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Release of adenosine from human neutrophils stimulated by platelet activating factor, leukotriene B_4 and opsonized zymosan

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ISOLATED human polymorphonuclear leukocytes (PMNL) stimulated by platelet activating factor (PAF), leukotriene B_4 (LTB_4) or opsonized zymosan (OZ) released adenosine measured by thermospray high performance liquid chromatography mass spectrometry in the cell-free supernatants. Stimulation by PAF or LTB_4 resulted in a bell-shaped concentration-effect curve; 5×10^{-7} M PAF, 10^{-8} M LTB_4 and 500 $\mu g\ ml^{-1}$ OZ induced peak adenosine release, thus cytotoxic concentrations did not elevate adenosine level in the supernatants. Therefore adenosine release was characteristic of viable cells. As calculated from concentration-effect curves, the rank order of potency for adenosine release was PAF > LTB_4 > OZ. These results suggest that adenosine, when bound specifically to membrane receptor sites, may initiate signal transduction, and, in co-operation with other inflammatory mediators, may modulate phagocyte function, e.g. production of chemoluminescence (CL).

Key words: Adenosine, Chemoluminescence, LTB_4, Opsonized zymosan, PAF, Polymorphonuclear leukocytes

Introduction

Platelet activating factor (PAF) is a phospholipid autacoid implicated as mediator in the pathogenesis of inflammation, thrombosis, immune disorders, septic shock and a great variety of physiological and pathophysiological conditions.[1,2] PAF as second messenger of diverse injurious stimuli releases eicosanoids and superoxide anions from leukocytes, macrophages and endothelial cells.[3]

Leukotrienes (LTs) are metabolites of arachidonic acid (AA) formed by 5′-lipoxygenase. One leukotriene, LTB_4, is a potent chemotactic and aggregating agent released from polymorphonuclear leukocytes (PMNL).[4,5] LTB_4 is also involved in a variety of pathophysiological processes, including γ-interferon production.[6]

Phagocytosis induced by opsonized zymosan (OZ) is one of the most widely used models for testing the function of stimulated PMNL.[7] Engulfment of particles via Fc, CR1 and CR3 receptors involves marked changes in cellular metabolism, leading to degranulation and production of superoxide anions.[8]

Adenosine is a natural nucleoside known to regulate various cellular functions, including neurotransmission[9] and local circulation.[10] These effects of adenosine appear to be mediated by two separate subtypes of binding sites, i.e., A_1 and A_2 receptors.[11] By interacting with one of these receptor subtypes, adenosine can initiate a transmembrane signal which then may inhibit or stimulate adenylate cyclase via A_1 or A_2 receptors, respectively. A third adenosine recognition site, termed the P site, located on the catalytic subunit of adenylate cyclase, is activated by relatively high concentrations of adenosine.[12] Near micromolar concentrations of adenosine, interacting with A_2 receptors, have been shown to inhibit PMNL functions.[13]

The present experiments were designed to study the production and release of adenosine from PMNL stimulated by PAF, LTB_4 and OZ. The results obtained suggest that adenosine released from PMNL may induce inhibitory action, which then, as a regulatory feedback signal, may protect the phagocytes from irreversible damage due to overstimulation during the inflammatory process.

Materials and Methods

Materials: Acetonitrile (HPLC grade, Baker Chemicals, Düsseldorf, Germany), adenosine, bovine serum albumin (BSA), 2-chloroadenosine, luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma Chemicals, St. Louis, MO), dextran (molecular mass 70 kDa, Macrodex) and zymosan (Mannozym, Human Serobacteriological Institute, Budapest, Hungary), Ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden), LTB_4 dissolved in 99.5% ethanol (Calbiochem, Lucerne, Switzerland), PAF (Bachem, Bubendorf, Switzerland), TC-199 medium (Parker medium, Institute of Hygiene, Budapest, Hungary),

trypan blue (Reanal, Budapest, Hungary), Uromiro (Bracco Chimica, Milan, Italy) were used, PAF was dissolved in phosphate-buffered saline (PBS), containing 1 mg ml^{-1} of BSA.

