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Adenosine and Deoxyadenosine Toxicity in Colony Assay Systems for Human T-Lymphocytes, B-Lymphocytes, and Granulocytes

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Summary. *Adenosine and deoxyadenosine toxicity was examined in colony assay systems for human T lymphocytes, B lymphocytes, and granulocytes. In the absence of deoxycoformycin, an adenosine deaminase inhibitor, no growth inhibition was observed in the three systems with concentrations of adenosine or deoxyadenosine of at least 200 μM. Deoxycoformycin itself had no growth-inhibitory effect at concentrations of at least 10 μg/ml. Combinations of deoxycoformycin (1 μg/ml) and either adenosine or deoxyadenosine gave growth inhibition in all three systems. Deoxyadenosine was the most toxic in all the systems, the LD_{50} values being 20–25 μM. The LD_{50} values for adenosine were 45–55 μM. There was no evidence of selective toxicity by adenosine or deoxyadenosine with these three colony assay systems. In the T-lymphocyte colony system deoxyadenosine appeared to be toxic to both the inducer/helper and the suppressor/cytotoxic T-lymphocyte subpopulations.*

Introduction

The initial clinical interest in adenosine deaminase inhibitors was in their potential use to increase the therapeutic effectiveness of various adenosine analogs in cancer treatment. The observations that lymphoid tissue has high levels of adenosine deaminase and that children with a genetic deficiency of adenosine deaminase often exhibit a syndrome called severe combined immunodeficiency disease led to the idea that adenosine deaminase inhibitors alone might exhibit antitumor activity in malignancies of lymphoid origin. An initial phase I study of the adenosine deaminase inhibitor, deoxycoformycin, revealed partial remissions in three of seven acute lymphoblastic leukemia patients [18]. Additional clinical studies with deoxycoformycin are in progress in various centers.

Inhibition of adenosine deaminase is not likely to be the direct cause of the observed toxicity, as in cultured cell systems adenosine deaminase inhibitors, such as deoxycoformycin, are usually not toxic at the concentrations required for essentially complete deaminase inhibition [1, 4]. Deoxycoformycin produces toxicity by potentiating the toxicity of adenosine and/or deoxyadenosine, which are normally rendered nontoxic through deamination. The mechanisms of toxicity of adenosine and deoxyadenosine are still under study and various alternative hypotheses have been presented [3, 5].

This study was designed to examine the relative toxicities of adenosine and deoxyadenosine in colony assays of human T-lymphocytes, B-lymphocytes, and granulocytes, and also to determine whether these colony assay systems demonstrated the apparent lymphoid specificity that has been observed in the animal and human studies [17, 18].

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Methods

T-lymphocyte colonies were grown from the mononuclear cell fraction of human peripheral blood, which was isolated by centrifugation through Ficoll-Hypaque. The mononuclear cell fraction was washed and resuspended in Dulbecco's minimum essential medium (MEM) containing 10% fetal calf serum (Gibco). Aliquots (0.1 ml) of a plating mixture containing cells, Dulbecco's MEM, 10% fetal calf serum, 0.8% methyl cellulose (4,000 centipoises), and 10% conditioned medium were plated in 96-well flat-bottomed Linbro microtitration plates [10]. The conditioned medium was prepared by incubating isolated T-lymphocytes at 1 million cells per ml in Dulbecco's MEM containing 10% fetal calf serum and 1% phytohemagglutinin (Wellcome HA15) for 72 h at 37° C [10]. The cells were removed by centrifugation and the conditioned medium stored at 4° C. The T-lymphocyte colonies that contained in excess of 20 cells were counted after a 4–5-day incubation in a humidified 37° C incubator with a 7.5% CO_2 atmosphere.

The overall lymphocyte-plating efficiency was in the range of 1%–1.5%. There was no observable difference in plating efficiency or growth pattern if monocytes were removed from the mononuclear fraction by adherence to glass petri dishes prior to plating. The number of colonies observed was proportional to the number of cells plated over the range studied, which was 2,000–20,000 cells per well.

The cells in the aforementioned colonies were characterized as T-lymphocytes by their ability to form rosettes with neuraminidase-treated sheep erythrocytes [19] and the presence of various membrane markers. A cell was classified as a T-lymphocyte if the observed rosette contained at least five sheep erythrocytes. Colony cells were also stained with the Ortho Pharmaceutical OKT3, OKT4, and OKT8 monoclonal antibodies and fluorescein-labeled goat antimouse immunoglobin (Meloy) as described in the Ortho product information. The fraction of labeled cells was determined by fluorescence microscopy with vertical illumination.

Colony growth of B-lymphocytes was achieved using the methyl cellulose system described by Izaguirre et al. [6]. T-lymphocytes and monocytes were depleted from the mononuclear cell fraction of human blood by rosette formation and adherence, respectively. Feeder cells consisted of T-lympho-

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cytes irradiated with 2000R from a caesium source. Colonies containing in excess of 20 cells were counted 6–7 days after plating.

Human granulocytic colonies were grown using the method of Pike and Robinson [12]. Colonies were counted after an incubation of 14 days.

