of liquid culture.

The Effects of Allergen

In our initial studies [10], we evaluated the effects of allergen sensitization and challenge in the airways on bone-marrow eosinophilia and ex vivo eosinopoiesis. This was done in BALB/c mice, which are one of the favorite strains for studies of allergy (subsequent studies would show that other strains may differ markedly in their hematological responses to sensitization and challenge, but the description that follows applies to BALB/c, unless specifically stated otherwise). Allergen sensitization did not seem to have any major impact on bone-marrow eosinophilia, nor in responses to eosinopoietic cytokines (IL-5 and IL-3 were used in that study). By contrast, allergen challenge of previously sensitized mice increased significantly the numbers of eosinophils in the bone-marrow, as well as the ex vivo responses to IL-3 in eosinophil colony formation assays and to IL-5 in liquid culture, when all three parameters were examined 24 h after exposure to allergen in the airways. In that study, the changes in the eosinophil compartment in vivo were paralleled by similar ex vivo changes in response to eosinopoietic factors. However, eosinophilia observed in vivo 24 h after challenge necessarily has a different kinetics from eosinopoiesis observed after 7 days in cultures from bone-marrow that had been collected 24 h after in vivo challenge. These phenomena may be related, but are not identical, and the ex vivo results probably reflect the potential for sustained eosinopoiesis, while the in vivo data possibly reflect both increased eosinopoiesis (which may be sustained over time, an issue that has not been examined by us) and simultaneous changes in export from bone-marrow. At this specific time point, however, we did not see a significant increase in circulating numbers of eosinophils over the control group of saline-challenged, sensitized mice.

Because of the rapidity with which these changes took place after challenge, we hypothesized that a circulating or neural mediator was involved. Plasma from sensitized donor mice challenged (but not from saline-challenged, similarly sensitized, controls), collected 24 h after airway challenge, and transferred to was able to upregulate responses to IL-5 in liquid culture of bone-marrow taken from the plasma recipients 24 h after transfer (i.e. 48 h after the original airway challenge). Most interestingly, plasma was equally effective in upregulating responses in naive recipients as in sensitized ones. This suggests ovalbumin sensitization is irrelevant to the effects of plasma in the bone-marrow, and its role seems to be restricted to the induction of the circulating mediator present in plasma of the appropriate donor. It also suggests that the effects of sensitization and challenge combined can be replaced by plasma transfer. Even though this experiment failed to provide positive evidence for a specific mediator, at least it provided important negative evidence: the absorption of active plasma samples in protocols designed to remove IL-5, IL-3 and GM-CSF, as well as circulating immune complexes, was unable to remove the activity.
The Effects of Glucocorticoids

Subsequent experiments [13] addressed the effects of glucocorticoids on eosinopoesis, using bone-marrow from both naive and sensitized/challenged mice. Glucocorticoids were evaluated because they are anti-inflammatory agents very effective in the control of allergic diseases and asthma, when given to whole animals as well as to patients, so it would be interesting to assess whether their effects on bone-marrow of allergic animals are consistent with their overall anti-allergic effects. Dexamethasone, contrary to our expectations, strongly enhanced eosinophil production in liquid culture, when bone-marrow from both naive and sensitized/challenged BALB/c was cultured in the presence of exogenous IL-5. The effects of dexamethasone were dose-dependent and synergistic with those of IL-5, so that enhancement always required IL-5 and was greatest at relatively low concentrations of dexamethasone, and undetectable at higher doses. Several different glucocorticoids, including the hormones hydrocortisone (cortisol) and corticosterone, which mediate the adrenal glucocorticoid responses to stress in humans and mice, respectively, were effective in proportion to their known glucocorticoid potencies. Furthermore, classical glucocorticoid receptor-mediated mechanisms were involved, because the effects of dexamethasone were blocked by mifepristone (RU 486). Even though a wide dose-range was explored, no inhibition of the effects of IL-5 was observed. Bone-marrow from both naive and allergic animals responded similarly, even though the ability to detect enhancement by dexamethasone was somewhat impaired by the already elevated background of sensitized/challenged bone-marrow (see above). Dexamethasone was also active in vivo: naive mice pretreated with dexamethasone showed a dose-dependent enhancement of their ex vivo responses to IL-5 in bone-marrow culture collected 2-24 h after drug treatment. This suggested that dexamethasone had a priming effect, as it was effective if given before IL-5. On the other hand, if dexamethasone was first added to the cultures 48 h after they were initially cultured in the presence of IL-5, it was no longer effective. This window of effectiveness suggested that the dexamethasone-responsive target was a cell that disappeared after a certain time in culture, as expected from a precursor.

Eosinophil colony formation was also enhanced greatly by dexamethasone, both administered in vivo as a priming agent, and present in vitro for the duration of the culture. In this case, the window of effectiveness was not evaluated, as the effectiveness of addition of dexamethasone to a semi-solid culture might be severely hindered by the gel in which cells were embedded. It was clear that dexamethasone upregulated colony formation in two distinct ways: it enhanced total colony formation (including colonies producing predominantly neutrophils) and it increased the frequency of eosinophil colonies.

Consistently with a priming mechanism, the administration of dexamethasone in vivo to naive animals did not increase bone-marrow eosinophilia, even though it enhanced their subsequent ex vivo responses to IL-5. Hence, dexamethasone did not abolish the need for IL-5, although it amplified responses to IL-5 when it was present. In this way, these observations remain strictly in agreement with the observed effectiveness of steroids as anti-allergic agents, because these are believed to inhibit production of IL-5 in vivo [14].

Based on this, one may appropriately question the biological significance of the observation that glucocorticoids enhance the effects of a cytokine, if the latter is itself the target of inhibition by glucocorticoids. However, even though the situation is not a simple one, because the dose-response relationships and the kinetics of cytokine inhibition, at both the transcriptional and translational levels, as well as the half-life of the preexisting cytokine, are decisive factors in determining whether or not IL-5 and glucocorticoids will be simultaneously present in the environment where eosinopoesis takes place, we favor the view that this effect of glucocorticoids is physiologically relevant [14], based on an entirely independent body of evidence, as detailed below.

