Endogenous and Exogenous Adenosine Inhibit Granulocyte Aggregation Without Altering the Associated Rise in Intracellular Calcium Concentration

By Keith M. Skubitz, Nicholas W. Wickham, and Dale E. Hammerschmidt

The effects of adenosine, adenosine deaminase (ADA), and an irreversible ADA inhibitor 2'-deoxycoformycin (DCF) on granulocyte aggregation in response to four different stimuli: the synthetic chemotaxin N-formyl-met-leu-phe (FMLP), zymosan-activated plasma (ZAP), the calcium ionophore A23187, and phorbol myristate acetate (PMA) were studied. Adenosine inhibited granulocyte aggregation in response to 10^{-7} mol/L FMLP in a dose-dependent fashion; inhibition in the presence of 1 μmol/L adenosine was 25% ± 3% (SD) and was 50% (the maximal inhibition observed) with 1 mmol/L adenosine. Quantitatively similar results were obtained when ZAP or A23187 was used as the aggregant but the response to PMA was not affected. ADA not only reversed the inhibition due to adenosine but actually augmented the aggregation to FMLP by 118% ± 9%. Similar results were obtained with ZAP and A23187 but not with PMA. These effects of ADA depended on its enzymatic activity as they could be blocked by preincubation with DCF. Fluorescent measurement of intracellular calcium in fura-2 loaded granulocyte suspensions established that neither adenosine nor ADA affected subsequent FMLP-stimulated calcium responses. Adenosine, therefore, may inhibit granulocyte responsiveness by blocking signal transduction at a point after calcium entry/mobilization but before activation of protein kinase C. Furthermore, the augmentation of responses seen with ADA suggests that endogenous adenosine may be a physiologic autocrine regulator of granulocyte function.

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DENOSINE, at physiologic concentrations, has been reported to alter the response of a variety of cells to stimulation. Recently, adenosine has been found to inhibit superoxide generation by human neutrophils and neutrophil adhesion to umbilical vein endothelial cells. Some adenosine analogues, but not adenosine, have been reported to inhibit lysosomal enzyme release as well. An important neutrophil response to stimulation is aggregation, a process implicated in the pathobiology of a number of diverse clinical syndromes. Limited earlier studies have not shown an effect of adenosine on neutrophil aggregation. To further define the effects of adenosine on neutrophil function, we have studied the effects of adenosine, adenosine deaminase (ADA), and 2'-deoxycoformycin (DCF), an irreversible adenosine deaminase inhibitor, on granulocyte aggregation. We used four different stimuli. Two were receptor-mediated and known to have separate receptors (the synthetic chemotactic agent, N-formyl-met-leu-phe [FMLP], and zymosan activated plasma [ZAP]), and two were agonists that bypass receptor-ligand coupling by different mechanisms (the calcium ionophore, A23187, and 12-0-tetradecanoyl-phorbolmyristate acetate [PMA]). The results demonstrate that adenosine is an inhibitor of granulocyte aggregation in response to FMLP, ZAP, and A23187. Little effect on aggregation in response to PMA was observed. Furthermore, ADA markedly augmented granulocyte aggregation in response to FMLP, ZAP, and A23187 suggesting that release of adenosine by neutrophils, under the conditions of the assay, had a prominent effect on neutrophil function and may serve an autoregulatory function. To further elucidate the mechanism of adenosine action, its effect on calcium flux induced by FMLP was tested; adenosine had no effect on the rise in intracellular-free calcium that follows stimulus activation.

MATERIALS AND METHODS

Granulocyte preparation. Granulocytes were prepared from heparinized (2 U/mL) normal human venous blood by a modification of the method of Hjorth et al, using sedimentation at 1 g in Hestastarch (2%) for 30 minutes at 23°C followed by separation on a two-stage Percoll (Pharmacia, Piscataway, NJ) gradient as previously described. Cells were suspended at appropriate concentrations in Hanks’ balanced salt solution (HBSS), pH 7.4 (MA. B. Products, Bethesda, MD) containing 5 mg/mL human serum albumin (HSA) (HBSS-HSA). Differential cell counts on Wright’s-stained cells revealed >95% polymorphonuclear cells.