2′-Deoxycoformycin (NSC 218321) was obtained from Drug Research and Development, National Cancer Institute, Bethesda, MD, USA.

Results

Cells growing in the T-lymphocyte colony assay were characterized as T-lymphocytes since over 90% of these cells both formed rosettes with neuraminidase-treated sheep erythrocytes, and were positive for the OKT3 membrane marker, which has been reported to react with 100% of peripheral T-lymphocytes [3]. Furthermore, these cells were also positive for either the OKT4 or OKT8 markers, which identify the inducer/helper or the suppressor/cytotoxic T-lymphocyte subsets, respectively [8, 13, 14]. The average ratio of inducer/helper to suppressor/cytotoxic T-lymphocytes growing in colony assays derived from five normal individuals was 1.6 ± 0.3.

Deoxycoformycin was added to the plating mixture for the T-lymphocyte colony assay to give a final concentration of 1 μg/ml. Plasma levels of 1 μg deoxycoformycin/ml have been obtained in phase I trials with a dose of 30 mg/m^2. One hour after plating 0.005-ml aliquots of either adenosine or deoxyadenosine were added to various wells to give the final concentrations shown in Fig. 1 and 2. The average dose response curves, using lymphocytes from eight normal donors, are shown in Fig. 1 and 2. Colonies from five wells were counted and the results averaged for each nucleoside concentration. Deoxycoformycin was included in the control wells and concentrations up to 10 μg/ml had no observable effect on colony growth. The majority of the control colonies contained in excess of 250 cells, whereas colony size was increasingly reduced with increasing nucleoside concentrations. No attempt was made to quantitate this latter observation. The LD_{50} values for adenosine and deoxyadenosine were 55 μ*M* and 23 μ*M*, respectively.

In the absence of deoxycoformycin, the concentrations of adenosine and deoxyadenosine used in this study had no inhibitory effect on colony growth. Thin-layer chromatography demonstrated that the adenosine and deoxyadenosine were deaminated within 12 h in the absence of deoxycoformycin.

In experiments with lymphocytes from three normal donors, deoxyadenosine did not appear to be selectively toxic to either the inducer/helper or the suppressor/cytotoxic T-lymphocyte subpopulations. The ratio of $OKT4^+$ to $OKT8^+$ cells growing in the presence of 20 μ*M* deoxyadenosine was 1.5, as against the aforementioned value of 1.6 for untreated controls.

The recently described method of Izaguirre et al. for obtaining colony growth of B-lymphocytes was used to assess adenosine and deoxyadenosine toxicity [6]. The overall plating efficiency in these experiments was 0.8%–1%. In excess of 90% of the cells growing in this system were negative for the OKT3 T-lymphocyte marker. Deoxycoformycin (1 μg/ml) was again required for the observation of adenosine or deoxyadenosine toxicity at reasonable concentrations. As with the T-lymphocyte colony growth experiments, deoxycoformycin itself had no observable effect on colony growth of B-lymphocytes. In five experiments using B-cells from normal donors, the average percentages of surviving colonies together with the standard deviations after treatment with adenosine at 10 μ*M*, 20 μ*M*, 40 μ*M*, and 80 μ*M* were 83 ± 5, 67 ± 6, 52 ± 13, and 39 ± 8, respectively. The corresponding values after treatment with the same concentrations of deoxyadenosine were 72 ± 4, 52 ± 10, 36 ± 8, and 7 ± 6, respectively. In these experiments the colonies in five wells were counted and averaged for each nucleoside concentration studied. The LD_{50} values for adenosine and deoxyadenosine in this B colony assay system were 47 μ*M* and 25 μ*M*, respectively.

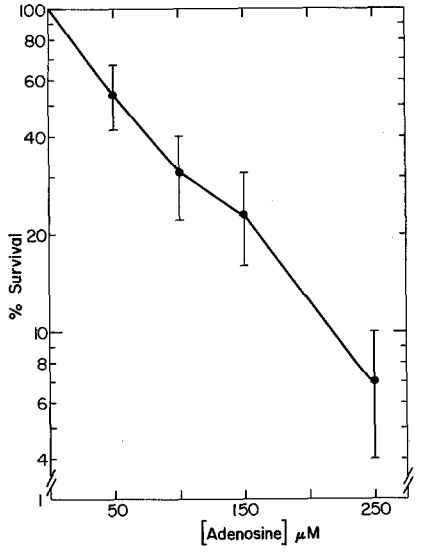

Fig. 1. Survival of T-lymphocyte colonies as a function of adenosine treatment in the presence of deoxycoformycin (1 μg/ml). The data presented are the average colony survival together with the standard deviation for experiments on cells from eight normal donors. Approximately 20,000 cells per well were plated in each instance