Separation of cells: Heparinized venous blood was obtained from healthy donors. After removal of mononuclear cells by Ficoll-Uromiro density gradient centrifugation, the neutrophil-rich pellet was sedimented by dextran. Residual erythrocytes were lysed by hypotonic saline (0.45%). The final cell suspension consisted of 95% PMNL and 5% mononuclears but no red blood cells.[14]

Measurement of adenosine: A thermospray high performance liquid chromatography mass spectroscopy (TSP-HPLC-MS)[15,16] was used to determine adenosine concentrations in cell-free supernatants of PMNL stimulated with various concentrations of PAF, LTB_4 or OZ.[17,18] Proteins in 1 ml cell-free supernatants were precipitated by addition of acetonitrile (1:10, v/v) and subsequent centrifugation at 1500 ***g*** for 10 min. Aliquots of acetonitrile solution, 25 μl of each, were injected directly onto the HPLC column. HPLC was carried out using a Water C 18 Nova-Pak RP column (15 × 0.40 cm I.D.) on a Model 640 gradient controller and Model U6K injector. A linear gradient from 20.5% to 70% acetonitrile in 0.05 M aqueous ammonium acetate (1 ml min^{-1}) was used. The water for HPLC was Milli-Q quality. Sample ionization was achieved by thermospraying the HPLC eluent into a VG-TRIO-2 quadripole mass spectrometer (VG MassLab, UK) via a VG thermospray/plasmaspray interface. The ion source temperature was held at 200°C, TSP probe temperature was 210°C. Adenosine shows a base peak corresponding to the $[M^+H]^+$ ion at $m/_z$ 268. The mass spectrometer monitored the eluent continuously at $m/_z$ 268.

Measurement of chemiluminescence: 2×10^6 PMNL were incubated in the presence of 5×10^{-7} M PAF, dissolved in PBS/BSA, and/or with 2-chloroadenosine in various experimental conditions at 37°C for 60 min. Chemiluminescence of PMNL was measured in PBS at a final volume of 1 ml in the presence of 10^{-7} M luminol using a Nuclear Chicago/300 liquid scintillation counter (Searle, Indianapolis, IN) in the off coincidence mode.[19,20]

Viability of cells: Viability was determined by the trypan-blue exclusion test to detect the percentage of viable cells at the beginning of the experiments and to check the cytotoxic effect of PAF, LTB_4 and OZ at the end of incubation with these stimulating agents.[21]

Statistical analysis: Data are expressed as means ± standard deviation (SD). Each adenosine determination was carried out in triplicate samples. Data were statistically analyzed by the tailed Student's *t*-test. Differences were considered significant when p was less than 0.05.

Results

Adenosine release: Adenosine release was studied in samples of 2×10^6 PMNL ml^{-1} incubated with various concentrations of PAF, LTB_4 and OZ in TC-199 medium under 5% CO_2 for 30 and 60 min. Adenosine content in the supernatants was determined by TSP-HPLC-MS, and measured values were compared to that obtained in non-stimulated cell suspensions. In supernatants obtained from non-stimulated cells adenosine concentrations were below the limit of detectability. PAF at 10^{-8} M concentration resulted in a well established adenosine release; maximal production was induced by 5×10^{-7} M, whereas after exposure to 10^{-4} M no detectable adenosine concentrations were measured in the supernatants (Figure 1). The cytotoxic effect of 10^{-4} M PAF was 85%. This bell-shaped concentration-effect curve indicates that extracellular appearance of adenosine may be due to intracellular metabolic processes rather than cell damage.

