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Drugs Elevating Extracellular Adenosine Administered *in vivo* Induce Serum Colony-Stimulating Activity and Interleukin-6 in Mice

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Summary

Our previous studies have shown that the combined administration of drugs elevating extracellular adenosine, i.e. dipyridamole (DP) and adenosine monophosphate (AMP), enhances murine hematopoiesis and potentiates the action of granulocyte colony-stimulating factor (G-CSF). In this study, colony-stimulating activity (CSA) of blood serum of mice treated with DP+AMP, G-CSF or all these drugs in combination, i.e. the ability of the sera to stimulate the growth of GM-CFC colonies, was assayed *in vitro*. Furthermore, the concentration of GM-CSF and IL-6 in the sera was determined. Administration of DP+AMP was found to enhance significantly serum CSA at all time intervals of serum sampling including 24 h after the last injection of the tested drugs. Additive effects of DP+AMP and G-CSF on serum CSA were noted at early intervals after administration of the drugs. Furthermore, IL-6 levels were significantly elevated in the sera of mice which were administered DP+AMP either alone or in combination with G-CSF. Our results show that the effects of DP+AMP are indirect, mediated through the induction of some cytokine(s) and/or growth factor(s) and that extracellular adenosine can act in cooperation with G-CSF. These findings contribute to the further elucidation of the role of adenosine in hematopoiesis.

Key words

Extracellular adenosine • Granulopoiesis • G-CSF • Serum colony-stimulating activity • GM-CFC • IL-6

Introduction

Adenosine and adenine nucleotides represent a universal system of intracellular signals that are capable of modulating a variety of cell functions including cell growth, development and maturation (Abbracchio 1996, Schulte and Fredholm 2003). There is evidence that adenosine and synthetic analogues of adenosine can act as potent regulators of the proliferation of normal and tumor cells from various sources (Ohana *et al.* 2001, Mujoomdar *et al.* 2003, Kronenwett *et al.* 2005) or may trigger apoptosis in cancer cells under certain circumstances (Kohno *et al.* 1996, Barry and Lind 2000). Studies performed in our laboratories have shown that adenosine signaling can also participate in the control of hematopoiesis. It has been demonstrated that elevation of extracellular adenosine under *in vivo* conditions induced by the combined administration of dipyridamole (DP)

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and adenosine monophosphate (AMP) enhances hematopoiesis in normal and myelosuppressed mice and synergizes with the effects of granulocyte colony-stimulating factor (G-CSF) (Hofer *et al.* 1997, 1999, 2001, 2002, Pospíšil *et al.* 1995, 1998, Weiterová *et al.* 2000). It has been noted that these hematopoiesis-stimulating effects are pleiotropic and result from enhanced cycling of progenitor cells (Pospíšil *et al.* 2001). Adenosine has been shown to potentiate stimulatory effects of certain hematopoietic growth factors also in an *in vitro* study (Hofer *et al.* 2006). The possibility to utilize adenosine receptor signaling in enhancing hematopoiesis has been confirmed by experiments of Fishman *et al.* (2000) and Bar-Yehuda *et al.* (2002) demonstrating the curative effects of adenosine and an adenosine A3 receptor agonist on hematopoiesis in cyclophosphamide-treated mice.

The extracellular regulatory actions of adenosine are mediated *via* four subtypes of G protein-coupled receptors distinguished as A1, A2A, A2B and A3 (Klotz 2000, Fredholm *et al.* 2001). Adenosine signaling observed in the above-mentioned studies modulates the growth of different populations of progenitor cells by activating different adenosine receptor subtypes. Besides directly affecting target cells *via* a receptor-dependent mechanism, adenosine signaling could also modulate hematopoiesis indirectly through its action on the regulatory network. It has been reported that activation of adenosine receptors modifies the production of secondary mediators such as cytokines and growth factors in an astrocytoma cell line, lipopolysaccharide-stimulated endothelial cells, monocytes and macrophages (Bouma *et al.* 1994, 1996, McWhinney *et al.* 1996, Jacobson 1998). In studies using selective agonists of adenosine receptors, modifications of cytokine release *in vivo* have been demonstrated (Hasko *et al.* 2000, Bar-Yehuda *et al.* 2002, Forrest *et al.* 2005). Moreover, Fishman *et al.* (2000) demonstrated the capability of the agonists of A1 and A3 adenosine receptors to induce G-CSF production, which led to hematopoiesis-stimulating effects.

