Journal of Cellular Biochemistry 80:241–247 (2000)

Role of the Phosphorolysis of Deoxyadenosine in the Cytotoxic Effect of the Combination of Deoxyadenosine and Deoxycoformycin on a Human Colon Carcinoma Cell Line (LoVo)

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Abstract In LoVo cells, phosphorolytic activity acting on deoxyadenosine plays a major role in the resistance to the cytotoxic effect of the combination of deoxynucleoside with deoxycoformycin. In fact, the observed dependence of toxicity on cell density appears to be related to the metabolic conversion of deoxyadenosine into adenine. The phosphorylation of the deoxynucleoside, which represents the first step towards the formation of the cytotoxic agent dATP, proceeds at a significantly lower rate as compared to the phosphorolysis of deoxyadenosine. The analysis of the levels of deoxyadenosine and its derivatives in the incubation media reveals that the rates of disappearance of deoxyadenosine and of formation of adenine increase in concert with the reduction of the effect on cell survival. J. Cell. Biochem. 80:241–247, 2000. © 2000 Wiley-Liss, Inc.

Key words: deoxyadenosine; deoxycoformycin; LoVo; purine enzymes; adenosine phosphorylase

The combination of deoxyadenosine (dAdo) and deoxycoformycin (dCF), a powerful inhibitor of adenosine deaminase (ADA) [Agarwal et al., 1977], has proven to affect the growth of a human colon carcinoma cell line (LoVo) [Camici et al., 1995]. The cytotoxicity seems to be dependent on phosphorylation of dAdo, because inhibitors of adenosine kinase activity significantly reduced the effect of the combination [Camici et al., 1995]. In general, when dealing with compounds that exert their effect after metabolic conversion into the effective cytotoxic agent (pro-drugs), the complete metabolic pattern of the pro-drug must be taken into account. Indeed, not only the "activation", but also the "inactivation" pathways play a crucial role in the determination of the efficacy of the cytotoxic agent. In particular, the efficacy of the combination of dAdo and dCF is likely due to the balance between the "activating" enzymes (adenosine kinase, deoxycytidine kinase) and "inactivating" enzymes (Fig. 1).

In the latter class, besides the nucleotidase and phosphatase activities, which subtract the nucleoside monophosphate from the subsequent phosphorylation steps that lead to the formation of the cytotoxic agent dATP, in LoVo cells we have recently isolated a phosphorolytic enzyme activity acting on dAdo [Bemi et al., 1998] distinct from methylthioadenosine phosphorylase, purine nucleoside phosphorylase, and S-adenosyl homocysteine hydrolase. In mammalian tissues, the existence of this activity appears to be a novelty because its presence has been reported in several biologic sources [Jensen, 1978; Trembacz and Jezewska, 1994], however, with the only exception of an adenosine phosphorylase activity described in Sarcoma 180 cells and rat liver [Divekar, 1976], not in mammals. Although the phosphorolysis of dAdo appears to be a secondary metabolic pathway in LoVo cells when the deamination

Grant sponsors: CNR (Target Project Biotecnologie), MURST.

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Received 27 July 1999; Accepted 5 May 2000

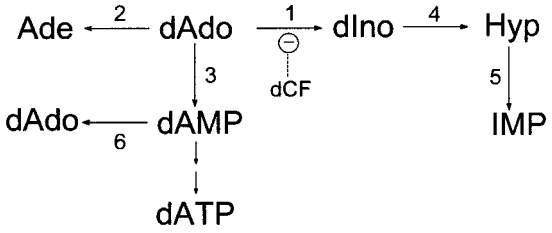

Fig. 1. Schematic diagram of the metabolism of deoxyadenosine (dAdo) in LoVo cells. **1**: Adenosine deaminase; **2**: adenosine phosphorylase; **3**: adenosine kinase and/or deoxycytidine kinase; **4**: purine nucleoside phosphorylase; **5**: hypoxanthine guanine phosphoribosyltransferase (HGPRT); **6**: 5′nucleotidase and/or alkaline phosphatase. - : Inhibition. Ade, adenine; dIno, deoxyinosine; dAMP, deoxyadenosine monophosphate; dATP, deoxyadenosine triphosphate; dCF, deoxycoformycin; Hyp, hypoxanthine; IMP, inosine monophosphate.

