Potent Toxicity of 2-Chlorodeoxyadenosine toward Human Monocytes In Vitro and In Vivo

A Novel Approach to Immunosuppressive Therapy

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Abstract

Lymphoid cells were thought to be uniquely susceptible to excess 2'-deoxyadenosine (dAdo), when exposed to inhibitors of adenosine deaminase (ADA). However, we now find that human monocytes are as sensitive as lymphocytes to dAdo or to the ADA-resistant congener 2-chloro-2'-deoxyadenosine (CldAdo). Monocytes exposed in vitro to CldAdo, or to dAdo plus deoxycoformycin rapidly developed DNA strand breaks. Both the DNA damage and the toxicity of CldAdo or dAdo toward monocytes were blocked by deoxycytidine, but not by inhibitors of poly(ADP-ribose) polymerase. A partial decrease in RNA synthesis and a gradual decline of cellular NAD were early biochemical events associated with monocyte DNA damage. Low CldAdo concentrations (5–20 nM) inhibited monocyte phagocytosis and reduced the release of interleukin 6. Higher CldAdo concentrations led to a dose- and time-dependent loss of monocyte viability.

Circulating monocytes disappeared within 1 wk in patients with cutaneous T cell lymphoma or with rheumatoid arthritis during continuous CldAdo infusion. The marked sensitivity of human monocyte function and survival to CldAdo in vitro, together with the monocyte depletion in patients receiving CldAdo chemotherapy, suggests that CldAdo or other dAdo analogues offer a novel therapeutic strategy for chronic inflammatory and autoimmune diseases characterized by inappropriate monocyte deployment or function. (*J. Clin. Invest.* 1990. 86:1480–1488.) Key words: 2-chlorodeoxyadenosine • DNA damage • immunosuppression • monocyte

Introduction

The naturally occurring purine 2'-deoxyadenosine (dAdo)[1] is highly toxic to certain lymphoid cells if adenosine deaminase (ADA) is absent or inhibited. Intracellular accumulation of dAdo nucleotides by thymic lymphoblasts and by mature lymphocytes has been implicated in the pathogenesis of the severe combined immunodeficiency associated with inherited ADA deficiency (1). 2-Chloro-2'-deoxyadenosine (CldAdo) is an ADA-resistant congener that is also markedly toxic to both normal and malignant lymphoid cells (2, 3). Unlike conventional antimetabolites that impede nucleic acid synthesis in proliferating cells, both CldAdo and dAdo are extremely toxic to mature, nondividing peripheral blood lymphocytes (4). Resting lymphocytes exposed in vitro to CldAdo, or to dAdo plus an ADA inhibitor, rapidly develop DNA strand breaks (5, 6). The DNA damage is followed by a progressive decrease in lymphocyte RNA synthesis, and by the accelerated consumption of NAD for poly(ADP-ribose) synthesis. Together, these effects contribute to a decline in ATP levels and to eventual cell lysis.

Cells of the mononuclear phagocyte system regulate both immunity and chronic inflammation, and hence represent a potential target for chemotherapeutic modulation. Derived from bone marrow myeloid precursors, monocytes give rise to a diverse array of tissue macrophages with specialized phagocytic and synthetic functions necessary for immune competence (7). Unlike lymphocytes, blood monocytes have little, if any, proliferative capacity (8). The recruitment and activation of mononuclear phagocytes contribute to the pathogenesis of inflammatory arthritis and other autoimmune disorders (9, 10). However, most immunosuppressive strategies have been directed toward the lymphocyte component of the immune response.

CldAdo is currently in clinical trial for the treatment of chronic lymphoid malignancies. The drug has activity in chronic lymphocytic leukemia, hairy cell leukemia, cutaneous T cell lymphoma, and autoimmune hemolytic anemia associated with lymphoma (11–13). We recently made the serendipitous observation that freshly isolated human monocytes were as sensitive as lymphocytes in vitro to CldAdo or to the combination of dAdo plus deoxycoformycin. We report here the biochemical characterization of CldAdo toxicity toward monocytes, and show that the drug induces dose- and time-dependent damage to monocyte DNA, with subsequent inhibition of RNA synthesis. The selectivity of CldAdo toxicity for monocytes is illustrated by comparison experiments using confluent human fibroblasts and normal donor neutrophils. In addition, we find that certain in vitro assays of monocyte function are markedly inhibited by sublethal concentrations of CldAdo, concentrations readily achieved during in vivo administration (13). Retrospective analysis of differential leukocyte counts from patients receiving CldAdo chemotherapy showed a dramatic fall in the absolute circulating monocyte number, verifying the significance of our in vitro findings. Thus, the in vitro and in vivo effects of CldAdo on monocytes that we describe herein offer a new approach for the treatment of certain chronic inflammatory and autoimmune diseases.

