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Adenosine Acts as a Chemoprotective Agent by Stimulating G-CSF Production: A Role for A1 and A3 Adenosine Receptors

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Adenosine, a ubiquitous nucleoside, is released into the extracellular environment from metabolically active or stressed cells. It binds to cells through specific A1, A_{2A}, A_{2B}, and A3 G-protein–associated cell-surface receptors, thus acting as a signal-transduction molecule by regulating the levels of adenylyl cyclase and phospholipase C. In this study, we showed that adenosine stimulates the proliferation of murine bone marrow cells in vitro. Pharmacological studies, using antagonists to the adenosine receptors, revealed that this activity was mediated through the binding of adenosine to its A1 and A3 receptors. This result was further corroborated by showing that the two selective A1 and A3 receptor agonists, *N*-cyclopentyladenosine (CPA) and 1-deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-*N*-methyl-β-D-ribofuranuronamide (IB-MECA) respectively, induced bone marrow cell proliferation in a manner similar to adenosine. Adenosine's interaction with its A1 and A3 receptors induced G-CSF production, which led to its stimulatory effect on bone marrow cells. These results were confirmed in vivo when we demonstrated that low-dose adenosine (0.25 mg/kg) acted as a chemoprotective agent. When administered after chemotherapy, it restored the number of leukocytes and neutrophils to normal levels, compared with the decline in these parameters after chemotherapy alone. It is suggested that low-dose adenosine, already in clinical use, may also be applied as a chemoprotective agent. J. Cell. Physiol. 183:393–398, 2000. © 2000 Wiley-Liss, Inc.

Adenosine, an ubiquitous nucleoside, is released into the extracellular environment from metabolically active or stressed cells. In order to reenter the cells, adenosine binds to specific G-protein associated A1, A_{2A}, A_{2B}, and A3 membranal receptors (Stiles, 1990; Linden, 1991). Since specific surface receptors for adenosine are found in nearly all cells, almost every organ system in the body is regulated by its local release, that is, regulation of the electrophysiological properties of the heart, sedation and suppression of neurotransmitters' release, and regulation of renin release as well as vascular tone in the kidney (Belardinelli et al., 1989; Clarke and Coupe, 1989; Collis, 1989; Dubey et al., 1997). Adenosine exerts various effects on the immune system including anti-inflammatory activity through the inhibition of cytokine release, inhibition of platelet aggregation, induction of erythropoietin production, and modulation of the lymphocyte function (Gilbertsen, 1987; Soderback et al., 1991; Bouma et al., 1994).

In a recent study, we showed that adenosine and other small molecules released by muscle cells, are responsible for the resistance of this tissue to the development of metastases. It was found that adenosine exerted a differential effect on tumor and normal cell growth. Whereas the proliferation of tumor cells was inhibited, normal cells such as bone marrow were stimulated following treatment with adenosine (Djaldetti et al., 1996; Fishman et al., 1998). Adenosine's capability to induce bone marrow cell growth, prompted us to test its effectiveness in preventing chemotherapy-induced myelotoxicity.

In this study, adenosine was administered to mice after chemotherapy and was shown to prevent the decline in the number of peripheral blood leukocytes and neutrophils by inducing G-CSF production.

MATERIALS AND METHODS

Mice

Male ICR or C57BL/6J mice aged 2 months, weighing an average of 25 g were used. The mice were purchased from Harlan Laboratories (Jerusalem, Israel). Standardized pelleted diet and tap water were supplied.

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Drugs

All drugs were purchased from Sigma Chemical Co. (St. Louis, MO), excluding the A3 adenosine antagonist and agonist, which were ordered from RBI (Natick, MA). Adenosine was dissolved in water and kept as a stock solution in a concentration of 1 mg/ml. Dilutions in RPMI medium were carried out and final concentrations of 100, 50, 25, 10, and 5 μM were used. The following antagonists to adenosine receptors were used: 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), an adenosine A1 receptor antagonist; 3,7-dimethyl-1-propargyl-xanthine (DMPX), an A2 receptor antagonist; 9-chloro-2-(2-furanyl)-5-[(phenylacetyl)amino]-[1,2,4]-triazolo[1,5-c] quinazoline (MRS-1220), an adenosine A3 receptor antagonist. Two selective adenosine agonists were utilized: *N*-cyclopentyladenosine (CPA), an A1 receptor agonist and 1-deoxy-1-[6-[[(3-iodophenyl)methyl]amino]-9H-purin-9-yl]-*N*-methyl-β-D-ribofuranuronamide (IB-MECA), an A3 receptor agonist. Antimurine G-CSF antibodies (rabbit antiserum purified by Protein A chromatography; Cytolab LTD, Weizmann Science Park, Israel) were used. Cyclophosphamide was purchased from Taro Pharmaceutical Industries Ltd. (Haifa Bay, Israel).