When our studies with addition of glucocorticoids were completed, we became aware of studies carried out by others in a novel experimental model of pulmonary eosinophilic inflammation [15], which involved surgical implantation of heat-coagulated egg white, as a subcutaneous pellet, followed by aerosol challenge of implant recipients with ovalbumin. It occurred to us that this model, characterized by intense tissue eosinophilia, both at the implant site and in the lungs, might present the two features that were necessary for evaluating the physiological significance of glucocorticoid effects in promoting eosinopoesis, namely stress with its accompanying rise in glucocorticoid hormones, on the one hand, and sustained peripheral eosinophilia (which in principle would be explained by a sustained increase in bone-marrow eosinopoiesis), on the other. We investigated therefore whether this was the case, and whether there was a causal link between elevated glucocorticoid hormones and the observed eosinophilia [16].

We were able to confirm that mice of the BALB/c strain, bearing subcutaneous implants of heat-coagulated egg white (the so-called EWI recipients, for egg white implant) presented significant bone-marrow eosinophilia over a 30-day period following the implant surgery. We observed, however, that a similar bone-marrow eosinophilia, although of shorter duration, was present in mice of the control group, which had been submitted to surgery (i.e., that was stressed) but had received no egg white implant (which is believed to be the source of allergen to which the sensitized animals will respond, when challenged later at the airways). Bone-marrow eosinophilia in both groups of mice was associated with greatly increased responses to IL-5 in liquid culture, as well as increased responses in colony formation assays, supporting the view that increased eosinopoesis was taking place in vivo. The simplest explanation for these findings was that surgical stress itself upregulated eosinopoesis, and that circulating glucocorticoid hormones played a role in this effect. We were able to confirm that surgery, independently of the presence of an egg white pellet, was a powerful stimulus for the release of stress hormones, by directly measuring plasma corticosterone levels. We further showed that blockade of this endocrine response to surgical stress prevented all the changes in bone-marrow eosinopoesis. This was shown by three independent approaches, namely: a) the blockade of glucocorticoid receptors by mifepristone;
b) the inhibition of glucocorticoid hormone production by a one-week course of metyrapone (which abolished, as expected, the glucocorticoid response to surgery, as well as the bone-marrow effects of surgery); and c) the surgical removal of the adrenal glands in animals in which any glucocorticoid-mediated stress response to the adrenalectomy procedure had been prevented by pretreatment with metyrapone. All effects of surgical stress on bone-marrow, both in vivo and ex vivo, were prevented in a concordant manner by all three experimental manoeuvres. By contrast, blockade of the adrenergic endocrine response to stress, which also depends on the adrenal glands, had no effect on the link between stress and bone-marrow eosinophilia.

It is clear therefore that under specific circumstances glucocorticoids can powerfully enhance eosinopoiesis in vivo and ex vivo, as part of a stress reaction. The observed dose-response relationships of various synthetic and natural glucocorticoids are compatible with these observations, with glucocorticoid levels comparable to those found in stress being able to enhance eosinopoiesis, while higher levels, compatible with those observed in patients receiving anti-inflammatory or immunosuppressive steroid regimens, were less effective or not effective at all. It is also clear, however, that the role of glucocorticoids in stress goes beyond the observed in vivo effects of dexamethasone alone, since the latter never induced bone-marrow eosinophilia, which is a major finding in the EWI model. In this respect, the effects of surgical stress are closer to those of ovalbumin sensitization and challenge (see above). Accordingly, the effects of surgical stress on bone-marrow in vivo could be duplicated by plasma transfer from surgically stressed donors to naive recipients, using the protocols previously employed in the study of sensitized/challenged mice (unpublished observations). It remains to be established whether surgical stress plays an additional role in promoting release of IL-5, or other eosinopoietic cytokines, in the short period following surgery, in a way that could account for increased eosinopoiesis in vivo.

These observations are in general agreement with reports of the association between stress-induced glucocorticoid hormones and eosinophilic inflammation [17]. They also prompt a reinterpretation of reports in which allergen challenge of asthmatic subjects was followed by rises in plasma cortisol [18], or in which an atopic predisposition correlated with increased, rather than decreased, cortisol response to acute stress [19]. The current interpretation of these findings is that elevated cortisol acts as an anti-allergic mechanism, to curtail the ongoing acute inflammatory response. However, our observation suggests that it also may influence bone-marrow so as to induce an increase in eosinophil production, and thereby positively influence future allergic reactions, just as they negatively influence the current one. We have already obtained evidence for such an effect (unpublished observations), and are currently carrying out studies aiming at establishing the immunoendocrine pathways responsible for linking allergen exposure in the airways to stimulation of eosinopoiesis in bone-marrow. If confirmed, these observations indicate that the allergen-induced increase in glucocorticoid stress hormones may have an impact on the current or subsequent allergic episodes. Such an impact on future events would be consistent with the priming mode of action of both dexamethasone and stress-induced glucocorticoids.

The Effects of Prostaglandin E₂

We have subsequently evaluated the effects of an important chemical mediator of inflammation, Prostaglandin E₂ (PgE₂), on eosinophil production in bone-marrow cultures [20]. PgE₂ dose-dependently inhibited production of eosinophils in IL-5-stimulated liquid cultures, and suppressed GM-CSF-dependent colony formation in agar. The effects of PgE₂ seemed to be duplicated by dibutyl cyclic AMP (dBCAMP), rolipram and forskolin, which share the ability to raise intracellular levels of cyclic AMP, suggesting that PgE₂ acted through activation of adenylate cyclase. Interestingly, in the course of these experiments we observed, for the first time, strain differences in responses to these pharmacological agents: while PgE₂ was able to suppress eosinopoiesis in liquid cultures from all strains tested, it did not suppress colony formation in cultures from BALB/c bone-marrow, although it was very effective on colony forming cells from a number of other strains. Subsequent work showed a similar lack of responsiveness to isoproterenol, another cyclic AMP elevating agent that acts through adenylate cyclase (manuscript in preparation). In BALB/c as in other strains, dbcAMP, rolipram, forskolin and cholera toxin, which all raise intracellular cAMP levels independently of the receptors for PgE₂ or isoproterenol, were similarly effective. This suggested that the defect in response was detectable at the progenitor level only, and was probably related to the ability of some G protein-coupled receptors to interact with adenylate cyclase. Whatever the mechanism of that defect is, it seems to have been compensated or corrected by the time precursors arise. Hence, this chance observation provided us with a pharmacological marker capable of distinguishing progenitors (i.e. colony-forming cell stage before the PgE₂-sensitive checkpoint) from precursors (i.e. noncolony-forming cell stage after the PgE₂-sensitive checkpoint), provided one uses cells from BALB/c bone-marrow; this strain-specific trait could be useful in experiments designed to define the unique properties that enable progenitors to initiate colony growth.

