Effects of Adenosine on the Functions of Circulating Polymorphonuclear Leukocytes during Hyperdynamic Endotoxemia

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Endotoxin-activated polymorphonuclear leukocytes (PMNL) adhere to the vascular endothelium and cause damage by the release of toxic superoxide anions ($O_2^-$). Because adenosine is a potent inhibitor of PMNL in vitro, the present study investigates the effects of this nucleoside on the functions of circulating PMNL in a standardized porcine model of hyperdynamic endotoxemia. Ten anesthetized pigs received an intravenous (i.v.) 330-min infusion of endotoxin (5 μg/kg of body weight per h). Another 10 pigs were also infused with endotoxin plus adenosine (150 μg/kg/min [i.v.]; this treatment was begun 30 min prior to the beginning of endotoxin treatment. Control groups (five animals per group) received either adenosine or physiological saline. Infusion of endotoxin caused severe neutropenia, shedding of L-selectin, upregulation of β2-integrins, increased binding of C3-coated zymosan particles, and subsequent phagocytosis by PMNL. While phagocytosis-induced production of oxygen radicals appeared to decrease, extracellular release of superoxide anions was strongly enhanced. Infusion of adenosine during endotoxemia had no effect on neutropenia, expression of adhesion molecules, C3-induced adhesion, phagocytosis, or intracellular production of oxygen radicals, whereas extracellular release of $O_2^-$ was strongly inhibited. Thus, i.v. infusion of adenosine during endotoxemia could be useful in protecting from $O_2^-$-mediated tissue injury without compromising the bactericidal mechanisms of PMNL.

Polymorphonuclear leukocytes (PMNL) constitute the first line of defense in the elimination of a broad spectrum of microorganisms. Following chemotactic stimulation, PMNL migrate to the site of infection by the complex interaction of adhesion molecules located on their cell surfaces and the vascular endothelium. The major adhesion molecule of the β2-integrin family, which is constitutively expressed on PMNL, is the complement receptor type III (CR3, CD11b/CD18, and Mac-1). This β2-integrin mediates adhesion not only to the vascular endothelium but also to the degraded complement fragment type 3, C3bi. Following CR3-mediated adhesion of PMNL to C3bi-opsonized particles, for example, phagocytosis ensues and intracellularly trapped microorganisms are killed by the simultaneous release of reactive oxygen radicals and proteolytic enzymes. During phagocytosis, the outer side of the neutrophil’s membrane becomes inverted, thus forming the inner side of the phagosome. As the active center of the transmembrane NADPH-oxidase complex is directed to the extracellular space, most of the oxygen radicals are generated into the phagosome, although small amounts may escape (11, 26). By contrast, nonparticulate (i.e., humoral) stimuli activate the NADPH-oxidase on the whole-cell membrane, leading to the release of superoxide anions mainly into the extracellular space (26, 27).

It is well known that bacterium-derived endotoxins prime and stimulate PMNL to attain enhanced cellular responses (12). Upon exposure to endotoxin, PMNL become more adhesive and, most importantly, the production of oxygen radicals into the extracellular space is significantly increased (11).

Thus, during endotoxemia, not only may bactericidal mechanisms be enhanced (4), but formation of oxygen radicals may also be diverted from the intracellular to the extracellular space, thereby increasing the risk of PMNL-mediated tissue destruction (11, 12).

The nucleoside adenosine has been shown to inhibit many PMNL functions in vitro. Acting via A2 receptors, the nucleoside decreases the expression of adhesion molecules (29), the Fc-receptor mediated phagocytosis (32), the production of superoxide anions (7), the degranulation of enzymes (24), and also the production of tumor necrosis factor alpha (TNF-α) (30). However, adenosine does not appear to inhibit all of these functions of PMNL with the same efficacies and to the same extents; while phagocytosis and the associated oxygen radical production are only moderately inhibited (8, 28, 32), extracellular release of superoxide anions is strongly suppressed (7, 8). Because the action of adenosine on PMNL has not been well characterized in vivo so far, we studied the effects of a continuous intravenous (i.v.) infusion of the nucleoside on selected functions of PMNL in a standardized porcine model of hyperdynamic endotoxemia (15). The results suggest that adenosine might be useful in protecting from inflammatory tissue destruction by inhibition of the extracellular release of toxic superoxide anions from PMNL without compromising functions known to contribute to the ingestion and killing of opsonized bacteria.

