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Effects of adenosine A_3 receptor agonist on bone marrow granulocytic system in 5-fluorouracil-treated mice

Michal Hofer [a,*], Milan Pospíšil [a], Antonín Vacek [a], Jiřina Holá [a], Vladimír Znojil [b], Lenka Weiterová [a], Denisa Štreitová [a]

[a] *Laboratory of Experimental Hematology, Institute of Biophysics, Academy of Sciences of the Czech Republic, Královopolská 135, 612 65 Brno, Czech Republic*
[b] *Institute of Pathological Physiology, Medical Faculty, Masaryk University, Komenského nám. 2, 662 43 Brno, Czech Republic*

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Abstract

The purpose of the experiments reported was to investigate effects of N^6-(3-iodobenzyl)adenosine-5'-*N*-methyluronamide (IB-MECA), a selective adenosine A_3 receptor agonist, on the granulocytic system in femoral marrow of mice depleted by the cytotoxic drug 5-fluorouracil. In the phase of the highest cell depletion IB-MECA was injected i.p. at single doses of 200 nmol/kg given either once or twice daily in 2- and 4-day regimens starting on day 1 after 5-fluorouracil administration; the effects were evaluated on days 3 and 5, respectively. The general effect of IB-MECA in all these experiments was an enhancement of the counts of morphologically recognizable proliferative granulocytic cells, interpreted as evidence of the differentiation of committed progenitor cells. A more expressive effect was observed after IB-MECA injected twice daily. It was found that the induction of the strong differentiation pressures by IB-MECA given twice daily shortly after 5-fluorouracil treatment can be counterproductive due to the preponderance of differentiaton processes over the proliferation control. In additional experiments, it has been shown that the use of the 2-day administration of IB-MECA given twice daily in the recovery phase, i.e., on days 5 and 6 after 5-fluorouracil administration, does not induce stimulatory effects. Thus, the dosing and timing of IB-MECA treatment determines its effectivity in stimulating granulopoiesis under conditions of myelosuppression.
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Keywords: Adenosine A_3 receptor; Granulopoiesis; Myelosuppression; 5-Fluorouracil; (Mouse)

1. Introduction

The concept of adenosine receptor signalling is widely explored in various areas of physiology and pathophysiology including the regulation of the proliferation and differentiation of cells (Abbracchio, 1996; Schulte and Fredholm, 2003). So far, four adenosine receptors, i.e. A_1, A_{2a}, A_{2b}, and A_3 coupled to G proteins have been classified (Fredholm et al., 2000). These receptors can play a role also in haematopoiesis. In earlier studies we have shown that elevation of extracellular adenosine enhances haematopoiesis in normal and myelosuppressed mice and synergizes with effects of the granulocyte colony-stimulating factor (Hofer et al., 1997, 1999, 2002; Pospíšil et al., 1995, 1998). Moreover, we have demonstrated that elevation of extracellular adenosine increases cycling of haematopoietic progenitor cells as inferred from the cytotoxic effects of 5-fluorouracil (Pospíšil et al., 2001). Other authors have found that the selective agonist of adenosine A_3 receptors, N^6-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide (IB-MECA), induces similar effects on granulopoiesis (Bar-Yehuda et al., 2002; Fishman et al., 2000a). Gessi et al. (2002) have shown that adenosine A_3 receptors are present in human neutrophils as well as in promyelocytic HL60 cells, thus suggesting that this receptor subtype can be functional at the early stages of myeloid differentiation. Thus, the adenosine A_3 receptor can be responsible for the above-mentioned findings. In our experiments we have demonstrated stimulatory effects of IB-MECA on the cycling of granulocytic progenitor cells (Pospíšil et al., 2004, 2005). Interestingly, it has been shown by Fishman et al. (2000b) and also in our laboratory (Hoferová et al., 2003), that IB-MECA induces cytostatic effects in various tumour cell

* Corresponding author. Postal address: Institute of Biophysics, Academy of Sciences of the Czech Republic, Královopolská 135, 612 65 Brno, Czech Republic. Tel.: +420 541517171; fax: +420 541211293.
E-mail address: hofer@ibp.cz (M. Hofer).

