Brief Definitive Report

Suppression of Leukotriene B_4 Biosynthesis by Endogenous Adenosine in Ligand-activated Human Neutrophils

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Summary

Adenosine (Ado) has been shown to suppress several functional responses of human polymorphonuclear leukocytes (PMNs). The current study investigated whether endogenous Ado regulates the biosynthesis of leukotriene (LT)B_4 in ligand-stimulated PMNs. Measurements of Ado in PMN resuspended in Hanks' buffered salt solution (HBSS) or plasma showed a cell concentration– and time–dependent accumulation of the nucleoside. The removal of endogenous Ado with either Ado deaminase or the blockade of its action by the Ado A_{2a} receptor antagonist, 8-(3-chlorostyryl) caffeine, markedly increased LTB_4 biosynthesis upon ligand stimulation in HBSS. Similarly, LTB_4 synthesis by ligand-stimulated PMNs in plasma (containing recombinant LTA_4 hydrolase to allow the conversion of protein-bound LTA_4) was strongly enhanced by addition of Ado deaminase. Addition of red blood cells to suspensions of PMNs in plasma mimicked the effect of adding Ado deaminase and LTA_4 hydrolase in enhancing LTB_4 biosynthesis upon ligand stimulation. This effect of red blood cells on LTB_4 biosynthesis was blocked by dipyridamole, an inhibitor of Ado transport, or captopril, an inhibitor of LTA_4 hydrolase. These results demonstrate that endogenous Ado efficiently downregulates ligand-stimulated LTB_4 biosynthesis in PMN suspensions, pointing out a potentially important regulatory function of Ado in inflammatory exudates. These results also unveil a dual role for red blood cells in upregulating LTB_4 biosynthesis, namely, the removal of endogenous Ado and the conversion of LTA_4 released by activated PMNs.

Leukotriene (LT)[1] B_4 is a biologically active mediator of inflammatory processes; indeed, LTB_4 is a potent activator of leukocyte functions, and in particular, is a potent chemokinetic and chemotactic agent for neutrophils, monocytes, and macrophages. PMNs, mast cells, monocytes, macrophages, and B lymphocytes are the main cell types possessing the 5-lipoxygenase (5-LO) and LTA_4-converting enzymes and can thus directly produce LTB_4 from endogenous arachidonic acid (1, 2). In blood, PMNs have been found to be the predominant cell producing LTB_4 (3–5). Interestingly, it was recently demonstrated that activated PMNs release directly in the extracellular milieu a large proportion of the LTA_4 generated (6); such release of LTA_4 by PMNs points to the potential importance of transcellular metabolism in the biosynthesis of the biologically active metabolites of LTA_4, LTB_4, and LTC_4. Thus, many cell types present in the neutrophil environment that do not express the 5-LO, such as endothelial cells, RBCs, T cells, and platelets, likely constitute important factors in the dynamics of LT synthesis in vivo (3, 7–9).

Adenosine (Ado) is a ubiquitous autacoid with a large spectrum of biological activities, including the modulation of leukocyte functions. Indeed, numerous studies have reported that acting via Ado A_2 receptors, Ado suppressed PMN functions such as superoxide anion synthesis, adhesion, and phagocytosis, as well as the synthesis of inflammatory cytokines in monocytes. In addition, numerous studies have also demonstrated that endogenous Ado, as well as exogenous Ado or Ado analogues, exert antiinflammatory effects in vivo in animal models (for a review see reference 10). For these reasons, Ado has recently been proposed to act as an endogenous antiinflammatory agent (10).

In agreement with this concept, we demonstrated in a previous study that Ado and Ado analogues are very potent inhibitors of the biosynthesis of LTB_4 both in whole blood and isolated PMNs stimulated with physiological agents (11). Because it is well established that Ado accumulates in leukocyte suspensions (as a consequence of the extracellular breakdown of ATP; reference 12) where its accumulation reaches a concentration that exerts suppressive effects on PMN functions, we sought to determine the putative role of endogenous Ado in regulating LTB_4 biosynthesis by ligand-activated PMNs in various environments.