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1. *Abbreviations used in this paper:* ADA, adenosine deaminase (EC 3.5.4.4); CldAdo, 2-chloro-2'-deoxyadenosine; dAdo, 2'-deoxyadenosine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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Methods

Reagents. Cell culture media and sera were obtained from Whittaker M.A. Bioproducts Inc., Walkersville, MD. CldAdo was synthesized enzymatically from 2-chloroadenine as previously described (3), and [8-^{3}H]CldAdo (6.5 Ci/mmol) was prepared by Moravek Biochemicals, Inc., Brea, CA. Deoxycoformycin was supplied by the Investigational Drug Branch of the National Cancer Institute. dAdo, 2′-deoxyguanosine, 2′-deoxycytidine, 2-chloroadenosine, 2-chloroadenine, 6-thioguanine, 6-mercaptopurine, uridine, methotrexate, nicotinamide, 3-aminobenzamide, 3,3′-diaminobenzidene, erythrosin B, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co., St. Louis, MO. Cycloheximide and 9-β-D-arabinofuranosylguanine were from Calbiochem-Behring Corp., La Jolla, CA. [5-^{3}H]Uridine (18 Ci/mmol), [4,5-^{3}H]leucine (50 Ci/mmol), and [methyl-^{3}H]thymidine-5′-triphosphate (17 Ci/mmol) were obtained from ICN Biomedicals, Inc., Irvine, CA. Klenow fragment of DNA polymerase I and poly(dA-dT) were purchased from Pharmacia LKB Biotechnology Inc., Pleasant Hill, CA. All other chemicals were of the highest grade commercially available.

Cell isolation and culture. Monocytes were isolated from heparinized normal donor blood by Histopaque-1077 (Sigma Chemical Co.) density gradient enrichment of mononuclear cells, followed by adherence to gelatin-coated flasks (14). At least 90% of the adherent cells were positive for α-naphthyl butyrate esterase (Sigma Diagnostics, St. Louis, MO). Cells were cultured at 0.5–1 $\times$ 10^6 cells/ml in RPMI 1640 medium containing 20% autologous plasma, supplemented with L-glutamine 2 mM and 2-mercaptoethanol 50 μM (complete medium). Monocytes in complete medium were incubated at 37°C in 5% CO_2 in air overnight to allow attachment and metabolic equilibration prior to the addition of test compounds. For DNA damage and biochemical assays, monocytes were detached by xylocaine 0.4% (Astra Pharmaceutical Products, Inc., Westborough, MA) or with trypsin 0.25% plus EDTA 5 mM.

Neutrophils were recovered after Histopaque sedimentation by hypotonic lysis of contaminating erythrocytes, and were suspended in complete medium for immediate use in the DNA strand break and deoxynucleotide formation assays.

The normal fetal lung fibroblast line GM 01380 was obtained from the Human Genetic Mutant Cell Repository, Camden, NJ and was cultured to confluence in DME containing 20% fetal bovine serum. B9.9 hybridoma cells used in the bioassay for interleukin 6 (IL-6) were maintained as previously described (15).

Toxicity assessment. Toxicity of various compounds towards cultured human monocytes was determined by a modification of the MTT reduction assay described by Mosmann (16). Monocytes in complete medium were distributed at 10^5 cells per well into 96-well flat-bottomed tissue culture plates. When indicated, cells were preincubated 1 h with deoxycoformycin or with protection compounds (e.g., deoxycytidine) before the addition of cytotoxic agents. After culture for up to 5 d, MTT 0.2 mg in 40 μl was added to each well and the incubation continued for 4 h. Plates were then centrifuged, and the supernatants were carefully aspirated with a finely drawn pipette. Acidified isopropanol (0.04 N HCl) 100 μl was added to each well, and the sealed plates, shielded from light, were placed at −20°C overnight to allow complete dissolution of the formazan precipitates. Viable cell numbers were determined using a microplate reader (model 600, Dynatech, Torrance, CA), measuring absorbance at 570 nm with a reference wavelength of 630 nm.