(This research was presented in part at the International Symposium Purines 96, Molecular, Pharmacological and Therapeutic Advances, Milan, Italy, 6 to 9 July 1996. Some of this research was conducted by Sandra Moritz in partial fulfillment of the requirements for a doctoral thesis from the medical faculty of the Ludwig-Maximilians University of Munich, Munich, Germany.)
MATERIALS AND METHODS

Anesthesia, surgical instrumentation, and hemodynamic measurements. Female pigs with a mean body weight of 24.9 ± 0.65 kg (mean ± standard error [SE]; n = 30) were put on a fast for 24 h, with free access to water. After intravenous premedication (atropine, 0.5 mg/kg of body weight; ketamine, 15 mg/kg; sodium pentobarbital, 5 mg/kg; fentanyl, 0.01 mg/kg; and pancuronium bromide, 0.2 mg/kg), and the animals were intubated with a cuffed endotracheal tube. The animals were placed in a supine position and were normotubated (arterial pressure of CO2 35 to 45 torr [1 torr = 1 mm Hg]) in a controlled mode (N2O-O2, 1:1; positive end-expiratory pressure, 5-cm H2O; respiratory rate, 12/min; ratio of inspiration to expiration, 1:2; tidal volume, 15 to 20 ml/kg; Servo ventilator 900; Siemens, Elema, Sweden). Anesthesia was maintained by ketaminous i.v. infusion of Ringer's solution (5 ml/kg/h) containing fentanyl (0.004 mg/ml) and sodium pentobarbital (0.3 mg/ml). Additional boluses of pancuronium bromide (0.1 mg/kg) and fentanyl (0.01 to 0.02 mg/kg) were administered when active movements or spontaneous breathing efforts were noted. Additional fluid was administered when the pulmonary capillary wedge pressure decreased to less than 10 mm Hg. Body core temperature was kept between 36 and 37°C with a heating pad. ECO lead II was recorded continuously (Hellige Recomed; Hellige, Freiburg, Germany). Catheters were placed by surgery under sterile conditions into the right side of the animals, in the regio colli, to infuse crystalloids and drugs i.v., to draw blood samples, and to record hemodynamic variables as previously described (15).

This study was approved by the local Animal Care and Use Committee. All experiments were conducted in accordance with the National Institutes of Health guidelines (19a).

Experimental design. A total of 30 pigs were randomly allocated to four groups. Group I (n = 10) received a continuous i.v. infusion of Salmonella abortus equi endotoxin (Pyroantigok Diagnostik, Walldorf, Germany) at a constant rate of 5 μg/kg/h. Group II (n = 10) received (in addition to the infusion of endotoxin) a continuous i.v. infusion of adenosine (Boehringer, Mannheim, Germany) at a constant rate of 150 μg/kg/min, which was begun 30 min prior to the infusion of endotoxin. Groups III and IV were control groups (n = 5 each) which received either physiological saline or adenosine only.

After implantation of the catheters, hemodynamic parameters were recorded and blood samples were taken (time point, −30 min). Thereafter, the infusion of adenosine was begun, and 30 min later (time point, 0 min), all parameters were determined again and the infusion of endotoxin was begun. In the control groups, application of physiological saline or adenosine was started at the same time points as those for the groups additionally receiving endotoxin. All infusions were maintained until the end of the experiments (330 min). Additional hemodynamic measurements and blood samples were taken at 30, 90, 150, 210, 270, and 330 min. Expression of adhesion molecules was also determined at 10, 20, 45, and 60 min after the beginning of endotoxemia.

The infusion rate of adenosine was chosen in accordance with the results of dose-response studies performed for endotoxic animals. These studies demonstrated that hemodynamic side effects due to continuously i.v. infused adenosine at a rate of 150 μg/kg/min.

Determination of concentrations of endotoxin in plasma. Concentrations of endotoxin in arterial plasma were measured as previously described (15).

Determination of concentrations of adenosine in plasma. Concentrations of adenosine in plasma were measured according to a modification of the method described by Ostyn et al. (23). Arterial blood samples (2 ml) were drawn with sterile 5-ml syringes, which were prefilled with 2 ml of ice-cold physiological saline containing dipyriramole at 2 × 10−4 M. The samples were centrifuged (5 min; 1,500 × g), and 2 ml of the supernatants were removed and mixed with 100 μl of perchloric acid (70%). Denatured proteins were removed by centrifugation at 10,000 × g for 2 min. Deproteinized samples were neutralized by the addition of an equivalent amount of KOH. The contents of the samples in adenosine were analyzed with a high-performance liquid chromatography (HPLC) system (Waters Millipore, Eschborn, Germany). The HPLC method that was used has been described previously (29). Adenosine eluting at 16.5 min was sampled by a Cynetic fraction sampler (Isco Inc.). Evaporation of the adenosine fraction, the dried pellet was dissolved in KH2PO4 buffer (0.1 M, pH 7.4), and the adenosine was enzymatically degraded to inosine by adenosine deaminase (1 U/ml, 37°C, 30 min). Thereafter, the amount of inosine enzymatically formed was determined by HPLC (29).