LTB_4 also increased adenosine content of supernatants in a similar manner (Figure 2). Peak release was observed at 10^{-8} M. The highest concentration of adenosine released by LTB_4-stimulated PMNL remained, however, below the values that were produced by PAF. This corresponded to a lower cytotoxicity of LTB_4; the cytotoxic effect of 10^{-5} M LTB_4 was only 20%. The calculated ratio of adenosine production, i.e., total adenosine released by PAF/total adenosine released

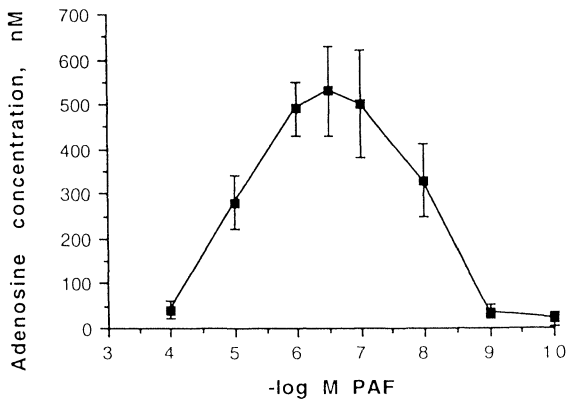

FIG. 1. Release of adenosine from human PMNL stimulated by various concentrations of PAF. 2×10^6 PMNL ml^{-1} were incubated in TC-199 medium at 37°C for 60 min. PAF was dissolved in PBS/BSA and 50 μl was added to each sample to reach its final concentration in 1 ml. Adenosine concentrations in cell-free supernatants were determined by TSP-HPLC-MS; means ± SD, $n = 3$.

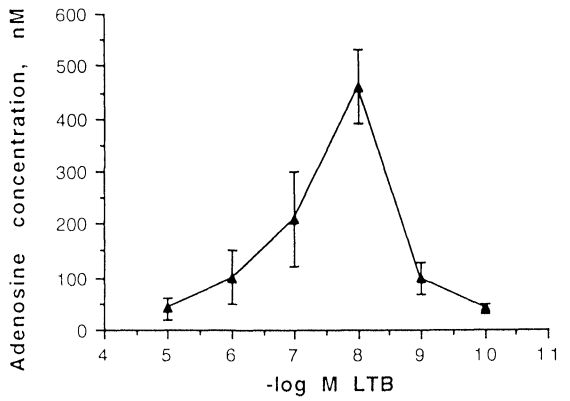

FIG. 2. Release of adenosine from human PMNL stimulated by various concentrations of LTB_4. 2×10^6 PMNL ml^{-1} were incubated in TC-199 medium at 37°C for 60 min. LTB_4 was dissolved in 99.5% ethanol then diluted with the incubation medium. The solvent itself, added in 3 μl to the 1 ml incubation medium, had no effect on adenosine release. Adenosine concentrations in cell-free supernatants were determined by TSP-HPLC-MS; means ± SD, $n = 3$.

by LTB_4, in the concentration ranges of 10^{-9} to 10^{-4} M or 10^{-10} to 10^{-5} M PAF or LTB_4 respectively was 2.25, indicating an efficacy for PAF higher than for LTB_4.

The kinetics of adenosine release were determined in PMNL stimulated with 5×10^{-7} M PAF, 10^{-8} M LTB_4 and 500 $\mu g\ ml^{-1}$ OZ at 37°C for 30 and 60 min. This entirely non-cytotoxic concentration of OZ was used since it was shown to induce the highest CL of PMNL.[22] As shown in Table 1, adenosine concentrations in the supernatants of cell suspensions stimulated for 30 min were lower than that measured after 60 min stimulation. Stimulation by OZ for 30 min did not produce any detectable elevation of adenosine concentration. The rank order of potency for adenosine release was PAF > LTB_4 > OZ.

Chemiluminescence: The inhibition by adenosine of CL in PMNL was demonstrated in transfer experiments. Supernatants of PMNL incubated in the presence of 5×10^{-7} M PAF, containing approximately 400 nM adenosine, were transferred to unstimulated cells, and the luminol induced amplification of CL in these cells was measured, or PAF induced CL was determined in the presence of 2-chloroadenosine, a stable analogue of adenosine. Preincubation of PMNL with PAF at 37°C for 60 min produced adenosine concentrations that were able to inhibit the CL of freshly added PAF when transferred to unstimulated cells. This stimulation was almost as intense as that was seen after addition of 10^{-6} M 2-chloroadenosine (Table 2).

