ADENOSINE PROMOTES NEUTROPHIL CHEMOTAXIS

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Adenosine modulates the function of a wide variety of cells by engaging specific receptors on the cell surface. We have recently demonstrated the presence of adenosine receptors on human neutrophils and shown that engagement of adenosine receptors on neutrophils inhibits superoxide and hydrogen peroxide production, adherence to endothelial cell monolayers, and killing of cultured endothelial cells by stimulated neutrophils (1–4). We and subsequently others have shown that adenosine inhibits neutrophil function by engaging a specific subset of adenosine receptors, A_2 receptors (2, 5–7). There are two types of cell surface adenosine receptors, A_1 and A_2, which are differentiated by their affinity for adenosine, the order of potency of adenosine analogues and, in many studies, opposing effects on cellular cAMP metabolism (8–10). Methylxanthines are competitive antagonists at both A_1 and A_2 receptors.

We now report the surprising observation that, in marked contrast to the inhibition of neutrophil functions that we have previously described, adenosine and its analogues promote neutrophil chemotaxis without affecting chemokinesis. Moreover, adenosine enhances chemotaxis by engaging A_2 receptors as indicated by the order of agonist potency. The paradoxical effect of adenosine A_2 receptor engagement on stimulated neutrophil function, inhibition of superoxide anion generation but promotion of chemotaxis, suggests that engagement of chemoattractant receptors stimulates movement and the respiratory burst by divergent pathways.

Materials and Methods

Materials. Adenosine, 2-chloroadenosine, Weigert's hematoxylin stain, and human serum albumin were obtained from Sigma Chemical Co. (St. Louis, MO). FMLP was obtained from Vega Biochemicals (Tuscon, AZ). N^6-(L)-phenylisopropyladenosine (PIA)[1] was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN). 5′-*N*-ethylcarboxamidoadenosine (NECA) and 8-*p*-sulfophenyltheophylline (8-PST) were obtained from Research Biochemicals, Inc. (Wayland, MA). Agarose was obtained from Bio-Rad Laboratories (Rockville Center, NY). Zymosan was obtained from ICN Biomedicals, Inc.

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[1] *Abbreviations used in this paper:* EC_{50}, concentration at which 50% effect occurs; NECA, 5′-*N*-ethylcarboxamidoadenosine; PIA, N^6-L-phenylisopropyladenosine; 8-PST, 8-*p*-sulfophenyltheophylline.

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(Cleveland, OH). Nitrocellulose filters were obtained from Millipore Corp. (Bedford, MA). Tissue culture plates were manufactured by Becton Dickinson Labware (Lincoln Park, NJ). Medium RPMI 1640 (10×) and sterile $NaHCO_3$ solution (7.5%) were obtained from Gibco Laboratories (Grand Island, NY).

Isolation of Neutrophils. Human neutrophils were isolated from heparinized whole blood by centrifugation through Ficoll-Hypaque and sedimentation through dextran (6% wt/vol) followed by hypotonic lysis of erythrocytes. This procedure allows study of populations of cells that are 98 ± 2% neutrophils with few contaminating erythrocytes or platelets (11). Neutrophils were suspended for all of the experiments reported here in PBS containing magnesium (0.5 mM) and calcium (0.9 mM).

Generation of Zymosan-activated Plasma (ZAP). Heparinized plasma was incubated with zymosan (1 mg/ml) for 30 min at 37°C, centrifuged at 1,000 *g* for 10 min, and the supernatant was collected and kept on ice until use. For use in chemotactic assays the ZAP was diluted 1:10 with incubation buffer (12).

Chemotaxis Assays. Chemotaxis through a filter was measured using the modified Boyden chamber technique of Zigmond and Hirsch (13) as modified by Axelsson et al. (14). Neutrophils (6×10^5) were preincubated at 37°C for 10 min with or without adenosine, adenosine analogues, and/or 8-PST and were then layered onto nitrocellulose filters (3-μm pore size) overlying chemotactic stimuli (e.g., ZAP, 10%) in an apparatus described by Axelsson et al. (14; a gift from Pharmacia AB, Uppsala, Sweden). The cells were then incubated for 35 min at 37°C in a 5% CO_2 atmosphere, after which the filters were removed, stained with hematoxylin, and mounted on microscope slides. The extent of chemotaxis was determined by the leading front technique. Before analysis the original codings on the slides were covered over and the slides coded so that the reader was unaware of the condition being examined. Each condition was tested in triplicate or quadruplicate and leading fronts were measured on three sites per filter. Random migration of cells towards buffer alone, chemokinesis, was subtracted from the distance travelled by cells towards the chemotactic stimulus, chemotaxis. Chemotaxis by cells incubated with adenosine or its analogues is expressed as percent of control chemotaxis. Data were then fit to a curve by means of the ALLFIT computer program on an Apple IIe desktop computer (15; Dr. Carl Johnson, University of Cincinnati College of Medicine, Cincinnati, OH).

