

Adenosine Receptors and Modulation of Natural Killer Cell Activity by Purine Nucleosides¹

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ABSTRACT

Natural killer (NK) cell activity is inhibited by the adenosine analogue tubercidin (Tub) and stimulated by the deoxyadenosine analogue 2-fluoro-1- β -D-arabinofuranosyladenine 5'-monophosphate (F-ara-AMP) in the spleen lymphocytes from mice treated with the drugs *in vivo* (T. Priebe *et al.*, *Cancer Res.*, 48: 4799, 1988). The present report demonstrates that the inhibition by Tub and stimulation by F-ara-AMP of NK cell activity are readily demonstrable in murine and human lymphocytes exposed to the drugs *in vitro*. In mouse spleen lymphocytes, NK cell activity is also inhibited by adenosine receptor A2 agonists, whereas potent A1 receptor agonists are more effective stimulators. Inhibition produced by adenosine, deoxyadenosine, and adenosine receptor agonists, but not by Tub, is partially prevented by the adenosine receptor antagonist 1,3-dipropyl-8-phenylxanthine amine congener. Agents that stimulate NK cell activity (deoxyadenosine, A1 receptor agonists, F-ara-AMP) do not increase further the 1.5-fold enhancement produced by a 10^{-6} M concentration of 1,3-dipropyl-8-phenylxanthine amine congener. The nucleoside transport inhibitor, *p*-nitrobenzylthioinosine 5'-monophosphate, has no effect on NK cell activity or intracellular ribonucleotide pools; however, it partially prevents Tub 5'-triphosphate formation, ATP depletion, and NK cell inhibition in mouse spleen cells treated with Tub. *p*-Nitrobenzylthioinosine 5'-monophosphate also partially prevents the F-ara-AMP stimulation of NK cell activity, but it does not influence the effects of adenosine or deoxyadenosine. The results obtained with the adenosine receptor agonists suggest roles for both A1 and A2 receptors in regulating murine NK cell activity. Tub inhibition of NK cell activity does not appear to involve adenosine receptors; however, inhibition by the other agents may be mediated via an A2 receptor (stimulatory for adenylyl cyclase). Since *p*-nitrobenzylthioinosine 5'-monophosphate inhibited the stimulation of NK cell activity by F-ara-AMP, this stimulation may occur via an intracellular "P" site (inhibitory to adenylyl cyclase).

INTRODUCTION

In the absence or inhibition of adenosine deaminase (EC 3.5.4.4), the substrates adenosine and dAdo³ accumulate (1). Since these nucleosides are growth inhibitory or cytotoxic to numerous cells, it has been assumed that the accumulation might play a role in the severe combined immunodeficiency associated with adenosine deaminase deficiency (2). To test the hypothesis that a particular lymphocyte population exists having unique sensitivity to these nucleosides, we examined properties of functionally distinct lymphocyte populations *in vitro* (3) and *in vivo* (4) for their susceptibility to adenosine, dAdo, or their analogues. Treatment of mice with the adenosine analogue Tub produced marked inhibition of NK cell activity

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³The abbreviations used are: dAdo, deoxyadenosine; CPA, *N*⁶-cyclopentyladenosine; F-ara-A, 2-fluoro-1- β -D-arabinofuranosyladenine; F-ara-AMP, 2-fluoro-1- β -D-arabinofuranosyladenine 5'-monophosphate; F-ara-ATP, 2-fluoro-1- β -D-arabinofuranosyladenine 5'-triphosphate; NBTIP, *p*-nitrobenzyl-6-thioinosine 5'-monophosphate; NECA, 5'-*N*-ethylcarboxamidoadenosine; PAA, 2-phenylaminoadenosine; PIA, *N*⁶-(*S*-phenylisopropyl)adenosine; Tub, tubercidin; XAC, 1,3-dipropyl-8-phenylxanthine amine congener; NK, natural killer.

