Synergistic Effect of Granulocyte Colony-Stimulating Factor and Drugs Elevating Extracellular Adenosine on Neutrophil Production in Mice

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Experimental evidence suggests that the activation of purinoceptors by extracellular adenosine can modulate proliferation and/or differentiation of hematopoietic cells. The present study was undertaken to investigate the potential interactions of this system of intracellular signaling with the effects of granulocyte colony-stimulating factor (G-CSF) on granulopoiesis in vivo. Elevation of extracellular adenosine in normal mice was induced by the joined administration of dipyridamole, a drug inhibiting the cellular uptake of adenosine, and adenosine monophosphate (AMP), an adenosine produg. The effects of dipyridamole, AMP, and G-CSF, administered either alone or in combinations, were evaluated. The agents were injected to mice in a 4-day regimen, and the hematoletic endpoints were determined 24 hours after the completion of the treatment. It was shown that the effects of G-CSF, ie, increases in peripheral blood neutrophils, granulocyte-macrophage progenitor cells (GM-CFC), and morphologically determined granulocytic cells in femoral marrow and a decrease in the marrow erythroid cells, can be enhanced by the combination of dipyridamole plus AMP administered 30 minutes before G-CSF. Furthermore, it was ascertained that the stimulatory action of dipyridamole plus AMP was expressed particularly at lower doses of G-CSF (1.5, 3, and 4.5 μg/dl). At higher doses of G-CSF (6 and 9 μg/dl), the interactions were no more evident. When combining dipyridamole, AMP, and 3 μg of G-CSF, peripheral neutrophils increased approximately 3.9- to 4.5-fold compared with an approximate 2.2-fold increase induced by G-CSF alone. The results indicate the possible therapeutic potential of combination therapy with G-CSF and drugs increasing extracellular adenosine.

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Granulocyte colony-stimulating factor (G-CSF) is a well-known hematopoietic regulator that accounts for the production, differentiation, and activation of neutrophilic granulocytes. In controlling granulopoiesis it acts in cooperation with other growth factors and regulatory signals that may increase or decrease its effects. In particular, possibilities of the enhancement of responses to G-CSF by other regulatory factors are of interest in view of the ability of this agent to reverse neutropenic states.

Experiments performed in vitro with a wide range of cell types have shown that purine nucleosides and nucleotides probably represent a universal and primitive system of intracellular signals that are capable of modulating several cellular functions including cell proliferation and differentiation. Attention was focused especially to adenosine and adenosine nucleotides, which bind to cell surface purinoceptors linked to the cAMP system. Because purinoceptors were shown to be expressed on myeloid cell lineages, these cell types might also be affected by regulatory signals of adenosine and adenosine nucleotides. For example, Gualtieri et al have shown that the addition of adenosine or adenosine monophosphate (AMP) to murine long-term bone marrow cultures resulted in increased granulopoiesis. It was reported that chronic treatment of myeloid progenitor cells with ATP analog modulated the cell phenotype towards differentiation. Adenosine was found to enhance DNA synthesis in a human myeloid leukemia-derived cell line. Yamaguchi et al described an enhancement of differentiation induction of mouse myelomonocytic leukemia cells by extracellular ATP.

We have shown previously that pharmacologically mediated adenosine receptor activation can modulate hematopoiesis even under in vivo conditions. We have found that the elevation of extracellular adenosine induced by the combined administration of dipyridamole, a drug inhibiting the cellular uptake of adenosine, and AMP to mice enhances granulopoiesis and exhibits radioprotective activity in terms of hematopoietic recovery. In these experiments, AMP was used instead of adenosine because of its greater solubility in water, which allowed us to administer small injection volumes and thus to avoid loading the mice with a large volume of fluid. It can be assumed that exogenously administered AMP is rapidly metabolized extracellularly to adenosine by cell surface ectonucleotidase activity. Our concept was based on the expectation that both AMP, serving as a source of exogenous adenosine, and dipyridamole, inhibiting adenosine uptake by cells and preserving it in the extracellular fluid, act jointly in enhancing extracellular adenosine level. Under these conditions, direct intracellular operation of adenosine can be ruled out and the effects obtained by such a treatment suggest that adenosine acts via cell surface receptors.

The present studies have been undertaken to determine the interactions of extracellular adenosine elevation with G-CSF in terms of their ability to enhance granulopoiesis in normal mice. The results of the experiments provide evidence of an enhancement of G-CSF-induced granulopoietic response by drugs elevating extracellular adenosine.

