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Adenosine and 2-Phenylaminoadenosine (CV-1808) Inhibit Human Neutrophil Bactericidal Function

G. E. HARDART,† G. W. SULLIVAN, H. T. CARPER, AND G. L. MANDELL*
Department of Medicine, University of Virginia, Charlottesville, Virginia 22908

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Adenosine is a natural autocoid and immunomodulator that serves an anti-inflammatory role. Stimulation of polymorphonuclear neutrophils (PMN) with soluble stimuli has been shown to inhibit the PMN oxidative burst. We examined the effects of adenosine and the adenosine analog 2-phenylaminoadenosine (CV-1808) on PMN bactericidal function. Adenosine (10 mM) and CV-1808 (10 to 100 μM) inhibited PMN killing of *Staphylococcus aureus*. There were more surviving bacteria after 240 min of incubation of PMN with *S. aureus* and adenosine (10 mM) or CV-1808 (100 μM) (254% ± 45% and 739% ± 88% of control, respectively) ($P < 0.05$) than there were in the control. In contrast, inosine (10 mM), the major degradation product of adenosine, did not affect killing. Adenosine and CV-1808 did not alter cell association of *S. aureus*, but *S. aureus*-activated PMN superoxide release was decreased by adenosine (10 μM) and CV-1808 (10 μM) to 67% ± 7% and 32% ± 12% that of the control, respectively ($P < 0.05$). Since adenosine inhibited PMN bactericidal function only at ~10,000 times peak physiological concentrations, endogenous adenosine levels would not be expected to adversely affect PMN bactericidal function. On the other hand, pharmacological concentrations of adenosine derivatives may decrease the oxidative burst and killing sufficiently to increase host susceptibility to infection.

Adenosine is a ubiquitous autocoid with potent and varied effects in many tissues, including the central nervous system (10), the heart and peripheral vasculature (25), and the immune system (15). Despite the variety of effects seen in these different sites, adenosine is believed to act through adenosine-specific receptors that are common to all these tissues (8, 17). Human polymorphonuclear neutrophils (PMN) have been shown to possess adenosine receptors (20).

Earlier work has shown that adenosine decreases the oxidative burst when PMN are stimulated by soluble stimuli (5–7, 20, 23). Adenosine derivatives are candidates as anti-inflammatory agents because of their ability to suppress the potentially harmful oxidative burst. However, superoxide and hydrogen peroxide, important components of the PMN oxidative burst, are essential for the destruction of certain microorganisms (19). We hypothesized that adenosine, by inhibiting PMN superoxide and hydrogen peroxide production, would inhibit PMN bactericidal function. We examined the effects of adenosine and the adenosine analog 2-phenylaminoadenosine (CV-1808) on the oxidative burst of neutrophils incubated with *Staphylococcus aureus* and quantitated cell association and killing of *S. aureus* by PMN.

MATERIALS AND METHODS

Reagents. Neutrophil isolation media (NIM) was obtained from Los Alamos Diagnostics (Los Alamos, N.M.), *S. aureus* Wood 46 was obtained from the American Type Culture Collection (ATCC 10832) (Rockville, Md.), Hanks' balanced salt solution (HBSS) was obtained from Whittaker Bioproducts (Walkersville, Md.), adenosine, deoxycoformycin, cytochrome *c*, and luminol were obtained from Sigma Chemical Co. (St. Louis, Mo.), and 2-phenylaminoadenosine (CV-1808) and 1,3-dipropyl-8-*p*-sulfophenylxanthine (DPPSX) were obtained from Research Biochemicals Inc. (Natick, Mass.).

PMN preparation. Purified PMN (~98% PMN; >95% viable as determined by trypan blue exclusion) containing less than one platelet per five PMN and <50 pg of lipopolysaccharide per ml (as determined by *Limulus* amebocyte lysate assay) were obtained from normal, heparinized (10 U/ml) venous blood by a one-step Ficoll-Hypaque separation procedure in neutrophil isolation media (11). The PMN were washed three times with HBSS.

Bacterial preparation. *S. aureus* Wood 46 was incubated for 18 h at 37°C in trypticase soy broth. The organisms were then washed with normal saline and adjusted by optical density (580 nm) to 5×10^8 organisms per ml.

Bactericidal assay. (i) Method 1. PMN (5×10^6) were suspended in HBSS (0.9 ml) containing adenosine or CV-1808 at various concentrations. The mixtures were tumbled at 37°C for 15 min before 0.1 ml of autologous serum and 5×10^6 *S. aureus* were added to each. After initial samples were taken and cooled in ice, the mixtures were returned to the tumbler and samples were taken at 60, 120, and 240 min. All samples underwent hypotonic lysis of the PMN in deionized water and were then vortexed for 2 min. Aliquots were plated on trypticase soy agar, and the number of CFU of *S. aureus* was counted after 24 h of incubation at 37°C.