while stimulating that of antibody production, whereas the dAdo analogue F-ara-AMP produced exactly opposite effects on these immune parameters (4). NK cells have been reported to suppress antibody-producing cells; therefore, the stimulation (Tub) and inhibition (F-ara-AMP) of primary antibody formation may occur indirectly due to effects of these compounds on NK cell activity (5). Experimental confirmation that these actions of Tub and F-ara-AMP on primary antibody response are mediated via NK cells has been obtained using a mutant of the C57BL mouse ("beige" mutation) lacking NK cell activity (5). Thus, NK cells appear to play an important role in the immunomodulation produced by these adenosine analogues. Consequently, it appears warranted to determine the mechanisms by which F-ara-AMP and Tub produce their opposite effects on NK cell activity. Experiments using a nucleoside transport inhibitor (NBTIP) and an adenosine receptor antagonist (XAC) suggest that the biochemical mechanisms for purine nucleoside regulation of NK cell activity are diverse. Preliminary reports of parts of this work have been presented (6, 7).

MATERIALS AND METHODS

NK Cell Assay. Effector cells were obtained from the spleens of 6- to 8 week-old male C3H/He mice (Harlan Sprague Dawley, Indianapolis, IN) and from peripheral blood of normal donors (Gulf Coast Blood Center, Houston, TX). Spleen cell suspensions were prepared in RPMI 1640 by passage through stainless steel mesh sieves. Spleen lymphocytes and human peripheral blood mononuclear cells were purified by Ficoll-Hypaque (Pharmacia, Inc., Piscataway, NJ) density gradient centrifugation. A Moloney virus-induced lymphoma of A/Sn origin, YAC-1, was used as the target cell for murine NK cell activity whereas the K562 cell line derived from a patient with chronic myelogenous leukemia in blast crisis was used as the target for human NK cell activity. Both cell lines were maintained as stationary suspension cultures in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% penicillin, and streptomycin. In either case, the target cells were labeled with ⁵¹Cr by incubating 5×10^6 cells with 100-150 μ Ci of sodium [⁵¹Cr]chromate (Amersham Corporation, Arlington Heights, IL) for 1 h at 37°C. The release of ⁵¹Cr was determined after 4 h incubation in RPMI 1640 supplemented as given above. At least four effector:target ratios were used to determine lytic units, *i.e.*, the number of effector cells required to achieve a given degree of specific target cell lysis (8). Purine nucleosides, analogues, or other agents were added directly to the test plates and were present during the 4-h incubation. The agents studied did not enhance or decrease spontaneous ⁵¹Cr release; *i.e.*, release was not altered by incubation of labeled target cells in the absence of effector cells (data not shown). In experiments using XAC or NBTIP as potential antagonists, the effector cells were preincubated for 30 min with these agents prior to addition of other drugs and target cells.

High Performance Liquid Chromatography Methods. Nucleotides were measured in mouse spleen lymphocytes using a Whatman Partisil SAX (strong anion exchange) column as described previously (9). After 4 h incubation, 5×10^6 cells were extracted with ice-cold 5% perchloric acid. The acid-soluble material was then neutralized with 10 N KOH, followed by centrifugation to remove the insoluble potassium perchlorate. The extract, equivalent to approximately 2×10^6 cells, was then applied to the column via a Rheodyne Model 7125 Sample Injector (Rheodyne Inc., Cotati, CA). Nucleotides were eluted from the column using a linear gradient from 5 mM ammonium dihydrogen phosphate,

pH 2.8, to 750 mM ammonium dihydrogen phosphate, pH 3.7, in 30 min at a flow rate of 2 ml/min. To measure F-ara-ATP, the pH of the upper buffer was lowered to 3.3 and a concave gradient was used (10). To determine the dephosphorylation of F-ara-AMP in the medium of cells incubated with the drug, samples of medium were applied directly to a reverse-phase column (C₁₈- μ Bondapak; Waters Associates, Milford, MA). The samples were eluted with a linear gradient from water to 20% methanol in water during 15 min at 2 ml/min. The eluting peaks were integrated using a Laboratory Data Control/Milton Roy (Riviera Beach, FL) M3000 Data System. Quantitation was accomplished by external standardization using ribonucleosides or ribonucleoside 5'-monophosphates.