Materials and Methods

Materials. Ado deaminase (EC 3.5.4.4., calf intestinal type VIII), captopril, dipyridamole, *N*-formyl-Met-Leu-Phe (fMLP),

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/97/10/1401/06 $2.00
Volume 186, Number 8, October 20, 1997 1401–1406
http://www.jem.org

and LPS (*Escherichia coli* 01110B4) were from Sigma Chemical Co. (St. Louis, MO). Ado deaminase was dialyzed against NaCl 0.9% before use. 2-*p*-(carboxyethyl)phenetylamino-5′-*N*-ethyl-carboxamido-adenosine HCl (CGS 21680) and 8-(3-chlorostyryl) caffeine (CSC) were from Research Biochemicals International (Natick, MA). 5′(*N*-ethyl)carboxamidoadenosine (NECA) was from ICN Biomedicals Canada Ltd. (Mississauga, Canada). rLTA$_4$ hydrolase was obtained from Sf9 cells infected with LTA$_4$ hydrolase using the baculovirus system (13). The 100,000 *g* supernatant of infected Sf9 cells was used directly as the source of LTA$_4$ hydrolase and contained a specific activity of 60 nmol LTB$_4$/mg of protein (12 mg of protein/ml). The 100,000 *g* supernatant of the wild-type Sf9 cells (uninfected cells) was used as control. Recombinant GM-CSF and TNF-α were provided by the Genetics Institute (Cambridge, MA) and Knoll Pharmaceuticals (Whippany, NJ), respectively.

Cells. Human PMNs were isolated as previously described (4). In brief, human venous peripheral blood was collected into heparinized tubes. RBCs were allowed to sediment at 1 *g* after mixing 4 volumes of blood and 1 volume of dextran 2% in HBSS. Mononuclear cells were removed by centrifugation on Ficoll-Paque cushions. Contaminating RBCs in the PMN pellet were eliminated by a 20-s hypotonic lysis in water. PMNs were resuspended in 10 mM Hepes-buffered HBSS (pH 7.4) containing 1.6 mM $CaCl_2$. RBCs obtained from dextran sedimentation were freed of contaminating leukocytes by repeated removal of the buffy coat after four successive centrifugations (200 *g*, 15 min at 20°C) and resuspensions in four volumes of 10 mM Hepes-buffered HBSS (pH 7.4) containing 1.6 mM $CaCl_2$. In some experiments, RBCs were treated with 1 mM captopril or its diluent (NaCl 0.9%) for 30 min at room temperature before the fourth washing.

5-LO Product Analysis. Incubations were stopped by adding cold (0°C) methanol/acetonitrile (50/50; vol/vol) containing 12.5 ng each of 19-OH prostaglandin B$_2$ and prostaglandin B$_2$ as internal standards to aliquots of cell suspensions and stored at −20°C until reverse phase HPLC (RP-HPLC) analysis. Denatured samples were centrifuged at 2,000 *g* for 10 min, and the supernatants were subjected to RP-HPLC using on-line extraction procedures as previously described for PMN suspensions in HBSS (14) or samples containing plasma (15). LTB$_4$, 6-*trans* isomers of LTB$_4$, ω oxidation metabolites of LTB$_4$, and 5(S)-hydroxy-6,8,11,14(E,Z,Z,Z)-eicosatetraenoic acid (5-HETE) were measured by photometry at 280 and 229 nm, using fixed wavelength UV detectors. The lower limits of detection were 0.5 ng at 280 nm and 1 ng at 229 nm.