Chemotaxis under agarose was measured by a modification of the method of Nelson et al. (16). Agarose (2% wt/vol, final) was dissolved in water and then mixed with an equal volume of a solution containing RPMI 1640 (2×) and human serum albumin (1.5% wt/vol) adjusted to a pH of 7.4 with $NaHCO_3$ solution (7.5%, wt/vol). The final solution was layered onto tissue culture plates and permitted to solidify. A series of three wells 3 mm apart were cut into the agarose using a template. An aliquot (10 μl) of a neutrophil suspension (3×10^7/ml) was placed in the center well and either buffer or a solution of FMLP was placed in the flanking wells. In preliminary experiments we determined that the optimal concentration of FMLP for chemotaxis was 10^{-7} M. Each condition was studied in triplicate. The plates were then incubated for 3–4 h at 37°C in a 5% CO_2 atmosphere, then fixed with a solution of gluteraldehyde (2%) and, after removal of the agarose, the cells were stained with Weigert's hematoxylin for 2 min. The plates were studied using an inverted microscope with the image projected onto a screen at a magnification of ×200. Chemotaxis was determined by quantitating the number of cells contained in an area measuring 25 × 25 mm at varying distances from the origin along the shortest line connecting the wells containing cells and chemoattractant. Each box was assigned a relative value (ranging from 1–6), depending upon its distance from the well containing the cells. Chemokinesis was determined similarly in the direction of the well containing buffer. A chemotactic index was calculated by multiplying the cells in each box by the appropriate weighting figure, calculating the sum of the weighted number of cells, and then subtracting the sum calculated for chemokinesis from the sum derived for chemotaxis. The data are expressed as a percentage of control.

Chemokinesis Determinations. Neutrophil chemokinesis was assessed using the checkerboard analysis technique of Zigmond and Hirsch (13). Buffer or varying concentrations

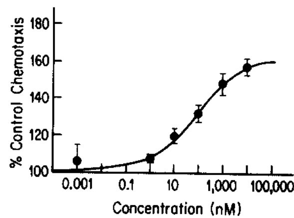

FIGURE 1. Adenosine enhances neutrophil chemotaxis. Neutrophils (6 × 10^5) were incubated for 10 min at 37°C with or without adenosine before introduction into the modified Boyden chamber. Chemotaxis to ZAP (10%) was quantitated as described in Materials and Methods. Each condition was tested in quadruplicate and leading fronts were measured on three sites per filter. Migration by cells incubated with adenosine is expressed as percent control chemotaxis. Points represent the mean percent control chemotaxis of three or four separate determinations on cells from different donors. The mean chemotactic index of control cells in these experiments was 52 ± 10 μm (± SEM). Dose-response curves were generated using the ALLFIT program.

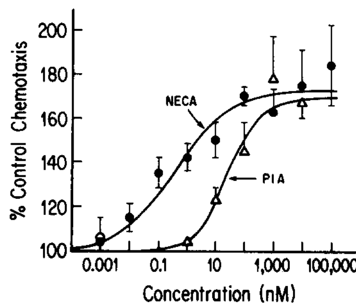

FIGURE 2. The adenosine analogues NECA and PIA enhance neutrophil chemotaxis. Neutrophils (6 × 10^5) were incubated for 10 min at 37°C with or without NECA (○) or PIA (△) before introduction into the modified Boyden chamber. Chemotaxis to ZAP (10%) was quantitated as described in Materials and Methods. Each condition was tested in quadruplicate or triplicate and leading fronts were determined on three sites per filter. Migration by cells incubated with NECA or PIA is expressed as percent control chemotaxis. Points represent the mean percent control chemotaxis of separate determinations on cells from different donors (NECA: n = 3–7; PIA: n = 3–4). The mean chemotactic index of control cells was 52 ± 5 and 47 ± 4 μm in the experiments studying the effect of NECA and PIA, respectively. Dose-response curves were generated using the ALLFIT program.

of NECA or 2-chloroadenosine were used on either side of the nitrocellulose filter (3-μm pore size) in the modified Boyden chamber. The neutrophils (6 × 10^5) were layered onto the nitrocellulose filters and migration was assayed as described above. Each condition was tested in duplicate and leading fronts were measured on three sites per filter.

