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 α2-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 β$_2$-integrin family, which is constitutively expressed on PMNL, is the complement receptor type III (CR3, CD11b/CD18, and Mac-1). This β$_2$-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, nonparticular (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.

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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 induction of anesthesia (intravenous; 0.06 mg/kg body weight; ketamine hydrochloride, 4 mg/kg; and azaperone, 12 mg/kg), anesthesia was induced i.v. (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 not ventilated (arterial pressure of CO2, 35 to 45 mmHg; C02 tension, 35 to 45 mmHg) 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). Hemostasis was maintained by an i.v. infusion of Ringer's solution (5 ml/kg/h) containing fentanyl (0.004 µg/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. Core body temperature was kept between 36 and 37°C with a heating pad. ECG lead II was recorded continuously (Hellige Recomed; Siemens-Elema, Sweden). Anesthesia was maintained by continuous i.v. infusion of Ringer's solution (5 ml/kg/h) containing fentanyl (0.004 µg/ml) and sodium pentobarbital (0.3 mg/ml). A cytological fraction sampler (Isco Inc.). Following evaporation of the adenosine solution, 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 fluorescence detector (excitation, 350 nm; emission, 480 nm) using a method that has been described by Ontyd et al. (23). Arterial blood samples (2 ml) were drawn with a syringe from a catheter placed in the aorta (Cournand model 9505, Berthold, Wildbad, Germany). Neutrophil-specific activity was calculated using the fluorescence of luminogenic probes. For determination of luminol-enhanced chemiluminescence activity, whole blood was diluted with autologous plasma to a final hemoglobin concentration of 0.5 g/dl. Diluted cell suspensions were incubated for 15 min at 37°C and stimulated for a further 15 min by the addition of C3-coated zymosan particles or humoral stimuli, i.e., humoral stimuli specific for the detection of oxygen-reactive metabolites by the chemiluminescence method. The final concentration of luminol was 10-7 M in a volume of 250 µl of suspended cells. Luminofluor-enhanced chemiluminescence activity was assessed according to the procedure described above for luminol-enhanced chemiluminescence, with the exception that the cells were not suspended in autologous plasma but in HBSS and that cytochalasin B was added at a final concentration of 1 µg/ml. Cells were incubated for 15 min at 37°C. Cell viability was assessed by Trypan blue exclusion. In addition, phorbol myristate acetate (PMA; final concentration, 10-7 M) was added to some samples as a positive control for the detection of oxygen-reactive metabolites. The final concentration of luminol was 10-7 M in a volume of 250 µl of suspended cells. For determination of luminol-enhanced chemiluminescence activity, the cells were suspended in autologous plasma to a final hemoglobin concentration of 0.5 g/dl. Diluted cell suspensions were incubated for 15 min at 37°C and stimulated for a further 15 min by the addition of either particles or suspension medium. The specific stimulus and its solvent, i.e., HBSS, was used for the suspension of opsonized zymosan particles. The difference between the activities determined after addition of either particles or suspension medium was considered to reflect C3-stimulated intracellular oxygen radical production. The final concentration of luminol was 10-7 M in a volume of 250 µl of suspended cells. Luminofluor-enhanced chemiluminescence activity was assessed according to the procedure described above for luminol-enhanced chemiluminescence, with the exception that the cells were not suspended in autologous plasma but in HBSS and that cytochalasin B was added at a final concentration of 1 µg/ml. Cells were incubated for 15 min at 37°C. Cell viability was assessed by Trypan blue exclusion. In addition, phorbol myristate acetate (PMA; final concentration, 10-7 M) was added to some samples as a positive control for the detection of oxygen-reactive metabolites. The final concentration of luminol was 10-7 M in a volume of 250 µl of suspended cells.

