ADENOSINE: A PHYSIOLOGICAL MODULATOR OF SUPEROXIDE ANION GENERATION BY HUMAN NEUTROPHILS*

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The responses of neutrophils to soluble and insoluble stimuli include aggregation, chemotaxis, lysosomal enzyme release, and the generation of active oxygen species such as superoxide anion. These processes, which have been described as stimulus-response coupling, can be influenced by ions, cyclic nucleotides, and metabolites of arachidonate (1). In recent years, a role for adenosine has been described in the stimulus-response coupling of a variety of cells and tissues. Adenosine receptors and/or hormone like responses to adenosine have been reported in adipocytes, neurons, hepatocytes, smooth muscle cells, coronary arteries, and heart muscle (2–7). These responses have also been described in circulating cells of the blood, i.e., lymphocytes (8–10), macrophages (11), basophils (12), and mast cells (13).

Adenosine in the presence of homocysteine thiolactone inhibits transmethylation reactions. These reactions are required for maintenance of high avidity receptors for the chemoattractant *N*-formyl-methionyl-leucyl-phenylalanine (FMLP)[1] on murine macrophages. Pike and Snyderman (11) found that generation of superoxide anion by macrophages in response to FMLP was inhibited by adenosine. These studies suggested that adenosine might regulate stimulus-response coupling in neutrophils as well. However, Marone et al. (14) were unable to demonstrate a significant effect of adenosine on the release of lysosomal enzyme (beta-glucuronidase) from human neutrophils stimulated by particle ingestion.

We have therefore examined the role of adenosine as a modulator of superoxide anion generation, degranulation, and aggregation in human neutrophils exposed to soluble stimulants. We report that adenosine, at physiological concentrations, is a potent and specific regulator of neutrophil superoxide anion generation but exerts little or no effect on either degranulation or aggregation. Further, we demonstrate that this effect is independent of the cellular uptake of

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[1] *Abbreviations used in this paper:* ADA, adenosine deaminase; Con A, concanavalin A; cyto C, cytochrome C; DCF, deoxycoformycin; EHNA, erythro-9-(2-hydroxy-3-nonyl) adenine; FMLP, *N*-formyl-methionyl leucyl-phenylalanine; LDH, lactate dehydrogenase; PMA, phorbol myristate acetate; ZTS, zymosan-treated serum.

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adenosine, does not appear to depend upon inhibition of transmethylation reactions, and is found with a variety of stimuli that activate neutrophils via different mechanisms.

Materials and Methods

Materials. Cytochalasin B was purchased from Aldrich Chemical Co., Milwaukee, WI. Concanavalin A (Con A), cytochrome C (cyto C) (type III), adenosine, 2-chloroadenosine, 2′-deoxyadenosine, inosine, hypoxanthine, FMLP, L-homocysteine thiolactone, epsilon aminocaproic acid, xanthine oxidase (grade III from buttermilk), and calf intestinal adenosine deaminase (ADA) (type I in ammonium sulfate) were obtained from Sigma Chemical Co., St. Louis, MO. Zymosan was obtained from ICN Nutritional Biochemicals, Cleveland, OH. Superoxide dismutase was obtained from Miles Laboratories, Inc., Elkhart, IN and dextrose (D-glucose) was obtained from Fisher Scientific Co., Fairlawn, NJ. Calcium ionophore A23187 (A23187), and 6-nitrobenzylthioinosine were purchased from Calbiochem-Behring Corp., San Diego, CA and phorbol myristate acetate (PMA) was obtained from Chemalog, S. Plainfield, NJ. Erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) was obtained from Burroughs Wellcome & Co., Research Triangle Park, NC. (8-^{14}C)-adenosine (50 mCi/mmol) was obtained from Schwarz/Mann Div., Becton, Dickinson & Co., Spring Valley, NY. Deoxycoformycin (DCF) was a gift from Dr. J. Douros of the National Cancer Institute. A lactate dehydrogenase (LDH) Statzyme kit was obtained from Worthington Biochemical Corp., Freehold, NJ. DEAE cellulose chromatography paper (DE 81) was supplied by Whatman Inc., Clifton, NJ.

Preparation of Cell Suspensions. Heparinized blood was obtained from normal volunteers. Purified preparations of neutrophils were isolated by means of Hypaque/Ficoll gradients (15) followed by dextran sedimentation and hypotonic lysis of erythrocytes (16). This procedure allowed studies of cell suspensions containing 98 ± 2% neutrophils with few contaminating erythrocytes or platelets. The cells were suspended in a buffered salt solution consisting of Na^+ (150 mM), K^+ (5 mM), Ca^{2+} (1.3 mM), Mg^{2+} (1.2 mM), Cl^- (155 mM), and Hepes (10 mM), pH 7.45. Release of cytoplasmic LDH was used as an indicator of cell death (17). Rupture of cells by detergent (Triton X-100) led to the release of total LDH, beta-glucuronidase and lysozyme (17).

Preparation of Zymosan-treated Serum (ZTS). Serum was obtained from normal donors and rendered 0.25 M with respect to epsilon aminocaproic acid. The serum was incubated for 15 min with zymosan (1 mg/ml) at 37°C, vortexing at 5 min intervals. The suspension was then spun at 2,000 *g* for 15 min and kept at 4°C until use. A final concentration of serum of 10% was used as a stimulant in subsequent experiments (18).

Superoxide Anion Generation. Superoxide anion generation was monitored by determination of superoxide dismutase-inhibitable reduction of cyto C in the presence or absence of cytochalasin B. Duplicate reaction mixtures containing 2×10^6 neutrophils, 75 nmol horse heart ferricytochrome C (type III), and various inhibitory compounds (e.g., adenosine) at designated concentrations in a final volume of 1 ml, were incubated for 10 min at 37°C. Cytochalasin B (5 μg/ml) was added where indicated and cells incubated at 37°C for an additional 5 min before exposure to stimuli (e.g., FMLP). 5 min after stimulation, cells were spun down at 4°C at 1,000 *g* in a Sorvall RC-3 centrifuge (Sorvall Instruments Div., Dupont Co., Newtown, CT) and the supernatants were collected. Absorption at 550 nm was determined in a spectrophotometer (Model 25; Beckman Instruments Inc., Fullerton, CA) and the nanomoles of superoxide anion generated were calculated as previously described (19).

