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Enhancement of Haemopoietic Spleen Colony Formation by Drugs Elevating Extracellular Adenosine: Effects of Repeated In Vivo Treatment

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Summary

The potential role of adenosine receptor signalling in the amplification of haemopoietic stem cells *in vivo* was investigated. Elevation of extracellular adenosine in mice was induced by the joint administration of dipyridamole, a drug inhibiting the cellular uptake of adenosine, and adenosine monophosphate, an adenosine prodrug. The response of haemopoietic stem cells to the drug treatment was measured by endogenous spleen colony-forming assay in sublethally gamma-irradiated animals. The combination of drugs was administered before irradiation either singly or repeatedly at 24 h intervals. The results demonstrated the possibility of enhancing the spleen colony formation by the drug treatment. The highest stimulatory effect on spleen colony counts and on the colony sizes occurred after 3–4 injections of the drugs. Higher spleen colony responses were observed under injection regimens terminated 3 h before irradiation, as compared to those terminated 24 h before the radiation exposure. The results are interpreted as an evidence of the expansion of the stem cell pool. A tolerance to this stimulatory action developed after more than 3 injections of the drugs.

Key words
Haemopoietic stem cells – Dipyridamole – Adenosine monophosphate

Introduction

Adenosine is now widely accepted as an agent participating in the regulation of physiological activity in a variety of mammalian tissues. In the extracellular space, it acts through the cell surface receptors coupled to the inhibition (A_1) or activation (A_2) of adenylate cyclase, which regulates the intracellular concentration of cAMP (Daly 1982, Stiles 1990). Elevation of extracellular adenosine mediates diverse effects including vasodilation, bradycardia, modulation of neurotransmission, inhibition of platelet aggregation, etc. (Newby 1984, Burnstock 1993). Another interesting feature of adenosine seems to be its ability to influence the kinetics of cell renewal systems. Based on *in vitro* experiments, purine nucleotides and nucleosides, including adenosine, have been proposed to represent a universal and primitive system of signals that are capable of modulating cell proliferation and differentiation in a wide range of cell types (Rathbone *et al.* 1992). In earlier studies, we demonstrated that these effects can also be induced *in vivo*. It has been shown that pharmacologically mediated adenosine receptor activation exhibits stimulatory effects on haemopoiesis in gamma-irradiated mice (Pospíšil *et al.* 1989, 1992, 1993a,b, 1995a). Furthermore, we have found that the elevation of extracellular adenosine in normal mice can synergize with the action of a granulocyte colony-stimulating factor, which controls granulopoiesis (Pospíšil *et al.* 1995b).

The purpose of the present study was to further examine the effects of extracellular adenosine on haemopoietic stem cells, using endogenous spleen colony formation in gamma-irradiated mice as the end point. The experimental procedure was based on the previously utilized (Pospíšil *et al.* 1989, 1992, 1993a,b,

1995a,b, Hofer *et al.* 1995) joint administration of dipyridamole (DP), a drug inhibiting adenosine uptake by cells (Plagemann *et al.* 1988), and adenosine monophosphate (AMP), acting as an adenosine prodrug. AMP was used instead of adenosine because of its greater solubility in water. It can be assumed that exogenously administered AMP is rapidly metabolized extracellularly to adenosine by cell surface ectonucleotidase activity (Gordon *et al.* 1989). Thus, DP and AMP can act jointly in enhancing extracellular adenosine levels. As was shown earlier, only the combination of DP with AMP induces expressive haemostimulatory effects (Pospíšil *et al.* 1989, 1995b). With regard to the practical implications of the potential haemostimulatory action of extracellular adenosine signalling, the effects of repeated administration of the drugs were investigated.

Materials and Methods

Animals

Three-month-old male (CBAxC57BL/10)F1 mice weighing 25 g on the average were used. Standardized pelleted diet and HCl-treated tap water (pH 2–3) were given *ad libitum*. The mice were kept under controlled light regimen (light:dark 12:12 h) and the temperature was maintained at 22±1 °C.