Antiserum to PMNL was used as a measure of the functional upregulation of 2-integrins (CD11b/CD18 and CD11c/CD18), the adhesion of C3-coated zymosan particles (1 mg/ml). The difference between the activities determined after addition of either particles or suspension medium was considered to reflect C3-stimulated intracellular oxygen radical production. The final concentration of luminol was 10-7 M in a volume of 250 µl of suspended cells. Luminofluor-enhanced chemiluminescence activity was assessed according to the procedure described above for luminol-enhanced chemiluminescence, with the exception that the cells were not suspended in autologous plasma but in HBSS and that cytochalasin B was added at a final concentration of 1 µg/ml. Cells were incubated for 15 min at 37°C. Cell viability was assessed by Trypan blue exclusion. In addition, phorbol myristate acetate (PMA; final concentration, 10-7 M) was added to some samples as a positive control for the detection of oxygen-reactive metabolites. The final concentration of luminol was 10-7 M in a volume of 250 µl of suspended cells. Luminofluor-enhanced chemiluminescence activity was assessed according to the procedure described above for luminol-enhanced chemiluminescence, with the exception that the cells were not suspended in autologous plasma but in HBSS and that cytochalasin B was added at a final concentration of 1 µg/ml. Cells were incubated for 15 min at 37°C. Cell viability was assessed by Trypan blue exclusion. In addition, phorbol myristate acetate (PMA; final concentration, 10-7 M) was added to some samples as a positive control for the detection of oxygen-reactive metabolites. The final concentration of luminol was 10-7 M in a volume of 250 µl of suspended cells.
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, \( r = 0.96 ± 0.01 \) and \( 0.93 ± 0.04 \), respectively; for the numbers of PMNL versus spontaneous and C5a-stimulated lucigenin-enhanced chemiluminescence activity, \( r = 0.96 ± 0.07 \) 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 \( \beta_2 \)-integrins of circulating PMNL during endotoxemia. After endotoxemia was induced, the expression of \( \beta_2 \)-integrins 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 adherence 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-
ticles to PMNL increased and reached the highest values within the following 90 min. When animals were additionally infused with adenosine, endotoxemia-induced adhesion of C3-coated particles to PMNL was moderately attenuated, but with no statistically significant differences (Fig. 3A). When phagocytosis was allowed to occur subsequently to adhesion, the percentage of PMNL phagocytosing C3-opsonized zymosan particles increased strongly during endotoxemia. However, the elevation of concentrations of adenosine in plasma did not affect the enhancement of phagocytosis induced by endotoxemia. There were no significant changes in the percentages of PMNL binding or phagocytosing C3-opsonized zymosan particles, neither within nor between the groups infused with physiological saline or adenosine alone (Table 1).

**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-eclited 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-CI-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 nonspecific adenosine receptor agonist 2-CI-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>
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<th>Time (min)</th>
<th>Concentration of adenosine (nmol/liter)</th>
<th>No. of leukocytes (10^3/µl)</th>
<th>No. of PMNL (10^3/µl)</th>
<th>L-Selectin (relative fluorescence U)</th>
<th>β2-Integrins (relative fluorescence U)</th>
<th>FITC-positive PMNL Adhesion</th>
<th>Phagocytosis (cpm/10^6 PMNL)</th>
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<tr>
<td>0</td>
<td>III 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>1.5 ± 0.5</td>
<td>12.4 ± 5.0</td>
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<td></td>
<td>IV 642 ± 54</td>
<td>17.1 ± 1.4</td>
<td>10.3 ± 0.8</td>
<td>78.7 ± 21.7</td>
<td>40.0 ± 1.6</td>
<td>2.9 ± 0.6</td>
<td>8.9 ± 3.7</td>
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<td>30</td>
<td>III 130 ± 78</td>
<td>16.7 ± 3.3</td>
<td>10.8 ± 2.6</td>
<td>88.9 ± 23.9</td>
<td>38.0 ± 2.5</td>
<td>2.3 ± 1.1</td>
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<td>IV 1,634 ± 201</td>
<td>17.4 ± 1.5</td>
<td>11.0 ± 1.4</td>
<td>60.2 ± 14.9</td>
<td>39.7 ± 0.9</td>
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secondary humoral and cellular inflammatory mediators, in the in vitro experiments recombinant human complement anaphylatoxin C5a and TNF-α 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 (IC50), was as follows: IC50 (C3 [5 × 10^3:1]) ≈ 1.1 × 10^{-7} M >> IC50 (C5a, TNF-α, and C5a plus TNF-α) ≈ 1.0 × 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-α (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-TNF-α-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 t-selectin on circulating PMNL decreased, whereas numerical 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 chemiluminescent 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, bissaccharonic 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 bactericidal 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
The IC50s of adenosine calculated from data published regarding, e.g., the inhibition of N-formylmethionylphenylalanine-stimulated cell responses (O22, degranulation, and adhesion molecule expression) are all approximately 1027 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 631027 M prior to the beginning of endotoxemia and continued to increase to 1026 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 unaltered 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 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...
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 IC_{50}s 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^{-2} M) that was determined for adenosine-infused endotoxic animals (≈10^{-2} 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 phorbolester 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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