Materials. Adenosine, dAdo, Tub, and deoxycoformycin were purchased from Sigma Chemical Co., St. Louis, MO. F-ara-AMP was provided by the Drug Synthesis and Development Branch of the National Cancer Institute. F-ara-ATP was a gift from Dr. William Plunkett of M. D. Anderson Cancer Center. F-ara-A was prepared from F-ara-AMP by treatment with snake venom 5'-nucleotidase, followed by membrane centrifugation to remove protein (Centriflow membrane filters; Amicon Corp., Lexington, MA). PIA and CPA were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN) and NECA, PAA, and XAC were purchased from Research Biochemicals (Natick, MA). NBTIP was a generous gift from Dr. A. R. P. Patterson of the University of Alberta, Edmonton, Alberta, Canada.

RESULTS

Our previous observation (4) that murine NK cell activity is inhibited by Tub and stimulated by F-ara-AMP *in vivo* is readily reproduced using lymphocyte preparations *in vitro* (Fig. 1). Moreover, the dose-response relationship for mouse spleen lymphocytes is remarkably similar to that obtained using human peripheral blood mononuclear cells. Because of this similarity, we used murine splenocytes to determine the mechanisms for these effects *in vitro*. To test whether adenosine receptors might regulate NK cell activity *in vitro*, mouse spleen lymphocytes were exposed to highly selective adenosine receptor agonists during the 4-h NK cell ⁵¹Cr release assay (Fig. 2). As predicted from the known inhibitory effect of cyclic AMP on NK cytotoxicity (11), the adenosine A₂ receptor agonists (NECA and PAA) that stimulate adenylyl cyclase were more

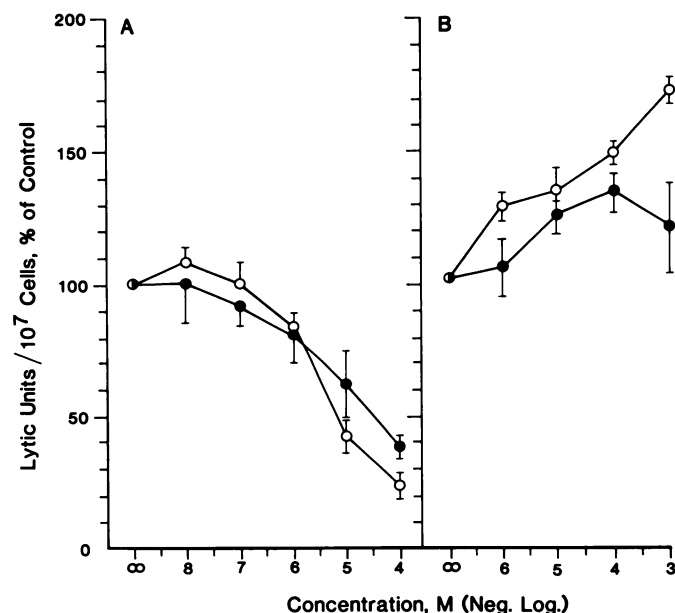


Fig. 1. Comparison of Tub and F-ara-AMP effects on NK cell activity in mouse and human lymphocytes. NK cell activity was determined in the presence of the indicated concentrations of tub (A) or F-ara-AMP (B) as described in "Materials and Methods." ●, human peripheral blood mononuclear cells; ○, mouse splenocytes. Points, mean values; bars, SE for at least 6 separate determinations.