Table 1. Adenosine concentrations in the supernatants of human PMNL stimulated by PAF, LTB_4 and opsonized zymosan

	Concentration of adenosine, nM means ± SD		
	Stimulation by		
Incubation, min	PAF 5×10^{-7} M	LTB_4 10^{-8} M	opsonized zymosan 500 $\mu g\ ml^{-1}$
30	405 ± 89	183 ± 81	ND
60	533 ± 151	351 ± 113	103 ± 41

2×10^6 PMNL ml^{-1} were incubated in TC-199 medium at 37°C. Adenosine concentrations in the cell-free supernatants were determined by TSP-HPLC-MS; $n = 3$, PMNL were used from three healthy donors, ND = not detectable.

Table 2. Effect of PAF and 2-chloroadenosine (2-c-Ado) on the chemiluminescence of human PMNL

Group	number of emitted photons, cpm means ± SD
1 PMNL in PBS/BSA 60 min (control)	1152 ± 143
2 PMNL in PBS/BSA 60 min plus PAF	2114 ± 342*
3 PMNL plus PAF in PBS/BSA, 60 min	1320 ± 211
4 PMNL plus supernatant (3) plus PAF	1457 ± 256
5 PMNL in PBS/BSA 60 min plus 2-c-Ado	1085 ± 117
6 PMNL in PBS/BSA 60 min plus 2-c-Ado and PAF	1188 ± 196

2×10^6 PMNL were incubated at 37°C. PAF dissolved in PBS/BSA and 2-chloro-adenosine (2-c-Ado) were added at final concentrations of 5×10^{-7} M and 10^{-6} M, respectively. In transfer experiments the cell-free supernatant of PAF stimulated (adenosine containing) PMNL (sample 3) was added to freshly prepared, unstimulated cells (sample 4). In sample 6, PAF was added to the cells after preincubation of PMNL with 2-c-Ado for 10 min. Chemiluminescence, measured in a scintillation counter by the off coincidence mode, was induced by 5×10^{-7} M PAF added to the cells at the end of incubation, except when the background chemiluminescence of PMNL incubated alone (sample 1) or in the presence of 2-c-Ado (sample 5) was determined; $n = 4$, PMNL of the same healthy donors were used in four separate experiments. *$p < 0.01$ as compared to control.

Discussion

Beyond the lipid character, common features in the effects of PAF and LTB_4 are the induction of chemotaxis, aggregation and superoxide anion production in PMNL.[1,23,24] This study shows that both PAF and LTB_4 release adenosine from PMNL, although the effect of PAF is more marked than that of LTB_4. The difference may be explained by the fact that PAF can also release LTB_4,[3,18] thus adenosine release induced by PAF presumably includes that released by LTB_4. Regardless of distinct receptor binding sites for PAF and LTB_4 on PMNL membrane,[6,24–26] these findings point to the similarity of signal transduction triggered by the two autacoids, suggesting their involvement in a common pathway of the inflammatory process.

The particles of OZ are internalized by PMNL via Fc, CR1 and CR3 receptors leading to degranulation of specific azurophil granules and

production of superoxide anion.[27] In our study, OZ proved to be the least effective in releasing adenosine from human PMNL. Stimulated PMNL produce PAF and LTB_4 at picomolar concentrations,[28] and this may explain the finding that adenosine concentrations are much lower in supernatants of cells stimulated with OZ than in supernatants stimulated with higher, micromolar concentrations of exogenous PAF or LTB_4. This can also be reflected by the kinetics of adenosine production in stimulated cells. While 30 min stimulation was found to be optimal for production of adenosine by PAF or LTB_4, this incubation period was insufficient in OZ-activated PMNL[17,29] to detect a measurable amount of adenosine in the supernatant, while 60 min stimulation resulted in a well established increase of adenosine concentration. Accordingly, adenosine production by PAF and LTB_4 was also more marked after 60 min than at 30 min stimulation.