(ii) Method 2. In a second set of experiments, the protocol was altered to minimize the addition of adenosine from the serum and the metabolism of adenosine by the PMN. The experiments were conducted as described for method 1, except that *S. aureus* was opsonized with 20% autologous serum, centrifuged ($2,000 \times g$ for 10 min), and then resuspended in HBSS before addition to the PMN. The PMN were not preincubated with adenosine prior to addition of the bacteria.

Supernatant-sediment differential centrifugation technique. The same basic technique described above for the bactericidal assay (method 1) was employed. However, before being plated on trypticase soy agar, the aliquots were centrifuged at 4°C at $150 \times g$. The resulting sediments were

* Corresponding author.
† Present address: Department of Pediatrics, Johns Hopkins Medical Center, Baltimore, MD 21205.

resuspended in deionized water, vortexed for 2 min, and plated on trypticase soy agar. The supernatants were vortexed for 2 min, and aliquots were plated on trypticase soy agar. The number of CFU of *S. aureus* was counted after 24 h of incubation at 37°C.

PMN oxidative burst. (i) Chemiluminescence. PMN (5×10^6) were incubated for 15 min in HBSS (0.9 ml) with or without adenosine or CV-1808. Each sample was run against a control without adenosine or CV-1808. Autologous serum (0.1 ml) and 2 μl of luminol (5×10^{-2} M in dimethyl sulfoxide) were added to the sample and the control, and both were placed in a Chronolog photometer (Havertown, Pa.) equipped with matched phototubes and were stirred at 37°C. Baseline chemiluminescence was measured for 2 min, then 5×10^6 *S. aureus* was added, and the resultant chemiluminescence was measured and recorded. *S. aureus* in the absence of PMN had no detectable chemiluminescence.

(ii) Superoxide release. The PMN and *S. aureus* were prepared as described above for the bactericidal assay (method 2). Catalase (0.062 mg/ml to prevent H_2O_2 reoxidation of cytochrome *c* [21]) and cytochrome *c* (120 μM) were added to the PMN with the *S. aureus*. Matched samples containing superoxide dismutase (200 U per sample) were also prepared. The PMN and *S. aureus* were tumbled (37°C) for 120 min. The samples were iced and centrifuged ($2,000 \times g$ for 15 min). The optical densities of the supernatants were read at 550 nm, and the nanomoles of superoxide dismutase-inhibitable superoxide released per 10^6 PMN in 120 min was calculated with the extinction coefficient of 2.11×10^4 cm^2/mol (27).

Adenosine assay. Samples were prepared as described above for the bactericidal assay (method 1). At the prescribed time intervals, aliquots were placed in a solution containing HBSS and 100 μM deoxycoformycin (to inhibit endogenous PMN adenosine deaminase) and then membrane filtered (pore size, 0.45 μm) to remove remaining cells and bacteria (Acrodisc; Gelman Sciences Inc., Ann Arbor, Mich.). Automated radioimmunoassay for adenosine was then performed by using the Gammaflo system (2). Antibody was prepared as described by Bredehorst et al., except that goats, not rabbits, were used as the antibody donor (1) and ^{125}I-aminobenzyladenosine was used as the radioligand in place of ^{3}H-adenosine (16).

Statistics. Statistical analysis of the difference between two means was evaluated by Student's *t* test. The level of significance was set at $P = 0.05$.

RESULTS

Adenosine and the analog CV-1808 inhibit PMN killing of *S. aureus*. Adenosine (10 mM) increased *S. aureus* survival to 254% of that of the control at 240 min (Fig. 1) ($P = 0.004$). Adenosine is readily broken down by the enzyme adenosine deaminase to the less active nucleoside, inosine. We found that inosine had no effect on PMN bactericidal activity for *S. aureus* (data not shown).

Substitution at the 2 position of adenosine yields compounds that are metabolically stable and resistant to the action of adenosine deaminase (9). One such analog, CV-1808, was a more potent inhibitor of PMN bactericidal function than was adenosine. CV-1808 (10 to 100 μM) significantly decreased killing by 120 min of incubation ($P < 0.05$) (Fig. 1).

