Suppressive Effect of 1-Methyladenosine on the Generation of Chemiluminescence by Mouse Peritoneal Macrophages Stimulated with Opsonized Zymosan

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ITOH, K., MAJIMA, T., EDO, K., MIZUGAKI, M. and ISHIDA, N. Suppressive Effect of 1-Methyladenosine on the Generation of Chemiluminescence by Mouse Peritoneal Macrophages Stimulated with Opsonized Zymosan. Tohoku J. Exp. Med., 1989, 157 (3), 205-214 — The preparation of an in vitro assay system for the immunosuppressive activities of modified nucleosides on macrophage (Mφ) functions is described. Briefly, Listeria-elicited mouse peritoneal Mφs (Lm-Mφs) were treated with nucleosides in vitro for 18 hr at less than 1 mM concentration and chemiluminescence (CL) was measured after stimulation with opsonized zymosan. To confirm the usefulness of this assay (in vitro test), the immunosuppressive activities of 1-methyladenosine (m1Ado) and adenosine (Ado) were determined by mouse Listeria infection (in vivo test), CL generation by Mφs obtained from the peritoneal cavity of nucleoside-treated mice (in vivo-in vitro test), and the in vitro test. The immunosuppressive activity of m1Ado detected by the in vitro test was confirmed by the in vivo and the in vivo-in vitro tests. As for Ado, no immunosuppression was detected by the in vivo and the in vivo-in vitro tests though a potent suppressive effect was detected by the in vitro test. The ineffectiveness of Ado can be explained by the in vivo conversion of Ado to an inactive form. Thus, the proposed in vitro test seems to be useful, with the provision that the known in vivo metabolism is taken into consideration. ——— immunosuppression; modified nucleosides; 1-methyladenosine; chemiluminescence; macrophage

Known immunosuppressive states in the later stages of cancer (e.g., skin test negative) have been examined to characterize the suppressive factors in body fluids (Kamo and Friedman 1977). In tumor-bearing animals, the same or related factors have also been found in higher concentrations, in particular in tumor ascitic fluids (Matsubara et al. 1980; Umenai et al. 1980; Takano et al. 1983). Although some of these factors have a high molecular weight (M.W.) (Tamura et al. 1981), others have a M.W. that is comparatively small. Ishida and his coworkers detected several bases and nucleosides in Ehrlich ascites fluids (Ishida
Two of them, which inhibited the augmentation of NK cells by interferon (IFN), were found to be uracil and uric acid (Sami et al. 1986). Another, which impaired the resistance of mice to *Listeria* infection, is now known to be 1-methyladenosine (m1Ado) (Takano et al. 1986).

Evaluation of immunosuppressive activities by antibacterial resistance in mice is complicated and time consuming. Consequently, we have endeavoured to establish a rapid and simple in vitro assay system, by utilizing the luminol-dependent CL generated by Mφs after stimulation with opsonized zymosan. Mφs were used because of the following observations: Suppressor Mφs may play the most critical role in T cell function after PHA or Con A stimulation (Shibata et al. 1983) and also in the induction of IFN γ by human peripheral mononuclear cells (Yamaguchi et al. 1984; Noda et al. 1986).

Thus the main purpose of this report is to evaluate our in vitro test by comparing it with the in vivo and the in vivo-in vitro tests. As for the immunosuppressive agents, we used mainly the modified nucleosides and bases employed in previous experiments (Takano et al. 1986).

**Materials and Methods**

*Mice used for immunosuppression test*

Male BALB/c mice were obtained from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Hamamatsu). They were maintained in an air conditioned room with free access to a basal diet and water, and used at 6 to 10 weeks old.

*Nucleosides and other chemicals*

All purine and pyrimidine derivatives were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Wako Pure Chemical (Osaka). 2′-Deoxy-1-methyladenosine (m1Ado) was synthesized from 2′-deoxyadenosine (dAdo) according to the method of Jones and Robins (1963). All these nucleoside derivatives were 99% or more pure according to HPLC analysis. Luminol was purchased from Wako Pure Chemical and Zymosan A from Sigma. Brain heart infusion (BHI) medium and heart infusion agar (HIA) were obtained from Difco Laboratories (Detroit, MI, USA).

*Bacteria used for immunosuppression experiments in mice*

*Listeria monocytogenes* (serotype 4b), isolated from the spinal fluid of a patient with *Listeria* meningitis was grown in BHI medium at 37°C for 15 hr and then centrifuged at 3,000 rpm for 30 min to collect organisms. The number of live organisms was counted by plating on HIA. The organisms were suspended at 1 × 10⁹ cells/ml in the BHI medium, and aliquots were stored at -80°C until use. A median lethal dose (LD₅₀) was 1-2 × 10⁹ when given intravenously (i.v.) to BALB/c mice.