Analysis of Ado. PMN incubations (1 ml) were stopped by adding 100 μl of 22% TCA. NECA was added (10 ng/sample) as an internal standard and the denatured cell suspensions were placed at −20°C for at least 30 min. The samples were then centrifuged at 2,000 *g* for 10 min and the supernatants were extracted on Sep Pak Cartridges (3 cc, C-18 sorbent) as follows. The samples were loaded on the cartridges which were washed with water; Ado was then eluted with 3.5 ml of methanol/water (50:50, containing 0.1% acetic acid). The eluates were evaporated to dryness using a Speed Vac evaporator. The residues were dissolved in 200 μl of methanol/water (25:75, containing 0.05% acetic acid). The samples were analyzed by liquid chromatography–mass spectrometry using nebulizer-assisted electrospray ionization in the positive mode and by monitoring the transitions m/z 309 and m/z 268 (protonated parent ions) to m/z 136 (protonated adenine), corresponding to the loss of the carbohydrate moieties from NECA and Ado. The samples (1–2 μl) were injected onto a C-18 column (Ultrasphere, 2 × 150 mm, 5-μ particles; Beckman, Fullerton, CA) and eluted at a flow rate of 200 μl/min using methanol/water (40:60, containing 0.1% acetic acid) as the mobile phase. Ado was quantitated by extrapolating the measured Ado/NECA ratio on a calibration curve generated from standard solutions containing 1 ng NECA and 0–4 ng Ado in 5 μl. The limit of detection for Ado was ~50 pg injected (signal to noise ratio ≥5).

Results

Ado Concentrations in PMN Suspensions. Isolated PMNs in suspension in salt buffers have been reported to release Ado at levels that influence their functions (12). We thus performed measurements of endogenous Ado concentrations in PMN suspensions under conditions used for the assessment of leukotriene biosynthesis. After the centrifugation and resuspension of PMNs in fresh buffers, aliquots were removed for up to 60 min and the concentration of Ado was measured. PMNs resuspended in HBSS or autologous plasma released Ado in a time– and cell concentration–dependent manner (Fig. 1). Cell-depleted plasma was found to contain 30 ± 3 nM (mean ± SE, $n = 6$) of Ado. The addition of 0.1 U Ado deaminase after the incubation of 2.0×10^7 PMN/ml for 15 min in HBSS reduced the concentration of Ado within seconds to <4 nM, and remained below this level for up to 30 min. Stimulation of PMNs with 0.6 μM platelet-activating factor (PAF) did not have any effect on the levels of endogenous Ado (not shown).

LTB$_4$ Biosynthesis in HBSS. We first examined the effect of endogenous Ado present in PMN suspensions in HBSS on the synthesis of LTB$_4$. Endogenous Ado was neutralized using two different approaches; the addition of a selective A$_{2a}$ receptor antagonist, CSC (16; Fig. 2 *A*), or Ado deaminase (Fig. 2 *B*) to the incubation media. The pretreatment of TNF-α/GM-CSF–primed neutrophils with increasing concentrations of either CSC or Ado deaminase before stimulation with 0.6 μM PAF resulted in a progressive enhancement of 5-LO product biosynthesis, as compared to cells stimulated in the absence of CSC or Ado deaminase.

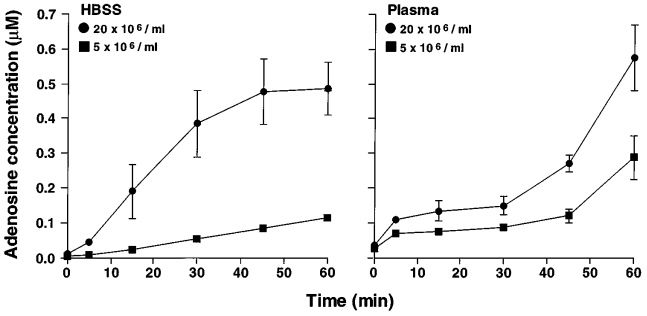

Figure 1. Time course of Ado accumulation in PMN suspensions. Freshly isolated PMNs were resuspended in HBSS (*A*) or autologous plasma (*B*) at concentrations of 5×10^6/ml (*squares*) or 20×10^6/ml (*circles*). At various time points, 1-ml aliquots of the cell suspensions were denatured with TCA and the Ado content was measured by liquid chromatography–mass spectrometry. Results shown are the means ± SD of triplicate incubations from one experiment representative of three. Error bars are not shown when smaller than symbols.