Endogenous spleen colony-forming unit (CFU-S) assays

Mice were given a total-body dose of 6.5 Gy from a ^{60}Co gamma-ray source at a dose rate of 0.28 Gy/min. On day 10 after irradiation, mice were sacrificed by cervical dislocation, spleens were removed and fixed in Bouin's solution. Optically visible surface spleen colonies were counted according to their diameter in three size categories: 0.5–1.0 mm, 1.0–2.0 mm, and >2.0 mm. In some experiments, also microscopically counted colonies were evaluated. The spleens were embedded in paraffin, and a single midline-longitudinal section was considered to be a representative sample of each spleen (Jenkins *et al.* 1969). Using a light microscope, these histologically evaluated colonies were counted in four size categories: <0.25 mm (more than ten cells were considered to be a colony), 0.25–0.5 mm, 0.5–1.0 mm, and >1.0 mm. Taking into account the number of colonies in individual size categories, and a roughly spherical shape of a colony, average colony volumes (mm^3) were estimated for the surface colonies and the histologically evaluated spleen colonies using a modification of the method used by Hellman and Grate (1971). This served for semiquantitative estimation of cell colony sizes.

Drug treatment

Dipyridamole (Sigma, USA) was dissolved in 0.4% tartaric acid and injected s.c. (0.4 ml) at a dose of 2 mg per mouse. Adenosine 5'-monophosphate sodium salt from yeast (Sigma, USA) was dissolved in distilled water and injected i.p. (0.2 ml) in a dose of 5 mg free acid per mouse. AMP was administered 20 min after DP. Tartaric acid and saline were used for control s.c. and i.p. injections, respectively. The combination of drugs was given either singly or repeatedly at 24 h intervals, and the injection regimen was terminated either 24 h or 3 h before the start of radiation exposure. Because the control values of colony numbers and spleen colony sizes belonging to different treatment regimens did not differ, they were pooled into one group. In order to ascertain the cardiovascular effects induced by DP+AMP, noradrenaline (Léčiva, Czech Republic) was injected s.c. (0.1 ml) in a dose of 1.4 mg per mouse immediately before AMP administration. Such a dose of noradrenaline was found to eliminate the hypotensive action of the combination of DP+AMP (Pospíšil *et al.* 1993b). The proliferative state of CFU-S was assessed by means of hydroxyurea, which destroys cells that are in the S phase at the time of its administration (Nečas *et al.* 1989). Hydroxyurea (Sigma, USA) was dissolved in saline and given i.p. (0.2 ml) in a dose of 27 mg per mouse 1 h before irradiation.

Statistics

All data are presented as means ± S.E.M. The data were subjected to analysis of variance followed by multiple comparisons using Tukey's test for unequal sample sizes (CSS: Statistica, V 3.1, StatSoft, Inc., Tulsa, USA, 1992). The significance level was set at $P<0.05$; for the sake of simplicity only this value was used in all comparisons.

Results

Data illustrating the effects of different treatment regimens on the optically visible surface spleen colonies are given in Figure 1 and Table 1. As shown in Fig. 1, already a single bolus of DP+AMP administered 3 h before irradiation significantly increased the number of colonies. Repeated drug administration of up to 3–4 injections further enhanced the colony formation. The stimulatory effect of repeated drug administration was also evident if the treatment was terminated 24 h before irradiation. However, the effects of treatment regimens terminated 3 h before irradiation were more pronounced. After more than three injections of the drugs, the stimulatory effects were gradually weakened and disappeared. As shown in Table 1, spleen colony size was increased after four injections of drugs terminated 3 h before irradiation. After 10 injections of drugs terminated 24 h before irradiation, the spleen colony size was decreased below the level of values found in mice treated four times.