process is operating, it becomes relevant in the presence of dCF. We have shown [Bemi et al., 1998] that the effect of the combination of dAdo and dCF on LoVo cell growth is strictly dependent on the initial cell density. We show here that the adenosine phosphorylase activity, which subtracts dAdo from the "activation" pathway, plays a major role in this dependence of cytotoxicity on cell density.

MATERIALS AND METHODS

Materials

Ham's F-12 medium, fetal calf serum (FCS), and anti-PPLO (pleuropneumonia-like organism) agent were from Gibco (Berlin, Germany). Mycoplasma-removing agent was provided by ICN, Costa Mesa, CA. The MycoCell kit was from International Microbio (Signes, France). The human colon carcinoma cell line LoVo and dCF were provided by Dr. O. Sanfilippo (Milan, Italy). Bisbenzamide, [8-^{14}C]adenine (55.6 mCi/mmol), and all purine compounds were from Sigma (St. Louis, MO). Polyethylene imine (PEI)-cellulose thin-layer plastic sheets (0.1 mm thick) from Merck (Darmstadt, Germany) were prewashed once with 10% NaCl and three times with deionized water before use. Scintillation liquid HiSafe III was purchased from LKB Pharmacia, Uppsala, Sweden. All the other chemicals were of analytical grade.

Cell Cultures and Preparation of Cell Extracts

Cells were grown as a monolayer in Ham's F-12 medium with 7% FCS and antibiotics (standard medium). The doubling time was approximately 32 h. When cells were in the logarithmic phase of growth, the monolayers were washed twice with phosphate-buffered saline (PBS) and the cells were scraped off with a cell scraper, collected, and centrifuged at 800*g* for 3 min. The pellets were further washed with PBS and stored at −75°C. For the preparation of the cell extracts used for the enzyme assays, the pellets were resuspended in approximately 3 vol of 50 mM Tris-HCl buffer, pH 7.4, subjected to ultrasonic treatment, and centrifuged at 45,000*g* for 1 h at 4°C. The supernatant (cell extract) was kept at −75°C.

Cytotoxicity Tests

The incubations were performed essentially as described by Bemi et al. [1998]. Various amounts of cells were suspended in 3 ml of standard medium in 35-mm dishes, to give cellular densities ranging from 3,000 to 50,000 cells/cm^2, and incubated in the absence (control) and in the presence of both 0.1 or 0.2 mM dAdo, with or without 1 μM dCF. Deoxycoformycin, when present, was added to the standard medium 30 min before dAdo. Identical experiments were performed in which adenine (Ade), at a final concentration of 0.1 mM, was added to the culture medium. Additional experiments were carried out in which the same amount of cells was plated in dishes of 60-mm diameter to give different surface densities, but equal number of cells per volume of medium. After 4 days of incubation, the standard medium was withdrawn, and 0.5 ml of PBS were added twice to wash the cell monolayer. A 0.5-ml portion of 0.025% trypsin containing 0.02% ethylenediaminetetraacetate (EDTA) was then added and kept for few minutes at 37°C. The cells were collected, diluted in an appropriate volume of standard medium, and counted in a Burker chamber. The results are reported as a percentage of cell survival in relation to the appropriate control in which both dCF and dAdo were absent. Preliminary experiments showed that ADA activity present in the standard medium and activity present in the cells at all cell densities was completely inhibited by 1 μM dCF [Bemi et al., 1998].