Lysosomal Enzyme Release. For the determination of enzyme release, neutrophils (2×10^6) were preincubated with inhibitors (e.g., adenosine) for 10 min at 37°C. Cytochalasin B (5 μg/ml) was then added and the mixture was incubated for an additional 5 min. Appropriate stimulus was added to a final volume of 1 ml and after 5 min incubation at 37°C the cell suspensions were centrifuged at 1,000 *g* in a Sorvall RC-3 centrifuge for 5 min at 4°C. Aliquots of the supernatants were taken for standard determinations of beta-glucuronidase (20) and lysozyme (21). Total enzyme activity and residual, cell-associated

activity were measured in selected reaction mixtures after cells were lysed by the addition of 0.2% Triton X-100. Appropriate control experiments were performed to determine whether there was preferential loss of enzyme activity in resting or treated cells or whether test reagents interfered with enzyme assays. Results are expressed as the percent of maximally stimulated enzyme release from control cell suspensions.

Neutrophil Aggregometry. Aggregation was monitored by determining changes in light transmission in a dual channel aggregometer (Payton Associates, Inc., Buffalo, NY) by the method of Kaplan et al. (22). 90 μl of a suspension of neutrophils (0.25×10^6) in buffer with designated test agent and cytochalasin B (5 μg/ml) were added to a siliconized microcuvette with siliconized stir bar, and allowed to equilibrate a 37°C. The cells were then stimulated with FMLP (0.1 μM) and the resulting changes in light transmission recorded continuously over time. The extent of the aggregation was determined by measurement of the areas under the resulting curves and is expressed as the percent of control aggregation (FMLP stimulated aggregation in the absence of additives).

Determination of Adenosine Concentration. Supernatants of neutrophils were prepared with and without EHNA (10 μM) and adenosine at varying concentrations, as for lysosomal enzyme release, and analyzed immediately. Before analysis, proteins were precipitated with 2.1 N perchloric acid, the pH readjusted to 7.0 with 2.21 N KOH and the samples spun in a Beckman microfuge at 4°C (900 *g*). Adenosine concentrations were determined by reverse-phase high pressure liquid chromatography as previously described (23). In brief, samples were applied to a C18 uBondapack column (Waters Associates, Inc., Milford, MA) and eluted with a 0–40% linear gradient (formed over 60 min) of 0.01 M ammonium phosphate (pH 5.5) and methanol, with a 1.5 ml/min flow rate. The adenosine peak was identified by retention time, the 250/260 UV ratio of adenosine, and disappearance of the peak after incubation with exogenous ADA (0.12 IU/ml for 2 h at 37°C).

Determination of ^{14}C-Adenosine Uptake by Neutrophil Suspensions. (8-^{14}C)-adenosine was added to suspensions of neutrophils or buffer at a final concentration ranging from 0.1 to 0.5 μM. Suspensions were incubated for 10 min at 37°C, stimulated with FMLP (0.1 μM) in the presence of cytochalasin B (5 μg/ml) and supernates collected after 5 min, as before. An equal volume of buffer or buffer containing dipyridamole (final concentration 10 μM) was added to neutrophil suspensions 5 min before the addition of labeled adenosine. Aliquots of the supernates were lyophilized and resuspended in 50 μl of distilled water. 20 μl of the resulting suspension was spotted on DEAE cellulose paper with solutions of carriers (AMP, hypoxanthine, inosine, and adenosine) and descending chromatography was performed with 1 mM ammonium formate. The carriers were visualized under short-wave UV irradiation, cut out, placed in scintillant (PPO-POPOP in toluene) and counted in a Beckman model LS 210 scintillation counter at ~75% efficiency (23).

Xanthine Oxidase-Hypoxanthine Generation of Superoxide Anion. Duplicate tubes containing a 1-ml solution of xanthine oxidase (1:100 dilution), hypoxanthine (2 mg/100 ml) (100 nM), and ferricytochrome C (75 nM) were prepared with one of the following: adenosine (100 μM), 2-chloroadenosine (100 μM), dipyridamole (10 μM), EHNA (10 μM), DCF (1 μM), or ADA (0.250 IU/ml). Tubes were incubated at 37°C for 5 min. Superoxide generation was monitored as above and expressed as nanomoles cyto C reduced.

Preparation of Adenosine Deaminase. 30–50 μl of purified calf intestinal ADA (1,000 U/ml) were dialyzed for at least 3 h at 4°C against 500 ml of phosphate-buffered saline. After dialysis the enzyme was diluted 1:40 in buffer and 10 μl of this solution was added to a 1 ml final volume of cell suspension. In those experiments in which ADA was inactivated, the dialyzed enzyme was diluted 1:40 in buffer containing DCF (100 μM). This mixture was allowed to incubate at room temperature for at least ½ h before use. These conditions were found to completely inactivate the enzyme, as determined by spectrophotometric assay (24).

Results

Exogenously Added Adenosine Inhibits Generation of Superoxide Anion by Neutrophils Stimulated with FMLP. Incubation of human neutrophils with adenosine at

concentrations ranging from 1 to 1,000 μM resulted in the inhibition of superoxide anion generation in response to FMLP (Fig. 1). Inhibition was dose dependent with maximal inhibition observed at 100 μM adenosine (50 ± 1%). At higher concentrations (1,000 μM), no increase in inhibition was observed. Significant inhibition could still be detected at 1 μM adenosine (36 ± 6% inhibition). When inhibitors of the enzyme ADA (EHNA, 10 μM, or DCF, 1 μM) were added together with adenosine, modestly increased inhibition of superoxide generation was observed (Fig. 1). Addition of DCF alone had no inhibitory effect on superoxide generation, while EHNA alone had a small but significant inhibitory effect.