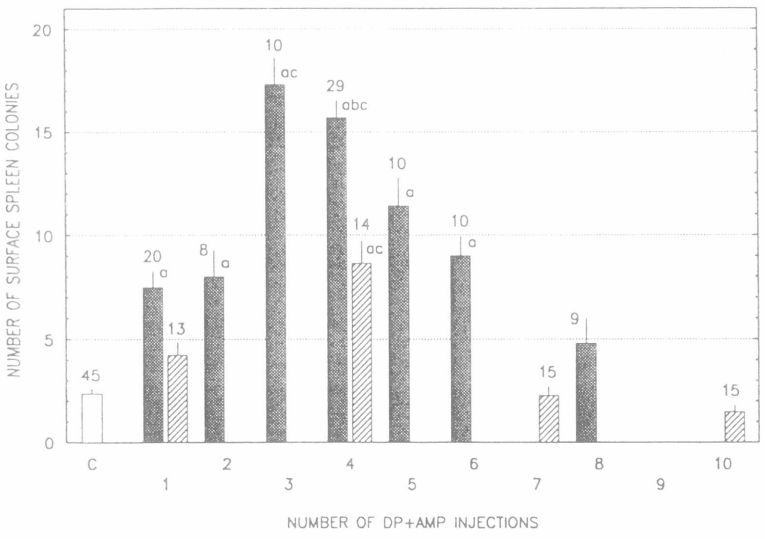

Fig. 1. *Numbers of surface spleen colonies in mice subjected to various treatment regimens. The empty column relates to controls (C); the full columns relate to mice irradiated 3 h after a single or the last injection, the hatched columns represent mice irradiated 24 h after a single or the last injection of drugs. The numbers at the top of the columns give numbers of mice receiving each treatment. Statistical significance (P<0.05): a – compared to control, b – compared to mice given the last injection 24 h before irradiation, c – compared to mice treated once.*

Table 1. Average volume of surface spleen colonies (mm^3) after DP+AMP treatment

Number of DP+AMP injections	Irradiation 3 h after a single or last injection	Irradiation 24 h after a single or last injection
1	0.787±0.084	0.699±0.114
2	0.686±0.178	n.d.
3	0.835±0.141	n.d.
4	1.281±0.126[a]	1.036±0.094
5	0.867±0.096	n.d.
6	0.765±0.099	n.d.
7	n.d.	0.772±0.177
8	0.832±0.185	n.d.
10	n.d.	0.272±0.052[d]
Controls	0.780±0.081	

For number of mice per group see Figure 1. Statistical significance (P<0.05): a – compared to controls, d – compared to mice treated four times, n.d. – not done.

In order to ascertain whether the effects observed on the surface spleen colonies are also induced in the class of the smallest colonies which are not registered by the naked eye, histologically determined colonies were enumerated under selected experimental conditions. The data given in Table 2 show that the numbers and sizes of histologically evaluated colonies correspond approximately to the findings obtained when investigating grossly visible surface colonies. Again, the highest stimulatory effect on colony numbers and spleen colony sizes was observed after the treatment regimen using four injections of drugs terminated 3 h before radiation exposure. About 90 % of histologically determined colonies were of the erythropoietic type. The treatment did not modify the differentiation pattern of spleen colonies.

Additional experiments (data not shown) were performed to elucidate some of the mechanisms which could participate in the observed effects. First, the possible role of the cardiovascular, i.e. hypotensive, effects of DP+AMP was tested in the regimen of four

injections which was terminated 3 h before irradiation. Noradrenaline was administered before each AMP injection in a dose eliminating these cardiovascular effects (Pospíšil 1993b). These experiments did not reveal any modification of enhanced colony formation, thus excluding the possible role of hypotensive and ensuing hypoxic effects. Second, hydroxyurea given shortly before irradiation to animals treated with DP + AMP either 24 h or 3 h before irradiation did not modify the enhanced colony formation. Thus, the role of an increased fraction of cells in the S phase at the time of irradiation could also be rulled out.

Table 2. Numbers of histologically evaluated spleen colonies and average volumes of these colonies after DP+AMP treatment

	n	Number of colonies	Spleen colony volume (mm^3)
Control	12	1.7±0.4	0.072±0.021
DP+AMP 1x, irradiation 24 h after the injection	7	2.3±0.8	0.084±0.020
DP+AMP 1x, irradiation 3 h after the injection	7	4.1±0.6	0.095±0.014
DP+AMP 4x, irradiation 24 h after the last injection	7	6.6±0.5[a,c]	0.105±0.013
DP+AMP 4x, irradiation 3 h after the last injection	22	10.8±0.7[a,b,c]	0.277±0.035[a]

n – number of mice. Statistical significance ($P<0.05$): a – compared to control, b – compared to mice given the last injection 24 h before irradiation, c – compared to mice treated once.