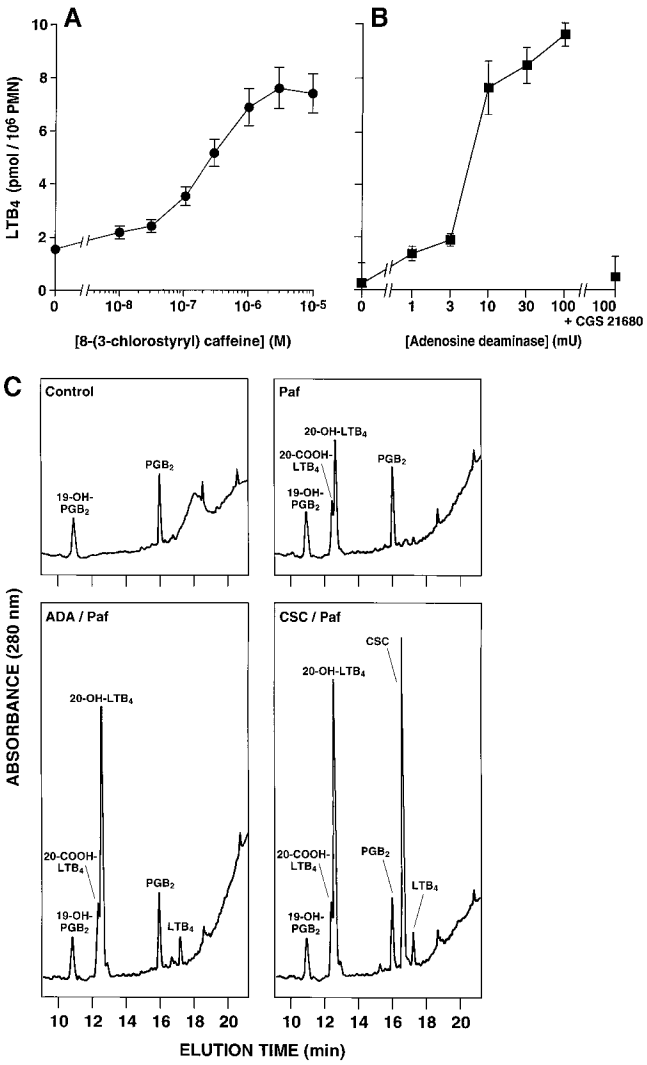

Figure 2. Effects of CSC and Ado deaminase treatment of PMN suspensions on LTB_4 biosynthesis. PMN suspensions in HBSS (10^7/ml) were preincubated with 700 pM GM-CSF + 1.2 nM TNF-α for 30 min, and then treated with various concentrations of CSC (*A*) or Ado deaminase (*B*) for 5 min and stimulated with 0.6 μM PAF. After 10 min of incubation in the presence (or absence) of PAF, incubations were stopped and 5-LO products were measured by RP-HPLC. In *B*, CGS 21680 was added at the concentration of 1 μM. Data are the means (± SD) of triplicate incubations from one experiment representative of three. (*C*) RP-HPLC profiling of 5-LO products generated by TNF-α/LPS–primed PMN suspensions stimulated (or not, control) with PAF, with or without earlier pretreatment with either 0.1 U deaminase Ado or 1 μM CSC. Conditions were as indicated for *A* and *B*; 5(S)-hydroxy-6,8,11,14(E,Z,Z,Z)-eicosatetraenoic acid (5-HETE) was not detectable in all conditions tested. Amounts of LTB_4 indicated represent the sum of 20-OH LTB_4, 20-COOH LTB_4, and LTB_4. *PGB_2*, prostaglandin B_2; *ADA*, adenosine deaminase.

The stimulatory effect of Ado deaminase was fully reversed by the addition of 1 μM CGS 21680, a selective A_{2a} receptor agonist (17). Fig. 2 *C* illustrates typical HPLC profiles of the 5-LO products generated by PMNs activated with PAF in the presence or absence of CSC or Ado deaminase. In the absence of PAF stimulation, the biosynthesis of LTB_4 was not detected.