*Preparation of peritoneal macrophage suspensions*

LM-Mφs used for the in vitro test were obtained from BALB/c mice 4 days after intraperitoneal (i.p.) injection of live *Listeria*, 1 × 10⁶ cells/mouse. Peritoneal Mφs used for the in vivo-in vitro test were obtained from mice 2 days after i.p. injection of nucleosides or saline-control.

These peritoneal cells were harvested after i.p. injection of 6 ml of cold RPMI 1640 medium supplemented with 100 U/ml penicillin G and 100 μg/ml streptomycin (RPMI),
followed by recovery (5 ml) within 2 min. The harvested cells were washed twice with RPMI and finally resuspended in RPMI at $1 \times 10^6$ cells/ml.

**In vivo test for detecting suppressive activities of nucleosides against Listeria infection (in vivo test)**

Evaluation of the effect of the nucleosides on *Listeria* resistance was performed according to the method previously described (Takano et al. 1983, 1986). Briefly, 0.2 ml aliquots of nucleosides dissolved in saline (0.02 mM) were given i.v. to BALB/c mice 24 hr before *Listeria* infection. The control mice were given 0.2 ml of saline. After i.v. challenge of 1 LD$_{50}$ dose of *Listeria*, the progress of the survivors was followed for 20 consecutive days.

**Measurement of luminol dependent CL generation by nucleoside treated macrophages (in vitro test)**

One ml aliquots of Lm-M$\phi$ suspension ($1 \times 10^6$ cells) prepared as described above were put in plastic vials and placed in a 5% CO$_2$ incubator at 37°C. Two hr later, nonadherent cells were counted and removed. The remaining adherent cells were washed three times with RPMI, then cultured with 1 ml of RPMI containing 5% heat-inactivated fetal calf serum (FCS-RPMI) and tested nucleosides dissolved in saline (0.3 mM) were added to the cultures. After 18 hr incubation, the nucleoside solution was discarded and adherent cells were washed three times with RPMI, then incubated with 1 ml of FCS-RPMI. Ten $\mu$l of luminol dissolved in RPMI (0.2 mg/ml) was added to each vial and set to a liquid scintillation spectrometer (Biolumat LB 9500T, Berthold, Germany). When, after 2 min, the background count became constant, 40 $\mu$l of Zymosan A opsonized with fresh autologous serum (25 mg/ml) was added to the vial, on the understanding that opsonized particles would react with Fc receptors of macrophages (Cheung et al. 1983). Photocounting of each vial was continued every minute for 30 min. M$\phi$ CL generation was assessed by the peak height and represented as counts per minute (cpm) per $10^6$ cells. The CL analysis was done in duplicate or quadruplicate and t test was used to assess the statistical significance.

**Results**

**Effect of m$^1$Ado on mouse Listeria infection (in vivo test)**

Based upon previous experimental results (Takano et al. 1986), m$^1$Ado and its related compounds, Ado, Ino, and m$^4$dAdo were tested at 0.02 mM concentrations (the most effective dose for the enhancement of *Listeria* infection) by i.v. injections of 0.2 ml solution, to evaluate the impairment of resistance or the enhancement of infection in mice challenged 24 hr later with 1 LD$_{50}$ of *Listeria* (Fig. 1). As a result, deaths in the control mice given saline as well as in those receiving Ado and Ino reached 50% by days 6–8. As had been expected, all mice receiving m$^1$Ado died within 5 days. Unexpected results were obtained with m$^4$dAdo, with mice proving rather resistant to the infection.

**Effect of intraperitoneal administration of m$^1$Ado on CL generation by harvested macrophages (in vivo – in vitro test)**

It is already known that M$\phi$s play an important role in the elimination of *Listeria* in mice (Mitsuyama et al. 1978). In confirmation of this, the suppressive effect of m$^1$Ado on the resistance of mice to *Listeria* infection was demonstrated (Takano et al. 1986), the mechanism being partly explained by the impaired
migration of Mφs to the peritoneal cavity. To transfer this in vivo observation to an in vitro experiment and also to discover whether the impairment can be found with harvested Mφs obtained from treated mice, 0.7 mM m'Ado or Ado (the optimal concentration determined by a preliminary test) were given i.p. to mice and peritoneal Mφs were harvested after 2 days. Their in vitro activities were examined after incubation for 2 or 18 hr in vitro. The latter incubation period was chosen to exclude the activities of neutrophils, if any (Table 1). The CL generated by peritoneal Mφs obtained from m'Ado-treated mice was approximately 30% of control Mφs, whereas Mφs obtained from Ado-treated mice generated as much CL as control Mφs. As far as the two analogues are concerned, the in vitro results were consistent with the in vivo results, shown before.