Evaluation of bone marrow cell proliferation in vitro

Bone marrow cells were obtained from the femur of C57BL/6J mice. Cells were disaggregated by passing through a 25G needle. [^{3}H]thymidine incorporation assay was used to evaluate the proliferative capability of the bone marrow cells. Cells (3×10^5/well) were incubated with RPMI medium, containing 10% fetal bovine serum (FBS; Biological Industries, Beit Haemek, Israel), in the presence and absence of adenosine, in 96-well microtiter plates for 48 h.

To explore the specific receptor through which adenosine exerts its activity, adenosine antagonists were introduced to bone marrow cultures. To test the effect of the antagonists on their own, they were administered to bone marrow cell cultures at concentrations of 0.01–10 μM. No toxicity was exerted by the antagonists at a concentration of 0.1 μM. Thus, we used this concentration to explore the receptor through which adenosine stimulates bone marrow cell proliferation.

CPA (0.1 μM), agonist to the A1 adenosine receptor, and IB-MECA (0.1 μM), agonist to the A3 adenosine receptor, were added to bone marrow cultures in the absence of adenosine.

Cultures containing cells suspended in RPMI medium and 10% FBS served as controls for the abovementioned detailed experiments. In the last 18 h of incubation, each well was pulsed with 1 μCi [^{3}H]thymidine. Cells were harvested and the [^{3}H]thymidine uptake was determined in an LKB liquid scintillation counter (LKB, Piscataway, NJ). Each drug was tested in triplicate and four different experiments were carried out.

Stimulation of cell proliferation was calculated as follows:

$$\%\ \text{of stimulation} = \frac{A \times 100}{B} - 100 \qquad (1)$$

where A is cell count of sample and B is cell count of control. According to this calculation control values will be 0% of stimulation.

Cell-cycle analysis

Flow cytometric analysis of the cell cycle of murine bone marrow cells was carried out by propidium iodide staining according to Krishan (1975). Cells, at a concentration of 3×10^5/ml, were cultured in RPMI medium supplemented with 10% FBS in the presence of adenosine at a concentration of 25 μM for 24 h at 37°C in a CO_2 incubator. Cells cultured in RPMI supplemented with 10% FBS served as control. At the end of the incubation period, the cells were washed three times with PBS and resuspended in staining buffer containing 0.1% bovine serum albumin (BSA), 50 μg/ml propidium iodide, 0.1% Triton X-100, and 1 mg/ml RNAase (boiled for 10 min). Samples were examined after 30 min of staining on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Results are expressed as percentage of control (each phase of the cell cycle in the sample is compared to the relative phase in the control).

Effect of adenosine on the production of human granulocyte-colony stimulating factor (G-CSF)

Human mononuclear cells were used to measure the effect of adenosine on G-CSF production. This was assayed in the human system using ELISA, as murine G-CSF cannot be quantitatively assessed. Conditioned medium was obtained by incubating 2.5×10^6 peripheral blood mononuclear cells (separated by Ficoll hypaque gradient from heparinized whole blood) in RPMI medium containing 10% FBS. Following incubation for 48 h, cell supernatants were centrifuged and kept at −20°C until assayed. G-CSF production was examined in the presence of adenosine (25 μM). G-CSF was evaluated using a commercial ELISA kit (R&D Systems, Minneapolis, MN).

Effect of antibodies against murine G-CSF on the response of murine bone marrow cells to adenosine

To test whether adenosine induces bone marrow cell proliferation via its capability to stimulate G-CSF production, anti-G-CSF antibodies (62.5 ng/ml; titer, 1:16,000) were added to a culture of bone marrow cells derived from ICR mice in the presence of adenosine (25 μM), CPA (0.1 μM), or IB-MECA (0.1 μM). Cell proliferation was measured as described earlier.