Total leukocyte count and differential staining. Arterial blood samples were drawn into EDTA tubes, and then blood cell counts (Coulter Counter T540; Coulter, Krefeld, Germany) were performed and smear detections were stained by the May-Grünwald-Diff-高尔丁法.

Determination of adhesion molecules and preparation of FITC-labeled, C3-coated zymosan particles. The numerical expression of T-selectin and β2-integrins on the cell surfaces of PMNL was determined and fluorescein isothiocya-

ne labeled, C3-coated zymosan particles were prepared as previously described (15). Since the majority of the C3 molecules are deposited on zymosan particles as C3bi (20), with the latter being strongly bound to 90° light scatter values. The values for adhesion and phagocytosis of the FITC-labeled zymosan particles are given as the fractions of fluorescent PMNL incubated at 4 and 37°C, respectively.

Determination of phagocytosis-induced intracellular oxygen radical production and extracellular release of superoxide anions. Phagocytosis-induced intracellular production of oxygen radicals was measured by luminol-enhanced chemiluminescence. Although the chemiluminescent probe luminol allows one to detect a large spectrum of oxidants, the most widely used compound has been shown to react mainly with hypochloric acid, which is generated during phagocytosis within the phagosome by the myeloperoxidase (MPO)-hydrogen peroxide-halide system of the phagocyte (9, 10).

Extracellular superoxide anion production was measured by lucigenin-enhanced chemiluminescence activity. Lucigenin-enhanced chemiluminescence activity has been reported chiefly to detect the extracellular release of superoxide anions from neutrophils (9, 14, 27).

To test the effects of adenosine on the luminol and lucigenin-enhanced chemi-
luminescence activity elicited by C3-coated zymosan particles or humoral stimuli, blood samples from healthy pigs were incubated with increasing concentrations of the ligands. As previously described, the effect of adenosine on C3-mediated adhesion (2-C-adenosine). The conditions of determination of luminol or lucige-
nin-enhanced chemiluminescence activity were as described above for the ex vivo measurements of the blood samples taken during endotoxemia. For the activa-
tion of cells by humoral stimuli, the human recombinant complement anaphy-
latotoxin C5a (final concentration, 4 ng/ml; Sigma Chemicals, St. Louis, Mo.) or TNF-α (final concentration, 100 U/ml; Boehringer) was added alone or in com-

bination. When both stimuli were used, TNF-α was added 5 min prior to the addition of C5a. In addition, phorbol myristate acetate (PMA; final concentra-
tion, 10−7 M) and ionophore A23187 (final concentration, 10−5 M) were used to activate PMNL via protein kinase C- and intracellular calcium-dependent pathways, respectively. The stimulus-induced chemiluminescence activity was calcu-
lated as the difference between the activities measured after the addition of the specific stimulus and its solvent, i.e., HBSS. When cell-free systems were used to generate luminol- or lucigenin-enhanced chemiluminescence, the addition of 2-C-adenosine or adenosine at concentrations of as much as 10−4 M had no effect. These results strongly suggest that the purine compounds do not nonspe-

cifically interefere with the detection of oxygen-reactive metabolites by the chemi-
luminescent probes.

Luminol- or lucigenin-enhanced chemiluminescence activities were monitored continuously, and the areas under the curves were calculated for integrated chemiluminescence activity measurement. Luminol- and lucigenin-enhanced chemiluminescence activity measurement was linearly related to the number of }

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PMNL present in the incubation vials. The means ± SEs of six linear-regression analyses, each of which comprises six data pairs, resulted in the following correlation coefficients: for numbers of PMNL versus spontaneous and C3-stimulated luminol-enhanced chemiluminescence activity, \( r^2 = 0.96 ± 0.01 \) and 0.93 ± 0.04, respectively; for the numbers of PMNL versus spontaneous and C5a-stimulated lucigenin-enhanced chemiluminescence activity, \( r^2 = 0.86 ± 0.07 \) and 0.98 ± 0.01, respectively. The range of the number of PMNL in the incubation volume was \((8.5 - 455.7) \times 10^6\). The percentage of monocytes among the leukocytes was less than 0.5%, which further decreased during endotoxemia, making the monocytes unlikely to contribute significantly to the chemiluminescence activity.

Statistics. The data were analyzed with commercially available software (SPSS software, version 4.0; SPSS Inc., Chicago, Ill.). Normal distribution was tested by the Kolmogorov-Smirnov test. Results are expressed as means ± SEs. Comparisons within the group were made by repeated-measure analysis of variance (ANOVA). Comparisons between groups were performed separately for all points in time studied applying one-way ANOVA. On finding significant \( F \) values, means were compared by the Student-Newman-Keuls test procedure. Significance was defined as \( P < 0.05 \).