LTB_4 Biosynthesis in Plasma. We next examined whether endogenous Ado also suppressed LTB_4 biosynthesis by PMNs resuspended in plasma. Stimulation of TNF-α/LPS–primed PMNs with fMLP resulted in the biosynthesis of only minimal amounts of LTB_4 (Fig. 3 *A*). The addition of Ado deaminase to the similarly primed PMN suspensions before fMLP stimulation failed to further increase the biosynthesis of LTB_4. In contrast, the addition of both Ado deaminase and $rLTA_4$ hydrolase resulted in a marked enhancement of LTB_4 biosynthesis. As observed with PMNs treated with Ado deaminase alone, the addition of $rLTA_4$ hydrolase alone failed to increase the synthesis of LTB_4 upon fMLP stimulation. The effect of adding Ado deaminase and $rLTA_4$ hydrolase to PMN suspensions in plasma on LTB_4 biosynthesis was mimicked by the addition of RBCs, which are known to uptake extracellular Ado (18) and contain LTA_4 hydrolase activity (7; Fig. 3 *B*). That the RBC LTA_4 hydrolase was involved in the biosynthesis of LTB_4 was confirmed by a pretreatment of RBCs with captopril, an inhibitor of LTA_4 hydrolase (19), which resulted in a near complete inhibition of the effect of RBCs. Similarly, the involvement of RBCs in lowering extracellular Ado was demonstrated by adding dipyridamole, an inhibitor of Ado transport (18), to the incubation media 15 min before fMLP stimulation. Dipyridamole also caused a marked inhibition of LTB_4 biosynthesis; that the inhibitory effect of dipyridamole was the consequence of an accumulation of Ado in the incubation media was assessed by the coaddition of Ado deaminase, which completely restored LTB_4 biosynthesis to the level observed with RBCs (data

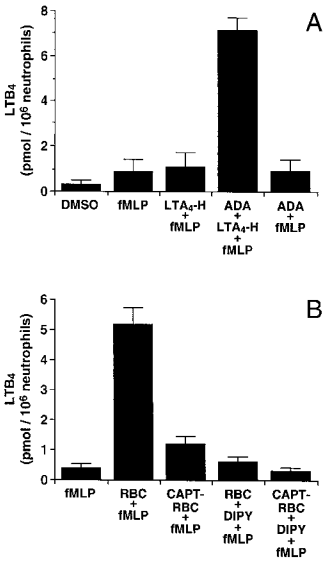

Figure 3. Effect of exogenous LTA_4 hydrolase, Ado deaminase, and RBCs on the biosynthesis of LTB_4 by PMNs in plasma. (*A*) PMN (5×10^6) in suspension in autologous plasma (0.5 ml) were treated with 1.2 nM TNF-α and 1 μg/ml LPS for 30 min at 37°C. 10 μl of the preparation of $rLTA_4$ hydrolase (see Materials and Methods) and/or 4 U of Ado deaminase (or its diluent, NaCl 0.9%) were added (per milliliter of incubation media) 1 and 5 min, respectively, before stimulation with 1 μM fMLP. After 15 min of stimulation, the incubations were stopped and LTB_4 production was measured by RP-HPLC. (*B*) PMNs (5×10^6) in suspension in autologous plasma (0.5 ml) were treated with 1.2 nM TNF-α and 1 μg/ml LPS for 30 min at 37°C. RBCs (0.5 ml of packed cells) treated or not with captopril (see Materials and Methods) were next added to PMNs in plasma; the incubation media were further treated or not with 30 μM dipyridamole for 25 min at 37°C, and then stimulated with 1 μM fMLP for 15 min. LTB_4 biosynthesis was measured by RP-HPLC. Results shown are the means ± SD of triplicate incubations from one experiment representative of three. *LTA_4-H*, LTA_4-hydrolase; *DIPY*, dipyridamole; *CAPT-RBC*, captopril-treated red blood cells; *ADA*, Ado deaminase.

not shown). The pretreatment of RBCs with captopril and addition of dipyridamole inhibited the biosynthesis of LTB_4 to the level observed in the absence of RBCs.