Although 1 mM adenosine did not significantly affect PMN bactericidal activity, it was not significantly removed from the medium or metabolized by the PMN. There was 1.28 ± 0.18 mM adenosine at the beginning of the experiments, and after 240 min there was 0.99 ± 0.03 mM adenosine in the medium ($P = 0.252$). The control samples contained 0.002 ± 0.001 mM adenosine at the beginning of the experiments and 0.003 ± 0.001 mM after 240 min.

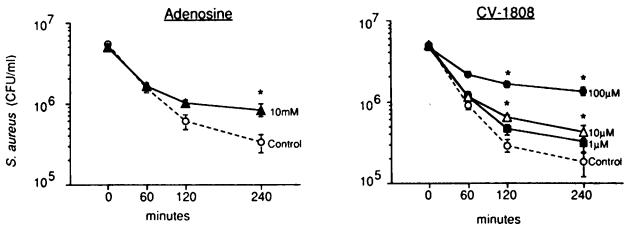

FIG. 1. Effects on PMN killing of *S. aureus* by adenosine (10 mM) and CV-1808 (1 to 100 μM). Results are expressed as mean ± standard error of the mean (each datum point is the mean from 6 to 8 separate experiments). Asterisks indicate that adenosine or CV-1808 increased the number of live *S. aureus* ($P < 0.050$).

Adenosine and CV-1808 do not affect cell association of *S. aureus* by PMN. In order to determine whether the inhibition of bactericidal function was due to a decrease in cell association of *S. aureus*, supernatant-sediment differential centrifugation experiments were conducted. In the first 120 min of incubation, the PMN removed >97% of the bacteria from the supernatant both in the presence and in the absence of adenosine (10 mM). Only after 240 min of incubation (by which time the bacteria could have replicated) did adenosine (10 mM) increase survival of bacteria in the supernatant ($P = 0.005$) (Fig. 2).

CV-1808 also had little effect on PMN removal of *S. aureus* from the supernatant fraction. There was >96% removal of bacteria from the supernatant fraction in the first 120 min of incubation, both in the presence and in the absence of CV-1808 (1 to 100 μM) (Fig. 2).

Conversely, the sediment counts reflecting cell-associated bacteria were higher with adenosine (10 mM; 120 to 240 min) and CV-1808 (1 to 100 μM; 60 to 240 min) than in the control, indicating increased survival of ingested organisms ($P < 0.05$) (Fig. 3).

Adenosine and CV-1808 decrease *S. aureus*-activated PMN oxidative burst. In order to determine whether the decrease in killing of *S. aureus* was accompanied by a decrease in PMN oxidative function, the *S. aureus*-activated oxidative burst was measured by using luminol-enhanced chemiluminescence as the assay system. Ten millimolar adenosine, the

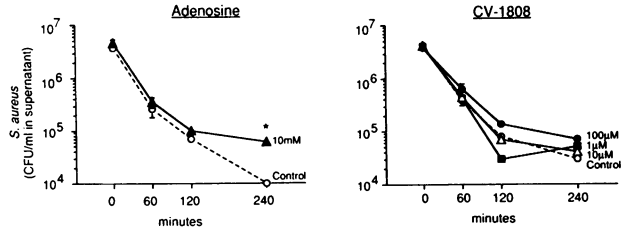

FIG. 2. Effects of adenosine (10 mM) and CV-1808 (1 to 100 μM) on cell association of *S. aureus* by PMN, as measured by the change in CFU of *S. aureus* in the supernatant (which represents cell-free *S. aureus*). Results are expressed as mean ± standard error of the mean (each datum point represents the mean from 3 to 6 separate experiments). The asterisk indicates that adenosine increased the number of live *S. aureus* in the supernatant fraction ($P < 0.050$).

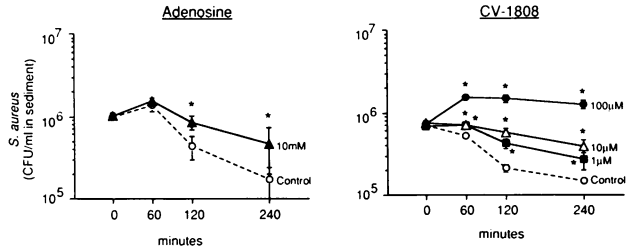

FIG. 3. Effects of adenosine (10 mM) and CV-1808 (1 to 100 μM) on killing of *S. aureus* by PMN, as measured by the change in CFU of *S. aureus* in the sediment (which represents cell-associated bacteria). Results are expressed as mean ± standard error of the mean (each datum point represents the mean from 3 to 6 separate experiments). Asterisks indicate that adenosine or CV-1808 increased the number of live *S. aureus* in the sediment fraction ($P < 0.050$).

concentration that diminished PMN killing of *S. aureus* (method 1), decreased the PMN oxidative burst stimulated by *S. aureus* (Fig. 4; Table 1).