Preparation of solutions. Adenosine (Sigma Chemical Co, St Louis) was dissolved in phosphate buffered saline, pH 7.4 (PBS) immediately before use. ADA (type VIII; Sigma) was obtained as a suspension in 3.2 mol/L (NH₄)SO₄ and was diluted in PBS immediately before use. DCF was a gift of the Warner-Lambert Company (Ann Arbor, MI), and was dissolved in PBS immediately before use. FMLP (Peninsula Laboratories Inc, San Carlos, CA) was dissolved in dimethylsulfoxide (DMSO) at 10 mmol/L, diluted in PBS to 1 mmol/L, and stored at –70°C until use. ZAP was prepared as previously described by incubating 2 mg of zymosan (ICN Pharmaceuticals, Cincinnati) per milliliter of heparinized (1 U/mL) human plasma for 30 minutes at 37°C followed by removal of zymosan by centrifugation at 10,000 g for 30 minutes at 4°C. Aliquots were stored at –70°C until use. PMA (Sigma) was dissolved in DMSO at 1 mg/mL and aliquots were stored at –70°C. Appropriate dilutions of PMA were prepared in PBS immediately before use. A23187 (Sigma) was dissolved in DMSO at 10 mmol/L and stored at –70°C until use.

Granulocyte aggregation. Granulocyte aggregation was performed as previously described. Fifty microliters of PBS containing the desired concentrations of adenosine, ADA, and DCF, were added to 400 μL of a granulocyte suspension (1.25 × 10⁷ cells/mL in HBSS-HSA) to give the final concentrations indicated. Aliquots were incubated for 5 minutes at 37°C; 10 μL was removed, to determine the nonadherent cell count. The percent change from the nonadherent cell count was used as the measure of aggregation.

From the Department of Medicine, University of Minnesota Medical School, and the Masonic Cancer Center, Minneapolis.

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Address reprint requests to Keith M. Skubitz, MD, Box 325, University Hospital, Minneapolis, MN 55455.

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HBSS-HSA), which was stirred at 37°C at 900 rpm in a Payton 300B aggregometer/recorder system (Payton Associates, Buffalo). After 2 minutes, 50 µL of HBSS containing the desired stimulus was added and the resulting light transmittance was recorded on a stripchart recorder. The state of granulocyte aggregation was also confirmed by light microscopy of aliquots taken from the aggregometer.

Determination of intracellular free calcium. Cells were loaded with the fluorescent intracellular Ca²⁺ chelator dye, fura-2, and the fluorescence intensity was determined as described.²¹ Briefly, granulocytes (2 x 10⁶/mL) were incubated for 40 minutes with occasional mixing at 23°C in HBSS containing 1 µmol/L of the acetoxy methyl ester of fura-2 (Molecular Probes, Junction City, OR). The cells were then washed with HBSS and resuspended at 10⁶ cells/mL in HBSS at 23°C. Cells were >98% viable after loading as determined by trypan blue dye exclusion. Three milliliters of cells were then placed in a thermostatically controlled (37°C) cuvette holder in a Spex model 1990 Fluorolog 2 scanning spectrophotometer (Spex Industries, Edison, NJ). The cells were kept in suspension with a magnetic stirrer and equilibrated for six minutes before adding 30 µL of adenosine, ADA, DCF, or HBSS as indicated. In some cases, cells were equilibrated for six minutes in the presence of adenosine or HBSS before the addition of FMLP (30 µL) (10⁻⁷ mol/L final concentration). The change in the ratio (R) of emission signals detected at 512 nm after excitation at 340 nm and 380 nm, respectively, reflects the change in free intracellular Ca²⁺ concentration and was calculated from the formula below using the dissociation constant (Kd) for intracellular fura-2 at 37°C of 224 nmol/L as published by Grynkiewicz et al.²²

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\frac{[Ca^{2+}]}{K_d (R - R_{min})} = \frac{F_{380}}{(R - R)} \frac{F_{340}}{F_{380}}
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The ratio in the presence of saturating concentrations of intracellular calcium (Rmax) was obtained most satisfactorily by permeabilizing the cells with Triton X-100 (Sigma) by the addition of 60 µL of a 1% solution to the cuvette. The ratio in the virtual absence of calcium (Rmin) was obtained using EGTA dissolved in NaOH and TRIS base (Sigma), such that the addition of 60 µL to the cuvette raised the pH to >8.5 with a final concentration of EGTA of 5 to 10 mmol/L.