In vivo studies

To examine the myeloprotective effect of adenosine, mice were injected intraperitoneally with 50 mg/kg body weight of cyclophosphamide. Adenosine (0.25 mg/kg body weight) was subcutaneously administered 48 and 72 h after the chemotherapy. Two control groups were treated with PBS or cyclophosphamide.

Each group contained 10 mice and each experiment was repeated three times. Ninety-six, 120, and 144 h after the chemotherapy, the mice were terminated, blood samples were taken, and bone marrow cells were harvested from the femur. White blood cell (WBC) counts were carried out in a Coulter counter and neutrophil cell counts were performed on smear prepara-

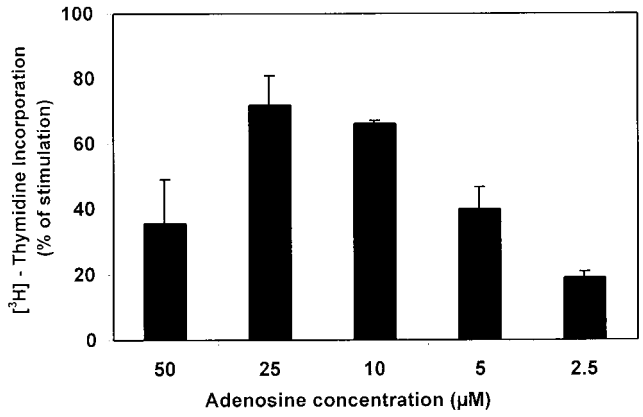

Fig. 1. Adenosine, at different concentrations, stimulates bone marrow cell proliferation, measured by [^{3}H]thymidine incorporation assay.

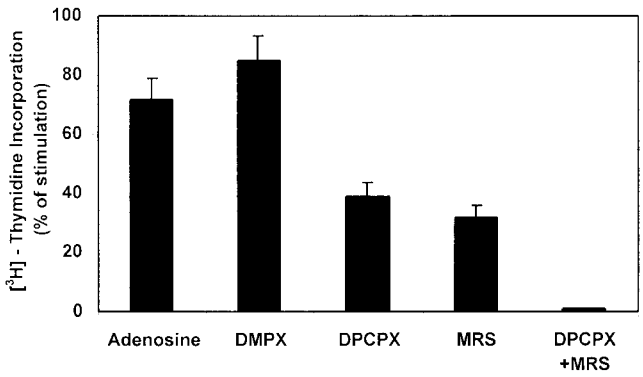

Fig. 2. Effect of adenosine receptor antagonists on bone marrow cell proliferation in the presence of adenosine (25 μM). When DPCPX (0.1 μM) and MRS-1220 (0.1 μM) were administered, the stimulatory effect of adenosine on bone marrow cells was decreased. When the two antagonists were combined, they canceled the stimulatory activity of adenosine on the proliferation of bone marrow cells.

tions stained with May–Grunvald–Giemsa solution. Bone marrow cell proliferation was tested ex vivo using [^{3}H]thymidine incorporation as specified previously.

Statistical analysis

Values in the figures and text are expressed as means ± SEM of at least five observations. Statistical analysis of data was carried out using the Students' t-test. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of adenosine, adenosine receptor antagonists, and agonists on bone marrow cell proliferation and cell cycle

Exposure of bone marrow cells to adenosine, at concentrations of 2.5–50 μM, stimulated (in a bell-shape curve) [^{3}H]thymidine incorporation in all concentrations tested (Fig. 1). The maximal stimulatory effect (72 ± 9%) was at a concentration of 25 μM. Cell-cycle analysis revealed an increase in the number of cells in the S phase (40 ± 6.3%) and in the G2 phase (185 ± 22.4%).

To find the adenosine receptor responsible for this stimulatory effect, three adenosine receptor antagonists were used (DPCPX, A1 antagonist; DMPX, A2 antagonist; MRS-1220, A3 receptor antagonist). The effect of the antagonists, in the absence and presence of adenosine, on bone marrow cell proliferation was examined. The addition of the antagonists, on their own, to cultures of bone marrow cells did not have any effect on bone marrow cell proliferation. However, when added in the presence of adenosine, DPCPX and MRS-1220 significantly decreased the stimulatory effect of adenosine on bone marrow cell proliferation ($P < 0.01$). The coadministration of these two antagonists (in the presence of adenosine) canceled the stimulatory effect of adenosine. However, the introduction of DMPX yielded slightly higher proliferation stimulation values than those of adenosine ($P < 0.06$) (Fig. 2). These findings indicate that the A1 and A3 receptors are responsible for the stimulatory effect of adenosine.