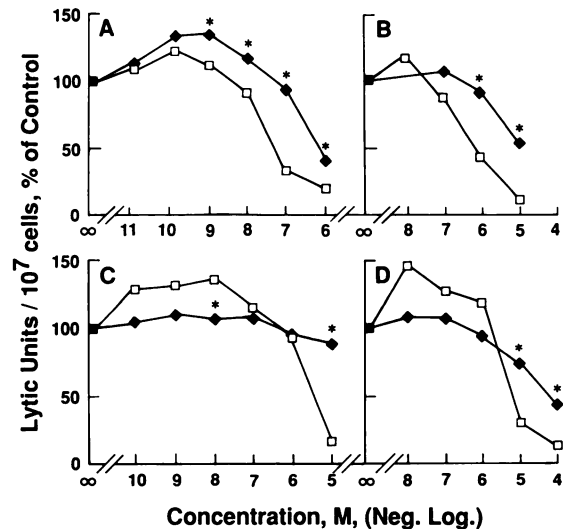


Fig. 2. Inhibition and stimulation of murine NK cell activity by adenosine receptor agonists. The agonists were added to the cultures at the beginning of the 4-h lytic assay. A, NECA; B, PAA; C, PIA; D, CPA. Asterisks, values significantly different from that obtained in the absence of XAC ($P \leq 0.05$, $n \geq 3$). □, adenosine receptor agonist alone; ◆, plus XAC, 10^{-6} M.

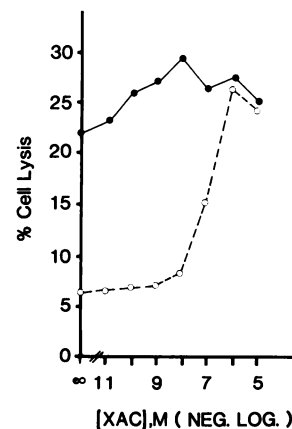


Fig. 3. Dose-response curve for XAC reversal of NECA-induced inhibition of NK cell activity in mouse splenocytes. Lymphocytes were incubated at an effector:target ratio of 100:1 as described in "Materials and Methods." ●, XAC alone; ○, NECA, 10^{-7} M.

potent inhibitors of NK cell activity than were the more selective adenosine A₁ receptor agonists (PIA and CPA), agents that are inhibitory to the enzyme. The adenosine A₁ receptor agonists were also more effective stimulators of the NK cell activity, and stimulation was observed at extremely low concentrations [*i.e.*, 10^{-10} to 10^{-8} M (Fig. 2, C and D)]. The possibility that these effects are mediated via adenosine receptors is supported by the known selectivities of the agonists used and that their effects are antagonized by the adenosine receptor antagonist XAC. The concentration of XAC selected [10^{-6} M] was the minimum required to prevent the inhibition due to NECA [10^{-7} M (Fig. 3)]. Although not indicated by these data, XAC alone at this concentration significantly stimulated NK cell activity $152 \pm 10\%$ of control, mean value \pm SE for 14 separate experiments). Therefore, in the experiments using XAC, the results given are compared to the control response using XAC alone. Thus, in terms of the possible antagonism of a stimulatory response it seems more appropriate to state that an agent either adds or does not add to the stimulation produced by XAC alone. Specifically, NECA (10^{-9} M) clearly does add to the XAC effect whereas PIA and CPA (10^{-10} to 10^{-8} M) do not (Fig. 2, A, C, and D). In the case of inhibitory responses due to the adenosine

receptor agonists, XAC clearly produces a partial antagonism in each case.