MATERIALS AND METHODS

Mice. Male (CBAxCS7BL/10)F1; mice 3 months of age and weighing an average of 25 g were used. The mice were produced in a conventional manner by the institute’s own farm. Standardized pelleted diet and HCl-treated tap water (pH 2-3) were given ad libitum. The mice were kept under controlled illumination conditions (light:dark 12:12) and the temperature was maintained at 22°C ± 1°C.
Drugs. Recombinant human G-CSF (Neupogen; purchased from F. Hoffman-LaRoche Ltd, Basel, Switzerland) was diluted with 5% glucose and injected subcutaneously at a single daily dose of 1.5, 3, 4.5, 6, and 9 pg per mouse in a volume of 0.1 mL. Dipyridamole (Sigma, St Louis, MO) was dissolved in 0.4% tartaric acid and injected subcutaneously at a single daily dose of 2 mg per mouse in a volume of 0.4 mL. Adenosine 5'-monophosphate sodium salt from yeast (Sigma) was dissolved in distilled water and injected intraperitoneally at a single daily dose of 5 mg free acid per mouse in a volume of 0.2 mL. Controls were injected with the same volumes of the pertinent vehicle.

Drug administration. In the combination treatment, dipyridamole was administered 20 minutes before AMP; G-CSF was administered 30 minutes after AMP (unless stated otherwise). Drugs were administered daily for 4 days at 9 to 10 am. Hematologic indices were determined 24 hours after the last administration of the drugs or of the vehicle.

Hematologic methods. Blood samples were taken from the tail vein. Mice were killed by cervical dislocation, femora were dissected, and marrow cells were flushed from the bone. Blood cell counts and number of nucleated cells of the bone marrow were determined using a Coulter Counter (model ZF; Coulter Electronics, UK), and differential counts were performed on smear preparations stained with the May-Grünwald-Giemsa method. The differential counts of marrow cells included the morphologically recognizable granulocytic (myeloblasts through segmented neutrophils) and erythroid (proerythroblasts through orthochromatic erythroblasts) precursor cells. Bone marrow hematopoietic progenitor cells committed to granulocyte-macrophage development (granulocyte-macrophage colony-forming cells [GM-CFCs]) were assayed using a semisolid plasma-clot technique. Briefly, femoral marrow suspensions were plated in quadruplicates using a 10% mouse lung-conditioned medium as a source of colony-stimulating factor. Colonies (>50 cells) were counted after 7 days of incubation in 37°C humidified environment containing 5% CO₂.

Experimental protocol. The main experiments compared several hematologic endpoints in mice treated with 4-day injection regimens and investigated 24 hours after the last treatment. The effects of dipyridamole, AMP, and G-CSF, administered either alone or in combinations, were evaluated. Because of technical circumstances, each individual experiment included parallel investigation of three drug-treated groups of 3 to 6 mice, together with the same number of control animals. Because seven experimental modifications were investigated in case of the peripheral blood cells and of the total cellularity of femoral marrow, the three drug-treated groups were differently combined in each experiment, and the experiments were performed eight times. Thus, each of the experimental modifications comprised pooled data from three to five experiments; controls were pooled from eight experiments. When investigating the counts of GM-CFCs and granulocytic and erythroid cells in femoral marrow, only three different experimental modifications were compared with the control and the experiment was repeated twice.

Statistics. In repeated experiments aimed at multiple comparisons, certain differences in the level of investigated indices appeared both among controls and among treated groups of mice (seasonal and other uncontrolled sources of variability). For the statistical treatment of these data, a special procedure was adapted involving a modified two-way analysis of variance for unbalanced and incomplete arrangements. Logarithmic transformation of the data was used to remove the inequality of variances and the asymmetry of the distributions. The procedure made it possible to normalize the results of the individual experiments into a common scale and to prove the multiplicative character of the effect of the treatment. The significance of differences in the effects of the treatment was analyzed by a sequentially rejective multiple test procedure. In some experiments, the nonparametric Mann-Whitney rank sum test was used. The significance level was set at $P < .05$.

RESULTS

Effects of different combinations of drugs on hematologic endpoints. The effects of dipyridamole, AMP, and G-CSF (3 μg/d), administered either alone or in combinations, were evaluated in terms of the ability of these agents to influence various hematologic indices. The drugs were administered in the 4-day treatment scheme, and their effects were determined 24 hours after the last treatment. The overall F value was used to define hematologic indices influenced significantly by the effects of the treatment. Statistically significant ($P < 10^{-5}$) effects were found in the case of peripheral neutrophils and lymphocytes as well as in GM-CFCs, granulocytic cells, and erythroid cells in the femoral marrow. The null hypothesis could not be rejected for peripheral erythrocytes and total cellularity of the femoral marrow; there were no significant differences in these indices between control and differently treated groups of mice (data not shown).

