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Research Article

# Suppression of Experimental Zymosan-Induced Arthritis by Intraperitoneal Administration of Adenosine

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**ABSTRACT** The anti-inflammatory effect of methotrexate (MTX) is mediated by the increasing extracellular concentration of adenosine. The response of the immune system to activation of cell membrane subclass receptors for adenosine initiates intracellular signaling pathways, which causes immunomodulatory responses. To test the direct immunomodulatory effects of systemic administration of adenosine on animal model of inflammation, zymosan induced arthritis (ZIA) in mice was employed. Sterile zymosan (30 μg) was injected directly into the knee cleft of 60 mice, divided into 3 equal groups. The experimental groups received, every second day, 0, 0.25, and 0.5 mg/kg adenosine intraperitoneally. Inflammatory intensity was evaluated clinically by knee swelling measurement, histology, the total white blood cell (WBC) count, and serum tumor necrosis factor-alpha (TNF-α) levels served as markers of systemic inflammatory reaction. As observed, only the mice that received the higher dose of 0.5 mg/kg adenosine developed a significantly milder arthritis. A 23.9% reduction ($P < 0.004$) of the mean knee diameter swelling was noted. The histological inflammatory parameters and WBC count in the 0.5 mg/kg adenosine-treated mice were also decreased ($6{,}555 \pm 510/mm^3$ vs. $9{,}250 \pm 530$ WBC/$mm^3$ in the untreated mice, $P > 0.006$; $9{,}188 \pm 588$ WBC/$mm^3$ in the 0.25 mg/kg adenosine-treated group). Serum TNF-α levels were significantly reduced (78.1 pg/ml in the 0.5 mg/kg adenosine-treated group vs. 124.3 pg/ml, in the control group, $P < 0.004$). The data indicate a remarkable clinical beneficial effect, as well as a local and systemic anti-inflammatory response that was noted on administration of 0.5 mg/kg adenosine every alternate day to mice with inflammatory arthritis. Drug Dev. Res. 57:182–186, 2002.


**Key words:** adenosine; inflammation; TNF-α; animal model; arthritis

## INTRODUCTION

The purine nucleoside, adenosine, mediates various physiological cellular activities such as cell growth, differentiation, and cell death [Ohana and Sitovsky, 2001; Virag and Szabo, 2001]. It is released into the extracellular environment from activated or

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metabolically stimulated cells and it binds to selective G-protein-associated membrane receptors designated $A_1$, $A_{2A}$, $A_{2B}$, and $A_3$ [Stiles, 1990; De Virgilio et al., 2001]. The cumulative effects of activated adenosine $A_{2A}$ and $A_3$ receptors on the immune system are considered anti-inflammatory. The modification of lymphocyte functions is expressed by the decrease of cytokine production such as tumor necrosis factor-alpha (TNF-α), IL-6, IL-8, and by the increase of the anti-inflammatory cytokine IL-10 secretion [Salvatore et al., 2000; Ohta and Sitkovsky, 2001]. Moreover, the cytokine milieu alters the expression and function of adenosine membranal receptors on inflammatory cells [Khoa et al., 2001].

Intermittent low-dose administration of methotrexate (MTX) is widely used as a disease-modifying agent for rheumatoid arthritis (RA), as well as for other forms of inflammatory arthritis [Cutolo et al., 2001; Choi et al., 2002]. The anti-inflammatory mechanism of MTX in acute and chronic arthritis is mediated by its capacity to increase the extracellular adenosine concentrations [Cronstein, 1997]. The MTX absorbed by the cells is converted to MTX-polyglutamate. This metabolite inhibits the enzyme 5-aminoimidazole-4, carboxamide ribonuclease (AICAR) transformylase and, as a consequence, intracellular AICAR accumulates and thus the extracellular concentration of adenosine is increased [Allegra et al., 1985; Cronstein et al., 1991]. The present study was designed to assess the direct anti-inflammatory effects and the arthritis-modulating potential of adenosine in zymosan-induced arthritis (ZIA) [Keystone et al., 1997].