RESULTS

Changes in concentrations of endotoxin and adenosine in plasma. After implantation of catheters (−30 min), the concentrations of endotoxin in plasma were less than the detection limit in all groups studied. Immediately thereafter, i.v. infusion of adenosine was begun at a rate of 150 \( \mu \)g/kg/min in groups II and IV. After an additional 30 min, i.e., at baseline (0 min), again no endotoxin could be detected in any group studied. Subsequently, i.v. infusion of Salmonella abortus equi endotoxin was begun in groups I and II at the rate of 5 \( \mu \)g/kg/h. Group III received physiological saline only. Although in control groups III and IV no plasma endotoxin could be detected throughout the whole observation period, concentrations of endotoxin steadily increased in groups I and II, reaching the highest values at the end of the experiments (330 min). The concentrations of endotoxin in plasma were slightly higher in animals that were additionally treated with adenosine, but the concentrations did not reach statistical significance (groups II and I, 131.3 ± 27.0 EU/ml versus 108.1 ± 27.4 EU/ml at 330 min, respectively; \( P = 0.21 \)).

Concentrations of adenosine in plasma did not significantly differ among the four groups when measured after implantation of catheters (Fig. 1; Table 1). As expected at this point, concentrations of adenosine in plasma were within the physiological range, i.e., 205.1 ± 45.5 nmol/liter (n = 30 [mean ± SE]). Upon infusion of adenosine, concentrations of the nucleoside in plasma rapidly increased in groups II (Fig. 1) and IV (Table 1). In the control (group IV), the concentrations of adenosine in plasma reached steady state within 60 min, whereas in animals additionally receiving endotoxin (group II), adenosine concentrations continuously increased until the end of the experiments (Fig. 1). Animals subjected to endotoxic shock only (group I) exhibited an increase in adenosine concentrations in plasma in the early phase of endotoxemia, which was followed by a decrease to less than the values determined prior to the infusion of endotoxin. In animals receiving physiological saline (group III), no significant changes in adenosine concentrations in the plasma occurred throughout the entire observation period.

Effect of adenosine on the number of leukocytes. Following the infusion of endotoxin, the numbers of circulating leukocytes and PMNL decreased to minimum values within the first 30 min and did not recover until the end of the experiments. Infusion of adenosine had no effect on the decrease in the numbers of circulating leukocytes induced by endotoxemia (data not shown). Under control conditions, i.e., in the absence of endotoxin, the number of circulating PMNL upon which the infusion of adenosine had no effect (Table 1) remained stable throughout the whole experimental period.

Effects of adenosine on the expression of adhesion molecules on circulating PMNL. Figure 2 shows the effects of adenosine on the expression of \( \alpha \)-selectin and \( \beta_2 \)-integrins of circulating PMNL during endotoxemia. After endotoxemia was induced, the expression of \( \alpha \)-selectin rapidly decreased within 30 min, while it took 60 min for the number of \( \beta_2 \)-integrins on circulating PMNL to increase. Infusion of adenosine prior to the onset of endotoxemia did not alter the expression of adhesion molecules on circulating PMNL. Adenosine also had no effect on changes in the expression of adhesion molecules induced by endotoxemia. Compared with the control group infused with physiological saline only, infusion of adenosine alone did not alter the expression of adhesion molecules (Table 1).

Effects of adenosine on the adhesion and phagocytosis of C3-coated zymosan particles. Only 30 min after the start of the infusion of endotoxin, the binding of C3-coated zymosan par-

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**FIG. 1.** Concentrations of adenosine in arterial plasma. Animals of group I received a continuous i.v. infusion of Salmonella abortus equi endotoxin, which was begun at baseline (0 min) at the rate of 5 \( \mu \)g/kg/h (\( \square \) [n = 10]). Animals of group II received, in addition to the infusion of endotoxin, a continuous i.v. infusion of adenosine at the rate of 150 \( \mu \)g/kg/min, which was begun 30 min prior to the infusion of endotoxin, i.e., immediately after the implantation of catheters (−30 min) (■ [n = 10]). §, \( P < 0.05 \) between groups (one-way ANOVA and post hoc Student-Newman-Keuls test); #, \( P < 0.05 \) versus means of previous levels (repeated-measure ANOVA).
Effects of adenosine on phagocytosis-induced intracellular oxygen radical production and on extracellular release of superoxide anions. Luminol-enhanced chemiluminescence activity stimulated by the addition of C3-coated zymosan particles to PMNL slightly increased during endotoxemia but did not reach statistical significance compared with values determined prior to the start of the infusion of endotoxin or compared with those values for the control groups. In accordance with the lack of any effect produced by adenosine on the adhesion and phagocytosis of C3-coated zymosan particles, the nucleoside did not inhibit C3-induced oxygen radical production (Fig. 4A). Under control conditions, infusion of adenosine alone also had no effect on C3-elicted oxygen radical production (data not shown).