Blood neutrophils and lymphocytes. Altogether, eight study groups (including controls) were subjected to comparison. Figure 1 gives the relative data obtained when analyzing
peripheral neutrophils. P values of the significance are given in Table 1. Neutrophils increased moderately, but significantly, after AMP, dipyridamole, and a combination of these agents. The combined treatment with dipyridamole plus AMP did not bring an advantage compared with the effects of dipyridamole or AMP administered alone. Treatment with G-CSF alone increased the mean neutrophil counts 2.16-fold. The effects of G-CSF on these cells was further enhanced significantly by the preceding administration of dipyridamole administered alone (3.24-fold increase). Coadministration of dipyridamole plus AMP with G-CSF yielded the best results. Under these conditions, peripheral neutrophils increased 4.46-fold. Interestingly, AMP alone significantly decreased the neutrophilia induced by G-CSF.

As regards the blood lymphocyte counts, the only statistically significant (P < .001) finding was the moderate 1.38-fold increase in mice treated with the combination of dipyridamole plus G-CSF compared with control (data not shown). The meaning of this effect is not clear.

Bone marrow hematopoiesis. Four study groups (including controls) were compared. Counts of GM-CFCs in femurs increased significantly after dipyridamole plus AMP and after G-CSF alone. The highest increase in counts of these cells was induced by the combined treatment with dipyridamole, AMP, and G-CSF (Fig 2, for statistical analysis see Table 2). Similar changes were observed in the granulocytic cells of the femoral marrow (Fig 3, for statistical analysis see Table 2). Because the total femoral cellularity was not influenced, increase in granulocytic cells was realized at the expense of erythroid cells (Fig 4, for statistical analysis see Table 2). Relative changes in granulocytic to erythroid ratio, compared with the control taken as 1.00, were 1.49 after dipyridamole plus AMP, 2.68 after G-CSF alone, and 4.01 after combining all the agents.

Effect of dipyridamole plus AMP on neutrophilia induced by different doses of G-CSF. Separate experiments evaluated the ability of the combination of dipyridamole plus AMP to stimulate neutrophilia induced by different doses of G-CSF. Drugs were administered in the 4-day scheme, and peripheral neutrophils were determined 24 hours after the last treatment. The results are shown in Fig 5. The effects of G-CSF administered alone were clearly dose-dependent. Administration of dipyridamole plus AMP before G-CSF further increased significantly neutrophil counts at 1.5, 3, and 4.5 μg of G-CSF. With doses of 6 and 9 μg of G-CSF, dipyridamole plus AMP failed to induce significant stimulatory effects.

Persistence of the stimulatory effect of dipyridamole plus AMP on neutrophilia induced by G-CSF. Pilot experiments

Further analysis of the stimulatory effect of dipyridamole plus AMP on neutrophilia was carried out. The combined treatment with dipyridamole plus AMP with G-CSF yielded the best results. Under these conditions, peripheral neutrophils increased 4.46-fold. Interestingly, AMP alone significantly decreased the neutrophilia induced by G-CSF.

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Effect of dipyridamole plus AMP on neutrophilia induced by different doses of G-CSF. Separate experiments evaluated the ability of the combination of dipyridamole plus AMP to stimulate neutrophilia induced by different doses of G-CSF. Drugs were administered in the 4-day scheme, and peripheral neutrophils were determined 24 hours after the last treatment. The results are shown in Fig 5. The effects of G-CSF administered alone were clearly dose-dependent. Administration of dipyridamole plus AMP before G-CSF further increased significantly neutrophil counts at 1.5, 3, and 4.5 μg of G-CSF. With doses of 6 and 9 μg of G-CSF, dipyridamole plus AMP failed to induce significant stimulatory effects.

Persistence of the stimulatory effect of dipyridamole plus AMP on neutrophilia induced by G-CSF. Pilot experiments
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were performed to investigate the persistence of the stimulatory action of dipyridamole plus AMP on the response of peripheral neutrophils to 3 μg/d of G-CSF. G-CSF was administered at intervals of 30 minutes and 1, 2, 3, and 4 hours after the treatment with dipyridamole plus AMP; neutrophil counts were determined 24 hours after the 4-day treatment scheme, as described above. It was ascertained that the combination of dipyridamole plus AMP retained its stimulatory effect on G-CSF response up to 2 hours; at later intervals, the effect decreased, but was still evident (data not shown).