High Performance Liquid Chromatography (HPLC) of the Incubation Media

The medium (100 μl) from the incubations performed as described for the cytotoxicity

tests in 35-mm dishes with dAdo both in the absence and in the presence of dCF was withdrawn at 0, 2, 5, 10, 24, 48 and 96 h of incubation. A control was also set up, in which dAdo either alone or with dCF was incubated for the above-reported times with standard medium with no cells. Cold methanol (150 μl) was added, kept at −20°C overnight, centrifuged, and the supernatant dried with a Speed-Vac evaporator. MilliQ water (100 μl) was then added, and the samples were analyzed by a Beckman System Gold apparatus, consisting of two HPLC pumps, a mixing chamber, an injector valve, and a UV/VIS detector. An Ultrosphere C-18 (Beckman) column (4.6 × 25 mm, 5 μm particle size) was used. The elution was performed according to Stocchi et al. [1985]. The flow rate was 1.3 ml/min, and ultraviolet detection was performed at 254 nm.

Contamination by Mycoplasma

Both an in situ DNA fluorescence method [Chen, 1977] and a method based on molecular hybridization [Hu et al., 1995] were used. The cell cultures were routinely treated with two different mycoplasma-removal agents, containing 4-oxo-quinoline-3-carboxylic acid derivative and Tylocine, respectively.

Enzyme Assays

The enzyme assays were performed using the cell extracts prepared as described above. The phosphorolytic activity on dAdo was measured as previously reported [Bemi et al., 1998]. Briefly, the reaction mixture contained 0.5 mM [8-^{14}C]adenine (12,000 dpm/nmol), 0.5 mM deoxyribose-1-phosphate (dRib-1-P; or ribose-1-phosphate, Rib-1-P), 5 μM dCF, 50 mM Tris-HCl, pH 7.4, and various amounts of cell extract. Aliquots withdrawn at 0, 10, 20, and 30 min incubation at 37°C were spotted on a PEI-cellulose thin-layer plastic sheet, developed with 0.1 M $(NH_4)_2SO_4$. The spots corresponding to dAdo (or Ado) were cut out and the radioactivity counted. One unit of enzyme is the amount of enzyme that catalyzes the conversion of 1 μmol of substrate per min under the adopted experimental conditions.

Other Methods

Protein content was determined according to Bradford [1979] using bovine serum albumin as standard. The statistical analysis was performed by using the *t* test for independent samples.

RESULTS

Enzyme Activities Involved in the Metabolism of dAdo in LoVo Cells

LoVo cells possess the enzyme activities that account for the conversion of dAdo into deoxyinosine (ADA), hypoxanthine (purine nucleoside phosphorylase, PNP), and (inosine monophosphate, IMP, hypoxanthine guanine phosphoribosyltransferase, HGPRT). In addition, adenosine kinase and deoxycytidine kinase are responsible for its phosphorylation, whereas 5′-nucleotidase and phosphatase activities catalyze the dephosphorylation of the mononucleotide [Camici et al., 1995; Bemi et al., 1999] (Fig. 1). The phosphorolytic cleavage of dAdo, catalyzed by phosphorylase activity, distinct from methylthioadenosine phosphorylase, PNP, and S-adenosyl homocysteine hydrolase, is also an alternative metabolic pathway of dAdo in LoVo cells [Bemi et al., 1998] (Fig. 1). Its specific activity was 11.4 ± 4 mU/mg.

Contamination by Mycoplasma

The possible contamination by mycoplasma, which is known to possess an adenosine phosphorylase activity [Hatanaka et al., 1975], has been tested accurately. Two different methods for the detection of contamination by mycoplasmas were tested on cells routinely treated with specific agents known to eliminate mycoplasma contamination. The in situ DNA fluorescence method, based on the use of bisbenzimide (Hoechst 33258), which binds to A-T regions of DNA, gave negative results. The method based on molecular hybridization using a cold biotinylated probe, which recognizes a region of DNA displaying a high degree of homology in all mycoplasmas, but does not interact with eukaryotic cells, gave negative results.