Concentrations of Exogenous and Endogenous Adenosine in Incubation Media of Neutrophil Suspensions. The increased inhibition by adenosine of superoxide generation in the presence of EHNA or DCF suggested that, in the absence of inhibitors of ADA, exogenously added adenosine was continuously deaminated in these suspensions. Inhibition of ADA by EHNA or DCF could have maintained the adenosine concentration. We therefore determined concentrations of adenosine in the media from neutrophil suspensions that had been incubated for 15 min with adenosine in the presence or absence of EHNA before stimulation with FMLP (Table I). Media of cells incubated with varying concentrations of adenosine (1–100 μM) in the absence of EHNA all contained less adenosine than had been added initially. When the cells were incubated with adenosine in the presence of EHNA, the concentrations of adenosine were higher than had been observed in the absence of EHNA, although in most cases still less than that added initially. Unexpectedly, when very low concentrations (0.1 μM) of adenosine were added, more adenosine was recovered than had been added, suggesting that adenosine was being generated. Indeed, media of cells incubated without any exogenous adenosine (both in the presence and absence of EHNA) contained

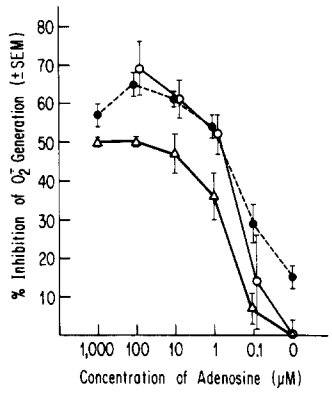

FIGURE 1. Effect of adenosine and inhibitors of ADA on generation of superoxide anion by FMLP-stimulated neutrophils. 2×10^6 neutrophils were stimulated with FMLP (0.1 μM) in the presence of cytochalasin B (5 μg/ml) after 15 min of incubation at 37°C with adenosine (△), adenosine plus EHNA (10 μM) (●), or adenosine plus DCF (1 μM) (○). Results are expressed as mean inhibition (percent) of maximally stimulated control superoxide anion. All points represent the mean of at least three separate determinations on cells from different donors. The mean control generation for these experiments was 16.4 ± 1.3 nmol cyto C-reduced/10^6 cells.

TABLE I

Adenosine Concentration in Media of FMLP-stimulated Neutrophils after Incubation in the Presence or Absence of EHNA and Adenosine

Concentration of adenosine added	Experiment 1 Concentration of adenosine recovered		Experiment 2 Concentration of adenosine recovered	
	−EHNA	+EHNA	−EHNA	+EHNA
μM	*μM*		*μM*	
100.00	65.00	72.60	89.60	91.16
10.00	3.28	8.70	7.60	10.20
1.00	0.59	0.80	0.71	1.10
0.10	0.25	0.30	0.20	0.21
0.00	0.28	—	0.14	0.18

Adenosine concentration was measured by high pressure liquid chromatography as described in Materials and Methods.

TABLE II

Effect of ADA and Inactivated ADA (ADA + DCF) on Superoxide Anion Generation by FMLP-stimulated Neutrophils

Treatment	*n*	Percent of control superoxide generation (±SEM)	*P* value*
ADA	11	155 ± 18	<0.006
ADA + DCF	3	81 ± 14	NS

2×10^6 cells were incubated for 10 min at 37°C with either ADA (0.25 IU/ml) or ADA incubated with its irreversible inhibitor DCF (100 μM) for 30 min. Cells were stimulated with FMLP (0.1 μM) in the presence of cytochalasin B. Control superoxide generation for these experiments was 26.9 ± 5.5 nmol cyto C-reduced/10^6 cells.

* *P* vs. control as determined by Student's *t* test. NS, not significant.

adenosine at concentrations ranging from 0.14 to 0.28 μM (Table I). Similar concentrations of adenosine were found in supernatants of cells that had not been stimulated with FMLP (0.17 μM).

Endogenous Adenosine Inhibits Superoxide Anion Generation by Neutrophils Stimulated with FMLP. To determine whether endogenously generated adenosine was of significance for the generation of superoxide anion, we incubated neutrophils with calf intestinal ADA at concentrations calculated to be in excess of those necessary to metabolize endogenously generated adenosine. Addition of 0.125–0.25 IU of ADA to neutrophil suspensions led to a consistent and sometimes dramatic increase in the generation of superoxide anion by neutrophils (Table II) as well as the disappearance of endogenously generated adenosine (Fig. 2 *A*). Enhancement of superoxide generation was abrogated when the ADA was preincubated with its irreversible inhibitor DCF (100 μM) (Table II), and adenosine was still present in the media (Fig. 2 *B*). Addition of ADA to unstimulated cells did not result in superoxide anion generation (data not shown).

Since deamination of adenosine leads to formation of hypoxanthine and inosine, we examined the effects of these compounds on generation of superoxide anion by neutrophils. Incubation of neutrophils with hypoxanthine (100 μM) or

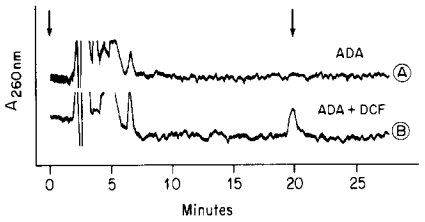

FIGURE 2. Chromatograms of neutrophil supernatants as determined by reverse-phase high pressure liquid chromatography. (*A*) Neutrophils (2×10^6) were incubated for 10 min at 37°C in the presence of 0.25 IU of ADA, then stimulated with 0.1 μM FMLP in the presence of cytochalasin B (5 μg/ml). (*B*) Neutrophils (2×10^6) were treated as above except that the ADA was incubated with DCF (100 μM) before incubation with cells. Adenosine is present at a final concentration of 0.18 μM. The first arrow indicates time of injection, the second arrow the elution time of authentic adenosine.