Hematopoiesis is regulated by a complex interplay between the bone marrow hematopoietic and microenvironmental cells. The production of cytokines plays a critical role in this process. The experiments with many types of agents have demonstrated that hematopoiesis-stimulating effects of these drugs positively correlate with the up-regulation of cytokine production such as granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor (GM-CSF), erythropoietin, thrombopoietin, stem cell factor, interleukin-1, interleukin-3 and interleukin-6 (IL-6) (Halaas *et al.* 1997, Son *et al.* 2003, Lebedev *et al.* 2004, Talmadge *et al.* 2004, Hanley *et al.* 2005). The involvement of adhesion molecules, which are expressed on hematopoietic stem/progenitor cells and cells of the bone marrow microenvironment, in the regulation of production GM-CSF and IL-6 has been described (Khaldoyanidi *et al.* 2002).

It has previously been reported in our laboratory that the addition of sera of mice administered various immunomodulatory agents to cultures of normal murine bone marrow cells can potentiate proliferation of hematopoietic progenitor cells for granulocytes and macrophages (GM-CFC) *in vitro*. These findings have added new information about the ways by which these immunomodulators exert their hematopoiesis-stimulating activity (Vacek *et al.* 2000, 2001). To obtain further information on the role of adenosine receptor signaling in hematopoiesis, we assessed the ability of sera of mice treated with drugs elevating extracellular adenosine given alone or in combination with G-CSF to stimulate proliferation of GM-CFC *in vitro*. It is known that colony-stimulating activity of sera is mediated by induced production of secondary substances stimulating the GM-CFC proliferation (Fedoročko *et al.* 2002). To obtain some data on the mechanisms through which the sera of mice treated with drugs elevating extracellular adenosine exert their colony-stimulating activity, the production of GM-CSF and IL-6 in the sera has been determined by the ELISA method.

Materials and Methods

Animals

B10CBAF1 male mice or ICR1 female mice aged 3 months and weighing an average 30 g were obtained from the breeding facility of the Medical Faculty, Masaryk University, Brno, Czech Republic. The mice were kept under controlled conditions; standardized pelleted diet and HCl-treated tap water were available *ad libitum*. The use and treatment of animals followed the European Community Guidelines as accepted principles for the use of experimental animals. The experiments were performed with the approval of the Institute Ethics Committee.

Drugs and their administration

Based on our earlier experience (Pospíšil *et al.* 1995, 1998, Hofer *et al.* 1999), elevation of extracellular adenosine was induced by the combined administration of

dipyridamole (DP), a drug inhibiting the cellular uptake of adenosine, and AMP, serving as a source of exogenous adenosine. DP (Sigma-Aldrich, St. Louis, MO) was dissolved in 0.4 % tartaric acid and injected subcutaneously at a dose of 2 mg per mouse in a volume of 0.4 ml. AMP from yeast (Sigma-Aldrich) was diluted with saline and given intraperitoneally at a dose of 5 mg free acid per mouse in a volume of 0.2 ml 20 min after the administration of DP. In the combination treatment, recombinant human G-CSF (Neupogen, F. Hoffman-LaRoche Ltd., Switzerland) was dissolved in 5 % glucose and injected subcutaneously at a dose of 1.5 μg per mouse in a volume of 0.1 ml 30 min after AMP. Respective vehicles were injected to the controls. In the repeated administration of drugs, 24-h intervals were used. DP+AMP, G-CSF, or all these drugs in a combination or respective vehicles were administered either singly or repeatedly in a 4-day treatment regimen.

Preparation of sera

One, 3, 5, 12 or 24 h after a single injection or the last one, peripheral blood samples were collected by cardiac puncture and sera of 5 mice were pooled in each experimental group. After two hours of blood incubation at room temperature and centrifugation, sera were removed and stored at <20 ºC until *in vitro* testing. Sera of animals in all experimental groups were added to the cultures of normal bone marrow cells, *in vitro* clonogenic assays for GM-CFC were performed and the colony stimulating activities of blood serum were evaluated.