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systems both in vitro and in vivo. The association of the stimulatory action of IB-MECA on haematopoiesis with its inhibitory action on the growth of tumour cells is promising from practical point of view, because myelosuppression belongs to undesirable complications of chemo- and radiotherapy of tumours. Thus, effects of IB-MECA on haematopoiesis deserve further attention. Recently, there has appeared a clinical study on the tolerability and pharmacokinetics of IB-MECA in healthy men (Van Troosteburg et al., 2004).

The purpose of the presented experiments was to investigate the effects of IB-MECA administration on the suppressed bone marrow granulopoiesis in mice pretreated with the cytotoxic drug 5-fluorouracil. Attention was focused on the timing of IB-MECA administration with the aim to define principles of the optimum treatment schedules.

2. Materials and methods

2.1. Animals

B10CBAF$_1$ male mice aged 3 months and weighing in 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 the 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's Ethics Committee.

2.2. Drugs

N^6-(3-iodobenzyl)adenosine-5′-*N*-methyluronamide (IB-MECA), the agonist of adenosine A_3 receptors, was dissolved initially in dimethyl sulphoxide, then diluted with sterile saline and injected i.p. at single doses of 200 nmol/kg in a volume of 0.2 ml. The final concentration of dimethyl sulphoxide was 2%. The schemes of administration of IB-MECA are given under Results. The choice of the dose of IB-MECA was based on our former experiments showing that this dose induces cycling of murine haematopoietic progenitor cells under in vivo conditions (Pospíšil et al., 2004, 2005). 5-Fluorouracil was diluted in saline and injected i.p. at a single dose of 100 mg/kg in a volume of 0.2 ml. The corresponding drug vehicles were used for control injections. All the drugs were obtained from Sigma (St. Louis, MO, USA).

2.3. Haematological methods

Blood samples were taken from the tail vein. Mice were then sacrificed by cervical dislocation. The femurs were dissected and marrow cells were flushed from the bone. Blood cell counts and numbers of nucleated cells of the bone marrow were determined using a Coulter Counter (model ZF; Coulter Electronics, UK). Differential counts were performed on blood and marrow smears stained with the May–Grünwald–Giemsa method. Based on the differentiation of marrow cells, the counts of proliferative (myeloblasts through myelocytes) and nonproliferative (metamyelocytes through segmented stages) granulocytic cells per femur were determined. Total number of granulocytic cells per femur represents the sum of the proliferative and nonproliferative cells. Bone marrow haematopoietic progenitor cells committed to granulocyte–macrophage development (granulocyte–macrophage colony-forming cells [CFC-GM]) were assayed using a semisolid plasma clot technique. Femoral marrow cell suspensions were plated in triplicate and incubated at 37 °C in humidified atmosphere containing 5% CO_2. CFC-GM were scored after 7-day incubation as colonies containing 50 or more cells. The numbers of CFC-GM per femur were calculated.

2.4. Statistics

The data are given as means ± S.E.M. Experiments were repeated twice to three times and the data were pooled. Mann–Whitney rank sum test was used for comparison of the effects and the Holm's method was applied to correct for multiple comparisons. The significance level was set at $P<0.05$.

3. Results

3.1. Effects of 5-fluorouracil alone on granulocytic cells

Fig. 1 demonstrates effects of 5-fluorouracil alone on the total number of granulocytic cells in femoral marrow compared to that obtained in untreated control mice. As shown, the cell system responded within the 7-day interval by phases of depletion and recovery. On the basis of such time course of the bone marrow cell response, the effects of IB-MECA were investigated separately in both these phases.