Another interesting feature of the effects of PgE₂ on bone-marrow concerns the timing of its action. If added to the liquid cultures after 48 h, PgE₂ had no effect. However, when PgE₂ is present from the beginning, its effects in suppressing eosinopoiesis would be most marked at later studies in culture, not within the initial 48 h. This is reminiscent of the window of effectiveness observed with dexamethasone, which has to act early in order to achieve an enhancing effect at later culture times, but does not prove that the mechanisms of action of these two different modulators are related.

The Effects of Nonselective Cyclooxygenase Inhibitors: Evidence for Regulation of Bone-Marrow Eosinopoiesis by Endogenous Prostanoids?

A further series of studies addressed the possibility that PgE₂, or other prostanoids, produced within the bone-marrow cultures, rather than added exogenously, would regulate eosinopoiesis ex vivo [21]. Our initial experiments showed that both indomethacin and aspirin, nonselective
inhibitors of cyclooxygenase, were able to dose-dependently upregulate eosinophil differentiation in IL-5-stimulated liquid cultures, as well as colony formation in GM-CSF semi-solid cultures. These effects were observed in bone-marrow of BALB/c as well as other unrelated strains. While these findings would be compatible with a role for endogenous prostanoids in regulating eosinopoiesis in bone-marrow culture, they are not conclusive in that respect, because some nonsteroidal anti-inflammatory agents are known to have other mechanisms of action in addition to their ability to inhibit cyclooxygenases [22-23]. They are even less conclusive as to whether the effects of these drugs could be accounted for by blockade of the endogenous production of PG_E_2, even though they are certainly compatible with this hypothesis. PG_E_2 is only one of possible cyclooxygenase derivatives that might play a role in eosinopoiesis, and nearly nothing is known about all the others which might play a role in our system. Besides, the ability of indomethacin to potentiate colony formation in the BALB/c strain is difficult to reconcile with a role for PG_E_2 alone, since colony formation in this strain is resistant to PG_E_2.

All these open questions arise from our ignorance about the presence and production of prostanoids and other arachidonic acid derivatives in bone-marrow culture. In the absence of biochemical evidence, pharmacological evidence is likely to remain suggestive.

Another important question concerns the cellular target for indomethacin inside the bone-marrow. By separating bone-marrow cells into adherent and nonadherent subpopulations, we were able to show that the ability to respond to indomethacin is a property of adherent cells, even though the ability to differentiate into eosinophils is associated with the nonadherent cells. The simplest explanation would be that indomethacin upregulates eosinopoiesis indirectly, by affecting an adherent cell type. Because macrophages are adherent cells, are present in bone-marrow at initial culture times, as the predominant adherent cell type, and are known to produce prostaglandins and other cyclooxygenase metabolites, we favor the view that the targets for the actions of indomethacin include bone-marrow macrophages. The possible role played by other adherent cell types remains to be determined. Indirect upregulation, if soluble mediators, rather than direct cell contact, were involved, would mean that the adherent cells in bone-marrow influence eosinophil differentiation from nonadherent precursors, either through indomethacin-sensitive secretion of an inhibitor (such as PG_E_2) or through indomethacin-induced secretion of a stimulator.

From what is known about the mechanism of action of both indomethacin and aspirin in other tissues, their ability to inhibit cyclooxygenase function would only account for the results if arachidonic acid, the biosynthetic precursor for prostanoid synthesis, were available for metabolism in the cells where indomethacin is acting. Hence, ongoing release of arachidonic acid by cytoplasmic Phospholipase A
_2 (cPLA
_2), as well as its availability for metabolism through the cyclooxygenase pathway would be required for such a potentiating effect [24].

Interestingly, at least one alternative mechanism would also require an activated cPLA
_2. It is known that in individuals with aspirin-sensitive asthma an increased production of cysteinyl-leukotrienes (Cys-LT) can be observed in the presence of aspirin because of a genetic defect that results in increased expression of LTC
_4 synthase in eosinophils [25-26; see, however, Ref. 27, for an alternative view]. In this case, excess LTC
_4 is produced (and causes symptoms) because there is release of arachidonic acid to start with (due to an active cPLA
_2), and because this free arachidonate is not consumed in cyclooxygenase-catalyzed reactions due to the irreversible inhibition of the enzyme by aspirin. Arachidonic, in these conditions, is believed to be available by metabolism through the 5-lipoxygenase/5-lipoxygenase activating protein (FLAP) pathway, which is also active constitutively in these subjects. A similar sequence of events is believed to occur in the presence of indomethacin. At least one laboratory has reported that Cys-LT enhances eosinopoiesis [28] in colony-formation assays. Although aspirin-sensitive asthma reflects events that take place in the lungs, the fundamental mechanisms described could, at least in principle, occur in the bone-marrow as well, and thereby influence eosinopoiesis through increased production of Cys-LT.