To confirm these results, the agonists CPA and IB-MECA were added to a culture of bone marrow cells.

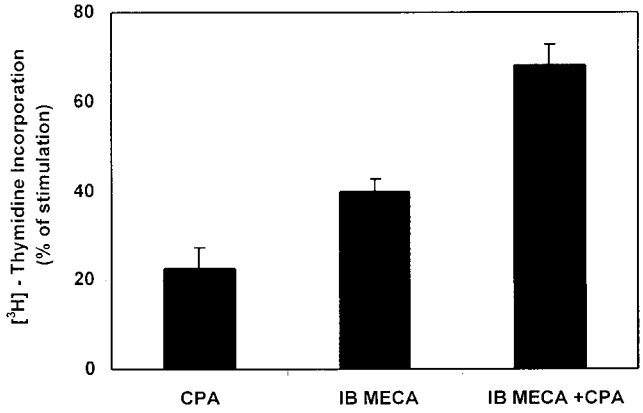

Fig. 3. CPA (0.1 μM) and IB-MECA (0.1 μM), adenosine A1 and A3 receptor agonists, respectively, stimulated the proliferation of bone marrow cells, measured by [^{3}H]thymidine incorporation assay. When the two agonists were given in combination, an additive stimulatory effect was observed.

Both agonists induced a statistically significant stimulation of bone marrow cell proliferation (CPA, $P < 0.01$; IB-MECA, $P < 0.001$). When the two agonists were introduced concomitantly, they exerted an additive stimulatory effect on bone marrow cell growth (Fig. 3).

Adenosine exerts its stimulatory activity via G-CSF production

Adenosine induced stimulation of G-CSF production by human peripheral blood mononuclear cells (control, 83.7 ± 10.2 pg/ml; adenosine 25 μM, 123.2 ± 8.9 pg/ml). The effect of adenosine on murine G-CSF production was examined using anti-G-CSF antibodies. The stimulation of bone marrow cell proliferation was abrogated when anti-G-CSF antibodies were added to these cultures. The anti-G-CSF antibodies also neutralized the stimulatory effect of CPA and IB-MECA, thus supporting the assumption that the effect of adenosine is mediated by its capability to induce G-CSF production (Fig. 4).

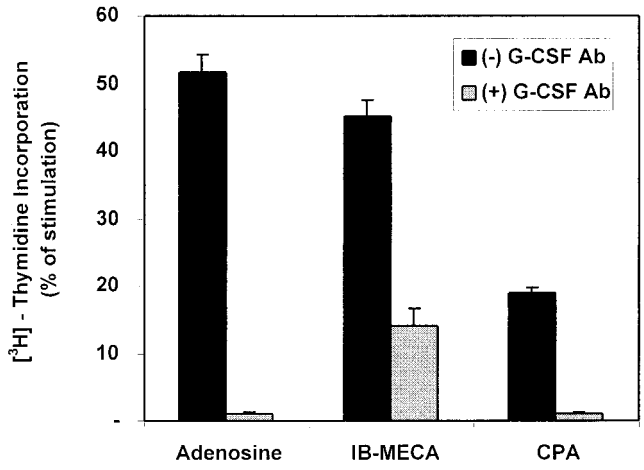

Fig. 4. Antibodies to G-CSF neutralized the stimulatory effect of adenosine and adenosine agonists on bone marrow cell proliferation (measured by [^{3}H]thymidine incorporation assay). (−) G-CSF stands for the control group, which is bone marrow cells in the presence of adenosine alone. (+) G-CSF stands for the bone marrow cells in the presence of adenosine and antibodies to G-CSF.