The viability of lymphocytes in suspension and of detached monocytes was determined by erythrosin B dye exclusion.

DNA strand break assay. The level of DNA strand breaks in monocytes exposed to CldAdo or to other agents was determined by the fluorescent assay for DNA unwinding in alkaline solution described by Birnboim and Jevcak (17). The unwinding rate of DNA in alkaline solution is proportional to the number of DNA strand breaks or alkali-labile sites. In this assay, the fluorescence of ethidium bromide bound to remaining double-stranded DNA after 1 h at pH 12.8 is expressed as a percentage of the total double-stranded DNA fluorescence from an aliquot not exposed to alkali. The percent double-stranded DNA after unwinding is taken as a measure of DNA strand breaks in control or in CldAdo-treated monocytes. Results for DNA damage are expressed in gray equivalents, derived from a standard curve for monocytes irradiated with 0–17.8 Gy at a dose rate of 1.29–4.45 Gy/min in an irradiator (Gammacell 1000, Atomic Energy of Canada Ltd., Kanata, Ontario).

Biochemical studies. Perchloric acid 0.5 M extracts of monocytes were neutralized with KOH and potassium phosphate buffer 0.33 M at pH 7.5. Cellular NAD content was measured by an alcohol dehydrogenase cycling assay (18) as previously described (6). Monocyte ATP was quantitated by anion exchange high performance liquid chromatography (HPLC) (3).

Monocyte synthesis of RNA and protein was estimated from the incorporations of [5-^{3}H]uridine and [4,5-^{3}H]leucine, respectively. Cells were incubated with radiolabeled compounds (20 μCi/ml per 10^6 cells) during the final hour of culture with or without CldAdo. Trichloroacetic acid 10% precipitates were collected onto cellulose acetate filters (Gelman Sciences, Inc., Ann Arbor, MI) and washed further with trichloroacetic acid and ethanol. Radioactivity was measured by liquid scintillation counting. [5-^{3}H]Uridine incorporation by control monocytes not exposed to CldAdo was 20–40 nCi/h per 10^6 cells.

Formation of CldAdo and dAdo nucleotides. [8-^{3}H]CldAdo was purified by reverse-phase HPLC immediately before use (3). Monocytes were exposed for varying times to [8-^{3}H]CldAdo 1 μM (6.5 μCi/ml per 10^6 cells). Nucleotides were extracted with perchloric acid 0.3 M, and the extracts were neutralized with 2 vol tri-*n*-octylamine 0.5 M (Sigma Chemical Co.) in Freon-FT, after the method of Kyhm (19). An aliquot of neutralized extract was treated with sodium periodate to destroy ribonucleotides (20). [8-^{3}H]CldAdo nucleotides were quantitated by anion exchange HPLC on an Ultrasil SAX column (Beckman Instruments, Inc., San Ramon, CA) using a complex gradient from $NH_4H_2PO_4$ 7.5 mM, acetonitrile 2% at pH 4.5, to $NH_4H_2PO_4$ 750 mM, acetonitrile 2% at pH 5.0, modified from the procedure described by de Korte et al. (21).

2′-Deoxyadenosine 5′-triphosphate (dATP) was measured by a modification of the DNA polymerase assay (22) in which [^{3}H]TTP is incorporated into a poly (dA-dT) template by the Klenow fragment of DNA polymerase I in the presence of sample dATP. For this assay, cellular deoxynucleotides were extracted by a modified two-step procedure using methanol 60% followed by perchloric acid and octylamine/Freon neutralization (23).