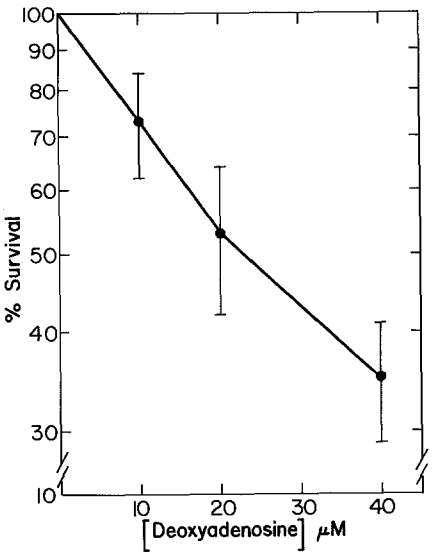

Fig. 2. Survival of T-lymphocyte colonies as a function of deoxyadenosine treatment in the presence of deoxycoformycin (1 μg/ml). The data presented are from simultaneous experiments on cells from the same eight donors as were used for the adenosine study of Fig. 1

Table 1. Effect[a] of adenosine and deoxyadenosine on granulocyte colony growth from human bone marrow

	Survival (%)							
Patient	Adenosine (μ*M*)				Deoxyadenosine (μ*M*)			
	25	50	100	200	25	50	100	200
J. Y.	35	8	NG	NG	12	6	NG	NG
P. A.	57	42	27	17	18	2	NG	NG
A. B.	98	77	75	58	53	38	7	6
H. L.	57	11	NG	NG	NG	NG	NG	NG
A. S.	71	61	43	–	6	1	NG	NG

[a] NG, no growth

Deoxycoformycin (1 μg/ml) was required to observe toxicity by adenosine and deoxyadenosine in the granulocytic colony assay of Pike and Robinson [12]. The colony survival data for granulocytic colonies from bone marrow aspirates of five patients with morphologically normal marrows are shown in Table 1. Control cultures contained between 100 and 250 colonies, depending on the particular marrow sample. The percentage colony survival for a given nucleoside concentration shows much more variability from patient to patient than was observed in the studies with T- or B-lymphocyte colonies. As in the lymphocyte systems, deoxyadenosine is somewhat more toxic than adenosine in this granulocytic system, with an LD_{50} for deoxyadenosine of less than 25 μ*M*. The LD_{50} for adenosine appears to be around 50 μ*M*.

Discussion

The results obtained in this study of human T-lymphocyte, B-lymphocyte, and granulocyte colony growth show that both adenosine and deoxyadenosine are toxic and inhibit cellular proliferation if their deamination is prevented by an adenosine deaminase inhibitor such as deoxycoformycin. In all three colony systems deoxyadenosine was approximately twofold more toxic than adenosine, with LD_{50} values in the range of 20–25 μ*M*. The toxicity of adenosine and deoxyadenosine in these human colony assay systems is similar to that reported in various human and animal cell culture systems [3, 9].

The mechanisms of toxicity of adenosine and deoxyadenosine are still being investigated, and complete agreement does not exist regarding them [3, 5]. However, inhibition of pyrimidine biosynthesis by nucleotides formed from adenosine, inhibition of ribonucleotide reductase by nucleotides formed from deoxyadenosine, and inhibition of *S*-adenosylhomocysteine hydrolase by unmetabolized adenosine and deoxyadenosine have all been proposed as pharmacologically important effects. The hypothesis that has received the most attention in the literature is that deoxyadenosine is the important compound in production of the clinical observations of the adenosine deaminase-deficient and the deoxycoformycin-treated patients. Furthermore, studies on adenosine deaminase levels in mature lymphocytes and in cultured human lymphoblastic cell lines have been interpreted as suggesting that T-lymphocytes may be more sensitive than B-lymphocytes to deoxyadenosine and adenosine toxicity.

The LD_{50} values for adenosine and deoxyadenosine obtained in this study are not sufficiently different to suggest that one of these nucleosides would be more likely to be the toxic compound. There was also no suggestion that T-lymphocytes may be more sensitive than B-lymphocytes. 2′-Deoxycoformycin treatment of animals and humans has been reported to give either very low blood levels (< 1 μ*M*) of adenosine and deoxyadenosine [7], or very sharp spikes of plasma deoxyadenosine [2]. Whatever the resulting pharmacology of adenosine or deoxyadenosine, deoxycoformycin induces lymphopenia and deoxyadenosine triphosphate accumulation in red blood cells of various animals, including man [7, 16]. In view of the induced lymphopenia occurring within 1–3 days, the extended lifespan of circulating lymphocytes, and the extremely low thymidine labeling index of mature lymphocytes, it is difficult for these authors to understand how the observed lymphocyte toxicity is due to inhibition of ribonucleotide reductase by nucleotides formed from deoxyadenosine.

There has been much recent interest in the use of various types of colony assay systems for predictive drug studies in clinical oncology [11, 15]. We had therefore anticipated that the granulocytic colony system would have been less sensitive to adenosine or deoxyadenosine toxicity, as deoxycoformycin-treated animals and humans exhibit little, if any, myelosuppression compared with the observed lymphopenia. However, the observed toxicity in the myeloid system was comparable to that in the lymphoid systems. One must conclude that either unknown pharmacological factors exist in vivo that have not been taken into account in this in vitro work, or that growth inhibition in these colony assay systems is not a valid end-point for the in vivo lymphoid toxicity produced by deoxycoformycin. In either case, this study indicates clearly that the use of colony assays for the clinical prediction of drug effects is not a method that can be used in a routine unquestioning manner.

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