The above results using adenosine receptor agonists and the adenosine receptor antagonist XAC suggest that these receptors regulate NK cytotoxicity in mouse spleen lymphocytes, *in vitro*. To determine whether adenosine, dAdo, Tub or F-ara-AMP may affect the cytotoxicity through similar mechanisms, analogous experiments were performed using these nucleosides (Fig. 4). Adenosine was the most potent inhibitor of NK cell activity, followed by Tub and dAdo. XAC at a concentration of 10^{-6} M partially antagonized the inhibition of NK cytotoxicity by adenosine and dAdo (Fig. 4, A and B); however, the nucleoside transport inhibitor NBTIP (10^{-5} M) did not alter the inhibition by either adenosine or dAdo. This concentration of NBTIP was without effect on NK cell activity when used alone, but was highly effective in preventing nucleotide formation from [3 H]Tub (Table 1). Although the adenosine receptor antagonist XAC failed to influence inhibition by Tub of NK cell activity, the nucleoside transport inhibitor did partially prevent this inhibition (Fig. 4C). dAdo (10^{-8} – 10^{-6} M) and F-ara-AMP were more effective stimulators of NK cell activity (Fig. 4, B and D). Neither nucleoside augmented the stimulation observed in the

presence of XAC. In contrast to an apparent enhancement of this property of dAdo, the nucleoside transport inhibitor prevented the stimulation produced by F-ara-AMP. Taken together, these data suggest that the stimulation and inhibition of NK cell activity produced by adenosine and dAdo might be mediated via adenosine receptors, independent of intracellular accumulation of adenosine or dAdo. On the other hand, prevention of the inhibition (Tub) or stimulation (F-ara-AMP) by NBTIP suggests that these agents must enter the cells to produce these actions. The ability of NBTIP to inhibit the cellular uptake of Tub by mouse spleen lymphocytes was confirmed by measuring Tub nucleotide formation in exposed cells (Table 1). A concentration of Tub that clearly inhibited NK cell activity [10^{-5} M (Fig. 4)] decreased the intracellular ATP pool after a 4-h incubation [Table 1]. This effect on ATP and the formation of the major intracellular metabolite of Tub, Tub triphosphate, were partially prevented by NBTIP (10^{-5} M). On the other hand, XAC (10^{-6} M) did not influence the cellular effects and metabolism of Tub. The stimulatory response of NK cells to F-ara-AMP was also observed with its dephosphorylated nucleoside form, F-ara-A (data not shown). Neither XAC nor NBTIP interfered with the conversion of F-ara-AMP to F-ara-A in the medium incubated with mouse spleen lymphocytes (Table 2). These compounds also did not prevent the formation of intracellular F-ara-ATP from either F-ara-A or F-ara-AMP. In contrast to Tub, F-ara-AMP or F-ara-A were without effect on intracellular ATP pools.

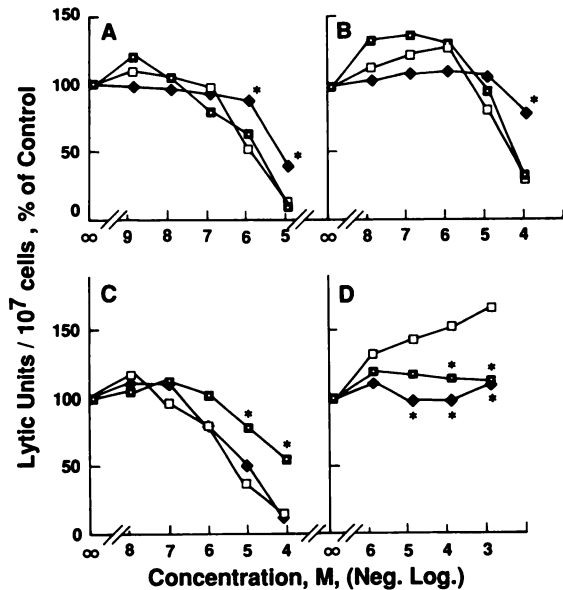


Fig. 4. Inhibition or stimulation of NK cell activity in mouse spleen lymphocytes by murine nucleosides. Drugs were added to the suspension of spleen lymphocytes during the 4-h lytic assay (see "Materials and Methods"). Asterisks, effect in the presence of XAC or NBTIP significantly less than when the nucleoside was present alone ($P < 0.05$, $n > 3$). A, adenosine; B, dAdo; C, Tub; D, F-ara-AMP; □, nucleoside alone; ◆, plus XAC, 10^{-6} M; ◇, plus NBTIP, 10^{-5} M. The adenosine deaminase inhibitor, deoxycoformycin (10^{-6} M), was present during incubation of the cells with adenosine or dAdo.