3.2. Effects of IB-MECA administered in the phase of cell depletion

IB-MECA at a dose of 200 nmol/kg per each injection was given i.p. on days 1 and 2 after 5-fluorouracil administration in

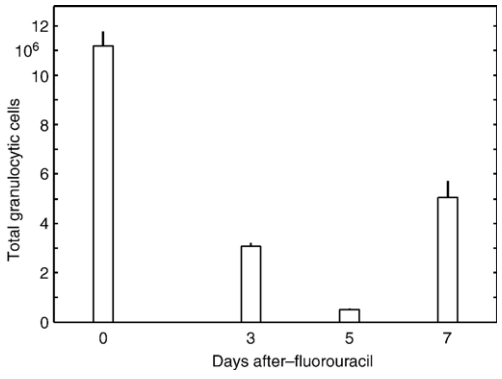

Fig. 1. Effects of 5-fluorouracil (100 mg/kg) alone on the counts of total granulocytic cells in femoral marrow of mice. Baseline values found in untreated control mice are given on day 0. Data are given as means ± S.E.M; 10 to 30 mice per group were used.

the first variant of experiments and on days 1 to 4 in the second one. In both these experiments the agonist was injected either once or twice daily. Thus, in the first variant of experiments mice received 2 or 4 injections, in the second one 4 or 8 injections of IB-MECA. The effects on granulopoietic indices in the femoral marrow were investigated 24 h after the last injection of the agonist, i.e. on days 3 or 5 after 5-fluorouracil administration.

Effects of the 2-day treatment with IB-MECA determined on day 3 after 5-fluorouracil administration are given in Fig. 2(A). The evidence of the stimulatory action of IB-MECA was provided by effects occurring in the pool of morphologically recognizable proliferative granulocytic cells. Increase of the counts of these cells was significant when administering the agonist both once and twice daily; a higher increase was observed after administering IB-MECA twice daily. While the counts of total granulocytic cells were significantly increased after administering IB-MECA once daily, treatment with the agonist twice daily did not influence this parameter significantly. Also counts of CFC-GM behaved similarly, exhibiting increase after IB-MECA given once daily; this effect was absent when administering this agonist twice daily. In an additional experiment it has been ascertained that the effects induced by 2-day IB-MECA treatment in the pool of proliferative granulocytic cells are reversible and fade quickly away because no significant modulation of these cells was observed when evaluated on day 5 after 5-fluorouracil administration (data not given). The factor of increase of the pool of proliferative granulocytic cells, attaining the value of 6.0 one day after 2-day treatment with IB-MECA given twice daily (i.e. on day 3 after 5-fluorouracil), declined 3 days after discontinuation of the treatment (i.e. on day 5 after 5-fluorouracil) to the value of 1.3.

Fig. 2(B) illustrates effects of the 4-day treatment with IB-MECA determined on day 5 after 5-fluorouracil administration. Results indicating enhancement of the counts of proliferative granulocytic cells after IB-MECA treatment coincide with those observed on day 3 after 5-fluorouracil administration, although they occur at a higher level of cell counts. In contrast to the effects observed on day 3 after 5-fluorouracil administration, significant increase of the counts of total granulocytic cells was observed under both regimens of IB-MECA treatment, i.e. its administration once and twice daily. These effects, however, occur at the lower level of cellularity, due to the progress in expressing the damage up to day 5 after 5-fluorouracil administration. Counts of CFC-GM were not influenced by IB-