We have evaluated the effects of drugs known to interfere with these mechanisms on the ability of indomethacin to upregulate eosinopoiesis in murine bone-marrow. Both MK886, an inhibitor of FLAP, and montelukast, an antagonist of the type 1 Cys-LT receptor, which mediates the bronchoconstrictory and inflammatory responses to Cys-LT in the lungs, were able to block the effects of indomethacin in bone-marrow cultures established from BALB/c mice in the presence of IL-5. Neither MK886 nor montelukast had a significant effect in the absence of indomethacin. The addition of Cys-LT (both LTC
_4 and LTD
_4) to cultures previously established with IL-5, indomethacin and MK886 still led to increased eosinopoiesis, showing that the effect of MK 886 does not arise from nonspecific toxicity to precursors. The effects of Cys-LT on eosinopoiesis were dependent on the presence of IL-5, as were the effects of indomethacin and aspirin (unpublished observations).

These limited pharmacological data, even though not yet supported by direct biochemical evidence, suggest that the shunting mechanism proposed for aspirin-sensitive asthma is operative in bone-marrow cultures in the presence of IL-5, and contributes to the observed effects of indomethacin. This is clearly not the case for other effects of indomethacin that, like this one, involve some positive effect of the drug in addition to its ability to prevent production of cyclooxygenase products [see for instance Ref. 29]. Because there are few experimental models in which the physiological relevance of this mechanism can be evaluated, there is an intrinsic interest in exploring further this possibility in bone-marrow cultures, in addition to its immediate explanatory value for the effects of indomethacin mentioned above.

Given our limited knowledge of how these pathways are regulated in bone-marrow, the goals of future investigation should include not only the direct demonstration of the presence of Cys-LT in bone-marrow cultures, as well as an increase in Cys-LT content in the presence of indomethacin, but also the localization and quantitation of the key enzymes in this pathway, namely cPLA
_2, 5-lipoxygenase with its co-
factor FLAP, and LTC4 synthase. It would be especially important to determine whether the cells that respond to indomethacin in the adherent cell population are those capable of producing Cys-LT through their expression of LTC4 synthase, and, alternatively, whether a transcellular biosynthetic pathway is operative in these conditions. At present, we have no idea of the stimuli that would account for the activation of cPLA2 and Cys-LT synthesis in these cultures. It would be important, therefore, to assess whether IL-5, which has been reported to activate cPLA2 in human eosinophils [26], is an activating stimulus for these key enzymes in bone-marrow.

A mechanism of action through which indomethacin upregulates eosinopoiesis by promoting Cys-LT synthesis is not incompatible, of course, with another mechanism by which the same drug simultaneously prevents production of PgE2 or other prostanoids capable of downregulating eosinopoiesis.

Cellular Actions of Glucocorticoids, Indomethacin and PgE2 on Developing Eosinophils: Modulation of Cell Adhesiveness, Cytological Maturation and Apoptosis

We have hitherto discussed the effects of these different agents in terms of numbers of eosinophils present after culturing bone-marrow for several days, or of numbers of colonies formed. However, this is just one of the aspects of their visible actions on hemopoiesis, which is preferentially discussed because it is easily quantifiable and therefore easily comparable between different treatments, from a statistical standpoint. Equally visible and specific imprints of their effectiveness are detectable, especially in the differentiation assays carried out in liquid culture, where morphology of individual cells and clusters can be readily assessed.

The effects of dexamethasone are most striking in terms of cell adhesiveness, nuclear morphology and overall cytological maturation. Mature eosinophils differentiated in bone-marrow culture are usually present as single cells, of uniform size, with a typical doughnut-shaped nucleus, and with a high density of eosinophil peroxidase-positive (EPO+) granules. By contrast, those which matured in the presence of dexamethasone are often present in large clusters containing exclusively EPO+ cells, which all resemble each other in being larger, with EPO+ granules less densely distributed, and a nucleus that is both large, irregularly shaped and of less condensed chromatin than usually observed in control cultures [13]. While they are unmistakably eosinophils, these cells give an overall impression of cytological immaturity. We have been able to ascribe the increased adhesiveness to an increased presence of α4 integrins on the cell surface (unpublished results). Cells maturing in the presence of IL-5, dexamethasone and a neutralizing monoclonal antibody to the murine α4 integrin chain were present in larger numbers than in (negative) control cultures with IL-5, but, unlike those in (positive) control cultures established with both IL-5 and dexamethasone, were predominantly single cells with mature morphology. These data suggest that while dexamethasone increases the numbers of cells committed to the eosinophil lineage, it also induces an increase in cell surface expression of α4 integrins, which helps to hold these developing cells together, and contributes to their maintenance of an immature phenotype.

In bone-marrow cultures exposed to PgE2, the picture is strikingly different. The reduction in eosinophil numbers is accompanied by the appearance of cells bearing the morphological hallmarks of apoptosis, including pyknosis and overall shrinking. The doughnut-shaped nucleus becomes a pyknotic mass and the average cell size is clearly diminished. These regressive changes affect only the eosinophils, as macrophages present in the same culture seem very healthy. Macrophages in these conditions are often usually loaded with EPO+ granules, which we interpret as remnants of phagocytosis of apoptotic cells and apoptotic bodies derived from eosinophils.

These extreme aspects are both lacking in cultures exposed to indomethacin. The only detectable change, in this case, is an increased number of morphologically mature EPO+ cells. This suggests that indomethacin and dexamethasone, even though they lead to similar increases in eosinophil numbers, act through distinct mechanisms. As predicted from the pharmacological data supporting Cys-LT as the mediator of the effects of indomethacin, the EPO+ cells present in cultures established with IL-5 and LTD4 (or LTC4) are mature and indistinguishable from those found in the presence of IL-5 and indomethacin.

Mechanisms of Modulation of Apoptosis by PgE2 and Dexamethasone: A Critical Role for Inducible NO Synthase

The morphological evidence of death by apoptosis in eosinophils developing in bone-marrow culture in the presence of IL-5 and PgE2, as well as the extensive literature reporting that dexamethasone and other glucocorticoids induce apoptosis in mature eosinophils [see Ref. 3 for a review], led us to directly evaluate the role of apoptosis in IL-5-stimulated bone-marrow cultures [30]. Bone-marrow cultures were established with IL-5, alone or in association with PGE2, dexamethasone or both. PGE2 inhibited eosinophil differentiation by selectively inducing apoptosis in developing eosinophils. This could be demonstrated by a variety of approaches, including the fragmentation of nuclear DNA as detected by the TUNEL technique, by the release of nucleosomal DNA in the extracellular medium, and by staining of apoptotic cells with Annexin V, which indicates the exposure of phosphatidylserine groups on the cell surface. On the other hand, dexamethasone failed to induce apoptosis in developing eosinophils in these conditions. As expected, PgE2 decreased and dexamethasone increased the number of eosinophils recovered after 7 days of culture, relative to cultures established with IL-5 alone. Dexamethasone, however, was able to protect developing eosinophils from PGE2-induced apoptosis. The resulting eosinophils resembled those in cultures established with IL-5 alone, both in morphology and in numbers.