Calculation of EC_{50} of Adenosine and Analogues for Chemotaxis. Enhancement data were fitted to a curve by the ALLFIT program on an Apple IIe desktop computer and the EC_{50} was calculated for the appropriate curves by means of the computer program (15).

Statistical Analysis of Variance between Dose-Response Curves of Promotion of Chemotaxis by Adenosine and its Analogues. F values were derived by use of the following formula: $[(SS_a\text{-}SS_b)/(df_a\text{-}df_b)]/(SS_b/df_b)$, where SS_a is the sum of the squares of the differences when the curve is forced to conform to external constraints, SS_b is the sum of the squares of the differences of the unconstrained curve, and df is degrees of freedom (17).

Results

Adenosine Promotes Neutrophil Chemotaxis. In our initial experiments we studied the effect of varying concentrations of adenosine on neutrophil chemotaxis stimulated by ZAP. We found that concentrations of adenosine ranging from 1 to 10,000 nM enhanced chemotaxis in a dose-dependent manner (Fig. 1).

Adenosine Enhances Chemotaxis by Engaging A_2 Receptors. To determine whether adenosine promotes chemotaxis by engaging an A_1 or an A_2 receptor, we next examined the effects of NECA, the most potent A_2 agonist, and PIA, a potent A_1 agonist, on neutrophil chemotaxis. NECA and PIA both enhanced chemotaxis in a dose-dependent manner (Fig. 2) with similar maximal enhancement. However NECA was a significantly more potent promoter of chemotaxis than either PIA or adenosine ($p < 0.01$, Fig. 2, Table I). Adenosine did not differ significantly from PIA with respect to promotion of chemotaxis. Thus, the

TABLE I
Adenosine Enhances Chemotaxis by Engaging an A2 Receptor

Adenosine analogue	EC_{50}	Maximal enhancement
	nM	%
NECA	00.34 ± 00.26	72 ± 6
PIA	25.00 ± 18.00	69 ± 11
Adenosine	59.00 ± 46.00	60 ± 7

Mean chemotaxis, as a percentage of control, was calculated at each concentration used and the data were fit to a curve by means of the ALLFIT program. The EC_{50} (concentration of ligand required to produce 50% of maximal enhancement of chemotaxis) and maximal enhancement figures were calculated from the dose-response curves by means of the computer program. The dose-response curves are shown in Figs. 1 and 2 and represent the results of three to seven separate determinations using the cells from different donors.

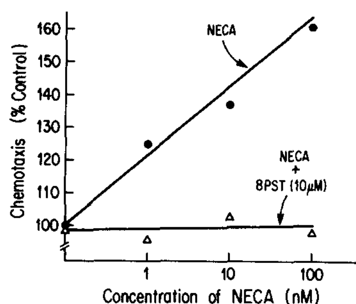

FIGURE 3. 8-PST reverses NECA-mediated enhancement of chemotaxis. Neutrophils (6 × 10^5) were incubated for 10 min with buffer, NECA at the indicated concentrations and/or 8-PST (10 μM). Chemotaxis to ZAP (10%) was quantitated as described in Materials and Methods. Each condition was tested in triplicate and leading fronts were determined on three sites per filter. Chemotaxis is expressed as percent control chemotaxis. These results are from a single experiment representative of three experiments. The chemotactic index of control cells in this experiment was 51 ± 3 μm.

order of potency of the adenosine analogues (NECA > PIA ≥ adenosine) is identical to that we had previously found for inhibition of superoxide anion generation and is characteristic of an adenosine A_2 receptor (2).

8-PST Abolishes NECAs Enhancement of Chemotaxis to ZAP. To provide further evidence that adenosine and its analogues act at specific adenosine receptors to enhance chemotaxis, we determined whether 8-PST, a potent adenosine receptor antagonist, could reverse promotion of chemotaxis by adenosine receptor agonists. Alone, 8-PST (10 μM) affected neither chemotaxis (98 ± 4% of control, n = 4) nor chemokinesis (99 ± 5% of control, n = 3). However, 8-PST (10 μM) completely abolished NECA-mediated (1–100 nM) enhancement of chemotaxis from a maximal enhancement of 71 ± 8% (concentration of NECA, 100 nM) to 1 ± 2% enhancement (n = 3, Fig. 3). These results are consistent with the hypothesis that NECA engages an adenosine receptor to enhance chemotaxis.