## MATERIALS AND METHODS

Six-week-old female C57Bl/6J mice were purchased from Harlan Laboratories (Jerusalem, Israel). Standardized pelleted diet and tap water were supplied. Experiments were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee at the Rabin Medical Center, Petah Tikva, Israel.

Acute arthritis of the right foot was induced by injection of 30 mg of sterile zymosan (*Saccharomyces cerevisiae*) dissolved in 0.2 ml of 0.9% sterile saline, directly into the arthricular cleft as previously described [Weinberger et al., 1996]. The inflammatory intensity, presented as joint swelling, was measured by Caliper (Mitutoyo Co., Tokyo, Japan). Histopathological sections of these decalcified whole joints were stained with hematoxylin-eosin. Slides were screened for the following arthritic characteristics: inflammatory cell infiltration, synovial cell lining hyperplasia, and pannus formation. The histological assessment of the knee inflammation intensity was graded 0–4 in each of the four following parameters:

1. Lymphatic follicle like formation;
2. Inflammatory cells infiltration;
3. Synovial cells hyperplasia;
4. Pannus formation.

The mean of these four parameters above all together designated "Total Arthritic Score" [Goldenberg et al., 1983].The white blood cell (WBC) count and the serum levels of the pro-inflammatory cytokine TNF-α served as humoral markers of the immune system activation. Tumor necrosis factor-α sera level was determined, in accordance with the manufacturer's guidelines, by ELISA kit "Quantikine M" (R&D Systems, Minneapolis, MN).

Twenty mice in each group were injected (ip) with adenosine (0, 200, and 400 mM/kg equivalent to 0, 0.25, and 0.50 mg/kg, respectively), daily, 1 week before the induction of arthritis, and continued alternate days for 4 weeks. When not specified, materials were purchased from Sigma Chemical Co. (St. Louis, MO).

### Statistical Analysis

The results were statistically evaluated using the Student's *t*-test. Comparison between the mean values of different experiments was carried out. The criterion for statistical significance was $P < 0.05$.

## RESULTS

### Clinical Observations

All the zymosan-injected mice developed knee arthritis. On day 28, the diameter of the zymosan-injected knee was compared to the contra lateral non-injected knee. No differences were found between the 0.25 mg/kg and control groups (0.67 and 0.66 mm respectively, $P = 0.36$). However, in mice that received 0.5 mg/kg adenosine, a significantly milder clinical arthritis was noted with an increase in diameter of only 0.51 mm ($P < 0.004$).

### Histopathological Studies:

All four histological parameters are presented together, comparing the non-treated group to the adenosine treated groups. Only the high dose of adenosine (0.5 mg/kg), injection i.p. suppressed the histological parameters of inflammation in the mice hind knees. As shown in Figure 1, there was a prominent difference in lymphatic accumulation in follicle-like formations, mean score of 0.8 in the 0.5 mg/kg adenosine-treated mice, opposed to the mean score of 1.3 in the saline and 0.25 mg/kg adenosine-treated groups ($P < 0.005$). A significant reduction of pannus