As can be seen in Fig. 4B, lucigenin-enhanced chemiluminescence significantly increased after the beginning of endotoxemia. In contrast to the lack of any effects of the nucleoside on the phagocytosis-induced intracellular production of oxygen radicals, infusion of adenosine strongly inhibited the endotoxemia-induced extracellular release of superoxide anions. The infusion of adenosine in the absence of endotoxin did not inhibit the ability of PMNL to release superoxide anions (Table 1).

Effects of 2-Cl-adenosine on intracellular oxygen radical production and on extracellular release of superoxide anions in vitro. In order to test whether adenosine might inhibit production of oxygen radicals in vitro as selectively as what was observed during endotoxemia, the metabolically stable nontoxic adenosine receptor agonist 2-Cl-adenosine was tested in vitro on the PMNL of healthy pigs with respect to the stimulus used and the site of reactive oxygen metabolites detected.

For stimulation of intracellular oxygen radical production, C3-coated zymosan particles were used at the concentration used for activation of PMNL in blood samples taken during the in vivo experiments. Because most of the detrimental effects of endotoxemia are thought to be caused by the production of

### Table 1. Concentrations of adenosine in plasma and PMNL functions for control group animals

<table>
<thead>
<tr>
<th>Time (min) and control group</th>
<th>Conc of adenosine (nmol/liter)</th>
<th>No. of leukocytes (10⁹/µl)</th>
<th>No. of PMNL (10⁴/µl)</th>
<th>L-Selectin (relative fluorescence U)</th>
<th>β-Integrins (relative fluorescence U)</th>
<th>% FITC-positive PMNL Adhesion</th>
<th>Phagocytosis O₂⁻-ind. Cl (cpm/10⁴ PMNL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>114 ± 22</td>
<td>18.6 ± 3.0</td>
<td>12.5 ± 2.6</td>
<td>88.1 ± 29.9</td>
<td>38.8 ± 2.9</td>
<td>1.0 ± 0.2</td>
<td>13.0 ± 6.4</td>
</tr>
<tr>
<td>30</td>
<td>130 ± 78</td>
<td>16.7 ± 3.3 b,c</td>
<td>10.8 ± 2.6 b,c</td>
<td>88.9 ± 23.9</td>
<td>38.0 ± 2.5</td>
<td>2.3 ± 1.1</td>
<td>9.0 ± 3.0 b,c</td>
</tr>
<tr>
<td>90</td>
<td>245 ± 113</td>
<td>15.7 ± 2.6 b,c</td>
<td>11.0 ± 2.3 b,c</td>
<td>96.6 ± 25.4 b</td>
<td>39.3 ± 2.5</td>
<td>2.3 ± 1.3 b,c</td>
<td>5.6 ± 1.2 b,c</td>
</tr>
<tr>
<td>150</td>
<td>195 ± 75</td>
<td>15.0 ± 2.0 b,c</td>
<td>9.7 ± 1.9 b,c</td>
<td>95.2 ± 30.3 b,c</td>
<td>36.5 ± 2.7</td>
<td>2.3 ± 0.1 b,c</td>
<td>7.3 ± 2.0 b,c</td>
</tr>
<tr>
<td>210</td>
<td>310 ± 155</td>
<td>13.9 ± 2.0 b,c</td>
<td>8.5 ± 1.6 b,c</td>
<td>113.4 ± 26.5 b,c</td>
<td>39.7 ± 3.4</td>
<td>3.0 ± 1.0 b,c</td>
<td>13.3 ± 4.6 b,c</td>
</tr>
<tr>
<td>270</td>
<td>280 ± 133</td>
<td>15.0 ± 2.2 b,c</td>
<td>9.1 ± 1.4 b,c</td>
<td>107.4 ± 29.0 b,c</td>
<td>42.0 ± 3.3</td>
<td>2.6 ± 1.0 b,c</td>
<td>11.8 ± 3.2 b,c</td>
</tr>
<tr>
<td>330</td>
<td>196 ± 21</td>
<td>15.3 ± 1.6 b,c</td>
<td>8.8 ± 1.2 b,c</td>
<td>111.5 ± 29.4 b,c</td>
<td>39.5 ± 2.9</td>
<td>2.5 ± 1.4 b,c</td>
<td>ND</td>
</tr>
</tbody>
</table>

a Animals in group III (n = 5) received the solvent of endotoxin and adenosine, i.e., physiological saline only. Animals in group IV (n = 5) were infused continuously with adenosine at a constant rate of 150 µg/kg/min, this treatment starting immediately after implantation of catheters (~30 min). Values are means ± SEs.
b P < 0.05 versus group I.
c P < 0.05 versus group II.
d P < 0.05 between groups tabulated (one-way ANOVA and posthoc Student-Newman-Keuls test).
e P < 0.05 versus means of previous levels (repeated-measure ANOVA).
secondary humoral and cellular inflammatory mediators, in the in vitro experiments recombinant human complement anaphylatoxin C5a and TNF-\(\alpha\) were used alone and in combination to induce a primed, receptor-dependent maximum release of superoxide anions.