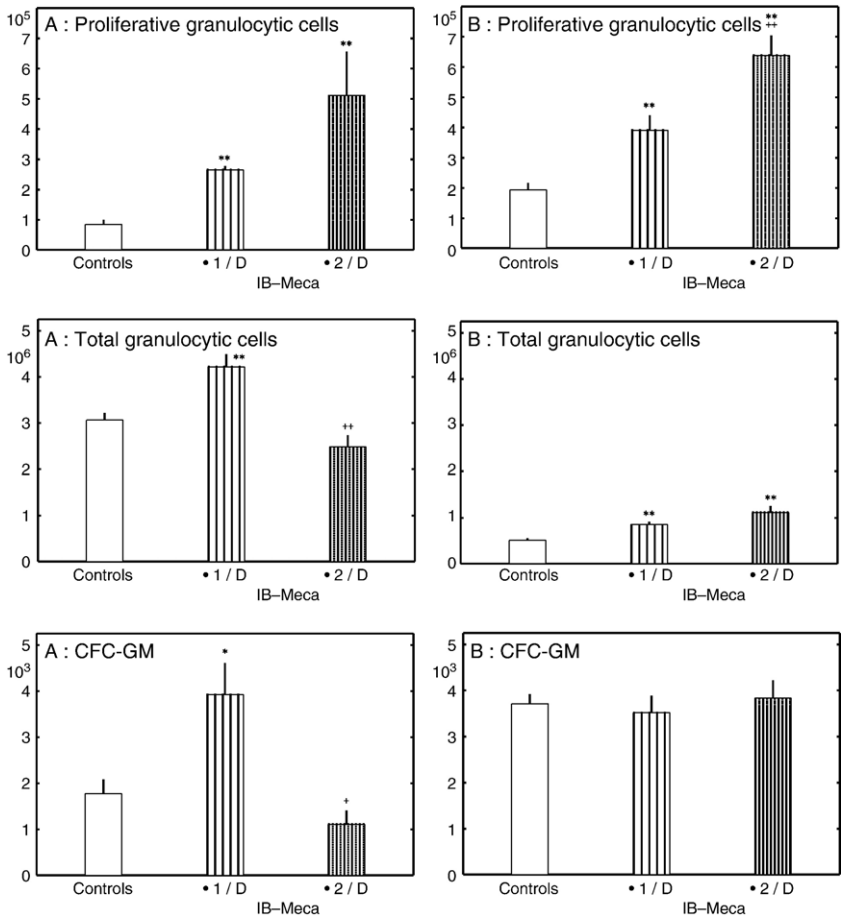

Fig. 2. Effects of 2-day (A) and 4-day (B) treatment with IB-MECA given once (*1/D) or twice (*2/D) daily at doses of 200 nmol/kg on the counts of proliferative granulocytic cells, total granulocytic cells and CFC-GM in femoral marrow of mice. The treatment started 1 day after 5-fluorouracil administration (100 mg/kg) and the effects were determined 1 day after the last injection of the agonist. The empty columns relate to vehicle-treated control mice receiving only 5-fluorouracil. Data are given as means ± S.E.M.; 20 to 30 mice were used in case of control groups, 10 mice were used in every group treated with IB-MECA. Statistical significance: $*P<0.05$, $**P<0.01$ vs. vehicle-treated mice, $^{+}P<0.05$, $^{++}P<0.01$ vs. mice treated with IB-MECA given once daily (Mann–Whitney test).

Table 1
Indices of granulopoiesis in untreated control mice and mice treated with vehicle or IB-MECA in the phase of granulocytic recovery after 5-fluorouracil administration

	Untreated controls	Day 7 after 5-fluorouracil	
		Vehicle	IB-MECA
			*2/day
Total granulocytic cells/femur ($\times 10^6$)	11.198±0.577	5.051±0.642	4.568±0.452
Proliferative granulocytic cells/femur ($\times 10^6$)	2.134±0.253	1.902±0.200	1.808±0.206
CFC-GM/femur	17,983±817	10,491±1133	11,270±1039
Neutrophils/μl blood	1130±45	82±25	96±73

The designation "untreated controls" relates to mice receiving neither 5-fluorouracil, nor IB-MECA. Vehicle or IB-MECA at dose of 200 nmol/kg given twice daily were injected on days 5 and 6 after 5-fluorouracil administration (100 mg/kg). The effects were ascertained 1 day after the last injection of vehicle or IB-MECA, i.e. on day 7 after 5-fluorouracil administration. Data are given as means±S.E.M. from 10 mice per group. No significant differences between the vehicle- and IB-MECA-treated mice were observed (Mann–Whitney test).

MECA treatment. Control and experimental values of CFC-GM reached similar higher level suggesting the beginning of recovery in the pool of progenitor cells.

In the experiments using 4-day treatment with IB-MECA peripheral blood neutrophils were also evaluated. The mean values±S.E.M. of neutrophils per 1 μl of blood were 112±19 for control, and 94±20 and 191±60 for IB-MECA given once and twice daily, respectively. The enhanced mean counts of neutrophils observed after IB-MECA given twice daily did not attain significance compared to controls.