Adenosine Analogues Do Not Affect Chemokinesis. Increased random movement in the presence of adenosine and its analogues might account for the apparent promotion of chemotaxis. We therefore performed a checkerboard analysis of the effects of NECA on random migration of neutrophils (chemokinesis). NECA alone did not promote random migration of neutrophils nor did it act as a chemotactic agent (Table II). Similar results were obtained when the adenosine

TABLE II
NECA Does Not Promote Chemokinesis

NECA above filter	NECA (μM) below filter					Control (10% ZAP)
	0	0.01	0.1	1.0	10	
μM			*μm*			
0.0	22 ± 2	29 ± 2	17 ± 1		20 ± 1	114 ± 4
0.01	23 ± 2	26 ± 2		23 ± 2	18 ± 1	
0.1	19 ± 4		23 ± 2	23 ± 2	21 ± 1	
1.0		21 ± 1	23 ± 1	18 ± 1		
10.0	23 ± 2	18 ± 1	23 ± 1		21 ± 1	

NECA, at the indicated concentrations, was added to buffer above (with PMNs) and/or below nitrocellulose filters in a modified Boyden chamber. The data are presented as the mean movement through the filter (± SEM) in micrometers and represent the measurement of three sites per filter in two separate filters.

TABLE III
NECA Promotes Chemotaxis to FMLP When Studied Using Chemotaxis under Agarose

Concentration of NECA	Control chemotaxis	
	Exp. 1	Exp. 2
M	%	%
10^{-4}	182	496
10^{-5}	165	526
10^{-6}	175	420
10^{-7}	157	383
10^{-8}	157	578
10^{-9}	ND	245

Neutrophils (3×10^5) were placed in wells cut into the agarose in the presence of buffer or NECA at the indicated concentrations. Chemotaxis to FMLP (10^{-7} M) was determined as described in Materials and Methods. These results represent the means of triplicate determinations. Mean control chemotactic index was 408 and 132 in Exps. 1 and 2, respectively.

receptor agonist 2-chloroadenosine (0.1–100 μM) was used instead of NECA (data not shown).

The Adenosine Analogue NECA Also Promotes Neutrophil Chemotaxis under Agarose. To confirm that engagement of adenosine receptors promotes chemotaxis we studied chemotaxis using a second method, chemotaxis under agarose, and a second chemotactic agent, FMLP. We found that NECA also promoted chemotaxis to FMLP when studied using this technique (Table III). Using this technique we also did not find any effect of NECA on chemokinesis (data not shown).

Discussion

We and others have previously demonstrated that adenosine and its analogues, by engaging a specific receptor on human neutrophils, inhibit a variety of neutrophil functions (1–4, 8–10). Therefore we were surprised when we observed that adenosine promotes rather than inhibits chemotaxis yet does not affect chemokinesis. Even more surprising is the observation that adenosine

promotes chemotaxis by engaging A_2 adenosine receptors; the same receptors that, when engaged, inhibit superoxide anion generation. No other agent, to our knowledge, promotes chemotaxis without affecting chemokinesis yet inhibits superoxide anion generation.

Adenosine is an autocoid that modulates the behavior of a wide range of mammalian cell types. Indeed, adenosine or its analogues have previously been reported to promote sperm motility by a mechanism that may involve cAMP but is probably not mediated by engagement of adenosine receptors (18–19). Moreover, adenosine is chemotactic for porcine endothelial cells, again by a mechanism that is not completely understood (20). The results of the studies reported here and previously suggest that adenosine and its analogues possess the general property of promoting motility. However, adenosine has been demonstrated to promote motility by a receptor-mediated mechanism only in the neutrophil.