As can be seen from Fig. 5, irrespective of the kind of stimulus used, 2-Cl-adenosine inhibited the intracellular production of oxygen radicals as well as the extracellular release of superoxide anions. Corresponding to the action of 2-Cl-adenosine at an A2 receptor site, all of the dose-response curves obtained were of sigmoidal shape, reaching maximal inhibitory effects in the micromolar range. The rank order of the stimulus-specific inhibitory potency of 2-Cl-adenosine, which was determined by the half-maximal inhibitory concentrations (IC\(_{50}\)), as follows: IC\(_{50}\) (C3 \([5 \times 10^3\) M]) = 1.1 \(\times 10^{-7}\) M \(\geq\) IC\(_{50}\) (C5a, TNF-\(\alpha\), and C5a plus TNF-\(\alpha\)) \(\equiv 1.0 \times 10^{-6}\) M. Thus, although 2-Cl-adenosine inhibited all of the stimuli tested, the nucleoside was 10 times more effective in inhibiting the extracellular release of superoxide anions elicited by humoral stimuli than in inhibiting the intracellular oxygen radical production associated with phagocytosis of C3-coated zymosan particles.

Since C3-coated zymosan particles were added at a suboptimal activating concentration, the effects of 2-Cl-adenosine on PMNL when stimulated at the much higher particle-to-cell ratio of 10^6:1 were also tested (Fig. 6). Under these conditions, 2-Cl-adenosine had only a minor effect on the intracellular production of oxygen radicals, compared with its strong inhibitory action on the superoxide anion production that was caused by the maximal stimulation of PMNL by the combination of C5a and TNF-\(\alpha\) (see legends to Fig. 5 and 6 for absolute values for chemiluminescence activities). Concerning the second messengers mediating stimulus response coupling in neutrophils, it has been reported that activation of protein kinase C plays an important role in phagocytosis (1), whereas C5a-stimulated activation of superoxide anion production has been demonstrated to be strongly dependent on an increase in intracellular calcium (25). Therefore, PMNL were activated either by PMA or by the ionophore A23187 which directly stimulates protein kinase C or induces an increase in intracellular calcium, respectively. Although PMA was a relatively weak stimulus compared with the ionophore, 2-Cl-adenosine had only a minor inhibitory effect on protein kinase C-dependent activation but a very strong effect on Ca-dependent superoxide anion production. These results are similar to those obtained for the effects of 2-Cl-adenosine on phagocytosis and on C5a-plus-TNF-\(\alpha\)-induced cell stimulation (Fig. 5).

**DISCUSSION**

In the present study, using a standardized porcine model of hyperdynamic endotoxemia (15), we have described the effects...
of a prophylactic, continuous i.v. infusion of adenosine on the functions of circulating PMNL. Bacterium-derived endotoxins are thought to be the major causative agents for the development of septic shock in patients. A vast body of evidence suggests that endotoxins exert their detrimental effects predominantly by the activation of the host's own humoral and cellular mediator systems, leading to products which are well-known to stimulate PMNL. Accordingly, the results of the present study show intense changes in the functions of PMNL after the start of endotoxin infusion. As a confirmation of findings previously published (15), severe leukopenia and neutropenia rapidly developed and the expression of \( \beta_2 \)-integrins increased (Fig. 2). In addition to an increase in the number of \( \beta_2 \)-integrins, the function of the major \( \beta_2 \)-integrin of the PMNL, CD11b/CD18 (which represents the receptor for the degradation product of the complement factor C3b, i.e., C3bi), was also upregulated, as shown by the enhanced binding of C3-coated, FITC-labeled zymosan particles (Fig. 3A). As a result, phagocytosis of C3-coated zymosan particles was also strongly increased during endotoxemia (Fig. 3B). When PMNL were stimulated with C3-coated zymosan particles at the ratio of particles to cells that was used for determination of phagocytosis, luminol-amplified chemiluminescence activity did not significantly increase during endotoxemia (Fig. 4). From these two results, the unchanged luminol-enhanced chemiluminescence activity that was calculated for a total of 10^3 PMNL, together with the increase in the fraction of PMNL undergoing phagocytosis (Fig. 3B), one may draw the conclusion that the luminol-enhanced chemiluminescence activity per phagocytosing cell is severely depressed during endotoxemia. In contrast to this, lucigenin-enhanced chemiluminescence activity is greatly increased (Fig. 4).