Statistics. Statistical significance was assessed by paired or unpaired Student's t test, as appropriate; corrected t tests were performed using one-way or two-way ANOVA-derived variances where multiple paired comparisons were available.²⁶⁻²⁹ Significance for nonparametric data and small sample sizes were confirmed, when appropriate by the Mann-Whitney U test.²⁴⁻²⁵

RESULTS

Effects of adenosine, ADA, and DCF on granulocyte aggregation. Adenosine caused a dose-dependent inhibition of granulocyte aggregation in response to FMLP (Fig 1). The degree of inhibition varied among granulocyte donors; the maximal inhibition observed was 54%, and was reliably achieved at or below 1 mmol/L adenosine. In two of ten experiments, complete dose-response curves were obtained, yielding 50% of the maximal inhibition (that observed with 1 mmol/L adenosine) at concentrations of 0.6 and 0.9 µmol/L, respectively. However, in some experiments 30% inhibition was observed at only 0.1 µmol/L adenosine. To confirm that the observed inhibition of granulocyte aggregation was due to adenosine, experiments were performed in which adenosine was preincubated with ADA for two minutes at 23°C before addition to the cells. ADA (0.25 U/mL final concentration in the aggregometer) completely abolished the inhibitory effect of 1 mmol/L adenosine (data not shown). In addition, ADA alone markedly augmented granulocyte aggregation in response to FMLP, suggesting that the presence of endogenous adenosine was limiting cell aggregation (Fig 1). Addition of the ADA inhibitor DCF reversed these effects, confirming that the effect of ADA on granulocyte aggregation was due to its enzymatic effects. The response of the cells to ADA also varied among all donors; the greatest augmentation of aggregation in response to FMLP caused by ADA alone was 330% and the smallest was 136%. Control experiments demonstrated that ADA that had been dialyzed to remove (NH₄)₂SO₄ was similarly effective, and that 0.24 mmol/L (NH₄)₂SO₄ (the concentration present in the ADA solution) had no effect on granulocyte aggregation in response to any of the stimuli used in this study (not shown).

Adenosine also caused a dose-dependent inhibition of granulocyte aggregation in response to ZAP (Fig 2). Again, half maximal inhibition occurred at ~1 µmol/L adenosine and the maximal inhibition observed was ~45%. As with FMLP stimulation, ADA markedly augmented granulocyte aggregation in response to ZAP; DCF abrogated the affect of ADA.

To further characterize the effects of adenosine on granulocyte aggregation we studied two stimuli that bypass receptor-ligand coupling by different mechanisms. Adenosine also caused a dose-dependent inhibition of granulocyte aggrega-
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Fig 2. Adenosine inhibits granulocyte aggregation in response to ZAP. A stirred suspension of granulocytes was preincubated at 37°C for two minutes in the presence of the indicated concentration of adenosine, ADA, and DCF in an aggregometer; ZAP (10% (vol/vol) final concentration) was then added, and granulocyte aggregation was quantitated by measuring the change in light transmittance at two minutes as described in the text. (Left) Dose-response curve for added adenosine. (Right) Bar graphs display experiments in which no exogenous adenosine was added: open bar, 0.25 U/mL ADA; hatched bar, 0.25 U/mL ADA and 5 × 10⁻⁷ mol/L DCF; solid bar, 5 × 10⁻⁷ mol/L DCF. Data are presented as percent of control values obtained in the presence of buffer alone. Each point represents the mean ± SD of three determinations. The inhibition by adenosine is statistically significant at a P value < .001 (F, 5.93). Similarly, augmentation by ADA was significant at P < .001 (F, 16.98). A duplicate complete dose-response experiment gave similar results; additional experiments using fewer doses in each confirmed inhibition of ZAP-stimulated aggregation.