This result suggested that the mechanism of action of dexamethasone was unrelated to its ability to prevent release of arachidonic acid from nuclear membranes by inducing lipocortin, which inhibits the activity of cPLA2. If such were the case, preformed PgE2 would escape the upstream blockade imposed by dexamethasone. An alternative target for
The Effects of Dexamethasone on Lung-Derived Eosinophil Precursors

More recently, we analysed the effects of dexamethasone on eosinophil precursors isolated from two different sites in the same animals, namely bone-marrow and lung. Our initial observations showed that the lungs of sensitized and challenged BALB/c mice contain hematopoietic progenitors predominantly committed towards the eosinophil lineage, and that the sensitivity of eosinophil precursors to dexamethasone differed between cells from these two sites [35]. However, because such cells are absent from the lungs of naive mice, and also because it was very important to rule out that the observed differences originated in the conditions used to isolate the cell populations for study, we carried out a systematic comparison with cells isolated following identical procedures from sensitized and challenged donors. The results differed strikingly: while in bone-marrow dexamethasone dose-dependently enhanced eosinopoiesis, in cells from lung it acted as a powerful suppressor of eosinophil differentiation. This correlated with the ability of dexamethasone to induce apoptosis in cells from lung, but not bone-marrow. Cells from lung were exquisitely sensitive to dexamethasone, even in the presence of high concentrations of IL-5. Most interesting, the effects of dexamethasone on both cell populations were dependent on precise timing, because it was ineffective in either cell population if added after the third day of culture [36]. We view these differences were interpreted as resulting from the cellular environment in which precursors differentiate, because differences due to strain, developmental stage and the procedures for immunization, challenge, isolation and culture were all controlled for in these experiments.

The Need for Further Experimentation

A comparison of our results with the picture emerging from the extensive literature on apoptosis in eosinophils and neutrophils generated by numerous other laboratories revealed a number of apparent inconsistencies, as detailed below. Although some of these might possibly be accounted for by the use of different experimental conditions (such as the presence or absence of IL-5 and other cytokines, the use of different drugs, or different methods or criteria for quantifying apoptosis), some clearly demand further research based on novel hypotheses. We have tried in the following discussion to point out those problems that would benefit from further experimental analysis. While some of the proposed studies are already under way in our own laboratory, some fall outside our means, but might perhaps prove interesting to other researchers.

Differences between Eosinophils and Neutrophils in Apoptosis Induced by Glucocorticoids: The Relative Contributions of Lineage and Development

Any comparison of data from different groups on this issue should take into account that neutrophils and eosinophils are studied differently in humans and mice, and that these differences are important in the interpretation of data. Most studies of apoptosis in peripheral (mature) granulocytes, both eosinophilic and neutrophilic, have been carried out with cells isolated from human blood. This is easily ex-
plained in the case of neutrophils, because they are abundant and easily purified from blood. For eosinophils, which are relatively scarce in blood and rather difficult to obtain in pure form, there is no such advantage; nevertheless, the bulk of research on eosinophil cell biology has been done with human eosinophils purified from blood [37], and the development of more efficient methods of purification has helped to consolidate this trend. By contrast, studies on murine peripheral neutrophils or eosinophils are plentiful when quantification of these cells in inflammatory sites is done, but not when cell biological analysis is the goal. Therefore, the literature on granulocyte apoptosis remains strongly biased towards human studies, and there always remains the possibility that some fundamental difference in results stems from the fact that human peripheral cells are being compared to murine cells derived from bone-marrow.

With this qualification in mind, however, most of the literature suggests that eosinophils and neutrophils differ fundamentally in their response to glucocorticoids. Both types of granulocytes are generally assumed to be programmed to die in the absence of survival-promoting cytokines [3; 38], and mitochondrial apoptotic mechanisms are central in both cases [39-41]. Despite this fundamental similarity, glucocorticoids are believed to delay neutrophil apoptosis and to accelerate eosinophil apoptosis [4; 42; for an exhaustive review of the literature on the effects of glucocorticoids on eosinophils, see Ref. 43]. It has been proposed that glucocorticoids do so by enhancing the activity of c-Jun terminal kinase following an initial step of oxidative damage to mitochondria [42; 44].

Our own observations on developing eosinophils from murine bone-marrow cultures are at variance with this general trend. In our studies, dexamethasone did not accelerate apoptosis of developing eosinophils from bone-marrow [30], although it did so in cultures established from allergic murine lung mononuclear cells [36]. The presence of IL-5 did not seem to protect the latter from apoptosis induced by dexamethasone. This suggests to us that in this respect developing eosinophils from murine bone-marrow resemble mature neutrophils from human blood in their resistance to apoptosis induction by glucocorticoids. It is clearly of interest to define whether this apparent exception to a previously established pattern reflects similar biochemical mechanisms.

The resistance of neutrophils to glucocorticoids has been ascribed by Strickland and colleagues [45], to the expression of the GRβ isoform of the human glucocorticoid receptor. GRβ does not bind glucocorticoids but inhibits the activity of GRα (the functional receptor). One could speculate that a similar biochemical mechanism would account for the resistance of developing murine eosinophils to glucocorticoids, since it has been invoked to explain glucocorticoid resistance in asthmatic patients [46], where increased eosinophil survival plays a major role [38]. However, this is unlikely, because murine neutrophils do not express GRβ, as shown by Strickland et al. [45]. Furthermore, in our experiments, both the enhancement of eosinopoiesis and the glucocorticoid-dependent resistance to apoptosis induction in the presence of PGE2 depend on an effective concentration of dexamethasone, and increased eosinopoiesis is blocked by mifepristone. This indicates that dexamethasone must act through GRα to exert its effects on developing murine eosinophils, which is incompatible with the mechanism proposed by Strickland et al.