Table 1 Reversal by NBTIP of Tub metabolism and depletion of intracellular ATP in mouse splenocytes

Treatment ^a	nmol/ 10^9 cells	
	ATP	Tub triphosphate ^b
Control	292 ± 58 ^c	
Tub		
10^{-5} M	82 ± 15	225 ± 12
+XAC, 10^{-6} M	81 ± 29	208 ± 29
+NBTIP, 10^{-5} M	210 ± 37	66 ± 5

^a Five × 10^6 spleen lymphocytes/ml were incubated for 4 h as described in "Materials and Methods." The cells were collected by centrifugation, extracted with perchloric acid, neutralized, and analyzed for nucleotide pools by high performance liquid chromatography.

^b Determined by absorbance at 280 nm. ATP was measured at 254 nm. The minimal detectable amount for the experimental method used was 15 nmol/ 10^9 cells.

^c Average value ± range for two separate experiments.

DISCUSSION

We have previously reported that Tub inhibits and F-ara-AMP stimulates NK cell cytotoxicity in the spleen lymphocytes from mice treated with these agents (4). These effects are also observed by treating mouse splenocytes with these agents *in vitro* (Fig. 1), suggesting a direct action on the NK cells. Further, these phenomena are also observed in human NK cells. Although not shown, preliminary data from experiments using cloned human and murine NK cell lines are also consistent with a direct effect of these analogues on NK cells. NK cell activity is not dependent upon cellular proliferation; *i.e.*, the lytic process is inherent and spontaneous. Thus, the mechanisms by which Tub and F-ara-AMP affect this function may differ from that by which they are cytotoxic or inhibit cell proliferation. One mechanism by which adenosine-related compounds might regulate NK cell activity involves adenosine receptors. Although evidence suggests the presence of an adenosine A2 receptor on murine and human T- and B-lymphocytes, T-helper and T-cytotoxic cells (12), basophils (13), mast cells (14), and neutro-

Table 2 F-ara-AMP metabolism by spleen lymphocytes

Treatment ^a	nmol/ 10^9 cells			F-ara-A ^c nmol/ml medium
	ATP ^b	GTP ^b	F-ara-ATP ^b	
Control	292 ± 58	44 ± 11		
F-ara-AMP				
10^{-4} M	258 ± 34	36 ± 2	10 ± 5	17 ± 2
+XAC, 10^{-6} M	186 ± 21	36 ± 2	5 ± 2	18 ± 3
+NBTIP, 10^{-5} M	252 ± 39	35 ± 2	16 ± 3	14 ± 3
F-ara-A				
10^{-4} M	184 ± 4	34 ± 1	26 ± 10	80 ± 9
+XAC, 10^{-6} M	229 ± 25	39 ± 3	32 ± 5	96 ± 10
+NBTIP, 10^{-5} M	242 ± 15	38 ± 2	46 ± 3	86 ± 1

^a Spleen cells were incubated in the presence of the agents shown as described in Table 1. Mean ± SE, $n = 3$.

^b Determined by high performance liquid chromatography of cell extracts using a UV absorbance detector at 254 nm as described in "Materials and Methods."