In experiments monitoring the production of GM-CSF and IL-6 in sera, peripheral blood from each mouse taken 3 or 5 h after a single injection of the tested drugs was placed into individual tubes. Sera were prepared and stored as described above until the implementation of ELISA assays.

Assessment of GM-CFC numbers

For GM-CFC determination, femoral bone marrow cells from untreated mice were withdrawn by flushing the femoral bone with Iscove's modification of Dulbecco's medium (IMDM) and counted with a Coulter counter (Model ZF; Coulter Electronics Ltd, Luton, Beds, UK). The cells were then plated in triplicates in a semisolid environment created by a plasma clot (Hofer *et al.* 2005, Vacek *et al.* 1990, Pospíšil *et al.* 2004) containing IMDM plus 20 % fetal bovine serum, 1 % conditioned medium containing recombinant murine interleukin-3 (rmIL-3) produced by a myeloma cell line (purchased from the Institute of Hematology and Blood Transfusion, Prague, Czech Republic), 10 % citrate bovine plasma, and $CaCl_2$ at a concentration of 1.5 mg/ml. Colonies of at least 50 cells were counted after 7 days of incubation in 37 ºC humidified environment containing 5 % CO_2. The experiments were repeated six times.

Colony stimulating activity (CSA) was defined as the ability of the sera of drug-treated mice to stimulate the growth of GM-CFC colonies, induced by a suboptimum concentration of rmIL-3. The dose of rmIL-3 was adjusted to a value that yields 30 % of maximum numbers of colonies in dose-dependent curve.

ELISA assays

Concentrations of murine IL-6 and murine GM-CSF in the sera were measured using commercially available ELISA kits (R&D Systems, Inc, Minneapolis, USA). The assay had sensitivities of 3.1 pg/ml for IL-6 and 1 pg/ml for GM-CSF. The experiments were repeated twice.

Statistics

The results are presented as means ± S.E.M. The experimental data were subjected to Kruskal-Wallis analysis of variance followed by detailed nonparametric analysis between groups or were evaluated by the t-test with Holms correction for multiple comparisons. The significance levels were set as $P<0.05$ and $P<0.01$.

Results

Effects of single administration of DP+AMP, G-CSF or combination of all the drugs on serum CSA

As statistically significant differences among the colony-stimulating activity (CSA) of sera of intact controls and controls injected by respective vehicles were not demonstrated, all control groups were linked together into one control group. In comparison with the controls, sera of mice given a single injection of the drugs elevating adenosine (DP+AMP) were found to have significantly enhanced CSA in all of the tested time intervals after administration of the drugs (1, 3, 5, 12, and 24 hours). The same was true for sera of mice administered G-CSF alone or given the combination of all the three drugs (Fig. 1A). As follows from Figure 1A, very similar results were obtained in the time intervals of 1, 3, and 5 h (denoted further as early time intervals); a considerable similarity was observed also in the time