TABLE III

Effect of Metabolites and Analogs of Adenosine on Superoxide Anion Generation by FMLP-stimulated Neutrophils

Agent (100 μM)	*n*	Percent of control superoxide generation (± SEM)	*P* value*
Hypoxanthine	3	93 ± 10	NS
Inosine	3	102 ± 8	NS
2′-Deoxyadenosine	3	91 ± 10	NS
2-Chloroadenosine	5	27 ± 7	<0.0007

2×10^6 cells were incubated with agents for 15 min at 37°C, then stimulated with FMLP (0.1 μM) in the presence of cytochalasin B (5 μg/ml). Control superoxide generation for these experiments was 26.9 ± 5.5 nmol cyto C-reduced/10^6 cells.

* *P* vs. control as determined by Student's *t* test. NS, not significant.

inosine (100 μM) neither increased nor diminished the generation of superoxide anion by FMLP-stimulated neutrophils (Table III).

Adenosine Inhibition of Superoxide Anion Generation is Reversible. Neutrophils were incubated in the presence of 100 μM adenosine for 15 min and ADA added at varying times before stimulation with FMLP. Addition of ADA as late as 1 min before stimulation with FMLP completely eliminated inhibition of superoxide anion generation. Enhancement of superoxide anion generation by ADA was also independent of the duration of incubation with enzyme before stimulation with FMLP (Table IV).

Structural Requirements for Adenosine Inhibition of Superoxide Anion Generation. To investigate the structural requirements for adenosine to inhibit generation of superoxide anion generation, the effects of two adenosine analogs were determined. 2-Chloroadenosine, a synthetic analog modified on the purine ring, resembled adenosine with respect to inhibition of superoxide anion generation (Fig. 3 and Table III). Additionally, at low concentrations (0.1 μM) similar to endogenous adenosine concentrations, 2-chloroadenosine, which is not metabolized by ADA (25), reversed the enhancing effects of ADA (98 ± 15% of control superoxide anion generation). In contrast, 2′-deoxyadenosine, a naturally occur-

TABLE IV

Effect of ADA on Superoxide Anion Generation by FMLP-stimulated Neutrophils: Reversibility of Adenosine Inhibition

Treatment and time before stimulation	Percent of control superoxide generation		
	Expt. 1	Expt. 2	Expt. 3
Adenosine 100 μM, ~15 min	46	18	50
Adenosine 100 μM, ~15 min + ADA, ~10 min	157	143	271
ADA, ~10 min	153	139	317
Adenosine 100 μM, ~15 min + ADA, ~5 min	157	137	274
ADA, ~5 min	163	142	296
Adenosine 100 μM, ~15 min + ADA, ~1 min	164	141	301
ADA, ~1 min	164	145	294

2×10^6 cells were incubated at 37°C with agents for the indicated period of time before stimulation with FMLP (0.1 μM) in the presence of cytochalasin B (5 μg/ml). Superoxide generation by cells in the absence of adenosine or ADA was, respectively, 19.5, 31.3, and 12.7 nmol of cyto C-reduced/10^6 cells.

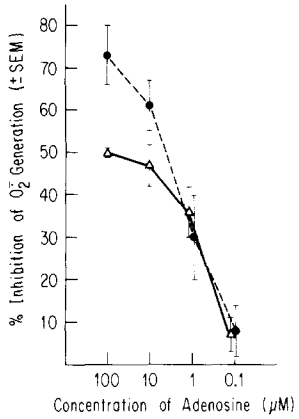

FIGURE 3. Effect of adenosine and 2-chloroadenosine on superoxide anion generation by FMLP-stimulated neutrophils. 2×10^6 neutrophils were stimulated with FMLP (0.1 μM) in the presence of cytochalasin B (5 μg/ml) after a 15 min incubation at 37°C with either adenosine (△) or 2-chloroadenosine (●). All points represent the mean of at least three separate determinations on cells from different donors. The mean control superoxide generation for these experiments was 26.9 ± 5.5 nmol cyto C reduced/10^6 cells.

ring analog modified in the ribose moiety, did not inhibit neutrophil generation of superoxide anion in response to FMLP (Table III).

Effect of Adenosine on Superoxide Anion Generation in Response to A23187, Con A, PMA, and ZTS. To determine whether the effect of adenosine was specific to FMLP, we examined the effect of adenosine on neutrophils stimulated by Con A (30 μg/ml), the ionophore A23187 (5 μM), and the tumor-promoting agent PMA (1 μg/ml). Incubation of neutrophils with adenosine at concentrations varying from 1 to 100 μM in the presence of EHNA (10 μM) led to significant dose-dependent inhibition of superoxide anion generation stimulated by FMLP, Con A, and A23187 (Fig. 4). Generation of superoxide anion in response to PMA (1 μg/ml) was much less affected by adenosine and a dose-dependent relationship could not be demonstrated (Fig. 4). ZTS stimulated neutrophils to

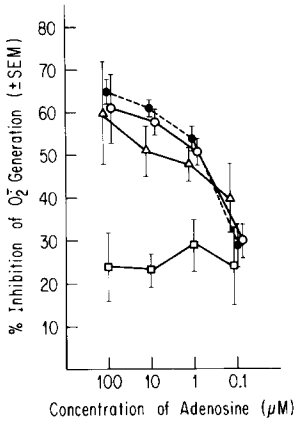

FIGURE 4. Effect of adenosine plus EHNA on superoxide generation by neutrophils stimulated with A23187, PMA, Con A, or FMLP. 2×10^6 neutrophils were stimulated with FMLP (0.1 μM) (●), PMA (1 μg/ml) (□), Con A (30 μg/ml) (○), or A23187 (5 μM) (△), in the presence of cytochalasin B (5 μg/ml) after a 15 min incubation at 37°C in the presence of adenosine and EHNA (10 μM). All points represent the mean of at least three separate determinations on cells from different donors. The mean control superoxide anion generation for these experiments was 16.4 ± 1.3 nmol cyto C reduced/10^6 cells for FMLP-stimulated cells, 21.8 ± 6 nmol cyto C reduced/10^6 cells for PMA-stimulated cells, 13.9 ± 0.7 nmol cyto C reduced/10^6 cells for Con A-stimulated cells, and 16.4 ± 6.5 nmol cyto C reduced/10^6 cells for A23187-stimulated cells.

generate superoxide anion (3.6 ± 0.7 nmol cyto C reduced/10^6 cells). Adenosine (100 μM) plus DCF (10 μM) inhibited this response by 52 ± 10% ($n = 7$, $P < 0.001$) and ADA increased generation of superoxide anion from 134 to 319% of control.