There are some well-characterized markers that distinguish between apoptosis in neutrophils and eosinophils, like the pattern of cleavage of procaspase 3 (reviewed in Ref. 2). More recently, Souza and colleagues [47] examined the caspase-catalyzed cleavage and activation of two kinases, mammalian sterile 20-like 1 and 2 (Mst1/Mst2) kinases, in eosinophils undergoing spontaneous apoptosis. They showed that cleavage of Mst1 from a larger precursor occurred during apoptosis, and depended on caspase activity, being enhanced by ligation of CD95 and suppressed by the survival-promoting cytokine, IL-5. Consistently with the central role of oxidative stress in spontaneous eosinophil apoptosis, cleavage of Mst1 was inhibited by catalase. Neutrophils lacked all steps of this sequence of events. No evidence, however, that Mst1 plays any active role in apoptosis, other than offering a substrate for caspases, was provided. Therefore, even though biochemical differences in the process of apoptosis clearly exist between these two types of granulocytes, it remains to be established whether any of them is related to the different patterns of responsiveness to glucocorticoids described for the neutrophil and eosinophil lineages.

The Relationship between NO, Eosinophilic Inflammation and Eosinophil Apoptosis

NO illustrates the difficulty in determining whether something that is unmistakably increased in inflammatory conditions will aggravate or attenuate inflammation. NO is increased in the asthmatic airways, but it is unclear to what extent this reflects the intensity of the inflammatory reaction or the effectiveness of anti-asthmatic drugs [26].

Nevertheless, several studies suggest that NO production in the airways plays a role in promoting eosinophilic infiltration. Feder et al. [48] showed that ovalbumin-induced pulmonary eosinophilia in sensitized and challenged B6D2F1/J mice was significantly reduced by L-NAME and aminoguanidine, suggesting that NO production is required for eosinophil infiltration in the allergen-challenged airways. However, the use of inhibitors with different degrees of selectivity for the NO synthase isoforms failed to support a role for inducible NOS in this model. The authors interpreted their findings as suggestive that after antigen challenge, the localized production of NO, possibly from pulmonary vascular endothelial cells, is involved in the extravasation of eosinophils from the circulation into the lung tissue. By contrast, Xiong et al. [49], using mice deficient for iNOS sensitized and challenged with ovalbumin, showed a marked decrease in eosinophil infiltration as well as other indicators of lung injury, relative to wild-type controls. Even though eosinophil maturation in the bone-marrow did not seem to be affected, the numbers of eosinophils both in blood and lung tissue were significantly reduced. iNOS-deficient mice presented greatly increased production of IFN-γ, which seemed to account for some of the observed differences, as the depletion of IFN-γ restored the allergic pathology in these animals. While it is clear that major differences exist between these reports, they agree on the fun-
damental finding that NO production underlies the eosinophil infiltration of the airways in allergen-challenged, sensitized mice.

Even more interestingly, this seems to work both ways. Iijima et al. [50], using a similar murine model, reported that the large increase in eosinophils following intranasal challenge of ovalbumin-sensitized A/J mice was associated with increased NO production and expression of inducible NO synthase. The use of a highly selective inhibitor indicated that iNOS accounted for much of the observed NO production, as well as for the eosinophil infiltration. They also found evidence for extensive tyrosine nitration, indicating protein modification by NO. On the other hand, both NO production and protein nitration were shown to be dependent on the eosinophil infiltration, with the use of the anti-IL-5 neutralizing antibody, TRFK-5. This report supports a role for iNOS in promoting eosinophil infiltration of the airways; it also suggests that eosinophils reinforce NO production either by expressing active iNOS themselves (as reported, for instance, by [51]) or by inducing iNOS expression in the surrounding tissue.

We think these findings are indicative that in an inflammatory site NO is being continuously produced, largely through iNOS, and interacts positively with infiltrating eosinophils, in such a way that it promotes eosinophil accumulation and is reinforced by it.

These observations clearly differ from our own in bone-marrow cultures, because we did not observe an effect of NOS inhibitors in the absence of prostaglandins, and because NO donors did reduce, not increase, eosinophil numbers. Because all of the preceding observations were made in mice, thus precluding species-specific differences in response, it is likely that these differences reflect fundamental features of mature (blood and tissue) and developing (bone-marrow) eosinophils, respectively. We would hypothesize that mature, infiltrating eosinophils would be resistant to apoptosis induced by NO, in contrast to the developing eosinophils found in bone-marrow of the same animals. If so, this would be consistent with the idea that the pattern of response to apoptotic stimuli is developmentally regulated in the eosinophil lineage.

The literature on the relationship between NO and apoptosis is extremely interesting, even though the interpretation of findings may be very complex, as NO seems to have both pro- and anti-apoptotic effects, depending on the precise conditions. The concentration range [52-53] is very important; because murine cells produce much larger amounts of NO through iNOS than usually is the case with human cells, the extrapolation of results involving endogenous NO production from mice to humans must be extremely careful, even though it is felt that studies on the effects of exogenous NO sources are not subject to the same constraints (reviewed in Ref. [54]). Furthermore, the exact source and conditions of release from different donor molecules, which differ in their rates of generation of NO as well as in their ability to simultaneously release other biologically active moieties [54], and the presence of other molecules, in addition to the sources of NO, which may modify its effects [34] are very important. In our own study [30], as well as in studies of a variety of hemopoietic cells [55-56; see 54, for a recent review], the effect of NO was proapoptotic. However, even among hemopoietic cells, exceptions can be found: in B-cell chronic lymphocytic leukemia, inducible NO synthase is associated with protection from apoptosis [57]. Where NO has been shown to induce apoptosis, however, it has often been reported to do so through the CD95-CD95L pathway [56; 58], in a way consistent with our own findings in bone-marrow culture. On the other hand, the CD95 pathway plays a major pathophysiological role in regulating neutrophil production in the bone-marrow under the influence of proinflammatory cytokines, including TNF-α and IFN-γ, which are known to promote NO synthesis [59].