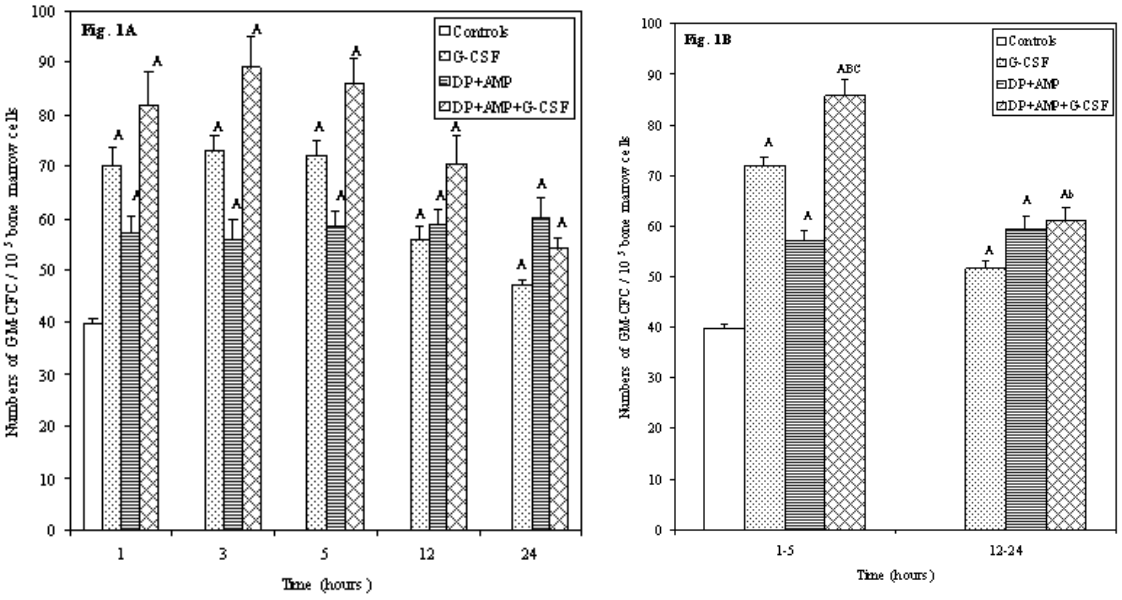

Fig. 1A, B. Numbers of GM-CFC per 10^5 bone marrow cells in cultures treated with serum of mice administered G-CSF, DP+AMP or a combination of DP+AMP+G-CSF in a single dose. Data are given as means ± S.E.M. Controls – sera of control mice. Time - time-interval between the last injection of the drugs and serum sampling (Fig. 1A – values in individual time intervals, Fig. 1B – values in grouped early and late intervals). Statistical significance: A, $p<0.01$, in comparison with controls. B and b, $p<0.01$ and $p<0.05$, respectively, in comparison with the cultures treated with serum of mice administered G-CSF alone. C, $p<0.01$, in comparison with the cultures treated with serum of mice administered DP+AMP.

intervals of 12 and 24 h (denoted further as late time intervals). Since appropriate statistical approaches revealed that it was possible to put together data from the early time intervals, as well as those from the late time intervals, the data were grouped in this way, and further statistical testing was performed in these newly formed groups of data from early and late time intervals. Figure 1B shows that in the early time intervals the CSA of sera of mice given a combination of DP+AMP+G-CSF was significantly higher in comparison with the CSA of control sera as well as with that of sera of mice given DP+AMP alone. In the late time intervals there was no significant difference between CSA of sera of mice given the drugs elevating adenosine (DP+AMP) alone and that of sera of mice administered the DP+AMP+G-CSF combination (Fig. 1B).

Effects of repeated administration of DP+AMP, G-CSF or combination of all the three drugs on serum CSA

When the drugs were administered repeatedly in a 4-day regimen and sera were taken at the intervals of 1, 3, 5, 12 and 24 h after the last injection of the drugs, the results obtained were very similar to those found at the same time intervals after a single injection of the drugs (Fig. 2A). The arrangement of the data made it possible to group them in the same way as performed with those obtained after a single drug injection; after putting the data together into the data groups from early and late time intervals, again a similar picture, including the lack of a statistically significant difference was obtained (Fig. 2B) between the value of the DP+AMP group and that of the DP+AMP+G-CSF group in the late time intervals.

The detection of IL-6 and GM-CSF in the sera of experimental animals

IL-6 and GM-CSF levels were determined in the sera of mice administered DP+AMP alone, G-CSF alone, or the combination of DP+AMP+G-CSF and assessed in the time intervals of 3 or 5 h after the injection. The results of IL-6 are summarized in Figure 3. A significant elevation of the IL-6 level was detected neither in the sera of control mice nor in those of mice given G-CSF alone. As shown in Figure 3, IL-6 levels were significantly elevated in the sera of mice which were administered DP+AMP either alone or in combination with G-CSF. No statistically significant differences were found between IL-6 levels in the sera of mice given DP+AMP and those of mice administered DP+AMP+G-CSF in combination. No detectable GM-CSF levels were determined in the sera of control mice as well as in the sera of mice administered the tested drugs (data not shown).