This allowed us to monitor continuously the fluorescence intensity immediately after the addition of FMLP. No difference in either the rate or the extent of the changes in fluorescence intensity following FMLP stimulation could be detected in the presence compared with the absence of adenosine (not shown).

DISCUSSION

Adenosine, at physiologic concentrations, modulates the functions of a variety of cells. 1-8 Adenosine has recently been shown to inhibit superoxide production by human neutrophils. 9-21 In addition, some adenosine analogues, but not adenosine, have been reported to inhibit lysosomal enzyme release by neutrophils. 22 Although adenosine has been found to inhibit neutrophil adhesion to umbilical vein endothelial cells, 23 limited earlier studies have not observed an effect of adenosine on neutrophil aggregation. 24,25 A cellular response that may play a role in the pathophysiology of a variety of clinical syndromes. 26-28 We found that adenosine inhibited granulocyte aggregation in response to both FMLP and ZAP, two ligands that stimulate neutrophils by binding to two distinct surface receptors. Half-maximal inhibition occurred at ~1 μmol/L adenosine, a physiologic concentration also present in many experimental systems. 29,30 Studies of the effect of a variety of adenosine analogs on superoxide generation suggest that the effect of adenosine on neutrophil function is mediated by binding to surface adenosine A2 receptors, which have been identified on human neutro-
and 5 x 10^{-5} \text{mol/L DCF}; \text{solid bar}, 5 \times 10^{-3} \text{mol/L DCF}. Data are presented as percent of control values obtained in the presence of buffer alone. Each point represents the mean \pm SD of three determinations. No statistically significant effect of adenosine, ADA, or DCF on PMA stimulated aggregation could be detected by ANOVA corrected t test. A duplicate complete dose-response experiment gave similar results; additional experiments using fewer doses of adenosine in each also showed no inhibition of PMA-stimulated aggregation.

The exact mechanism whereby adenosine exerts its effect on neutrophil function is not known. While a number of agents that inhibit neutrophil function do alter the binding of the stimulus to its cell surface receptor, adenosine has been found to have no effect on FMLP binding to neutrophils. Increases in intracellular-free calcium that are induced by FMLP and ZAP, but not by PMA, may be an important part of stimulus-response coupling in neutrophils. The results of the present study indicate that adenosine had no detectable effect on the rise in intracellular-free calcium induced by FMLP binding to the neutrophil surface, suggesting that inhibition by adenosine occurred at a step in stimulus-response coupling distal to the calcium transient.

To further characterize the effects of adenosine on granulocyte aggregation, we studied aggregation in response to the calcium ionophore A23187 and the protein kinase C activator, PMA, both of which presumably bypass receptor ligand coupling by different mechanisms. Adenosine inhibited granulocyte aggregation in response to A23187 but not to PMA, which stimulates aggregation without causing an increase in intracellular calcium. This is in agreement with other studies demonstrating inhibition by adenosine of superoxide generation in response to FMLP, ZAP, and A23187, but no effect of adenosine on superoxide generation when PMA was the stimulus. In addition, adenosine has been reported to have no effect on superoxide production by neutrophils stimulated by latex beads, another stimulus reported to act without inducing a rise in intracellular-free calcium.

The reason that the inhibitory effect of adenosine on granulocyte aggregation was not observed in previous studies is unclear. However, we did find marked variability in the magnitude of inhibition observed among different granulocyte preparations. In addition, ADA was found to markedly augment granulocyte aggregation. This suggests that the endogenous release of adenosine by neutrophils has a prominent effect on neutrophil function under the conditions of this assay. The effect of ADA on aggregation was also observed to vary from one cell preparation to another. Thus, differences in the baseline endogenous release of adenosine from different neutrophil preparations may explain earlier findings that adenosine did not influence granulocyte aggregation. These results also suggest that endogenous release of adenosine by neutrophils (and also platelets) may serve an important autoregulatory role in neutrophil function.

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