Discussion

We recently reported that Ado and analogues (particularly A_{2a} agonists) are potent inhibitors of LTB_4 biosynthesis in whole blood, as well as in isolated PMNs and monocytes (11). It is also recognized that Ado accumulates in PMN suspensions (12). These observations led us to hypothesize that endogenous Ado present in PMNs suspensions might exert a suppressive effect on the biosynthesis of LTB_4. Measurements of Ado concentrations in PMN incubation media (HBSS and plasma) clearly indicated that after 15–30 min of incubation in all experimental conditions tested, Ado reaches concentrations (25–400 nM; Fig. 1) likely to severely impact LTB_4 synthesis, given the IC_{50} of 80 and 60 nM measured previously for the inhibition of LTB_4 synthesis in blood (11) and HBSS (our unpublished data), respectively. Accordingly, we found that the removal of endogenous Ado using Ado deaminase or the blockade of its effect with the A_{2a} receptor antagonist CSC, strikingly increased the biosynthesis of LTB_4 in response to ligand stimulation. For PMNs activated in the presence of plasma, however, the addition of Ado deaminase to the incubation media was not efficient in increasing the biosynthesis of LTB_4. On the basis of the recent report that activated PMNs release LTA_4 in the extracellular milieu (6), we postulated that LTB_4 biosynthesis by PMN suspensions in plasma was inhibited by the trapping of its precursor LTA_4 by the plasma proteins, which prevented the uptake and further metabolism of LTA_4 by the PMN LTA_4 hydrolase. Indeed, it has been previously shown that LTA_4 binds to serum albumin, which results in a dramatic increase of its half-life (20). Accordingly, the addition of $rLTA_4$ hydrolase and Ado deaminase, but not of either enzyme alone, resulted in a significant increase of LTB_4 biosynthesis by PMNs in plasma. Our results therefore demonstrate that LTB_4 biosynthesis by PMNs in plasma is limited by the inhibitory constraint generated by endogenous Ado which prevents the formation of LTA_4. The removal of Ado (using Ado deaminase) is, however, insufficient to allow efficient LTB_4 biosynthesis since LTA_4 released by PMNs is bound to plasma proteins. The addition of $rLTA_4$ hydrolase in PMN suspensions in plasma drastically accelerates and enhances the generation of LTB_4. The mechanism by which Ado suppresses LT synthesis in PMNs remains to be defined. We can exclude, however, the idea that the inhibitory effect of Ado on LT biosynthesis in PMN results from an effect of the nucleoside on the release or uptake of LTA_4. Indeed, in the experimental system used (Fig. 2), the formation of LTB_4 (and LTB_4 metabolites) depends on the reuptake of extracellular LTA_4 which is then converted by the cytosolic LTA_4 hydrolase (6). Therefore, inhibition of initial LTA_4 release by the activated PMNs would be expected to facilitate LTB_4 formation, whereas inhibition of extracellular LTA_4 uptake would result in the formation of the 6-*trans* isomers (nonenzymic hydrolysis products) of LTB_4, which was not observed in our experiments. Another possible site of action of Ado in the regulation of LTB_4 biosynthesis could be at the level of the conversion of LTB_4 to its ω oxidation products. However, as seen in Fig. 2 *C*, endogenous Ado suppresses the formation of both LTB_4 and 20-OH LTB_4, the sum of LTB_4, and its metabolites being two- to threefold greater in incubations performed in presence of Ado deaminase or CSC. Interestingly, previous studies have shown that elevated extracellular concentrations of Ado inhibits external Ca^{2+} influx in PMNs stimulated by either PAF or fMLP (21). Since LTB_4 biosynthesis is highly dependent on ligand-stimulated Ca^{2+} influx (22), it is therefore conceivable that the inhibitory effects of Ado might be related to the modulation of arachidonate release and/or 5-LO activation, both of which are Ca^{2+} dependent (22, 23). Studies are in progress to assess this hypothesis.