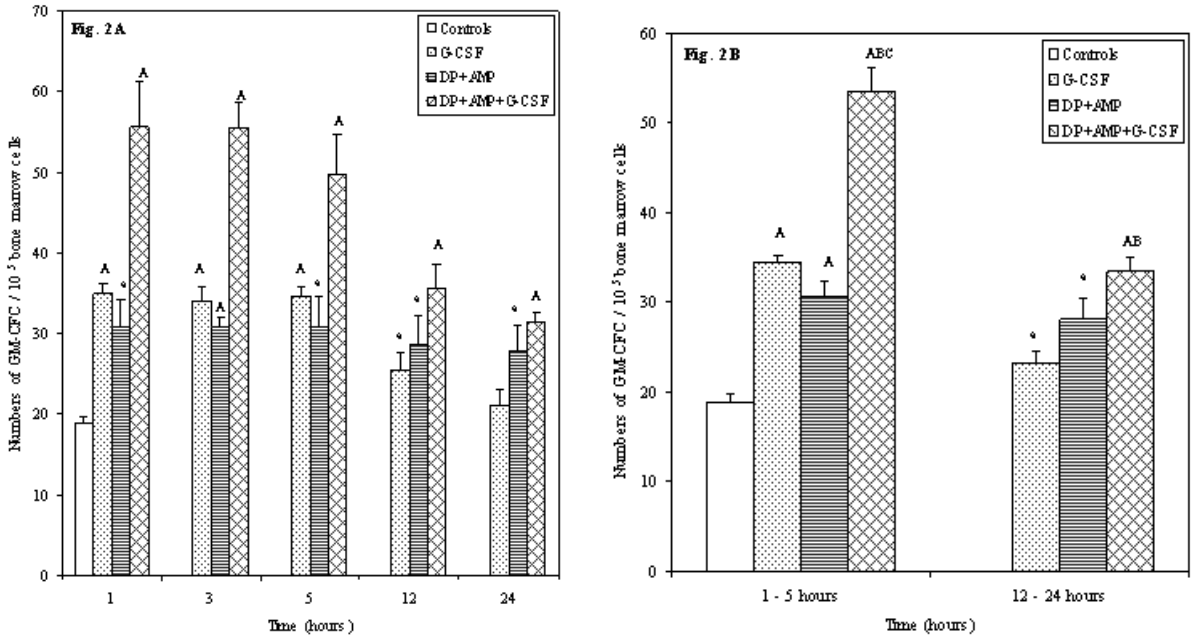

Fig. 2A, B. Numbers of GM-CFC per 10^5 bone marrow cells in cultures treated with serum of mice administered G-CSF, DP+AMP or a combination of DP+AMP+G-CSF in a 4-d treatment regimen. Data are given as means ± S.E.M. Controls - sera of control mice. Time – time interval between the last injection of the drugs and serum sampling (Fig. 2A – values in individual time intervals, Fig. 2B – values in grouped early and late intervals). Statistical significance: A and a, $p<0.01$ and $p<0.05$, respectively, in comparison with controls. B, $p<0.01$, in comparison with the cultures treated with serum of mice administered G-CSF alone. C, $p<0.01$, in comparison with the cultures treated with serum of mice administered DP+AMP.

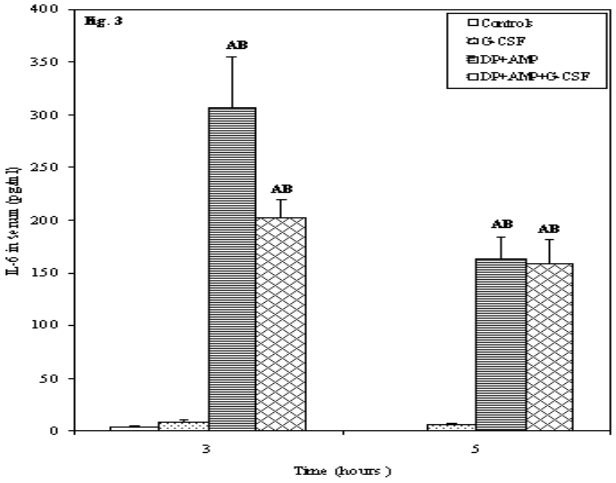

Fig. 3. IL-6 in sera of mice administered G-CSF, DP+AMP or a combination of DP+AMP+G-CSF in a single dose. Data are given as means ± S.E.M. Controls - sera of control mice. Time – time interval between the last injection of the drugs and serum sampling. Statistical significance: A, $p<0.01$, in comparison with controls. B, $p<0.01$, in comparison with serum of mice administered G-CSF alone. Results represent data of two experiments.