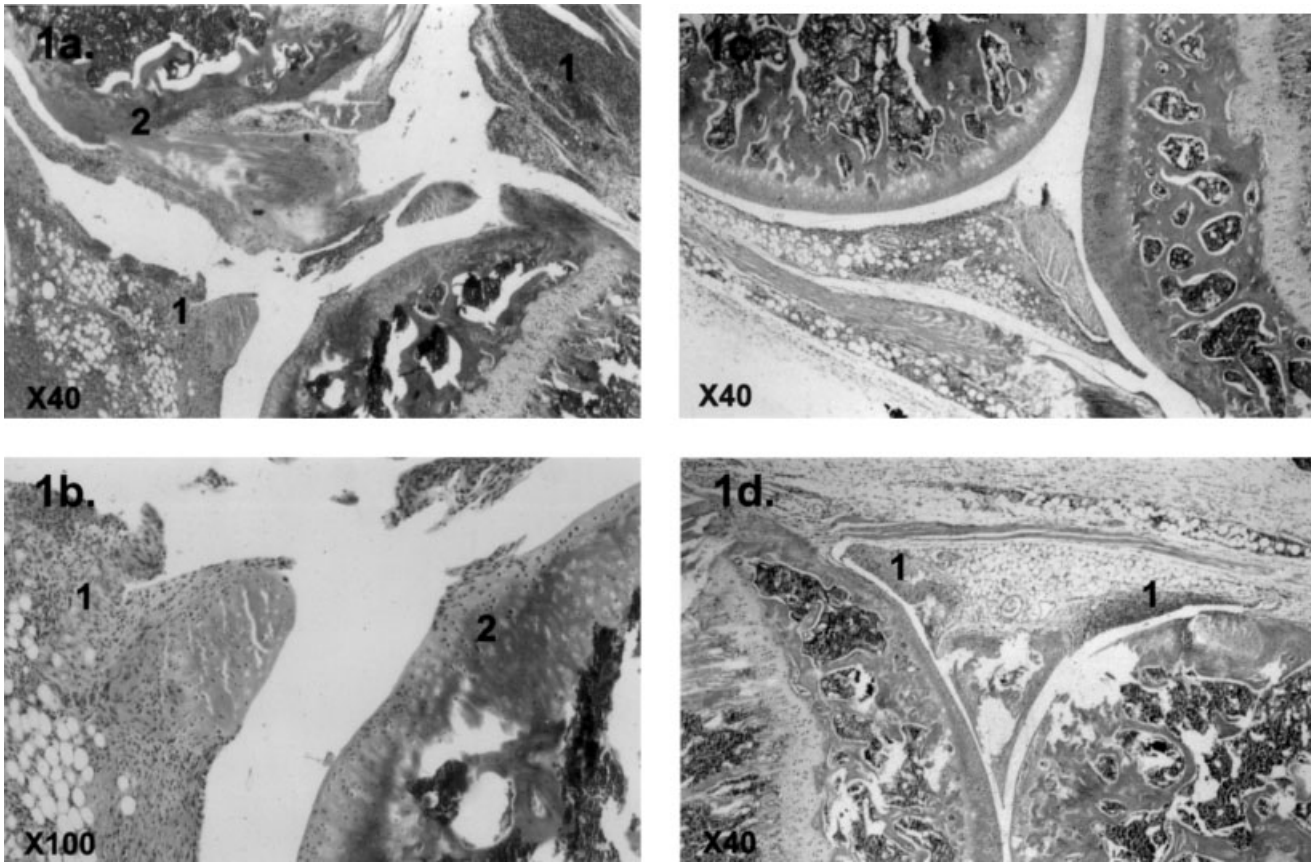


**Fig. 1.** Histological sections of mice knees, stained with hematoxylin-eosin. **a,b:** Zymosan-induced arthritis (ZIA), magnification $\times 40$, $\times 100$, respectively. 1: Synovitis characterized by massive leukocyte infiltration, tissue edema and synovial cell hyperplasia. 2: Cartilage degradation, cortical bone deformation, and bone marrow architecture disruption. **c:** Adenosine-treated mouse with ZIA. Normal joint appearance. **d:** Adenosine-treated mouse with ZIA depicting mild localized synovitis (1) with intact joint structure.

formation was also observed from a mean score of 1.1 in the adenosine-treated group vs. 0.2 in the control group ($P < 0.001$). There was a reduction, though not significant, in the mean synovial thickening score 0 of synovial hyperplasia observed in the 0.5 mg/kg adenosine-treated group as opposed to a mild thickening score of 0.4 in the NaCl and 0.25 mg/kg adenosine-treated groups ($P < 0.47$). The mean score for synovial inflammatory mononuclear and plasma cell infiltration was 0.9 in the 0.5 mg/kg adenosine-treated group and 2.6 in the NaCl and 0.25 mg/kg adenosine-treated groups, respectively, a significant difference ($P < 0.007$).

The mean of all the histopathological parameters together designated as "Total Arthritic Score" was significantly reduced in the 0.5 mg/kg adenosine-treated mice (Fig. 2).