Adenosine exerts a chemoprotective effect in vivo

Cyclophosphamide (50 mg/kg) was used to evaluate the chemoprotective effect of adenosine administered 48 and 72 h after the cytotoxic drug. Mice treated with cyclophosphamide only exhibited a decline in the number of white blood cells in comparison with the values in the control group (Fig. 5a). Following the administration of chemotherapy, the number of WBCs was significantly higher in the adenosine-treated group, compared with the values in the group treated with chemotherapy alone. The percentage of neutrophils declined in the chemotherapy treated group, whereas it was restored in mice administered with chemotherapy and adenosine (Fig. 5b). There were no changes in the number of red blood cells, hemoglobin level, and platelet number in the groups treated with adenosine. A marked increase in ex vivo bone marrow cell proliferation was observed in the group treated with cyclophosphamide and adenosine (153 ± 18.4%) in comparison with the control group.

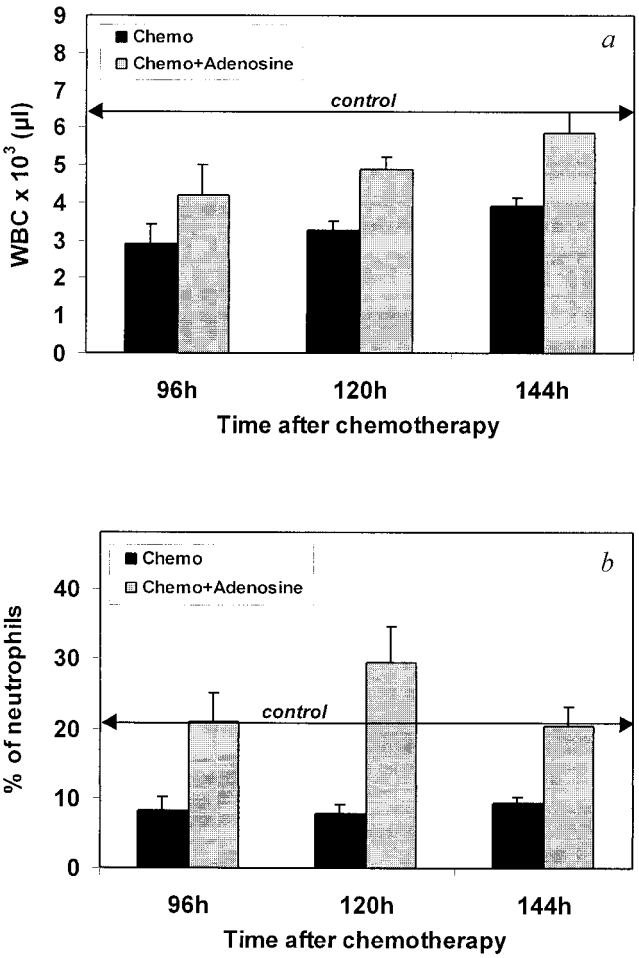

Fig. 5. The in vivo effect of adenosine on the myeloid system in mice treated with chemotherapy. Chemotherapy alone decreased the number of leukocytes and neutrophils. Adenosine, administered after chemotherapy, increased the number of total white blood cell count (**a**) and restored the percentage of neutrophils (**b**). Each group contained five mice and five experiments were carried out.

DISCUSSION

The results of the present study show that extracellular adenosine stimulated the proliferation of bone marrow cells in vitro. This activity is mediated by adenosine's capability to induce G-CSF production. When adenosine was administered to mice after chemotherapy, it acted as a chemoprotective agent.

Stimulation of bone marrow cell proliferation by adenosine was shown by [^{3}H]thymidine incorporation and by cell-cycle analysis, indicating the induction of cell division by adenosine. It took place through A1 and A3 receptor activation as DPCPX and MRS-1220 (adenosine A1 and A3 receptor antagonists, respectively) inhibited the stimulatory effect of adenosine. Moreover, the stimulatory effect of adenosine could be mimicked by CPA and IB-MECA (adenosine A1 and A3 receptor agonists, respectively). Both agonists caused a stimulatory effect on bone marrow cell proliferation, although IB-MECA exerted a more pronounced effect. This could result from a different affinity of the agonists to the receptors. However, blocking the A2 receptor by DMPX resulted in an increased proliferation, which exceeded that of the adenosine alone. It may be suggested that blocking the A2 receptor directed more adenosine toward the A1 and A3 receptors, resulting in stimulation of bone marrow cell proliferation.