Monocyte function assays. Monocyte phagocytosis was tested using autologous erythrocyte targets, sensitized with a subagglutinating titer of rabbit anti–human erythrocyte IgG (Cappel Laboratories, Malvern, PA). Monocytes at 2 $\times$ 10^5 cells per microwell were exposed to subtoxic concentrations of CldAdo for 72 h. Complete medium was then replaced with medium containing 10% fetal bovine serum and a 0.25% suspension of sensitized erythrocytes. After incubation for 4 h at 37°C, phagocytosis was quantitated by the spectrophotometric method of Jungi (24). This assay measures the peroxidation of diaminobenzidine catalyzed by hemoglobin in detergent lysates of the mononuclear phagocytes.

The spontaneous secretion of IL-6 by cultured monocytes was measured by the hybridoma growth factor bioassay as described previously (15). The proliferation of a B9.9 murine hybridoma subclone is dependent on the presence of IL-6 (25). Supernatants collected from monocytes cultured up to 72 h with CldAdo were dialyzed against HBSS to remove the drug, then diluted 1:12 into wells containing B9.9 cells. To reduce the stimulatory effects of contaminating lipopolysaccharide, polymyxin B sulfate 12.5 μg/ml (Burroughs Wellcome Co., Research Triangle Park, NC) was added to all reagents used to prepare monocytes for these experiments.

Statistical analysis. Results of replicate assays for monocyte NAD, ATP, and [^{3}H]uridine incorporation were compared for statistical significance using the paired-sample *t* test.

Clinical studies. Daily blood counts were obtained from nine pa-

tients undergoing experimental CldAdo chemotherapy for cutaneous T cell lymphoma, and from one patient receiving CldAdo for rheumatoid arthritis. CldAdo was administered at 0.1 mg/kg per day for 5–7 d by continuous intravenous infusion (11). The treatment was conducted on a General Clinical Research Center ward and was approved by our institutional review board.

Results

In vitro toxicity

The MTT reduction assay is admirably suited to the measurement of drug toxicity towards monocytes. Contaminating, mature lymphocytes produce little MTT formazan in the absence of a mitogen (26), and red cells produce none (16). Adherence of the cultured monocytes allows plasma-rich medium to be removed before addition of acid isopropanol, avoiding spectrophotometric variations due to precipitate turbidity. The MTT reduction assay detected as few as 2,000 unactivated monocytes, and the absorbance of MTT formazan at 570 nm was linearly related to monocyte number. Assay results were confirmed by microscopic inspection and dye exclusion in order to distinguish cell lysis from inhibitory effects of the test compounds on mitochondrial function.

Human monocytes were exquisitely sensitive in vitro to CldAdo at the same nanomolar concentrations that lyse resting peripheral blood lymphocytes (Fig. 1) (4). In contrast, nondividing, confluent human fibroblasts were impervious to the drug. CldAdo toxicity toward monocytes became evident at drug concentrations ≥ 10 nM, and was both dose- and time-dependent. A 5-d exposure of monocytes to CldAdo 27 nM resulted in a 50% cell lysis (IC_{50}), compared to simultaneous control cultures without the drug (Table I). Monocytes were also sensitive to dAdo during short-term culture, but only if the ADA inhibitor deoxycoformycin was also present. Deoxycoformycin 1–5 μM alone caused up to a 50% decrease in MTT reduction that was not accompanied by a proportional decrease in viable cell number (95% of control by dye exclusion).

As reported previously for lymphocytes and lymphoid cell lines (3, 6), monocyte sensitivity to CldAdo or to dAdo was substantially prevented by deoxycytidine (Fig. 1). This result implicates deoxycytidine kinase activity in the formation of toxic CldAdo nucleotides. However, the addition of nicotinamide or 3-aminobenzamide did not prevent the lysis of monocytes exposed to CldAdo (result not shown). This is in contrast to the toxicity delay caused by nicotinamide and other inhibitors of poly(ADP-ribosyl)ation in CldAdo-treated lymphocytes (6). The addition of uridine 100 μM as a source of pyrimidines also failed to prevent the toxicity of CldAdo towards monocytes in vitro.