The preparation of a completely platelet free human PMNL suspension is practically impossible. The rate of platelet contamination in our PMNL suspensions was ordinarily 1:1. The estimated amount of adenosine possibly derived from aggregated platelets was 50 nM. To aggregate platelets but not neutrophils, ADP was added to cells suspended in TC-199 medium, and adenosine release was measured in the supernatants 60 min later (data not shown). From these results the conclusion can be drawn that the major part of adenosine released by PAF, LTB_4 and OZ is derived from PMNL.

Adenosine is produced by the breakdown of intracellular ATP, and an increased consumption of ATP results from the stimulation of phagocytes via the pathway of ATP synthesis from ADP:

$$2ADP \longrightarrow ATP + AMP \quad (1)$$

$$AMP \xrightarrow{5'\text{-nucleosidase}} \text{adenosine}[22] \quad (2)$$

We assume that, at a certain degree of ATP depletion in activated cells, this process may lead to accumulation of adenosine at nearly micromolar concentrations in the extracellular space, because some adenosine molecules may escape from the rapid breakdown by adenosine deaminase located on the external surface of cells.[30] These molecules may then bring important signal transduction for regulating the function of surrounding cells. Adenosine binding A_2 receptor subtypes and P sites, has been shown to inhibit PMNL functions, e.g. intracellular killing or generation of oxygen derived free radicals.[31] As previously described,[11] this effect is related to an activation of adenylate cyclase with a concomitant elevation of intracellular cAMP level. Intracellular cAMP raised by eicosanoids, in particular prostacyclin (PGI_2), has been shown to downregulate eicosanoid production in platelets[32,33] and vascular endothelium.[34] PAF has been shown to release AA by conversion not only to the lipoxygenase product LTB_4,[3,18] but also through the cyclooxygenase pathway to prostaglandin E_2 (PGE_2)[35] and PGI_2.[36] All these events have been shown to augment the number of emitted photons in luminol induced CL.[37] Eicosanoid synthesis by itself is therefore associated with increased light emission[38] and indomethacin can block such CL.[39] Consequently, the elevation of intracellular cAMP by PGs, the endproducts of AA metabolism, is accompanied by decreased CL as a consequence of feedback inhibition brought about either directly as a result of decreased precursor conversion or through the NADPH oxidase system.[40] Thus, CL of phagocytes can be modulated not only by adenosine released from stimulated cells, but also by AA and its biologically highly active endproducts depending upon the stage of their production. At the same time, a great variety of interactions may occur among inflammatory mediators, for example, adenosine can block LT synthesis.[41] An elevated intracellular cAMP level, induced by either adenosine,[42] PGE_2[41] or PAF[43] may be an important signal transduction to downregulate CL,[44] thus being a sensitive marker for the metabolic and functional state of phagocytes. Another important aspect of this autoregulatory feedback network is that cAMP has also been implicated in the inhibition of PAF release.[45] On the other hand, the extracellular nucleotides, such as ATP, ADP and AMP, may induce the opposite effect, i.e., stimulation of adhesiveness and other functions of PMNL.[46]

This regulatory and inhibitory role for exogenously applied and endogenously released adenosine has been described in stimulated PMNL.[47–49] Our data confirm these observations and support the view that production and release of adenosine may regulate the function of activated PMNL and other phagocytes. In the autocatalytic feedback network of inflammatory mediator release,[50] adenosine may therefore be regarded as an important signal molecule downregulating the production of PAF, LTB_4 and other lipid mediators, and through this mechanism activated PMNL and other phagocytes may be protected from potentially irreversible damage due to overstimulation.[51,52] This modulatory action, however, takes place in co-operation with other mediators, mainly AA derivatives.

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