Maa et al. [32] studied the relationship between NO production and the differences in rates of spontaneous apoptosis between eosinophils from asthmatic patients and those of normal subjects. They found that iNOS immunoreactivity and spontaneous release of nitrate were higher in eosinophils from asthmatic patients than in those from healthy controls. However, this was accompanied by increased constitutive expression of the antiapoptotic protein Bcl-2, which correlated with increased eosinophil survival. Even though this indicates that NO is not sufficient for inducing eosinophil apoptosis in these conditions, inhibition of NOS by the nonselective inhibitor, L-NAME, further enhanced expression of Bcl-2 and decreased rates of spontaneous apoptosis, suggesting that NO, if anything, opposes rather than promotes the increase in eosinophil survival, and therefore tends to limit the lifespan of eosinophils. The authors also obtained evidence for a role of ERK and p38 MAPK in maintaining the elevated levels of Bcl-2 in eosinophils from asthmatic subjects, which are consistent with evidence from other studies [60]. The findings of Maa et al. contrast with those of Hebestreit et al. [33], and several other studies that report protection from apoptosis by NO in eosinophils (reviewed in Ref. [54]). They are also at variance with studies on other cell types pointing to a critical role of NO in countering the mitochondrial changes associated with apoptosis [61]. They are, nevertheless, consistent with our hypothesis that eosinophils in sites of allergic inflammation would not undergo apoptosis even in the presence of the elevated levels of NO produced through iNOS, as they seem to be protected by increased levels of Bcl-2.

Interestingly, the ability to increase expression of Bcl-2 has been suggested to mediate the survival-promoting effects of IL-5 in some [62-63] but not all studies [64; 65; see Ref. 38 for a perspective on this issue]. This would argue against a role for IL-5 alone in the Bcl-2-dependent increase in eosinophil lifespan described in asthmatic subjects, even though it is possible that a synergistic combination of survival factors, including IL-5 and GM-CSF [38], accounts for increased survival associated with Bcl-2 expression. At any rate, in murine bone-marrow cultures, the presence of IL-5 is clearly not sufficient to render developing eosinophils resistant to induction of apoptosis by NO [30].

The Effects of Cysteinyl-Leukotriene Receptor Antagonists and the Role of Cys-LT

Cowburn et al. [66] have examined the relationship between the effects of IL-5 and glucocorticoids on eosinophils, on the one hand, and the production of Cys-LT, on the other. IL-5 enhanced Cys-LT production by human eosinophils in
vitro, along with the expression of FLAP. Interestingly, the proportion of FLAP+ expressing eosinophils was also increased by dexamethasone. Neither IL-5 nor dexamethasone altered 5-LO expression, but IL-5 significantly increased 5-LO immunofluorescence localizing to eosinophil nuclei. Compared with normal subjects, allergic asthmatic patients had a greater proportion of circulating FLAP+ expressing eosinophils and a smaller IL-5-induced increase in FLAP immunoreactivity. The authors concluded that IL-5 increases FLAP expression and translocates 5-LO to the nucleus in normal blood eosinophils in vitro, these effects being associated with an enhanced capacity for Cys-LT synthesis and resembling the in vivo increases in FLAP expression in eosinophils from allergic asthmatics. It is difficult, however, to evaluate the relationship between these effects and the promotion of eosinophil survival by IL-5, especially because dexamethasone, which induces apoptosis in human peripheral eosinophils, and decreases Cys-LT synthesis [67], duplicates some of them. However, it is clearly of interest to evaluate whether an increased production of Cys-LT secondary to IL-5 exposure influences eosinophil lifespan. It is also of interest that dexamethasone had at least one effect on FLAP expression that correlated with increased Cys-LT synthetic capacity, raising the possibility that in specific circumstances glucocorticoids may positively, rather than negatively, influence Cys-LT production.

Two reports from the literature on the effects of Cys-LT receptor antagonists are consistent with the view that Cys-LT play a physiological role in promoting eosinophil survival. In both cases, mature human eosinophils were used; in both, Cys-LT seemed to be continuously produced and to exert antiapoptotic effects on eosinophils; accordingly, the interruption of these autocrine/paracrine loops by Cys-LT receptor antagonists resulted in induction of apoptosis.

Lee et al. [68] examined the role of eosinophil-derived Cys-LT in the maintenance of eosinophil survival, and the involvement of leukotrienes in the paracrine stimulation of eosinophil survival by mast cells and lymphocytes. Using eosinophils and autologous lymphocytes from peripheral blood of asthmatic subjects, they showed that Cys-LT (LTC\textsubscript{4} and LTD\textsubscript{4}) and granulocyte–macrophage colony-stimulating factor (GM-CSF), among other agents, effectively promoted eosinophil survival. Blockade of Cys-LT receptors with Pobilukast, as well as inhibition of 5-lipoxygenase (5-LO) with BW A4C and of FLAP with MK 886, not only abolished the effects of GM-CSF and of various survival-promoting conditioned media, but increased basal rates of eosinophil apoptosis. This was interpreted as consistent with the involvement of an autocrine Cys-LT pathway that supports eosinophil survival in response to a range of survival stimuli.

More recently, Abadoglu and colleagues [69] have evaluated whether the effectiveness of montelukast in limiting airway eosinophilia in patients with mild persistent asthma was linked to the induction of apoptosis in eosinophils as assessed in induced sputum samples. Their results indicate that montelukast treatment is associated with an increase in eosinophil apoptosis in vivo.

Despite these reports, the question of whether Cys-LT do extend eosinophil survival is far from settled, as a recent study by Murray et al. [70] failed to detect an antiapoptotic effect of LTD\textsubscript{4} on human eosinophils, even though evidence for activation of the eosinophils by this agonist was obtained.