Adenosine Does Not Affect Neutrophils by Inhibition of Transmethylation Reactions. The addition of homocysteine thiolactone (100 μM) to EHNA (10 μM) and adenosine has previously been shown to inhibit generation of superoxide anion by murine macrophages in response to FMLP (11). This combination of compounds inhibited transmethylation reactions and diminished the avidity of receptors for FMLP in these cells. Neutrophils were therefore incubated with adenosine and EHNA (10 μM) with or without homocysteine thiolactone. In contrast to findings in murine macrophages, the addition of homocysteine did not increase the inhibition of superoxide anion generation over and above that found in the presence of adenosine and EHNA alone (Fig. 5).

Cellular Uptake of Adenosine Is Not Required For Inhibition of Superoxide Anion Generation. Dipyridamole (10 μM) inhibits adenosine transport in a variety of cells (26). When this agent was added to suspensions of neutrophils 5 min before the addition of labeled adenosine (0.1 μM), uptake of labeled adenosine from the medium by neutrophils was completely inhibited (Table V). When higher concentrations (0.25–0.5 μM) of labeled adenosine were added, no consistent inhibition of uptake could be demonstrated (data not shown). Essentially all radioactivity counted in these supernates was present as adenosine and little or none was found as inosine, hypoxanthine, or adenine nucleotides. Although dipyridamole blocked uptake of adenosine from the medium, it did not appear to inhibit efflux of adenosine from neutrophils into the medium, since the

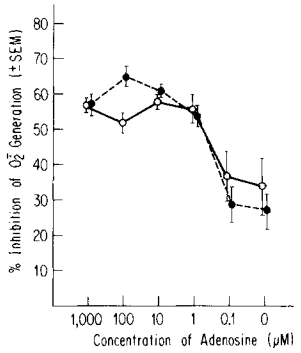

FIGURE 5. Effect of adenosine, EHNA (10 μM), and homocysteine thiolactone (100 μM) on superoxide anion generation by FMLP-stimulated neutrophils. 2 × 10^6 neutrophils were stimulated with FMLP (0.1 μM) in the presence of cytochalasin B (5 μg/ml) after a 15 min incubation at 37°C in the presence of adenosine plus EHNA (10 μM) (●) or adenosine plus EHNA (10 μM) plus homocysteine thiolactone (100 μM) (○). All points represent the mean of at least three separate determinations on cells from different donors. The control superoxide anion generation for these experiments was 16.4 ± 1.3 nmol cyto C reduced/10^6 cells.

TABLE V

Extracellular ^{14}C-Adenosine Remaining in the Medium After Incubation With Neutrophils in the Presence and Absence of Dipyridamole

	n	Counts per minute (±SEM)	Percent blank (±SEM)	*P* value
Blank	3	3,050 ± 82		
PMN + 0.1 μM adenosine	3	1,335 ± 338	45 ± 12	<0.03*
PMN + 0.1 μM adenosine + 10 μM dipyridamole	3	2,791 ± 225	92 ± 7	NS‡

2 × 10^6 cells were incubated with dipyridamole (10 μM) for 5 min at 37°C. ^{14}C-adenosine was added and the cells incubated for 10 min before stimulation with FMLP (0.1 μM) in the presence of cytochalasin B (5 μg/ml). Supernatants were collected and assayed as described in Materials and Methods.

* PMN + adenosine vs. PMN + adenosine + dipyridamole (Student's *t* test).

‡ PMN + adenosine + dipyridamole vs. blank (Student's *t* test).

preincubation of neutrophils with dipyridamole led to much greater concentrations of adenosine (labeled and unlabeled) in the medium (Table VI). Since dipyridamole did not inhibit transport at higher concentrations of adenosine, the drug does not appear to affect viability. When neutrophils were incubated with high concentrations of adenosine (10 μM) after preincubation with dipyridamole (10 μM) there was no significant effect on the generation of superoxide anion by neutrophils stimulated with FMLP (Fig. 6). At a concentration of added adenosine at which uptake was blocked (0.1 μM), dipyridamole significantly enhanced the effect of adenosine on superoxide anion generation. Dipyridamole (10 μM) had no significant effect on superoxide anion generation by neutrophils stimulated with FMLP in the presence of buffer alone. Similarly, the enhancement of superoxide anion generation found after incubation of neutrophils with

TABLE VI

Adenosine Concentration in Media of FMLP-stimulated Neutrophils After Incubation in the Presence or Absence of Adenosine and Dipyridamole (DIP)

Concentration of adenosine added	Concentration of adenosine recovered			
	Experiment 1		Experiment 2	
	−DIP	+DIP	−DIP	+DIP
μM		μM		
0.10	0.12	0.42	0.18	0.58
0.00	0.11	0.29	0.18	0.55

Neutrophils (2×10^6/ml) were incubated with buffer or buffer containing dipyridamole (final concentration, 10 μM) for 5 min at 37°C. Equal volumes of buffer or buffer containing adenosine at the indicated concentrations were added and cells were incubated for 10 min at 37°C. Cells were stimulated with FMLP (0.1 μM) in the presence of cytochalasin B (5 μg/ml). Supernatants were collected and adenosine concentration measured by high pressure liquid chromatography as described in Materials and Methods.