As a second measure of PMN oxidative activity we assayed superoxide release. We observed that adenosine (10 μM) decreased superoxide release from PMN stimulated with *S. aureus* for 120 min by 33% ($P = 0.003$) (Table 2). Adenosine (10 μM) inhibition of the PMN oxidative burst was partially prevented by the adenosine receptor antagonist DPPSX (30 μM; $P = 0.013$). This suggests that the effect of adenosine on the PMN oxidative burst is receptor mediated (Table 3).

Despite the 33% decrease in superoxide release, PMN bactericidal function in parallel experiments without serum (bactericidal assay method 2) was not decreased by adenosine (10 μM). There was 41% ± 14% killing in the control samples and 44% ± 13% killing in the samples containing adenosine (10 μM) after 120 min ($P = 0.264$).

CV-1808 (100 μM) decreased PMN chemiluminescence to 22% of control chemiluminescence ($P = 0.001$) (Table 1) without damaging the PMN (94% trypan blue exclusion). In parallel bactericidal experiments (method 1), CV-1808 (100 μM) decreased PMN killing of *S. aureus* to 69% of that in the control after 240 min of incubation ($P = 0.011$) (Fig. 1).

We observed that CV-1808 (10 μM) decreased superoxide release from PMN stimulated with *S. aureus* for 120 min by 68% ($P = 0.003$) (Table 2). The adenosine receptor antagonist DPPSX (30 μM) did not significantly prevent CV-1808

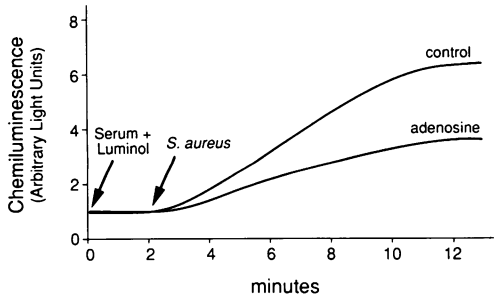

FIG. 4. PMN (5×10^6) were incubated with luminol (1×10^{-4} M) for 2 min. *S. aureus* (5×10^6) was added at the 2-min mark. Chemiluminescence was recorded in HBSS (1 ml) containing 10% fresh autologous serum with and without adenosine (10 mM).

TABLE 1. Effects of CV-1808 and adenosine on PMN chemiluminescence stimulated by *S. aureus*[a]

PMN treatment	Concn	Chemiluminescence (*n*)[b]
None		100
CV-1808	10 μM	107.0 ± 15.6 (3)
CV-1808	100 μM	19.5 ± 5.9 (8)[c]
Adenosine	1 mM	71.7 ± 8.6 (4)
Adenosine	10 mM	60.3 ± 7.6 (7)[c]

[a] PMN (5×10^6 per ml) were incubated (37°C) in HBSS with or without CV-1808 or adenosine for 15 min as described in the text, and then *S. aureus* (5×10^6 organisms per ml) was added and chemiluminescence was recorded.
[b] Results are expressed as mean percentage of control chemiluminescence ± standard error of the mean.
[c] $P < 0.050$.

(10 μM) from affecting the *S. aureus*-stimulated PMN superoxide release ($P = 0.278$) (Table 3).

DISCUSSION

Studies examining adenosine's effects on PMN have shown it to play an anti-inflammatory role. It is an inhibitor of superoxide generation when PMN are stimulated by formylmethionyl (fMet)-Leu-Phe, concanavalin A, calcium ionophore A23187, zymosan-treated serum, and to a lesser extent, phorbol myristate acetate (5). The adenosine analog 2-chloroadenosine has been shown to inhibit hydrogen peroxide production by PMN stimulated by fMet-Leu-Phe and concanavalin A but not by phorbol myristate acetate (6). Recently, it has been reported that adenosine decreases the oxidative response to soluble stimuli more than to *S. aureus* (14). In our experiments, adenosine (10 μM) and the adenosine analog CV-1808 (10 μM) decreased the PMN oxidative burst stimulated by *S. aureus*. The 33% decrease in superoxide release in the presence of adenosine (10 μM) was not sufficient to decrease PMN bactericidal activity. Hence, it appears that the PMN oxidative burst is more sensitive to adenosine than PMN bactericidal function.