^c Determined in the media by reverse-phase high performance liquid chromatography as described in "Materials and Methods."

phils (15), the presence of this or other adenosine receptors on NK cells has not been reported. It has been reported, however, that various chemical manipulations that mimic cyclic AMP (*i.e.*, dibutyryl cyclic AMP) or lead to its accumulation (prostaglandin E_1 , cholera toxin, isoproterenol) are inhibitory to NK cell function (16). Thus, adenosine receptor A2 agonists (stimulatory for adenylyl cyclase) would be anticipated to inhibit NK cell function whereas adenosine receptor A1 agonists (inhibitory for cyclase) might stimulate this immune response. To test this possibility, we investigated the effects of selective adenosine receptor agonists on NK cytotoxicity. PIA and CPA represent selective Ado A1 receptor agonists, whereas NECA and PAA have high affinity for the A2 receptor as well (17). The order of potency for inhibition of mouse NK cell activity was NECA and PAA > PIA and CPA (Fig. 2), consistent with inhibition being due to agonist activity at an adenosine A2 receptor. In each case, further evidence that this inhibition of NK cell activity was mediated via adenosine receptors was obtained by the partial reversal seen with the adenosine receptor antagonist, XAC. The involvement of an adenosine A1 receptor in the regulation of NK cell activity is suggested by the more effective stimulation of NK cell activity seen with low concentrations of PIA and CPA (Fig. 2). Although direct evidence exists for the presence of adenosine A2 receptors on human and mouse lymphocytes (12, 14), the type(s) of receptor(s) on NK cells *per se* have not been determined. Marone *et al.* (18) found that L-PIA inhibited cyclic AMP production by 10–25% in peripheral blood mononuclear cells, and these investigators suggested that the A1 receptor might be expressed only on a subpopulation of lymphocytes.

The functional experiments reported herein demonstrate that the adenosine receptor agonists modify NK cell activity in the manner anticipated from known effects of cyclic AMP on this function. Adenosine receptors could, therefore, account for the actions of Tub and F-ara-AMP. The data obtained, however, clearly indicate that the inhibition (Tub) or stimulation (F-ara-AMP) of NK cell activity *in vitro* does not occur via A2 or A1 receptors. Specifically, the inhibition by Tub was not prevented by XAC, although the transport inhibitor NBTIP did partially reverse this effect of Tub. This result suggests that Tub must enter the cell in order to inhibit NK cell activity. Possible mechanisms for such an intracellular effect of Tub include depletion of nucleotide stores (Table 1) or formation of a Tub analogue equivalent of cyclic AMP (19). Both NK cytotoxic factor production and secretion are energy-dependent processes (20). Thus, depletion of ATP by Tub is considered to be a plausible mechanism for the inhibition of NK cytotoxicity *in vitro* by this adenosine analogue. In agreement with a mechanism not involving adenosine receptors, Tub (7-deazaadenosine) was reported not to activate either the extracellular (R site) or intracellular (P site) regulatory receptors of adenylyl cyclase (17).

The stimulation of NK cell activity by F-ara-AMP was also prevented by NBTIP, suggestive of an intracellular site for its action as well. Since F-ara-A and its nucleotides represent "ribose-modified" analogues, it seems plausible that this stimulation is mediated via the intracellular "P site" for adenylyl cyclase (inhibitory to the enzyme), although other possibilities cannot be ruled out at this time. Inhibition of NK cell activity observed with adenosine and dAdo was blocked by XAC, but not by NBTIP, consistent with a possible interaction of these nucleosides with an extracellular adenosine type A2 receptor.

In summary, results from functional studies of NK cell activity in mouse spleen lymphocytes *in vitro* suggest that extracellular adenosine receptors and possibly an intracellular P site modulate this immune function. Further, the results indicate that inhibition due to adenosine and dAdo might be mediated via an adenosine type A2 receptor. Although the results obtained using Tub (inhibition of NK cell activity) suggest a mechanism not involving adenosine receptors, that obtained for F-ara-AMP are consistent with interaction at an intracellular "P" site. The possible presence of these receptors and their normal coupling with adenylyl cyclase in NK cells is currently being tested using cell membrane preparations from cloned human and murine NK cells for which the cell identity is certain.

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