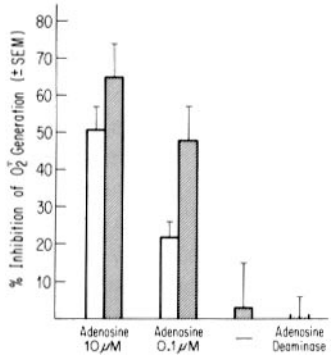

FIGURE 6. Effect of adenosine on superoxide anion generation by FMLP-stimulated neutrophils after blockade of adenosine uptake. 2×10^6 neutrophils were incubated for 5 min at 37°C in buffer alone (open bar) or in the presence of dipyridamole (10 μM) (hatched bar). Adenosine, at the indicated concentration, an equal volume of buffer, or buffer containing ADA was added and cells were incubated for 10 min at 37°C. The cells were then stimulated with FMLP (0.1 μM) in the presence of cytochalasin B (5 μg/ml). Results are expressed as the percent inhibition of control superoxide anion generation. Mean control superoxide generation for these experiments was 14.2 ± 1.3 nmol cyto C reduced/10^6 cells and 21.4 ± 3.8 nmol cyto C reduced/10^6 cells for ADA-treated cells.

ADA was not affected by dipyridamole.

Adenosine Inhibits Superoxide Anion Generation in the Absence of Cytochalasin B. Each of the previous experiments had been done in the presence of cytochalasin B in order to enhance superoxide generation and lysosomal enzyme release. Essentially similar results were obtained from experiments performed in the absence of cytochalasin B. Adenosine inhibited generation of superoxide anion by non–cytochalasin B-treated neutrophils stimulated with FMLP (Table VII). A dose-response curve of adenosine inhibition was not found in these experiments. ADA treatment of neutrophils also produced enhancement of

TABLE VII

Effect of Adenosine and ADA on Superoxide Anion Generation by FMLP-stimulated Neutrophils in the Presence and Absence of Cytochalasin B

Treatment	Percent of control superoxide generation					
	+cytochalasin B	*n*	*P* value*	−cytochalasin B	*n*	*P* value*
Adenosine 100 μM	50 ± 1	4	<0.006	29 ± 11	3	<0.015
Adenosine 10 μM	53 ± 5	4	<0.006	26 ± 11	3	<0.015
Adenosine 1 μM	64 ± 6	5	<0.006	23 ± 8	3	<0.009
Adenosine 0.1 μM	93 ± 4	4	NS	27 ± 16	3	<0.03
ADA	155 ± 18	12	<0.03	251 ± 23	4	<0.015

2×10^6 cells were incubated for 15 min at 37°C in the presence of adenosine or ADA with and without cytochalasin B (5 μg/ml) before stimulation with FMLP (0.1 μM). Mean control superoxide anion generation in the presence of cytochalasin B was 16.4 ± 1.3 nmol cyto C reduced/10^6 cells. Mean control superoxide anion generation in the absence of cytochalasin B was 5.4 ± 1.4 nmol of cyto C-reduced/10^6 cells.

* *P* vs. control as determined by Student's *t* test.

superoxide anion generation in the absence of cytochalasin B. Since stimulated neutrophils generated far less superoxide anion in the absence of cytochalasin B, the extent of inhibition or enhancement cannot be quantitatively compared with the results obtained in the presence of cytochalasin B.

ADA Enhanced Superoxide Anion Generation in the Presence of Glucose. One possible source of endogenous adenosine in our preparations was breakdown of intracellular ATP in neutrophil suspensions incubated without glucose. The addition of glucose (5 mM) did not enhance superoxide anion generation by FMLP-stimulated neutrophils. When cells were incubated with ADA in the presence of glucose, there was 161 ± 15% of control superoxide anion generation (14.7 ± 4.5 nmol cyto C reduced/10^6 cells, $n = 3$). Cells from the same donors incubated with ADA in the absence of glucose generated 187 ± 32% of control superoxide anion (14.6 ± 5.0 nmol cyto C reduced/10^6 cells).

Controls for Viability and Superoxide Anion Generation. Adenosine, 2-chloroadenosine, and ADA had no effect on cell viability, as determined by LDH release. In three separate experiments, control cells released 3.0 ± 0.9%, and adenosine-, 2-chloroadenosine-, and ADA-treated cells released 3.2 ± 1.0%, 2.5 ± 1.2%, or 3.3 ± 0.7%, respectively, of total cell LDH. Nor did these three compounds affect superoxide anion generation in the simple in vitro generating system using hypoxanthine and xanthine oxidase. In the presence of adenosine, 2-chloroadenosine, and ADA, there was 99 ± 1, 87 ± 10, and 107 ± 3%, respectively, of control superoxide generation (23 ± 6 nmol of cyto C reduced).

Effect of Adenosine on Lysosomal Enzyme Release and Aggregation. Release of lysosomal enzymes and aggregation by neutrophils in response to FMLP (0.1 μM) was much less affected by adenosine, ADA, or 2-chloroadenosine than was the generation of superoxide anion. No statistically significant effect of adenosine (100 μM or 10 μM) plus EHNA (10 μM), ADA, or 2-chloroadenosine on lysozyme release could be demonstrated (Table VIII). Beta-glucuronidase release was modestly inhibited by 100 μM or 10 μM adenosine plus 10 μM EHNA as well as by 100 μM 2-chloroadenosine (Table VIII). ADA did not have a significant effect on beta-glucuronidase release. Neither the rate nor the extent of neutrophil

TABLE VIII

Effect of Adenosine, 2-Chloroadenosine, and ADA on Release of Beta-Glucuronidase and Lysozyme by FMLP-stimulated Neutrophils

Treatment	*n*	Percent of control release of:	
		Lysozyme	Beta-glucuronidase
Adenosine 100 μM + EHNA 10 μM	4	79 ± 11	73 ± 6*
Adenosine 10 μM + EHNA 10 μM	4	99 ± 9	75 ± 9‡
2-Chloroadenosine 100 μM	4	88 ± 10	70 ± 6§
ADA	4	150 ± 30	135 ± 19

2×10^6 cells were incubated at 37°C with agents for 15 min before stimulation with FMLP (0.1 μM) in the presence of cytochalasin B (5 μg/ml). Supernatants were collected and assayed as described in Materials and Methods.