3.3. Effects of IB-MECA administration in phase of cell recovery

IB-MECA was injected twice daily at days 5 and 6 after 5-fluorouracil administration and the effects were determined on day 7. Results of this experiment are demonstrated in Table 1, which includes also parameters of granulopoiesis obtained in untreated control mice. With exception of the blood neutrophils, indices of the femoral bone marrow granulopoiesis in mice treated with 5-fluorouracil alone demonstrate an increase as compared to those obtained on day 5 after treatment with 5-fluorouracil alone (see Fig. 2B). The counts of proliferative granulocytic cells even attain the norm and thus suggest a high rate of recovery. None of the determined parameters was significantly influenced by the IB-MECA treatment.

4. Discussion

In order to investigate the effects induced by different intensities of the activation of adenosine A_3 receptors, accumulation of different numbers of single identical doses of IB-MECA within the same time intervals was employed. The reason for such approach is the possibility that the simple increase in the dose of the agonist in the single injection needs not induce straightforward alteration of the effect. Administration of increasing doses of the agonist in single injections in vivo induces often a bell-shaped response probably due to the loss of selectivity and/or activation of feed-back regulatory mechanisms (Calabrese, 2001; Pospíšil et al., 2004).

Effects of the 2- and 4-day treatment with IB-MECA, investigated 3 and 5 days after 5-fluorouracil administration, can be defined as stimulatory in terms of the enhancement of the counts of morphologically recognizable proliferative granulocytic cells. Such effects were observable in all the variants of the experiments and corresponded to the intensity of the stimulus. It seems that the phenomenon of the receptor desensitization, which is known to arise after activation of adenosine A_3 receptors in cell systems in vitro (Trincavelli et al., 2002) does not influence expressively the action of IB-MECA in our experiments. In terms of the hierarchical model of haematopoiesis, these effects can be due to an increasing rate of differentiation of the committed progenitors, i.e. their influx into the compartment of the morphologically recognizable precursor cells. On day 3 after 5-fluorouracil administration, when using the weak stimulus of IB-MECA given once daily, the effects in the pool of proliferative granulocytic cells are associated with the increase of both the counts of total granulocytic cells and of CFC-GM, suggesting that the proliferation (self-renewal) potential of haematopoietic progenitor cells is maintained and could act as overcompensation of their loss by differentiation. However, such a relationship is absent under the strong differentiation stimulus induced by IB-MECA given twice daily. Here, probably, the potential of progenitor cells for expansion is counterbalanced by a higher rate of cell removal from the population than the proliferation can provide for. Due to such a strong imbalance between proliferation and differentiation, the amplification potential of the system is limited. It is known that such effects occur particularly in the early time intervals after the action of cytotoxic drugs when administering differentiation-enhancing agents like cytokines and growth factors and that such a treatment can be counterproductive (Moore, 1992). It has been shown that the primitive, slowly cycling stem cells, which resist the toxic effects of 5-fluorouracil and feed the pool of committed progenitor cells, are stimulated into the proliferation with a certain delay, i.e. between days 3 and 5 after 5-fluorouracil administration (Harrison and Lerner, 1991). For these reasons, the balance inside the system can be renewed at day 5 after 5-fluorouracil administration, when the increase of the counts of proliferative granulocytic cells is already associated with the increase of the counts of total granulocytic cells. At this time interval, CFC-GM regenerate to an appreciable extent and are evidently able to balance the losses induced by differentiation.

Concerning the lack of significant effects in peripheral blood neutrophils after IB-MECA treatment it should be noted that their values are a less representative indicator of granulocyte production due to their short life span (several hours) and to the impossibility to measure their fractions stored within the intravascular compartments. Another negative effect, i.e. the inefficiency of IB-MECA under the conditions of the recovering granulopoiesis as compared to the effects observed in the phase of depletion suggests that the action of the agonist is conditioned by the functional state of the cell population. It is possible that in the recovering system there arises a saturation

phenomenon due to the cumulation of fully activated cytokine mechanisms of the positive control with signals produced by the stimulation of adenosine receptors.

In conclusion, our data indicate that IB-MECA acts as a stimulator of the bone marrow granulocytic system and can have a promising therapeutic potential under myelosuppressive states. Timing, and intensity of the agonist's action in terms of the frequency of its administration, as well as the functional state of target cell population are important conditions determining the resulting effects.

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