Discussion

The counting of endogenous haemopoietic spleen colonies in irradiated mice presents a simple method providing information about the size of the haemopoietic stem cell pool (Till and McCulloch 1963). The method is based on the ability of surviving haemopoietic cells to give rise to countable spleen colonies which were found to be clonal. More or less mature stem cells can form spleen colonies (Wolf and Priestley 1986, Nečas and Znojil 1989). Colonies appearing 10 days after irradiation, as used in this study, seem to be derived from the population of cells with a relatively high degree of self-replicative capacity and thus a lower degree of maturation. The size of the CFU-S pool surviving irradiation can be influenced either by the amplification mechanisms acting before or shortly after irradiation, or by actions modifying the radiosensitivity of these cells. Because the radiation damage to cells is inherent in the endogenous colony assay, modifications of CSU-S radiosensitivity could be a factor complicating the interpretation of our results. We have previously found (Pospíšil *et al.* 1993a) that the combination of DP+AMP administered 15 min before irradiation can decrease the radiosensitivity of CFU-S by inducing transient hypotension and ensuing hypoxia in the haemopoietic tissue. However, such an effect was no longer evident when animals were irradiated 1 h or later after drug treatment, i.e. at the time when the effects of hypoxia disappear. As is shown in this study, coadministration of noradrenaline in a dose, which eliminated the cardiovascular response to DP+AMP (Pospíšil *et al.* 1993b), did not influence the enhanced colony formation. Another possibility of modifying the radiosensitivity of CFU-S is their different position in the cell cycle. It was found that CFU-S in the late S phase of the cycle are more radioresistant (Chaffey and Hellman 1971).

Experiments of this study using hydroxyurea assay did not provide any evidence of larger numbers of CFU-S in the S phase shortly before irradiation. These findings confirmed the assumption that the possible radiosensitivity modifying effects of drugs did not play any role in the enhanced colony formation observed in the present experiments.

Taking the above evidence into account, the enhanced spleen colony formation observed after DP+AMP treatment has to be interpreted as a consequence of the expansion of the stem cell pool. Two mechanisms can account for this effect. The traditional concept considers the cycling activity of cells as the main mechanism of the positive control of haemopoiesis. Recently, another regulatory mechanism of cell amplification, programmed cell death (apoptosis), has been recognized also to play important role (Koury 1992, Nečas *et al.* 1995). Haemopoietic growth factors were found to promote viability of cells, to suppress apoptosis, and thus to contribute to the production of daughter cells without the necessity to increase cell cycle activity. It remains to be elucidated which of these mechanisms of cell amplification participates in the observed effects. As has been shown, the amplification effects can be enhanced by repeated administration of the drugs, the optimum effect being achieved after 3–4 injections. This suggests that the individual single effects are cumulative. In the case that treatment had been terminated 24 h before irradiation, the greater number of surviving CFU-S probably results from a larger population of CFU-S which are present at the time of irradiation. Considering the effects observed after the injection regimens which were terminated 3 h before irradiation, it is tempting to speculate that the last stimulus manifests its effects in the early postirradiation period, and acts by modifying the apoptotic and repair processes. Such a hypothesis seems to be supported by the earlier results from our laboratory indicating that the postirradiation elevation of extracellular adenosine by DP + AMP administration to mice decreases the early destruction of cells and increases cell repair, as indicated by the thymidine levels in the plasma, the amount of soluble polynucleotides in the thymus, and the rejoining processes of DNA strand breaks (Boháček *et al.* 1993). A similar mechanism could account for the higher efficacy of the treatment terminated 3 h before irradiation, as compared to that terminated 24 h before irradiation. The elevation of endogenous spleen colony counts induced by DP+AMP approximately correlated with the increase in colony size. These effects suggested that beside the amplification of the stem cell pool also other mechanisms enhancing the proliferation inside the clones forming the colonies can be induced by elevating extracellular adenosine. Cooperation of these two processes can be of advantage in increasing the production of functional cells.

An important finding of this study is the development of tolerance to various stimulatory factors. Development of refractoriness to the effects of adenosine agonists is a known phenomenon explained by the down-regulation of cell receptors (Stiles 1990). Another possibility may concern the elimination of cells expressing adenosine receptors, due to their transition into more mature cell compartments with restricted colony-forming ability.

In conclusion, our results clearly suggest the ability of drugs elevating extracellular adenosine to amplify the pool of primitive haemopoietic cells. They indicate that it is possible to enhance the effects by repeated administration of drugs and that it is necessary to respect the development of tolerance to their action. Further investigations are needed to define the type of adenosine receptors responsible for the observed effects and to elucidate the signal transduction pathway mediating the response in haemopoietic cell compartments. Nevertheless, our findings point at a promising possibility to utilize drugs acting *via* adenosine receptor signalling in the haemopoietic protective and curative strategies.

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Reprint Requests

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