* $P < 0.001$ vs. control as determined by Student's *t* test.

‡ $P < 0.01$ vs. control (Student's *t* test).

§ $P < 0.02$ vs. control (Student's *t* test).

TABLE IX

Effect of Adenosine, 2-Chloroadenosine, and ADA on Aggregation of Human Neutrophils Stimulated by FMLP

Agent	Percent of control aggregation			
	Experiment I		Experiment II	
	+Cyto B	−Cyto B	+Cyto B	−Cyto B
Adenosine 100 μM	90	97	88	110
2-Chloroadenosine 100 μM	91	88	87	88
Adenosine deaminase 0.125 IU/ml	93	91	89	78

0.25×10^6 cells were incubated with agents in the presence or absence of cytochalasin B (cyto B) (5 μg/ml) for 3 min before stimulation with FMLP (0.1 μM). Aggregation was measured as described in Materials and Methods.

aggregation induced by FMLP was altered by adenosine, 2-chloroadenosine, or ADA (Table IX).

Discussion

These experiments demonstrate that adenosine preferentially inhibited generation of superoxide anion by neutrophils stimulated with FMLP, Con A, A23187, and ZTS. The effects of adenosine were evident at concentrations ranging from 1 to 1,000 μM with maximal inhibition at 100 μM. Cellular uptake of adenosine was not required for adenosine-induced inhibition since inhibition was maintained despite the addition of dipyridamole, which blocks nucleoside uptake. Nor was deamination of adenosine required for inhibition since both DCF and EHNA, inhibitors of ADA, did not interfere with adenosine inhibition of superoxide anion generation. Further substantiation that the metabolism of adenosine was not necessary for inhibition was provided by the inhibitory effect of 2-chloroadenosine, which is not significantly metabolized (25).

The most striking finding in this study was that endogenously generated adenosine was present in the extracellular milieu in amounts that inhibited superoxide anion generation. Adenosine was present in the media of suspensions

of neutrophils at concentrations of 0.1–0.3 μM. Elimination of this adenosine by incubation of cells with exogenous ADA led to marked enhancement of neutrophil generation of superoxide anion in response to FMLP. Since inhibition of ADA with DCF abrogated the enhancement (and prevented degradation of adenosine), the enhancement was not due to a nonspecific effect of added protein. Nor was this enhancement due to the generation of hypoxanthine or inosine by the deamination of adenosine, since the addition of these compounds at 100 μM did not affect neutrophil superoxide generation. The increase of superoxide anion generation that we found after removal of low levels of endogenously generated adenosine was unexpected and suggested that previous measurements performed under similar conditions underestimated the capacity of neutrophils to generate superoxide anion in response to various ligands.

In vivo plasma concentrations of adenosine are at least 0.3 μM (27). The concentrations of adenosine present in plasma are greater than or equal to those we have found in vitro in neutrophil suspensions. Since removal of these lower concentrations of adenosine found in vitro enhanced generation of superoxide anion by neutrophils, the concentrations of adenosine found in plasma are sufficient to exert an inhibitory effect in vivo.

An equilibrium between cellular uptake of adenosine and efflux of adenosine into the medium must be present in these cell preparations. Despite the disappearance from the medium of more than half of the labeled adenosine added to neutrophil suspensions, higher concentrations of adenosine than those added were found in these supernates. Adenosine could have been released into the extracellular medium by contaminating platelets, disrupted cells, or the intracellular metabolism of ATP to adenosine. Borregaard and Herlin (28) have recently demonstrated the presence of large concentrations of adenine nucleotides in human neutrophils (1.9 fmol ATP/cell). The cellular ATP concentration represents an equilibrium between catabolism to adenosine and adenine nucleotides and phosphorylation of these compounds (0.75 fmol ATP generated/cell/min) (28). These fluxes of adenosine and adenine nucleotides could result in leakage of relatively modest amounts of adenosine into the extracellular milieu as well as uptake of adenosine from the surrounding medium. The concentrations of adenosine found in the extracellular medium of these suspensions represents ~5% of the cellular ATP pool. Massive catabolism of ATP to adenosine in these preparations was not a likely source of extracellular nucleoside since glucose, an exogenous energy source able to prevent the large scale catabolism of ATP in neutrophils, did not affect the ability of ADA to enhance superoxide anion generation (29). Although, in vitro, neither platelets, disrupted cells, nor fluxes in nucleotide metabolism were eliminated as a source of adenosine in these preparations, in vivo, multiple tissue sources are able to contribute to adenosine concentrations in plasma (30).

The combination of adenosine, EHNA, and homocysteine thiolactone has been shown to inhibit transmethylation reactions in murine macrophages (11). Transmethylation reactions have been shown to be necessary for maintenance of high avidity receptors for FMLP in these cells. Thus, when transmethylation reactions were inhibited by adenosine, EHNA, and homocysteine thiolactone, responses to FMLP, such as the generation of superoxide anion, were found to

be diminished. We did not find any additional effect of homocysteine thiolactone on the generation of superoxide anion by neutrophils. Although we used concentrations of adenosine, EHNA, and homocysteine thiolactone that inhibit transmethylation reactions in murine macrophages (11), we did not demonstrate directly that transmethylation reactions were inhibited. Pike and Snyderman (31) have also been unable to demonstrate that inhibition of transmethylation reactions altered the interactions of human neutrophils with FMLP.