In order to interpret the biological consequences of these findings, it is worthwhile to summarize briefly the basic chemical characteristics of the chemiluminogenic probes luminol and lucigenin. Luminol is a lipophilic compound of low molecular weight that can easily cross cell membranes, while lucigenin is a water-soluble, biscationic molecule of higher molecular weight that probably does not enter cells readily (9). The types of oxygen-derived reactive metabolites detected by both agents also differ significantly. In PMNL, luminol-amplified chemiluminescence activity is reflective of the halide-MPO-H_2O_2 system, which is active mainly within the phagosome (9, 10). Moreover, when luminol is used in the presence of serum proteins, e.g., albumin, which does not readily gain access to intracellular sites but which totally inhibits light emission from a cell-free MPO-H_2O_2 system, luminol-amplified chemiluminescence can be considered to detect only intracellular peroxidase oxygenation activity (3). Accordingly, in our experiments, measurements of luminol-amplified chemiluminescence were performed with whole-blood preparations diluted with simultaneously sampled autologous plasma. In order to eliminate the plasma’s intrinsic opsonic capacity on zymosan-induced chemiluminescence activity, zymosan particles precoated with C3 were added. In contrast to luminol, lucigenin, as has been well documented, reacts almost exclusively with superoxide anions released into the extracellular space (9, 27). Because luminol-enhanced chemiluminescence elicited by opsonized bacteria has been shown to correlate closely with the bactericidal capacity of PMNL (2, 17, 22), the endotoxemia-induced dysfunction of circulating PMNL observed in our experiments may be characterized as a deficit in the ability of the PMNL to kill microorganisms through oxygen-dependent mechanisms, whereas the production of superoxide anions which are potentially toxic to tissues is substantially increased. Modulation of leukocyte dysfunction during endotoxemia should, therefore, be directed at inhibiting the phagocytes’ cytotoxic reactions without compromising bacterial mechanisms.

In our search for such an agent, adenosine appears to be promising. This nucleoside has been shown in vitro and in vivo to be involved in the regulation of numerous functions of the PMNL with a dose-response profile that might well allow modulation of cellular responses known to contribute to endothelial cell injury during inflammatory states. Acting via A2 receptors, adenosine was shown to inhibit the numerical upregulation of \( \beta_2 \)-integrins, shedding of t-selectin (29), and adhesion to endothelial cells (6). In addition, adenosine inhibits the release of proteolytic enzymes (24) and superoxide anions (7). Moreover, in vitro studies demonstrated that the vascular endothelium releases endogenous adenosine in amounts sufficient to provide protection by inhibiting leukocyte adhesion (5) and the production of cytotoxic oxygen radicals (13).

The concentrations of adenosine able to inhibit the PMNL responses mentioned above are well within the physiological
range. The IC50s of adenosine calculated from data published regarding, e.g., the inhibition of N-formylmethionylphenylalanine-stimulated cell responses (O2, [7], degranulation [24], and adhesion molecule expression [29]) are all approximately 10^-7 M, which is within the concentrations determined for the plasma of healthy subjects. Compared with the relatively strong inhibition of these PMNL responses, adenosine appears to inhibit oxygen radical production induced by the phagocytosis of particulate stimuli to a much lesser extent. Phagocytosis has been reported to be uninhibited in the presence of adenosine even at millimolar concentrations (21) or to be only moderately inhibited in a subpopulation of PMNL that were allowed to become adherent (32).

When adenosine was infused continuously, concentrations in plasma increased to 6 x 10^-3 M prior to the beginning of endotoxemia and continued to increase to 10^-2 M by the end of the experiments (Fig. 1). Although these concentrations of adenosine in plasma are in the range of the binding affinities of A2 receptors (19), the infusion of adenosine failed to prevent leukopenia and neutropenia, both of which developed rapidly after the start of the infusion of endotoxin (data not shown). Accordingly, the nucleoside neither inhibited the shedding of T-selectin nor prevented the numerical and functional upregulation of β2-integrins, the latter being indicated by the altered binding of C3-coated zymosan particles to PMNL (Fig. 2 and 3).

These results are at variance with the aforementioned observations obtained from in vitro experiments. However, while in vitro studies adenosine was tested as to its effects on the alterations of adhesion molecules induced by one well-defined stimulus, the infusion of endotoxin must be expected to generate a large spectrum of inflammatory activators which together could activate PMNL in a complex way.

As was to be expected from the lack of any effect of adenosine on C3-mediated adhesion in our model, this nucleoside also had no effect on the marked increase in the percentage of phagocytosing PMNL induced by endotoxemia (Fig. 4B). In addition, the intracellular production of oxygen radicals induced by phagocytosis of C3-coated zymosan particles was not inhibited by adenosine. Thus, it appears that exogenously applied adenosine does not inhibit complement receptor type III-dependent adhesion, phagocytosis, or associated intracellular oxygen-dependent killing mechanisms of PMNL. However, one may argue that phagocytosis was measured after washing PMNL free of plasma and, therefore, that this measurement was taken in the absence of adenosine. In addition, even if plasma had not been replaced by HBSS in the assay, extracellular concentrations of adenosine would have rapidly decreased due to the very short life span of the nucleoside (amounting to a few seconds [18]). The observation that in control animals the spontaneous release of superoxide anions from PMNL was not inhibited by the infusion of adenosine (Table 1) is further evidence of an efficient elimination of the nucleoside. In contrast, the infusion of adenosine strongly inhibited the enhanced extracellular release of superoxide anions elicited by endotoxemia, even when measured in the absence of the nucleoside in vitro.