Adenosine (1 mM) was not significantly metabolized by the PMN but still did not decrease either PMN chemiluminescence or bactericidal function. An explanation for this observation is not readily apparent, but these results indicate that the lack of activity of adenosine at concentrations ≤1 mM in the method 1 bactericidal assays and chemilumines-

TABLE 2. Effects of CV-1808 and adenosine on PMN superoxide release stimulated by *S. aureus*[a]

PMN stimulation	Concn (μM)	Superoxide release (nmol/10^6 PMN/2h) (*n*)[b]
None		1.64 ± 0.41 (19)
S. aureus plus		
Nothing		3.62 ± 0.67 (18)
CV-1808	1	1.95 ± 0.24 (11)[c]
CV-1808	10	1.17 ± 0.43 (13)[d]
Adenosine	10	2.47 ± 0.38 (19)[d]

[a] PMN (5×10^6 per ml) were incubated (37°C) in HBSS or HBSS containing CV-1808 or adenosine and *S. aureus* (5×10^6 organisms per ml), as described in the text, for 2 h.
[b] There was no superoxide released in the presence of *S. aureus* and absence of PMN (−0.050 ± 0.22 nmol/2 h). Results are expressed as mean ± standard error of the mean.
[c] $P = 0.116$.
[d] $P = 0.003$.

TABLE 3. Effects of CV-1808 and adenosine on PMN superoxide release stimulated by *S. aureus*: modulation by the adenosine receptor antagonist DPPSX[a]

PMN stimulation	Concn (μM)	Superoxide release (nmol/10^6 PMN/2h)[b]
None		0.94 ± 0.41
S. aureus plus		
Nothing		2.73 ± 1.00
DPPSX	30	3.84 ± 0.92
CV-1808	10	0.59 ± 0.46
CV-1808 + DPPSX		1.49 ± 0.55
Adenosine	10	1.66 ± 0.50
Adenosine + DPPSX		2.36 ± 0.46[c]

[a] PMN (5×10^6 per ml) were incubated (37°C) in HBSS or HBSS containing DPPSX for 15 min and then with or without CV-1808, adenosine, and *S. aureus* (5×10^6 organisms per ml), as described in the text, for 2 h. DPPSX prevented adenosine inhibition of PMN superoxide released in response to *S. aureus*.

[b] Results are expressed as mean ± standard error of the mean; $n = 5$ for all experiments.

[c] $P = 0.013$.

cence experiments cannot be explained by adenosine metabolism.

The adenosine receptor antagonist DPPSX prevented adenosine inhibition of the PMN oxidative burst. This suggests that the effect of adenosine on the PMN oxidative burst is receptor mediated. The lack of a complete elimination of adenosine activity by DPPSX or of a significant effect of DPPSX on CV-1808 activity may be explained by the observation that alkylxanthines are both adenosine receptor antagonists and phosphodiesterase inhibitors. By inhibiting phosphodiesterase, alkylxanthines raise PMN cyclic AMP. Elevation of cyclic AMP can decrease PMN superoxide release (3, 12). Elevation of cyclic AMP from phosphodiesterase inhibition may be masking the activity of DPPSX as an adenosine receptor antagonist.

We found that inhibition of bactericidal function was not achieved through a reduction of *S. aureus* cell association. Nishida et al. reported that phagocytosis of latex particles and sheep erythrocytes by PMN was not affected by adenosine (22). We observed little effect on cell association of *S. aureus* with adenosine (10 mM) or CV-1808 (1 to 100 μM).

In vitro, adenosine inhibits the ability of PMN to kill *S. aureus* at millimolar concentrations. In vivo, concentrations of adenosine in plasma are only 0.2 to 0.4 μM (26). Although it has yet to be determined whether higher concentrations of adenosine occur in microenvironments, it appears doubtful that endogenous adenosine would reach a level that could have a significant effect on microbicidal function.

A decrease in PMN degranulation might contribute to the observed decrease in bactericidal activity with adenosine and adenosine analogs. Adenosine's effect on degranulation is in dispute; some studies show no significant effect (4, 5, 13), while others show an inhibition of degranulation by adenosine analogs when PMN are stimulated by soluble stimuli (24).

We observed that the decrease in bactericidal function was associated with a significant decrease in the oxidative burst of PMN, as measured by luminol-enhanced chemiluminescence and superoxide release. PMN and their oxidative products have been implicated as mediators of pathologic tissue damage in many inflammatory disorders. The damaging effects of such diverse disease states as sepsis, myocardial infarction, and the adult respiratory distress syndrome are now believed to be mediated, at least in part, by an inflammatory neutrophil response (18). Adenosine and its analogs may be useful in treating these disorders by mitigating the damage caused by neutrophils, but adenosine analogs have the potential to decrease PMN bactericidal activity.

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