Another finding of the current study is the involvement of a dual mechanism in the regulation of the biosynthesis of LTB_4 by RBCs. Indeed, RBCs have previously been shown to enhance the biosynthesis of LTB_4 through the transcellular metabolism of LTA_4 by RBC LTA_4 hydrolase (8). However, the results of our studies with PMN in plasma suggested that the presence of RBC LTA_4 hydrolase cannot fully account for the increased production of LTB_4, and that the reported capacity of RBCs to efficiently take up Ado (18) may contribute to the ability of RBCs to enhance the synthesis of LTB_4 by ligand-stimulated PMNs in plasma. In fact, both RBC-mediated events proved to be determinant in the stimulatory effect of RBCs since both captopril, an inhibitor of LTA_4 hydrolase, and dipyridamole, an inhibitor of adenosine transport, efficiently reversed the effect of RBCs on LTB_4 synthesis, in full agreement with the data obtained by the simultaneous addition of LTA_4 hydrolase and Ado deaminase to PMNs activated in plasma.

The fact that endogenous Ado exerts a negative regulation of LTB_4 biosynthesis by ligand-stimulated PMNs has led to an underestimation of the potential of this cell type to respond to stimulation by physiological agonists such as fMLP and PAF (24) and has also contributed to the generation of controversial data concerning the ability of PMNs to produce LTs in response to such stimuli. Indeed, it is likely that differences in experimental conditions used by different investigators, such as PMN concentration and preincubation temperature and time, directly impact on Ado concentration in the cell suspensions and therefore, on cell responsivity to the stimuli. Moreover, while LTB_4 synthesis by PMNs stimulated by soluble agonists such as fMLP and PAF is highly sensitive to inhibition by Ado, the synthesis of LTB_4 by PMNs stimulated by the ionophore A23187 is much less sensitive to Ado inhibition (11). It seems important to point out that in an in vivo context, the inhibition of PMN LTA_4 biosynthesis by Ado likely has consequences not only on LTB_4 formation, but also on the biosynthesis of cysteinyl LTs and lipoxins, since these may be generated, at least in part, from the transcellular metabolism of PMN-derived LTA_4 by endothelial cells (25) and platelets (26).

In summary, the current study demonstrates the regulatory role of endogenous Ado on ligand-stimulated LT biosynthesis by PMNs and strongly emphasizes that an elevated level of endogenous Ado in physiological settings can have profound consequences on the ability of PMNs to produce LTA_4, the direct precursor of the lipid mediators LTB_4, LTC_4, and lipoxins, which have been shown to modulate phagocyte functional responses and inflammatory events. Our observations also support the recently proposed concept that Ado is a natural antiinflammatory agent (10). Indeed, it has become increasingly apparent that the antiinflammatory mechanism of methotrexate and sulfasalazine, two potent antiinflammatory drugs, involves an increase of Ado concentration at sites of inflammation (10, 27). Most importantly, these studies showed that leukocyte accumulation at inflammatory sites was diminished and that these effects of the drugs could be antagonized by Ado deaminase or Ado receptor antagonists. In view of the ability of Ado to suppress LTB_4 biosynthesis, it is tempting to speculate that the mechanism by which these antiinflammatory agents act might include the inhibition of LTB_4-dependent extravasation of leukocytes. Further studies are needed to characterize the consequences of increasing Ado levels on LTB_4-mediated inflammatory processes. Finally, taken together, these recent observations and the previously reported inhibitory effects of Ado on PMNs and monocyte functions support that A_{2a} receptor agonists or agents that can regulate Ado biosynthesis, metabolism, or transport may represent a novel class of potent antiinflammatory agents.

This work was supported by the Arthritis Society of Canada.

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Received for publication 16 June 1997 and in revised form 25 July 1997.

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