Discussion

The results of the present experiments indicate that sera of mice administered DP+AMP and thus enhancing adenosine receptor-mediated actions are able to potentiate the production of GM-CFC in the cultures of normal bone marrow cells, i.e. that the sera exhibit CSA. Furthermore, it follows from our results that DP+AMP can act in cooperation with G-CSF, a granulopoiesis-stimulating hematopoietic growth factor, in inducing the colony growth. These findings are consistent with results of studies performed previously in our laboratories, which showed that administration of DP+AMP stimulates hematopoiesis in myelosuppressed mice (Hofer *et al.* 1995, 2002). Moreover, the elevation of extracellular adenosine was found to mobilize significantly both GM-CFC and granulocytes into the peripheral blood after both single or repeated administration of DP+AMP (Hofer *et al.* 2003). It has also been shown that DP+AMP and G-CSF synergize to enhance granulopoiesis in normal mice (Pospíšil *et al.* 1995) and to increase hematopoietic recovery from myelosuppression resulting from exposure of mice to ionizing radiation and/or cytostatic drugs (Pospíšil *et al.* 1998, Hofer *et al.* 1999, 2001, Weiterová *et al.* 2000). It has been noted that these hematopoiesis-stimulating effects are pleiotropic and result from enhanced cycling of progenitor cells (Pospíšil *et al.* 2001). The possibility of utilizing adenosine receptor signaling in enhancement of hematopoiesis has also been confirmed by experiments of Fishman *et al.* (2000) and Bar-Yehuda *et al.* (2002) demonstrating the

curative effects of adenosine and an adenosine A3 receptor agonist on hematopoiesis in cyclophosphamide-treated mice.

In the present study, the effectiveness of sera of mice taken in early (1-5 h) and late time intervals (12-24 h) after administration of the tested drugs was investigated. Evaluating CSA in the early time intervals a few hours after administration of the tested drugs opens the question of whether CSA of the serum in the early time intervals is mediated by factors induced by the drugs in recipient's organism, or if simply the effects of these drugs still persisting in the serum are observed. Adenosine is short-lived in the organism. The data in humans show that the half-life of adenosine in plasma or whole blood is only a few seconds (Klabunde 1983, Möser *et al.* 1989). In our study, AMP was used instead of adenosine because its greater solubility in water allowed to achieve effective plasma concentrations without an excessive fluid load. It is known that AMP is rapidly metabolized extracellularly to adenosine *via* ecto-5′-nucleotidase, an enzyme present in a variety of cells (Gordon *et al.* 1989). The half-life of degradation of adenosine nucleotides to adenosine in the extracellular space is again very short; adenosine becomes undetectable within tens of seconds (Shapiro *et al.* 1992, Meghji 1993, Dunwiddie *et al.* 1997). Moreover, the experiments examining the relation between adenosine production after administration of AMP to rats and increased blood flow suggested that AMP is rapidly transported to the active site for adenosine (Obata *et al.* 1996). As proposed in a study of kinetics of cell growth by Gualtieri *et al.* (1986), the addition of adenosine to long-term cultures results in similar effects as that observed with AMP. The data in literature thus provide evidence for rapid degradation of administered AMP and its metabolite adenosine. They seem to exclude the possibility that the effects of the drugs elevating extracellular adenosine on CSA of the serum observed in our experiments could be due to direct effects of adenosine persisting in the serum from the administered AMP till the time intervals of sampling. Therefore, the effects of extracellular adenosine may be supposed to be indirect, mediated by some cytokine(s) and/or growth factor(s) whose production is triggered by the adenosine receptor-mediated action. This holds for the action of DP+AMP alone, as well as for the effects of these drugs when given concomitantly with G-CSF. To obtain further support for this assumption, we added DP and AMP to the cultures of bone marrow cells in concentrations corresponding to levels of these drugs in the serum of mice at the time of administration. In comparison with controls, changes in the numbers of GM-CFC were not observed (data not given). Results of this additional experiment show that even when DP and AMP were present in the serum at the time of the sampling still in the same concentrations as were those attained immediately after the administration of the drugs, they were not able to induce the observed CSA effect alone. Less clear is the conclusion when evaluating the effects of G-CSF from this point of view. Data obtained in humans show the half-life of G-CSF is about 1-3 h (Akizawa *et al.* 1995, Kuwabara *et al.* 1996, Watari *et al.* 1997, Molineux 2004, Hernandez-Bernal *et al.* 2005). This implies that the serum of mice could probably contain sufficient concentrations of G-CSF capable of stimulating the growth of colonies from GM-CFC directly. However, it has to be noted that in our study aimed at the effects of extracellular adenosine, G-CSF served predominantly for the assessment of a possible enhancement of its granulopoietic effects by the drugs elevating extracellular adenosine, not for analyzing the mechanisms of its action.