Our demonstration that adenosine and its analogues promote chemotaxis conflicts with previous reports on the effect of adenosine on chemotaxis. Nishida et al. (21) found that concentrations of adenosine and PIA (200–2,000 μM) much higher than those used here (1 pM to 100 μM) did not affect neutrophil chemotaxis. Garcia-Castro et al. (22) were also unable to demonstrate that adenosine affected chemotaxis of rabbit neutrophils directly. The discrepancy between our results and those reported by Nishida et al. and Garcia-Castro et al. probably arises from the different methods used to measure chemotaxis by these groups and us. We have used the leading front technique to assess chemotaxis through a nitrocellulose filter whereas Nishida et al. (21) and Garcia-Castro et al. (22) have quantitated the number of cells adherent to the underside of the nitrocellulose filter. This latter method would underestimate chemotaxis if the migrating neutrophils did not remain adherent to the filter. We have previously reported that an adenosine receptor agonist, 2-chloroadenosine, inhibits adherence of stimulated neutrophils to cultured endothelial cells (3). Diminished adherence of neutrophils to the filters due to adenosine or its analogues could also contribute to the inability of previous investigators to detect adenosine-enhanced chemotaxis by quantitating the number of adherent cells on the underside of the filter. Measurement of chemotaxis by determining the leading front of neutrophils within the filter after a shorter incubation period avoids underestimation of chemotaxis due to nonadherence of neutrophils. Furthermore, we confirmed our findings by use of a second technique, chemotaxis under agarose. Finally, we found that adenosine and its analogues promoted chemotaxis to two different stimuli.

The mechanism by which adenosine receptor engagement inhibits superoxide anion generation and adherence to endothelial cells but enhances chemotaxis remains obscure. We have examined several mechanisms by which adenosine receptor engagement could alter signal transduction in the neutrophil. Adenosine does not interfere with stimulated calcium movements in neutrophils. Engagement of adenosine A_2 receptors induces an increase in cAMP content in several cell types (5–7). We and others have been able to demonstrate that both adenosine and NECA induce a rapidly reversible (5 min) increase in neutrophil cAMP content but only in the presence of a phosphodiesterase inhibitor (9, 23). The chemoattractant FMLP also elicits increased intracellular cAMP concentrations

in neutrophils. The increment in neutrophil cAMP concentration that follows stimulation by FMLP is enhanced by NECA in the presence of the phosphodiesterase inhibitor (23). However the role of cAMP in neutrophil motility is only poorly understood, with some groups reporting that agents that increase cellular cAMP content promote chemotaxis while others report the opposite (24–30). Adenosine receptor engagement could lead to alterations in stimulated phospholipid metabolism, activation of intracellular kinases, tyrosinylation of the cytoskeleton or other triggering mechanisms for stimulus response coupling in the neutrophil.

While adenosine is released from many different types of cells or tissue it is released in greater quantity from damaged cells (1, 30–32). We have found that adenosine, at concentrations similar to those found in the circulation (33), possesses the unique ability to promote neutrophil chemotaxis while inhibiting neutrophils from releasing potentially toxic oxygen metabolites into the extracellular milieu. Thus, adenosine may promote migration of neutrophils to sites of infection or tissue necrosis yet prevent the migrating neutrophils from damaging healthy tissues en route.

Summary

We have previously (1–4) demonstrated that adenosine, by engaging specific receptors on the surface of neutrophils, inhibits generation of toxic oxygen metabolites by activated neutrophils and prevents these activated neutrophils from injuring endothelial cells. We now report the surprising observation that engagement of these same neutrophil adenosine receptors *promotes* chemotaxis to C5 fragments (as zymosan-activated plasma [ZAP]) or to the bacterial chemoattractant FMLP. When chemotaxis was studied in a modified Boyden chamber, physiologic concentrations of adenosine promoted chemotaxis by as much as 60%. Adenosine receptor analogues, 5′*N*-ethylcarboxamidoadenosine (NECA) and N^6-phenylisopropyladenosine (PIA), also promoted chemotaxis; the order of agonist potency was consistent with that of an A_2 adenosine receptor (NECA > PIA ≥ adenosine). A potent antagonist at adenosine receptors, 8-*p*-sulfophenyltheophylline (10 μM), completely reversed NECA enhancement of chemotaxis but did not affect chemotaxis by itself. Neither NECA nor 2-chloroadenosine, a nonselective adenosine receptor agonist, alone was chemotactic or chemokinetic by checkerboard analysis. NECA also promoted chemotaxis quantitated by a different technique, chemotaxis under agarose, to the surrogate bacterial chemoattractant FMLP. These data suggest that engagement of adenosine A_2 receptors uniquely modulates neutrophil function so as to promote migration of neutrophils to sites of tissue damage while preventing the neutrophils from injuring healthy tissues en route.

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