Dependence of dAdo and dCF Cytotoxicity on LoVo Cell Density

The results show that dAdo alone, at all cell densities, did not exert a significant effect on cell survival; however, the presence of dCF in the culture medium affected significantly the growth rate, depending on the adopted experimental conditions (Fig. 2). In general, an increase in the cell density led to a reduction of the effect of the combination on cell growth. The presence of Ade

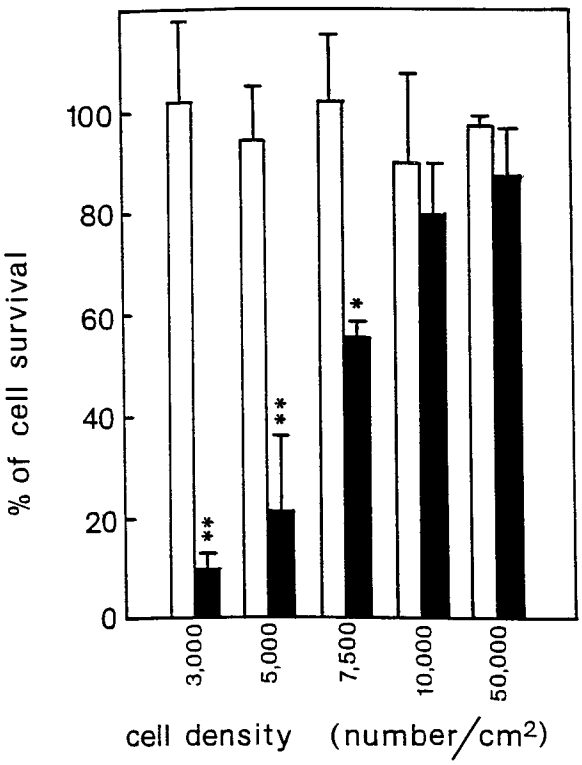

Fig. 2. Dependence of the toxicity of the combination of deoxyadenosine (dAdo) and deoxycoformycin (dCF) on cell density. Dishes of 35 mm diameter were used, and the incubations were performed with 0.1 mM dAdo either alone or in combination with 1 μM dCF. White columns: dAdo alone. Black columns: dAdo plus dCF. Results are reported as mean percentage of cell survival in relation to the appropriate control in which neither dAdo nor dCF were present. Error bars indicate ± SD. For the control incubations, 100% corresponds, after 4 days, to a total number of cells of ≈200,000 (initial density 3,000 cells/cm^2), 400,000 (5,000 cells/cm^2), 550,000 (7,500 cells/cm^2), 750,000 (10,000 cells/cm^2), and 1,400,000 (50,000 cells/cm^2). $^{**}P < 0.001$ $^{*}P < 0.01$.

in the culture medium did not affect the cytotoxicity tests. In fact, the differences between the tests performed in the absence and in the presence of Ade were not statistically significant ($P > 0.2$ for all initial cell densities). In particular, the changes in the mean percentage cell survival in the presence of Ade were 1% (initial density 3,000 cells/cm^2), 8% (5,000 cells/cm^2), and 5% (7,500 cells/cm^2). Also, the same degree of cytotoxicity was observed by plating the same number of cells per volume of medium in different diameter dishes (results not shown).

External Purine Metabolite Levels in the Incubation of LoVo Cells with dAdo

The levels of purine metabolites present in the media incubated with dAdo, withdrawn at different times, were evaluated by an HPLC technique (Fig. 3). In the absence of dCF, dAdo concentration decreases with time, and the first compound to appear in the medium is deoxyinosine (dIno), followed by hypoxanthine (Hyp). No Ade was measurable in all the assayed incubation media. The analysis of the media in a control incubation (in which dAdo was incubated in the culture medium without cells) indicates that Hyp is contained in the standard medium (approximately 0.027 mM). It also indicates that in the absence of cells, the ADA activity present in the serum is responsible for the deamination, though at a slower rate, of dAdo added to the culture medium (Fig. 3A).

External Purine Metabolite Levels in the Incubation of LoVo cells with dAdo and dCF in Combination

In the presence of dCF, the decrease in dAdo concentration in the medium is paralleled by the increase in Ade concentration (Fig. 4). Again, Hyp is present in the culture medium and its level does not change significantly with time. Overall, the rates of dAdo disappearance and of Ade formation increase with the increase in cell density.