The observations and considerations of the indirect effect of the drugs elevating extracellular adenosine brought us to search for the substance(s) responsible for the mediation of adenosine's membrane receptor action. In these experiments, increased IL-6 levels in the serum sampled at 3 or 5 h after single administration of DP+AMP were found. IL-6 is a pleiotropic cytokine, thought to play a key role in the regulation of hematopoiesis (Rodriguez *et al.* 2004, Nakamura *et al.* 2004). Although IL-6 alone does not stimulate *in vitro* proliferation of hematopoietic cells, it can synergize with other factors to induce proliferation of hematopoietic progenitor cells (Ikebuchi *et al.* 1988, Ogawa 1993, Patchen *et al.* 1993).

There is increasing evidence that adenosine signaling plays a role in the regulation of processes in the cytokine network. It was found that adenosine increased the release of IL-6 from human lung fibroblasts, rat peritoneal macrophages, adrenal and ovarian cells (Ritchie *et al.* 1997, Zhong *et al.* 2005) and augmented its production by monocytes stimulated with IL-1β or lipopolysacharides (Bouma *et al.* 1994, Song *et al.* 2005). It was reported that cAMP-dependent protein kinase is involved in the regulation of IL-6 production and the increased cAMP level causes a significant potentiation of IL-6 release from monocytes, fibroblasts and LPS-treated macrophages (Vellenga *et al.* 1991, Martin and Dorf 1991, Tang *et al.* 1998). Recently it has been shown in

studies with selective adenosine receptor agonists that adenosine receptor stimulation has a differential effect on the synthesis and release of IL-6 and may induce its release in cell cultures (Straub *et al.* 2002, Forrest *et al.* 2005). Our findings thus provide new data about the role of cooperation between regulatory actions of adenosine signaling and those of IL-6. On the other hand, GM-CSF, a hematopoietic cytokine with a specific ability to stimulate the proliferation and differentiation of GM-CFC, was not detected in our experiments in sera of mice given the drugs elevating extracellular adenosine. These results correspond to the findings from studies with drugs elevating cAMP level, suggesting a significant relationship between cAMP elevation and the inhibition of GM-CSF production (Shichijo *et al.* 1999, Seldon and Giembycz 2001, Lazzeri *et al.* 2001). In the present study, we limited our search for the mediators of the effects of extracellular adenosine to IL-6 and GM-CSF. However, it can also possible that other colony-stimulating factors and/or cytokines are responsible for observed CSA in addition to IL-6 found in our experiments. This field is open for further studies.

A generally lower level of the values of GM-CFC per 10^5 bone marrow cells was observed in experiments evaluating repeated administration of the drugs. This finding may probably be explained by seasonal variations in GM-CFC numbers because the experiments testing single and repeated administration were performed in different periods of the year.

Our present results add new information on the mechanisms of hematopoiesis-stimulating effects of receptor-mediated action of extracellular adenosine. Furthermore, the observed prolonged effectiveness of the combination of DP+AMP manifested by the elevation of CSA up to 24 hours after a single administration of the drugs brings further support for the statement that the action of extracellular adenosine on hematopoiesis should not be neglected and that it might find a use in clinical practice.

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