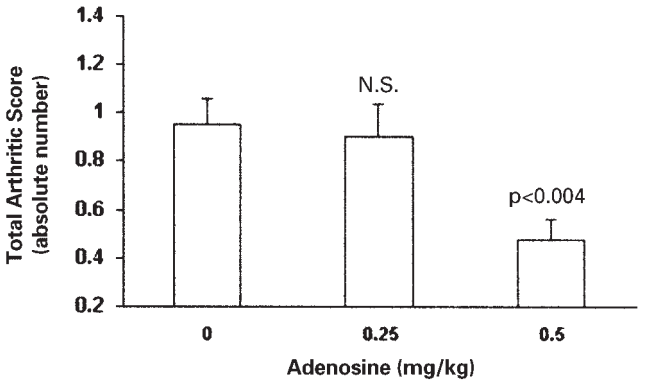


**Fig. 2.** "Total Arthritic Score"—mean of all the 4 histopathological parameters of inflammation. Treatment of 0.5 mg/kg of adenosine (ip) revealed a significant decrease in the inflammatory characteristic of arthritis, while a non-significant (N.S.) difference was observed at 0.25 mg/kg, in comparison to normal knee structure.

## Inflammatory Markers

WBC counts served as an indicator for systemic inflammatory response as depicted in Figure 3a. It clearly demonstrated an anti-inflammatory effect of 0.5 mg/kg adenosine. WBC count of naive mice was 6,335 $\pm$ 350/mm$^3$, while the group treated with 0.5 mg/kg adenosine had a WBC count within the normal range (6,560 $\pm$ 510/mm$^3$, $P > 0.36$). NaCl-treated mice and those treated with 0.25 mg/kg adenosine had a

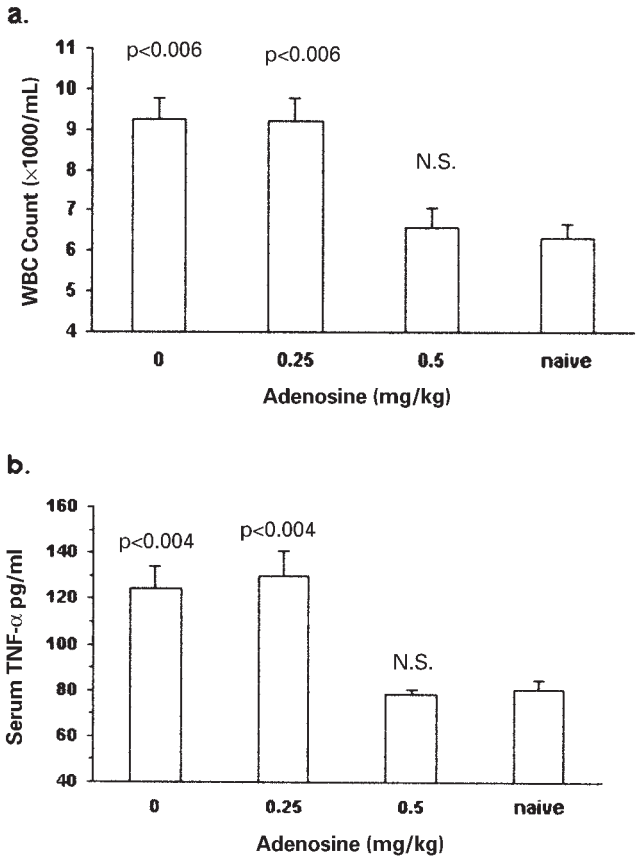


**Fig. 3.** **a:** Mean WBC counts of: 0, 0.25, 0.5 mg/kg i.p. Adenosine alternate day treated and naive mice (20 in each group). In ZIA mice, a marked increase in WBC counts was observed. A similar increase in the WBC counts was noted in the 0.25 mg/kg treated mice, while 0.5 mg/kg adenosine-treated mice had normal WBC counts. **b:** Mean serum TNF-α of: 0, 0.25, 0.5 mg/kg i.p. Adenosine alternate day treated and naive mice (20 in each group). Serum levels of TNF-α measured exhibited a similar phenomenon as the WBC counts. Treatment of 0.5 mg/kg adenosine blocked the increase of TNF-α in ZIA mice, whereas treatment of 0.25 mg/kg adenosine had no such effect.

significantly increased WBC count of 9,259 $\pm$ 530 and 9,188 $\pm$ 588/mm$^3$, respectively ($P > 0.006$), indicating a systemic inflammatory response. The measurement of TNF-α levels showed a similar result (Fig. 3b). A mild reduction (80.2 pg/ml) in circulating TNF-α levels was observed in mice treated with 0.5 mg/kg adenosine compared to naive mice, whereas the NaCl-treated and 0.25 mg/kg adenosine groups had marked elevation of TNF-α levels (124.3 and 129.8 pg/ml, respectively, $P < 0.005$).