**Table 1.** In vitro CL generated by peritoneal macrophages obtained from m'Ado and Ado treated mice (in vivo – in vitro test)

<table>
<thead>
<tr>
<th>Treatment (in vivo)</th>
<th>CL peak height (cpm/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 hr</td>
</tr>
<tr>
<td>Saline</td>
<td>4687 ± 114</td>
</tr>
<tr>
<td>m'Ado</td>
<td>1067 ± 254**</td>
</tr>
<tr>
<td>Ado</td>
<td>5538 ± 121</td>
</tr>
</tbody>
</table>

Peritoneal Mφs were obtained from mice receiving i.p. injection of 0.7 mM of m'Ado or Ado in 0.5 ml saline 2 days ahead. Then, Mφs were incubated for 2 or 18 hr before CL generation test was conducted.

Values are means ± s.d.

* *p < 0.05; **p < 0.01 against values in saline.
Suppressive effect on CL generated by macrophages when m¹Ado was added in vitro (in vitro test)

The effect of in vitro treatment with m¹Ado on the CL generated by Lm-Mφs was then examined to establish a rapid and simple assay system. In this test, an optimal concentration of m¹Ado was chosen 0.3 mM, which was the most effective and non-toxic dose, as shown in Fig. 2. Lm-Mφs were incubated in the presence of 0.3 mM m¹Ado and the result is illustrated in Fig. 3, which shows the suppressive effect of m¹Ado. The suppression reached almost 70% at 18 hr. After an 18-hr incubation with m¹Ado, the culture medium was replaced by fresh FCS-RPMI and incubated again for another 48 hr. The suppressive effect of m¹Ado diminished almost to nothing after 24 hr incubation (Fig. 3), indicating that the suppressive effect of m¹Ado was reversible and not lethal. The immunosuppressive effect of m¹Ado had been revealed not only in the in vivo Listeria challenge test, but also in the in vivo-in vitro test and again in this in vitro test.

Screening results of immunosuppressive nucleosides by means of in vitro test with Lm-Mφs

When the suppressive effect of 10 compounds including immunosuppressive nucleosides and their components was examined on the CL generated by Lm-Mφs (Table 2), m⁶Ado and Ado were found to possess suppressive effects comparable to

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**Fig. 2.** The suppressive effect of m¹Ado on CL generated by *Listeria*-elicited peritoneal macrophages (Lm-Mφs) in vitro. Lm-Mφs were treated with indicated doses of m¹Ado and incubated for 18 hr. Then, the CL generated by Lm-Mφs stimulated with opsonized zymosan was determined. Means ± s.d. of CL generation are shown.
that of m'Ado. As had been expected, m'dAdo and Ino did not reveal any suppressive effect. On the other hand, dAdo and 1-methylinosine (m'Ino) showed a moderate suppressive effect, which requires further examination and explanation. 1-Methyladenine (m'Ade), a base moiety of m'Ado, was shown to have a slight suppressive effect, although D-ribose, a sugar moiety of m'Ado, had no suppressive effect. 2-Chloroadenosine (2-ClAdo) had a potent suppressive effect probably due to its known cidal effect on Mφs (Ohtani et al. 1982; Saito and Yamaguchi 1985). In summary, the results obtained are almost consistent with our prior hypothesis. The details are left for the discussion.

![Graph](image)

**Fig. 3.** Time course of CL generated by Lm-Mφs in vitro in the presence and after the removal of m'Ado. Lm-Mφs were treated with 0.3 mM m'Ado and CL generation was determined at indicated hr. Means ± s.d. of CL generation are shown.

**Table 2.** Suppressive effect of tested nucleosides and other compounds on the CL generated by peritoneal macrophages (in vitro test)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Suppression (%)</th>
<th>Compound</th>
<th>Suppression (%)</th>
</tr>
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<tbody>
<tr>
<td>m'Ado</td>
<td>69.2 ± 12.2</td>
<td>m'Ino</td>
<td>50.9 ± 7.4</td>
</tr>
<tr>
<td>m'Ado</td>
<td>85.7 ± 5.7</td>
<td>Ino</td>
<td>3.4 ± 2.5</td>
</tr>
<tr>
<td>Ado</td>
<td>73.7 ± 8.0</td>
<td>m'Ade</td>
<td>25.6 ± 3.8</td>
</tr>
<tr>
<td>m'dAdo</td>
<td>7.4 ± 3.2</td>
<td>Ribose</td>
<td>5.1 ± 1.2</td>
</tr>
<tr>
<td>dAdo</td>
<td>40.5 ± 6.1</td>
<td>2-ClAdo</td>
<td>99.1 ± 0.3</td>
</tr>
</tbody>
</table>