The latter report is more consistent with our own inability to demonstrate an effect of montelukast per se in bone-marrow cultures, even though montelukast clearly blocked the effects of indomethacin and of exogenously added LTD\textsubscript{4}.

If, however, Cys-LT do have a survival-promoting effect on eosinophils, as suggested by Cowburn et al. [66] and by Lee et al. [68], we think the discrepancies between studies could arise from a difference between mature and developing eosinophils in the role played by Cys-LT, rather than a difference between eosinophils of human and murine origin. Cys-LT would be produced by peripheral eosinophils and promote their survival in an autocrine manner; by contrast, they would not be produced by developing eosinophils in bone-marrow culture (in the absence of indomethacin), even though the receptor for Cys-LT is expressed and can respond to Cys-LT exogenously added or produced in the presence of indomethacin. We believe this hypothesis is worthy of further experimentation.

The Complex Role of Prostaglandins in Eosinophilia and Eosinophil Apoptosis, and its Possible Relationship to the Effects of NO and Cysteinyl-Leukotrienes

The area in which we think novel experimentation will be most profitable, however, is that of the relationship between prostaglandins, NO and Cys-LT in the context of bone-marrow eosinopoiesis. References dealing with these relationships in other systems are scarce and somewhat contradictory. We will review below some which seem to us most relevant to the issue at hand.

Martin et al. [71] evaluated the mechanism of the anti-inflammatory actions of PGE\textsubscript{2} in a rat model of allergic asthma, using BN rats sensitized to ovalbumin and challenged by an ovalbumin aerosol. PGE\textsubscript{2}, administered intratracheally before the challenge, inhibited dose-dependently both the early and the late responses, as well as the increased eosinophilia of bronchoalveolar lavage fluid. Interestingly, the levels of Cys-LT in the fluid were also reduced by PGE\textsubscript{2}. The authors concluded that PGE\textsubscript{2} was a potent anti-inflammatory agent capable of reducing Cys-LT synthesis. This report is consistent with the observations by Gavett and colleagues [72] that mice deficient in both the 1 and 2 isoforms of PGH synthase (and therefore severely deficient in the production of PGE\textsubscript{2}) presented more intense allergic reactions. The bulk of the production of PGE\textsubscript{2} in these conditions was ascribed to PGHS-1. By contrast, Profita et al. [73] found that PGE\textsubscript{2} concentrations in the induced sputum supernatants of asthmatic subjects were directly correlated with the percentage of eosinophils, as well as the concentrations of eosinophil cationic proteins. Asthmatic subjects showed enhanced macrophage expression of cyclooxygenase-2, and the use of specific cyclooxygenase-2 inhibitors provided evidence that this isoform was responsible for the synthesis of PGE\textsubscript{2} in the samples from asthmatic subjects. These supernatants were able to extend the lifespan of purified eosinophils, and this activity seemed to be due to PGE\textsubscript{2}, as suggested from immunoprecipitation experiments. The authors accordingly concluded that PGE\textsubscript{2} generated
through cyclooxygenase-2 is a major factor contributing to chronic eosinophilic inflammation in asthmatic subjects.

These reports differ in many respects, and we will make no attempt to reconcile them. Because very different experimental conditions were used, they do not necessarily contradict each other. They highlight, however, the importance of defining whether PgE₂ attenuates or aggravates eosinophilic inflammation at sites of allergic challenge, and whether modulation of eosinophil apoptosis is involved in either effect.

This is all the more important since Marnett et al. [74] have shown that the ability of macrophages to synthesize PgE₂ is linked to their ability to produce NO. iNOS-deficient animals had severely diminished capacity to produce PgE₂, even though they had normal cyclooxygenase-2 expression, and generated large amounts of other cyclooxygenase products. This suggests a biochemical link between PgE₂ and NO that is clearly of interest to explore in the context of bone-marrow regulation, in view of the pharmacological link that we have detected in bone-marrow culture. An additional reason for doing so is that some nonsteroidal anti-inflammatory agents, including aspirin, which upregulates eosinophil production in bone-marrow cultures, were shown by Amin et al. [75] to be able to inhibit iNOS expression and the generation of NO through iNOS at therapeutic concentrations.

A recent report by Que et al. [76], however, prompts a reevaluation of the contribution of NO in asthma, and necessarily of its interaction with PgE₂ and other mediators subject to regulation by nonsteroidal anti-inflammatory agents. Their study indicates that NO is mainly protective against the development of airway hyperreactivity, through its ability to covalently modify proteins through the formation of S-nitrosothiols involving cysteine residues. Because the S-nitrosothiols are deemed to mediate the beneficial effects of NO, their concentration, rather than that of NO itself, seems to be the critical determinant, and strategies aiming at their preservation, such as inhibition of the enzyme S-nitrosothioglutathione reductase, responsible for their catabolism, provide a new therapeutic rationale in asthma. Most importantly, Que et al. provided evidence that iNOS played a major role in the formation of protective S-nitrosothiols. Taken together with the evidence provided by Marnett et al. [74], Martin et al. [71] and Gavett et al. [72], this indicates that NO and PgE₂ may be acting together, and reinforcing each other, in order to limit the damage caused by allergic inflammation. While this view is consistent with our own results from bone-marrow culture [30], it remains to be established how that relates to the effects reported for both agents in promoting mature eosinophil survival and migration into inflammatory sites.

CONCLUDING REMARKS

Studies of eosinophil production in murine bone-marrow culture, carried out over the last decade, have provided many results that vary markedly from those obtained with mature eosinophils with respect to the mechanisms of action of a variety of well-known anti-inflammatory drugs. The data suggest that the pattern of response of eosinophils to dexamethasone, indomethacin and montelukast is strongly influenced by their developmental stage, and possibly by other cells present in their microenvironment. Major actors of the inflammatory process, such as NO, PgE₂ and stress-induced glucocorticoids, may have their actions determined in part by the developmental stage of the eosinophils. Future research should aim at comparison of different developmental stages of the eosinophil lineage within the same species, either murine or human, and focus on definition of the cellular response programs that underlie such strikingly different outcomes.

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