Relationship Between Cytotoxicity and Temporal Changes in dAdo Concentration

The area under the curve (AUC), obtained by joining the points indicating the concentrations of dAdo in the medium at different times of incubation, is proportional to the length of time dAdo remains in the culture medium. We have combined the data of Figures 2 and 4, and those obtained in additional incubations performed at various cell densities with 0.2 mM dAdo (results not shown). A plot of cytotoxicity against the AUC for the concentration of dAdo can be obtained (Fig. 5).

DISCUSSION

In this article we confirm and extend our previous observations indicating that the toxic effect of the combination of dAdo and dCF on LoVo cells was dependent on the initial cell density [Bemi et al., 1998]. Indeed, a gradual increase in the cell survival is observed, as compared to the control, paralleling the increase in cell density (Fig. 2).

The analysis of the purine metabolites in the incubation media gives an indication of the

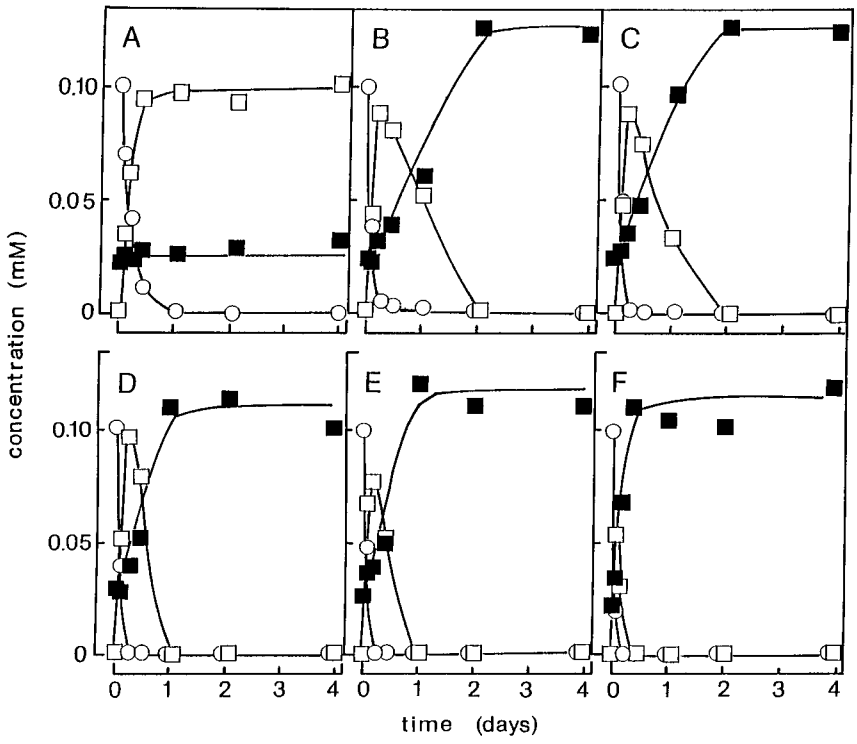

Fig. 3. Time course of the levels of various purine metabolites present in the medium of incubation of LoVo cells with deoxyadenosine (dAdo). Dishes of 35 mm diameter were used, and the incubations were performed with 0.1 mM dAdo alone. Each point is the average of at least two independent determinations. Deoxyadenosine (○); hypoxanthine (■); deoxyinosine (□). Panel **A**: no cells; **B**: 3,000 cells/cm^2, **C**: 5,000 cells/cm^2, **D**: 7,500 cells/cm^2, **E**: 10,000 cells/cm^2, F: 50,000 cells/cm^2.