**DISCUSSION**

The observed effects of G-CSF on granulopoiesis, including an increase in peripheral blood neutrophils and an expansion of the bone marrow pool of myeloid cells, are consistent with earlier reports describing the action of this growth factor in mice.\(^{18-23}\) Also, the concomitant decrease of erythroid cells in bone marrow, suggesting a decline of marrow erythropoiesis after G-CSF treatment, is a known phenomenon related probably to the lineage-competitive effects. In mice, the loss of marrow erythropoiesis seems to be effectively compensated by splenic erythropoiesis.\(^{22,23}\)

Our results show that all the investigated effects of G-CSF treatment can be enhanced by the action of drugs elevating extracellular adenosine. Moreover, they suggest that adenosine uptake by cells and intracellular metabolism of this nucleoside do not play any positive role in these effects. This is indicated by the positive effects of dipyridamole, an inhibitor of adenosine uptake by cells,\(^{12}\) when administered alone or, in particular, in combination with AMP, a source of exogenous adenosine. Dipyridamole retains the exogenously administered adenosine in extracellular spaces. Effects of dipyridamole administered alone can be explained by its ability to increase the extracellular level of endogenously produced adenosine.\(^{24}\) As shown in the case of peripheral neutrophil counts, the presence of dipyridamole in the combination appears to be necessary for inducing the stimulatory effects. AMP alone even antagonizes the effects of G-CSF. It is possible that when AMP is administered without dipyridamole, adenosine liberated from this nucleotide is rapidly transported into cells. Through an intracellular regulatory unit, "P-side," adenosine can mediate an action quite different from its extracellular receptor operation.\(^{22,23}\) Tanaka et al\(^{26}\) have observed that adenosine induced typical apoptotic death of the human leukemia HL-60 cells and that this effect could be suppressed by dipyridamole.

Extracellular adenosine is generally believed to exert its physiologic effects by binding to specific membrane recepto...
tors coupled to the inhibition (A₁) or activation (A₂) of adenylyl cyclase, which regulates the intracellular concentration of cAMP. Because evidence exists suggesting that A₁ receptors are also linked to additional effector systems, including guanylyl cyclase, the role of this transducing pathway cannot be excluded. The present study does not address the type of adenosine receptors activated. Also, mechanisms of interactions of adenosine and G-CSF effects remain to be established. Without their understanding it is difficult to explain the lack of the stimulatory action of dipyridamole plus AMP at relatively higher doses of G-CSF. This effect might suggest that the signals produced by high stimulation with G-CSF encompass signals produced by stimulation of adenosine receptors. Hypothetically, interactions of both factors can be based on their sequential action within a particular cell lineage, on the coalescence of the initially differing signaling pathways into a common pathway, on changes in receptor properties, etc. However, it is reasonable to speculate that both G-CSF and adenosine receptors can operate through a common signaling pathway of cyclic nucleotides. Matsuda et al suggested that, after the binding of G-CSF to its surface receptors, the activation of the guanine triphosphate-binding protein/adenylate cyclase system can be involved in the proliferation of murine immature myeloid cells. As shown by Byron, both cAMP- and cGMP-associated signals can provide proliferative stimuli to multipotential hematopoietic stem cells. Kurland et al have shown that the proliferative response of murine committed granulocyte-macrophage progenitor cells to the action of colony-stimulating factor can be antagonized by agents elevating cAMP and potentiated by agents increasing cGMP. Treatment of mature neutrophils with GM-CSF was found to increase their cGMP content. While analyzing the role of cAMP and cGMP in HL-60 cell differentiation, concluded that elevation of both cAMP and cGMP above a critical level is necessary for inducing differentiation in this cell line.

The receptor action of adenosine exerts multiple physiologic effects. Adenosine participates in the autoregulation of blood flow, inhibits platelet aggregation, and increases production of erythropoietin. It can also have anti-inflammatory properties and act as an endogenous activator of the cellular antioxidant defense system. Our results add one more activity to this list of extracellular adenosine activities, i.e., its ability to enhance the response of myeloid cells to G-CSF. Because the effects of relatively low doses of G-CSF have been especially enhanced by the combination of dipyridamole plus AMP, extracellular adenosine could play the role of an anchor factor-like activity proposed by Lowry et al as a general mechanism allowing hematopoietic growth factors to exert their effects at physiologically relevant levels.

Our experiments show that the interactions of the signaling by extracellular adenosine and of the G-CSF effects can be induced pharmacologically under in vivo conditions. When considering this possibility, toxicity aspects have to be mentioned. The doses of dipyridamole and AMP, based on our previous experiments, exhibit low toxicity. Doses of 5 mg of AMP and of 2 mg of dipyridamole represent 1/16 and 1/40 of LD₅₀ in mice, respectively. Both agents are clinically available drugs. Dipyridamole is widely used as a drug decreasing platelet aggregation. AMP has been used as a vasodilator and a cardioprotective agent. Thus, the clinical use of drugs increasing extracellular adenosine for enhancing effects of therapy with G-CSF and, consequently, reducing its cost needs might be a promising possibility.

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