## DISCUSSION

The data indicate additional direct support for the anti-inflammatory properties of adenosine. Our study showed that systemic administration of adenosine in a dose up to 0.25 mg/kg has no local or systemic anti-inflammatory effect. A 0.5-mg/kg dose of adenosine every alternate day significantly ameliorated the local inflammatory process in the knees of the ZIA-induced mice. Moreover, it clearly demonstrated a systemic anti-inflammatory effect of adenosine, reflected by the WBC counts and the reduction of TNF-α secretion. This cytokine plays a major role in the development, chronic activity, and complications in RA [Taylor, 2001; Fabris et al., 2002]. The effects of adenosine on the inflammatory process are mediated by its diverse receptors distributed on the outer membrane surface of all members of the immune system cells, the vascular endothelium [Deguchi et al., 1998] and the connective tissues cells [Nataka, 1993]. Thus, adenosine has the potential to change the interactions between all cellular components at the inflammatory site [De Virgilio et al., 2001; McPherson et al., 2001; Bodin and Burnstock, 1998]. In recent years, many of the mechanisms and effects of the adenosine receptors on different immune system cells were elucidated. The effect of activating $A_1$ adenosine receptors on polymorphonuclear (PMN) cells promoted chemotaxis and increased their adherence to the endothelium, thereby exerting a proinflammatory effect. On the other hand, stimulation of the A2 receptors on the same cells displays an inhibitory effect on PMN functions, phagocytosis, and chemotaxis [Cronstein, 1994]. Macrophage $A_{2A}$ receptor activation inhibits production of the pro-inflammatory cytokines: TNF-α, IL-6, IL-8 [Bouma, 1994]. Introduction of sub-inflammatory stimuli doses to the naive mice generated severe inflammatory response and death in $A_{2A}$ receptor deficient mice, indicating that the physiological role of $A_{2A}$ receptor activation is essential to terminate tissue-specific and systemic inflammation [Ohta and Sitkovsky, 2001]. Activation of $A_{2A}$ and $A_{2B}$ receptors inhibited T-cell proliferation [Huang et al., 1997; Mirabet et al., 1999]. $A_{2B}$ receptor activation blocked PMN transendothelial migration [Wakai et al., 2001]. $A_3$ receptor agonist suppressed the production of the macrophage pro-inflammatory chemokine MIP-1α [Szabo et al., 1998] and inhibited eosinophil migration [Knight et al., 1997]. Extracellular adenosine accumulation induced by blockage of the enzyme adenosine kinase, suppressed TNF-α production in human mononuclear cells [Eigler et al., 2000]. In this study, we tested the net effect of adenosine itself in an animal model of acute knee arthritis. The beneficial effect noted is in line with the findings that $A_3$ receptor activation reduced joint inflammation in collagen induced arthritis animal model [Szabo et al., 1998] and decreased inflammation intensity and matrix metalloproteinase expression in the adjuvant arthritis rat model [Boyle et al., 2001]. Adenosine also has direct

analgesic properties unrelated to its anti-inflammatory capacity. Intravenous adenosine infusions were given on a weekly basis and continued for 7 months without any severe side effects or signs of tolerance to relieve neuropathic pain [Gyllenhammar and Nordfors, 2001]. Alternate day injections of adenosine or long-acting adenosine agonists might be additional medication in the combat against uncontrolled inflammation. Moreover, in view of the low rate of serious adverse effects, adenosine or its derivatives might be used in the future as substitutes for MTX as disease-modifying agents in various types of inflammatory arthritis.

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