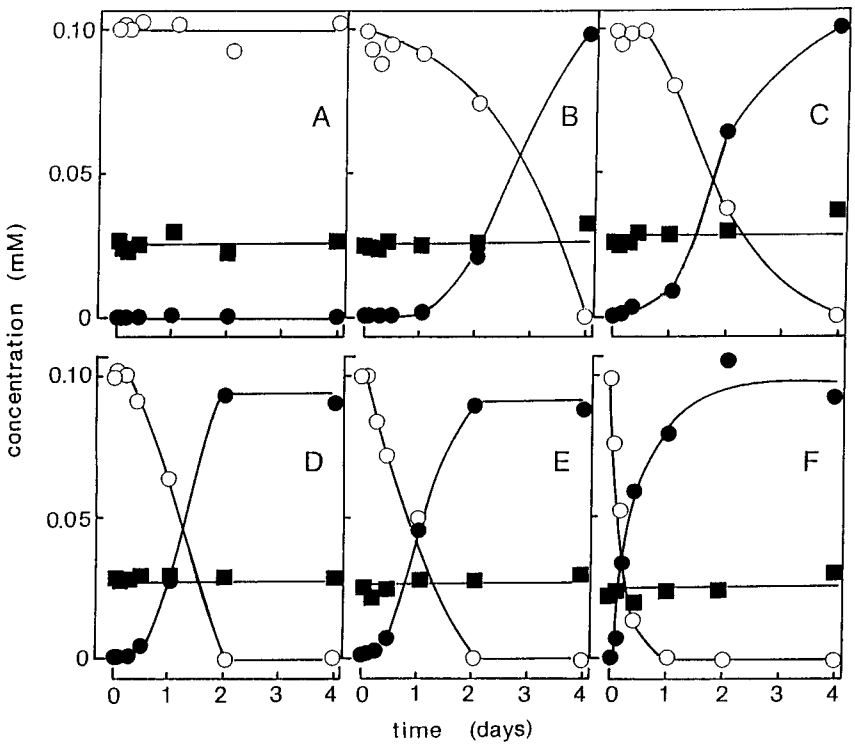

Fig. 4. Time course of the levels of various purine metabolites present in the medium of incubation of LoVo cells with deoxyadenosine (dAdo) plus deoxycoformycin (dCF). Dishes of 35 mm diameter were used, and the incubations were performed with 0.1 mM dAdo and 1 μM dCF. Each point is the average of at least two independent determinations. Deoxyadenosine (○); adenine (●); hypoxanthine (■). Panel **A**: no cells; **B**: 3,000 cells/cm^2, **C**: 5,000 cells/cm^2, **D**: 7,500 cells/cm^2, **E**: 10,000 cells/cm^2, **F**: 50,000 cells/cm^2.

metabolic pathways undertaken by dAdo, both in the absence and in the presence of dCF. Indeed, with the exception of the phosphorylated derivatives, which are formed and trapped inside the cell, the other dAdo derivatives can cross the cell membrane by concentrative or equilibrative transport mechanisms [Griffith and Jarvis, 1996]. The results indicate that an almost complete recovery of the purine ring in the incubation medium is obtained,

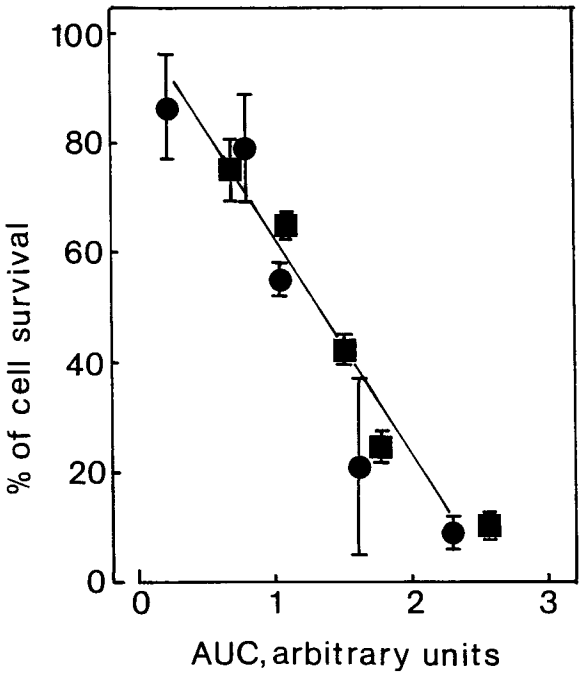

Fig. 5. Cytotoxicity of the combination of deoxyadenosine (dAdo) and deoxycoformycin (dCF) as a function of the area under the curve (AUC) for dAdo. Circles: values obtained from incubations with 0.1 mM dAdo; squares: values obtained from incubations with 0.2 mM dAdo. Error bars indicate ± SD.