Control value with saline-treated Mφs was 5120 ± 315 cpm/10^6 cells (means ± s.d.) in four determinations.
DISCUSSION

The present study was conducted to see whether evaluation of the immunosuppressive effect of the principles detectable in cancer ascitic fluids (e.g. m1Ado) can be made by means of a simple in vitro test. For this purpose, Listeria-elicited (both FcRI and FcRII activated) Mφs (Majima et al. 1989) were treated with these principles in vitro for 18 hr and examined for CL after the addition of opsonized zymosan, on the supposition that it would bind to the Fc receptors of Mφs.

M1Ado, known to have a potent suppressive activity on the resistance of mice to Listeria infection (in vivo test), was found to suppress the CL generated by Mφs obtained from pretreated mice (in vivo-in vitro test), as well as Lm-Mφ CL generation in in vitro treatment (in vitro test). On the other hand, m1dAdo, which has an enhancing effect or no suppressive effect on the resistance to Listeria infection in mice, showed no suppressive or enhancing effect on Lm-Mφ CL generation in the in vitro test. With this compound, m1dAdo, we had already found another in vivo enhancing effect on the primary antibody response to a T-dependent antigen (unpublished data). The enhancing effect of m1dAdo on resistance to Listeria infection might be attributed not to the activation of Mφs but to that of T or B cells. Ino, showing no suppressive effect on resistance to Listeria infection, had no inhibitory effect in the in vitro test.

The only exceptional or discordant finding in the three tests was obtained with Ado. The compound had a suppressive effect neither on the resistance of mice to Listeria infection nor on CL generated by Mφs obtained from Ado-treated mice. However, the compound showed a potent inhibitory effect comparable to the effect of m1Ado when treated in vitro. From a theoretical point of view, the ineffectiveness of Ado in the in vivo and the in vivo-in vitro tests can be explained by the conversion of Ado to an inactive form, probably by purine metabolic enzymes in the body fluid (Agarwal et al. 1975). On the other hand, the fact that m1Ado revealed a potent suppressive effect both in vivo and in vitro suggests that m1 Ado might be resistant to these purine metabolic enzymes. In fact, it is well known that m1Ado is an inhibitor of adenosine deaminase (ADA) (Agarwal et al. 1975), and m1Ado has an etiological role in an immunological disorder, that mimics adenosine deaminase deficiency syndrome.

It is also known that Ado plays an important role in the modulation of cellular functions (Berne 1980; Schimmel 1980). In this study, adenosine and its analogues such as m1Ado, m6Ado and dAdo other than m1dAdo showed potent suppressive activities on the CL generated by Mφs in vitro (Table 2). It is reported that Ado suppresses the production of active oxygen metabolites in human polymorphonuclear leukocytes (PMN) (Cronstein et al. 1985; Pasini et al. 1985; Roberts et al. 1985) and the lysosomal enzyme secretion of mouse peritoneal Mφs (Riches et al. 1985). Intracellular physiological change induced by Ado was
mediated by its specific receptors and modulated by the level of intracellular cAMP (Van Calker et al. 1978, 1979; Londos et al. 1980; Daly et al. 1981). The presence of adenosine receptors has recently been established with human and murine lymphocytes (Birch and Polmar 1986; Samet 1986), human PMN (Cronstein et al. 1985), and rabbit Mφs (Hasday and Sitrin 1987). It is noteworthy that the suppressive effect of m'Ado and Ado on Lm-Mφs is not diminished by pretreatment with dipyridamole, an inhibitor of adenosine uptake (Paterson et al. 1981) (data not shown). The suppressive effect of the two compounds was not reversed by simultaneous treatment with Ino, which does not by itself have any suppressive effect on CL generation in vitro (Table 2). Moreover, an in vitro suppressive effect did appear when noneffective dose of m'Ado and Ado were added simultaneously (data not shown). From these observations, the suppression of CL generation by m'Ado and Ado might be mediated by their binding to specific receptors shared by the two compounds. Identification of the subtypes of Ado receptor(s) on Lm-Mφ and elucidation of the mechanism of the suppression caused by m'Ado and Ado are now in progress.

References


Suppression of Macrophage Function by 1-Methyladenosine