The maximal stimulatory effect of adenosine on bone marrow cell proliferation took place after 48 h. Thus, we presumed that this effect was mediated through the production of a growth factor, first released by one population of bone marrow cells and then affecting the myeloid progenitors. G-CSF, a hematopoietic growth factor, released spontaneously by mononuclear cells, is known to induce myeloid progenitor cell proliferation and differentiation. Indeed, adenosine stimulated G-CSF production by human peripheral blood mononuclear cells. Moreover, antibodies to G-CSF inhibited the stimulatory effect of adenosine and its agonists on mu-

rine bone marrow cell growth, thus indicating that these activities were mediated through the induction of G-CSF production. The binding of adenosine and its agonists to the A1 and A3 receptors is known to activate the Gi protein cascade (Stiles, 1991), which inhibits adenylate cyclase activity and the production of cAMP. Earlier studies have shown that the proliferation of various cell types and the production of some cytokines is differentially regulated by cAMP. Although in some cells (hepatocytes, epithelial cells, Swiss 3T3 cells) cAMP elevation induces proliferation (Pastan et al., 1975), in others, such as myeloid progenitor cells, it suppresses growth (Nagao et al., 1984; Nilsson and Olsson, 1984; Lee, 1999). Increased levels of cAMP were shown to inhibit the proliferation of the NFS-60 myeloid cell line as well as human and murine committed granulocyte/macrophage progenitors (Ward et al., 1996; Kurland et al., 1997; Wilson et al., 1998). In addition, increased cAMP levels induce the production of IL-6, IL-8, and TNF (Parmely et al., 1993; Robson et al., 1995), whereas other cytokines such as IL-2 and GM-CSF are upregulated when the cAMP level is decreased (Bastin et al., 1990; Derigs et al., 1994; Patil and Borch, 1995). We presume that the binding of adenosine to its A1 and A3 receptors, known to inhibit adenylate cyclase and consequently decrease cAMP levels, induced stimulation of G-CSF production and bone marrow cell proliferation.

The anti-inflammatory effects of adenosine are attributed mainly to its ability to inhibit TNF production. Kitabayashi et al. (Kitabayashi et al., 1995; Wagner et al., 1998) reported that G-CSF inhibited TNF-α production. It is suggested that TNF-α inhibition by adenosine is mediated by its capability to stimulate G-CSF production.

The in vivo studies, which demonstrated an increase in leukocytes, neutrophil numbers, and ex vivo bone marrow cell proliferation, confirmed the in vitro results. Neutrophil counts were restored to normal values when adenosine was administered after chemotherapy; thus, the myelotoxic effects of chemotherapy were prevented. The in vivo studies showed that only the myeloid system was stimulated by adenosine. To the best of our knowledge, this is the first report that describes the presence of adenosine receptors on myeloid bone marrow progenitors, although such receptors have been defined on mature granulocytes, i.e., neutrophils (Cronstein, 1992).

Pospisil et al. (1993, 1995, 1998) showed that administration of adenosine monophosphate (AMP) combined with dipyridamole and G-CSF, prior to radiation, led to a radioprotective effect by the stimulation of hematopoiesis in the bone marrow and spleen of the treated mice. Jackson et al. (1978) and Epstein and Preisler (1984) treated mice with the antimetabolite 6-thioguanine and demonstrated that adenosine exerted a protective effect on myeloid progenitor cells. In these studies, adenosine or AMP was administered, but at a much higher daily dose (500 mg/kg body weight) than used in the present work. Thus, the results we hereby report are the first to demonstrate the use of a low-dose adenosine alone (0.25 mg/kg body weight) as a stimulator of bone marrow cell proliferation and differentiation in vitro and in vivo. Evidence is provided that adenosine, through A1 and A3 receptor activation, augments the production of G-CSF and thus stimulates bone marrow myeloid cell recovery.

G-CSF is clinically used to reduce the length of neutropenia following chemotherapy and bone marrow transplantation. It stimulates the proliferation and differentiation of hematopoietic progenitors and also controls the functional activities of neutrophils and macrophages (Itoh et al., 1984; Ikebuchi et al., 1988). In the present study, low-dose adenosine increased WBC counts and restored the number of neutrophils. Adenosine is already used in the treatment of patients with arrhythmias (Belardinelli et al., 1989). It is suggested, therefore, that adenosine be offered as a more cost-effective treatment with fewer side effects than G-CSF.

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