Release of adenosine from human neutrophils stimulated by platelet activating factor, leukotriene B$_4$ and opsonized zymosan

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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.

Measurement of adenosine: A thermospray high performance liquid chromatography mass spectrometry (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 ml of each, were injected directly onto the HPLC column. HPLC was carried out using a Water C 18 Nova-Pak RP column (15 x 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 quadrupole 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 x $10^6$ PMNL were incubated in the presence of 5 x $10^{-7}$ 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 x $10^6$ 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 x $10^{-7}$ 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. 2. Release of adenosine from human PMNL stimulated by various concentrations of LTB₄. 2 × 10⁶ PMNL ml⁻¹ were incubated in TC-199 medium at 37°C for 60 min. LTB₄ 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.

Table 1. Adenosine concentrations in the supernatants of human PMNL stimulated by PAF, LTB₄ and opsonized zymosan

| Group | Concentration of adenosine, nM means ± SD |
|-------|-----------------------------------------|
| Incubation, min | PAF | LTB₄ | opsonized zymosan |
| 30 | 5 × 10⁻⁷ M | 10⁻⁸ M | 500 μg ml⁻¹ |
| 60 | 533 ± 151 | 351 ± 113 | ND |

2 × 10⁶ 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 plus PAF | 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 × 10⁶ PMNL were incubated at 37°C. PAF dissolved in PBS/BSA and 2-chloroadenosine (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 PMNL 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.

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, 10⁻⁸ M LTB₄ and 500 μg 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. 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 a detectable elevation of adenosine concentration. The rank order of potency for adenosine release was PAF > LTB₄ > OZ.

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Beyond the lipid character, common features in the effects of PAF and LTB₄ are the induction of chemotaxis, aggregation and superoxide anion production in PMNL. This study shows that both PAF and LTB₄ release adenosine from PMNL, although the effect of PAF is more marked than that of LTB₄. The difference may be explained by the fact that PAF can also release LTB₄, thus adenosine release induced by PAF presumably includes that released by LTB₄. Regardless of distinct receptor binding sites for PAF and LTB₄ on PMNL membrane, 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. In our study, OZ proved to be the least effective in releasing adenosine from human PMNL. Stimulated PMNL produce PAF and LTB₄ at picomolar concentrations, 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 LTB₄. 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 LTB₄, this incubation period was insufficient in OZ-activated PMNL 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 LTB₄ 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, LTB₄ 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:

\[
2\text{ADP} \rightarrow \text{AMP} + \text{AMP} \rightarrow \text{AMP} \rightarrow \text{adenosine} \quad (2)
\]

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. These molecules may then bring important signal transduction for regulating the function of surrounding cells. Adenosine binding A₂ receptor subtypes and P sites, has been shown to inhibit PMNL functions, e.g. intracellular killing or generation of oxygen derived free radicals. As previously described, 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 (PGI₂), has been shown to downregulate eicosanoid production in platelets and vascular endothelium. PAF has been shown to release AA by conversion not only to the lipoxygenase product LTB₄, but also through the cyclooxygenase pathway to prostaglandin E₂ (PGE₂) and PGI₂. All these events have been shown to augment the number of emitted photons in luminol induced CL. Eicosanoid synthesis by itself is therefore associated with increased light emission and indomethacin can block such CL. 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. Thus, CL of phagocytes can be modulated not only by adenosine released from stimulated cells, but also by AA and its biologically highly active end-products 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. An elevated intracellular cAMP level, induced by either adenosine, PGE₂ or PAF, may be an important signal transduction to downregulate CL, 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.

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.

This regulatory and inhibitory role for exogenously applied and endogenously released adenosine has been described in stimulated PMNL. 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, adenosine may therefore be regarded as an important signal molecule downregulating the production of PAF, LTB₄ and other lipid mediators, and through this mechanism activated PMNL and other phagocytes may be protected from potentially irreversible damage due to overstimulation. This modulatory action, however, takes place in cooperation with other mediators, mainly AA derivatives.

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Adenosine release by stimulated neutrophils

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Mediators of Inflammation 1:1992 271