Our experiments suggest that adenosine exerted its effects at the cell surface, possibly at a specific receptor, since addition of an agent shown to block adenosine uptake did not interfere with the inhibitory effect of exogenous adenosine or with the enhancing effect of removal of endogenous adenosine. Indeed, dipyridamole increased the inhibition of superoxide anion generation at a low concentration of exogenous adenosine at which it completely inhibited uptake of the nucleoside. Uptake of adenosine does not appear to be a requirement for inhibition of neutrophil function, which is strong evidence in support of an effect at the cell surface.

Adenosine had no effect on neutrophil aggregation or secretion of lysozyme and only minimal effect on release of beta-glucuronidase. These experiments indicated that adenosine did not affect the entire sequence of stimulus-response coupling in the neutrophil but exerted, instead, a selective effect on generation of superoxide anion generation. These findings agree with those of Marone et al. (14) who were unable to demonstrate significant inhibition by adenosine of beta-glucuronidase release from neutrophils that had been exposed to particles.

Based on observations in murine macrophages, Tritsch and Niswander (32, 33) have proposed that fluxes through the purine salvage pathway are essential for the generation of superoxide anion generation. They suggested that the activity of intracellular ADA, the primary enzyme in this pathway, was directly correlated with and quantitatively critical for generation of superoxide anion. Thus, an increase in the concentration of adenosine, inosine, or hypoxanthine, the substrates in the proposed cascade, should enhance superoxide anion generation. However, we have shown that superoxide anion generation was inhibited by adenosine and not affected by hypoxanthine or inosine. If ADA were critical for superoxide generation, then inhibition of this enzyme should have inhibited superoxide anion production. We demonstrated minimal inhibition of superoxide anion generation by EHNA and no inhibition by DCF. Therefore, intracellular fluxes through the purine salvage pathway cannot be directly involved in superoxide anion generation by the human neutrophil.

Although adenosine was a potent inhibitor of neutrophils stimulated by A23187, Con A, FMLP, and ZTS, it had minimal effect on superoxide anion generation in response to PMA. This is not surprising since PMA activates neutrophils by mechanisms that differ from other stimuli (e.g., lack of enhancement by extracellular calcium [35] and different patterns of membrane remodelling [35]). The data suggested that activation of the superoxide anion-generating system by A23187, Con A, FMLP, and ZTS but not by PMA involves an adenosine-sensitive step, the nature of which is as yet undetermined.

The most consistent explanation for our results is that a receptor for adenosine is present on human neutrophils. Two major types of adenosine receptors have

been described (36). The P receptor, which is best demonstrated in cell lysates, is active at high (millimolar) concentrations of adenosine and is specific for adenosine analogs that have not been modified in the purine portion of the molecule. The R receptor is active in whole cells, present in cell membranes, active at low concentrations of adenosine, and is specific for adenosine compounds that have not been modified in the ribose portion of the molecule. The inhibition of superoxide anion generation by adenosine satisfies a number of criteria for receptor-ligand interactions including the following: (*a*) adenosine acts at the cell surface; (*b*) there is a maximum inhibition achieved despite increasing adenosine concentration, i.e., saturability; and (*c*) adenosine and 2-chloroadenosine are equally effective inhibitors for neutrophils, whereas 2′-deoxyadenosine is not, indicating structural specificity. The dramatic effect of 2-chloroadenosine and the lack of inhibition of neutrophils by 2′-deoxyadenosine would argue strongly for the presence of an R receptor. We have not, as yet, undertaken binding studies of labeled ligand to neutrophils to further investigate this hypothesis.

The products of activated neutrophils are potentially toxic to the host. The existence of circulating antiproteases and inactivators of active oxygen species attests to the need for protection from inflammatory mediators released by neutrophils. It is not surprising, therefore, that a mechanism exists to regulate the production of superoxide anion. We have demonstrated the role of adenosine, at physiological concentrations, as a specific inhibitor of superoxide anion generation by stimulated neutrophils. We conclude that alterations of local adenosine concentrations may influence the generation of potentially toxic oxygen metabolites by neutrophils.

Summary

The effects of adenosine were studied on human neutrophils with respect to their generation of superoxide anion, degranulation, and aggregation in response to soluble stimuli. Adenosine markedly inhibited superoxide anion generation by neutrophils stimulated with *N*-formyl methionyl leucyl phenylalanine (FMLP), concanavalin A (Con A), calcium ionophore A23187, and zymosan-treated serum; it inhibited this response to PMA to a far lesser extent. The effects of adenosine were evident at concentrations ranging from 1 to 1,000 μM with maximal inhibition at 100 μM. Cellular uptake of adenosine was not required for adenosine-induced inhibition since inhibition was maintained despite the addition of dipyridamole, which blocks nucleoside uptake. Nor was metabolism of adenosine required, since both deoxycoformycin (DCF) and erythro-9-(2-hydroxy-3-nonyl) adenine did not interfere with adenosine inhibition of superoxide anion generation. The finding that 2-chloroadenosine, which is not metabolized, resembled adenosine in its ability to inhibit superoxide anion generation added further evidence that adenosine metabolism was not required for inhibition of superoxide anion generation by neutrophils. Unexpectedly, endogenously generated adenosine was present in supernatants of neutrophil suspensions at 0.14–0.28 μM. Removal of endogenous adenosine by incubation of neutrophils with exogenous adenosine deaminase (ADA) led to marked enhancement of superoxide anion generation in response to FMLP. Inactivation of ADA with

DCF abrogated the enhancement of superoxide anion generation. Thus, the enhancement was not due to a nonspecific effect of added protein. Nor was the enhancement due to the generation of hypoxanthine or inosine by deamination of adenosine, since addition of these compounds did not affect neutrophil function. Adenosine did not significantly affect either aggregation or lysozyme release and only modestly affected beta-glucuronidase release by neutrophils stimulated with FMLP. These data indicate that adenosine (at concentrations that are present in plasma) acting via cell surface receptors is a specific modulator of superoxide anion generation by neutrophils.

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