thus suggesting that an equilibrative mechanism is likely involved in the passage of the purine compounds across the cell membrane, and that an equilibrium is reached between the intracellular and extracellular concentration of the compound. Indeed, the very small volume of the cellular component as compared to the volume of the medium can account for the small amount of the purine ring taken up by the cells as compared to that remaining in the medium. Analysis performed using [8-^{14}C]deoxyadenosine, in fact, demonstrated that approximately only 1% of the total radioactivity was incorporated [Bemi et al., 1998]. However, as shown in the present study, at the end of the incubations the deoxynucleoside was completely metabolized. In the absence of dCF, the results indicate that ADA readily converts dAdo into dIno (Fig. 3), which, in turn, is phosphorolytically cleaved to Hyp and dRib-1-P by PNP. The transient formation of dIno indicates that the purine compound is an intermediate that is formed from dAdo and is subsequently converted to Hyp. Because preliminary experiments demonstrated that the presence of FCS in the standard medium was unavoidable to allow the cells to grow, owing to the ADA activity present in the medium (0.4 U/ml), when dCF is absent, dIno is formed even in the absence of cells, though at a slower rate (Fig. 3A). However, the appearance of Hyp in the medium can be certainly ascribed to PNP present in LoVo cells. When dCF is present in the medium (Fig. 4), the disappearance of dAdo is paralleled by the formation in the medium of Ade, thus indicating that the deoxynucleoside undergoes a phosphorolytic cleavage.

In a previous article [Bemi et al., 1998], we demonstrated that the phosphorolytic cleavage of dAdo in LoVo cells is exclusively due to a specific adenosine phosphorylase activity, which, by ion-exchange chromatography, can be separated from methylthioadenosine phosphorylase, purine nucleoside phosphorylase, and S-adenosyl homocysteine hydrolase. The assay of adenosine phosphorylase has been reported as a test to detect a contamination of cultured cells by mycoplasma [Bonissol et al., 1988]. In the LoVo cell line, however, the multiple specific assays used to detect and remove mycoplasmas revealed that, overall, a contamination can be excluded. The formation of phosphorylated derivatives of dAdo cannot be monitored by measuring their levels in the medium because they are not freely accessible to the membrane lipid double layer. Indeed, the cytotoxic effect of dAdo appears to be dependent upon its phosphorylation [Camici et al., 1995]. Our data indicate that the rates of disappearance of dAdo and of formation of Ade increase in concert with the reduction of the effect on cell survival. The mechanism of the density-dependent cytotoxicity is not related to a protective effect of Ade or to a density-dependent cell signaling, since both the addition of Ade to the culture medium and the plating of the same number of cells per volume at different surface densities did not significantly affect the degree of cytotoxicity. On the other hand, the degree of cytotoxicity was nearly directly proportional to the AUC for the concentration of dAdo (Fig. 5), independently of the initial concentrations of dAdo. This indicates that the toxicity is related to the length of time dAdo remains in the medium. In fact, at the cell densities in which we observe a cytotoxic effect of the combination of dAdo and dCF, dAdo is slowly consumed and remains in the incubation medium at high concentration for a rather

long period of time (i.e., high AUC values). This means that each cell is continuously provided with dAdo, which is presumably both phosphorolytically cleaved and phosphorylated. Because each cell is supplied with dAdo for a long period of time, it is likely that the phosphorylated cytotoxic-derived compound dATP, accumulates and reaches an intracellular concentration that is incompatible with the cell growth. Our results also indicate that, even though both phosphorylation and phosphorolysis of dAdo can occur, the phosphorolysis appears to be the major metabolic pathway of the deoxynucleoside, when the deamination process is inhibited.

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