To answer whether adenosine can selectively moderate the

![Figure 5](https://iai.asm.org/)

**FIG. 5.** Effect of 2-Cl-adenosine on the C3-induced luminol-enhanced and C5a-, TNF-α-, TNF-α plus C5a-stimulated, lucigenin-enhanced chemiluminescence activities of blood PMNL from healthy pigs. 2-Cl-adenosine inhibited chemiluminescence activity irrespective of the stimulus used. The IC50 of 2-Cl-adenosine was determined for each stimulus from its dose-response curve by logistic regression analysis by using the Marquardt-Levenberg algorithm. The rank order of the stimulus-specific IC50 of 2-Cl-adenosine is given by the equation IC50 (C3 [5 x 10^7:1]) = 1.1 x 10^-3 M > IC50 (C5a, TNF-α, and C5a plus TNF-α) = 1.0 x 10^-6 M. In the absence of 2-Cl-adenosine, stimulus-induced control chemiluminescence activities were as follows: 4.55 ± 1.16 cpm/10^3 PMNL for C3 particle-to-cell ratio of 5 x 10^7:1, 1.6 ± 0.74 cpm/10^3 PMNL for 4 ng of C5a per ml, 2.5 ± 1.0 cpm/10^3 PMNL for 100 U of TNF-α per ml, and 13.5 ± 2.52 cpm/10^3 PMNL for TNF-α plus C5a (n = 6). Straight vertical line, IC50; medium dashed line, estimate of the inhibitory effects of the highest concentration of adenosine (10^-6 M) determined in endotoxic animals infused with adenosine.
functions of porcine PMNL also in vitro, the effects of 2-Cl-adenosine were studied. This metabolically stable compound can be compared to native adenosine with respect to its non-selective action at adenosine receptor sites (19). When the IC₅₀s are compared (Fig. 5), it is clear that 2-Cl-adenosine inhibited the intracellular production of oxygen radicals elicited by the phagocytosis of C₃-coated PMNL to a much lesser degree than the extracellular release of superoxide anions stimulated by inflammatory mediators known to be generated during endotoxemia (31). Moreover, taking the highest concentration of adenosine in plasma (10⁻²⁶ M) that was determined for adenosine-infused endotoxic animals (10⁻²⁶ M), one may extrapolate from the dose-response curves (Fig. 5) that the C₃-caused oxygen radical production might have been inhibited in vivo by only 10%, whereas superoxide anion production elicited by humoral stimuli might have been reduced by 50%. However, the even less-pronounced inhibitory effect of 2-Cl-adenosine on C₃-stimulated oxygen radical production could not be overcome by an increase in the strength of cell stimulation, because at a higher ratio of particles to cells, maximum inhibition by 2-Cl-adenosine was further decreased (Fig. 6). Since activation of protein kinase C has been suggested to play an important role in C₃-mediated phagocytosis (1), the effects of 2-Cl-adenosine on PMNL when activated by the phorbol ester PMA, which is known directly to activate protein kinase C, were also studied. As expected, 2-Cl-adenosine only slightly inhibited the release of superoxide anions induced by PMA. Interestingly, C₃-mediated phagocytosis has also been demonstrated to occur completely independently of the presence of intracellular calcium, because C₃-induced phagocytosis was not affected by buffering intracellular ionized calcium to concentrations of less than 10 nM (16). In contrast, activation of PMNL by the complement anaphylatoxin C₅a is greatly dependent on an increase in intracellular calcium (25). Because adenosine has been suggested to inhibit predominantly cellular activation by calcium-dependent stimuli (28, 32), the effect of 2-Cl-adenosine on superoxide anion production when stimulated by the ionophore A23187 was also tested. Compared to PMA, ionophore-stimulated superoxide anion production was much more strongly inhibited by the adenosine derivative. Although the mechanisms of cell priming are far from being understood, the elevation of intracellular calcium concentrations in resting and stimulated PMNL has been shown to be related to the phenomenon of the priming of PMNL by lipopolysaccharides (12).

Thus, it is conceivable that adenosine strongly inhibits the extracellular release of superoxide anions during endotoxemia by the selective attenuation of calcium-dependent priming effects without affecting protein kinase C-dependent bactericidal mechanisms.

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REFERENCES


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