Table I also lists the toxicity of other purine antimetabolites towards human monocytes in short-term culture. 2-Chloroadenine and 2-chloroadenosine were at least 200-fold less toxic than CldAdo, confirming that CldAdo toxicity is not due to degradation with subsequent salvage into ribonucleotide metabolite pools. 2′-Deoxyguanosine was essentially nontoxic to monocytes in vitro, although an inhibitor of purine nucleoside phosphorylase was not included in the assay. Both 6-thioguanine and 9-β-D-arabinofuranosylguanine were toxic at ~ 1 μM. However, 6-mercaptopurine was at least 10-fold

Table I. Toxicity of dAdo and Purine Antimetabolites toward Human Monocytes In Vitro

Purines and analogues	IC_{50} at 5 d
	μM
CldAdo	0.027
2-Chloroadenosine	5.97
2-Chloroadenine	15.18
dAdo	>100.00
dAdo + deoxycoformycin 5 μM	0.21
2′-Deoxyguanosine	>200.00
9-β-D-Arabinofuranosylguanine	0.90
6-Thioguanine	0.96
6-Mercaptopurine	9.70

Toxicity after 5 d was measured by the MTT reduction assay (16). IC_{50} is the drug concentration that decreases monocyte viability to 50% of control culture viability without drugs. The results represent the mean values from three or more experiments.

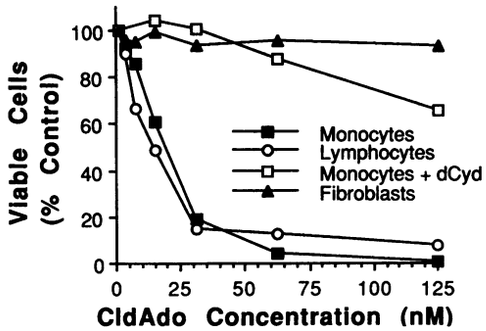

Figure 1. CldAdo toxicity toward nondividing cells. Freshly isolated monocytes (■) and lymphocytes (○), and normal human GM 01380 fibroblasts at confluence (▲) were cultured for 5 d with various concentrations of CldAdo. Deoxycytidine 100 μM was added to duplicate monocyte cultures (□). Viable monocytes and fibroblasts were measured by the MTT reduction assay (16), and viable lymphocytes were enumerated by dye exclusion. Results are expressed as the percentage of viable cell number in control cultures without CldAdo.

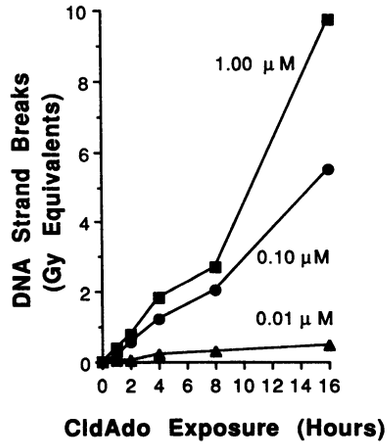

Figure 2. DNA damage in monocytes exposed to CldAdo. Monocytes were treated with CldAdo 0.01 μM (▲), 0.10 μM (●), or 1.00 μM (■) for 1–16 h. DNA strand breaks were determined by the fluorescent assay for DNA unwinding in alkaline solution (17). DNA strand breaks in CldAdo-treated cells are expressed in gray equivalents, derived from a standard curve for DNA damage in irradiated monocytes.

less effective. Neither methotrexate nor 5-fluoro-2′-deoxyuridine were toxic to monocytes in vitro at concentrations in excess of 10 μM (results not shown).

DNA strand breaks in monocytes

DNA strand breaks appeared promptly in human monocytes treated in vitro with CldAdo or with dAdo plus deoxycoformycin. As shown in Fig. 2, DNA breaks accumulated with time during CldAdo exposure, and the level of DNA damage was dose-dependent. The DNA unwinding assay detected DNA strand breaks in monocytes exposed to as little as 10 nM CldAdo or dAdo. At higher concentrations of either deoxynucleoside, significant DNA damage occurred in monocytes within the first hour of culture. Treatment of monocytes for 1 h with CldAdo 1 μM, or with dAdo 1 μM plus deoxycoformycin, resulted in DNA damage equivalent to that produced by 0.38 Gy γ-irradiation. By 4 h, the level of DNA strand breaks induced by CldAdo was equivalent to 1.78 Gy. Deoxycoformycin alone caused some accumulation of DNA strand breaks in monocytes, but the dose response was highly variable (results not shown). Among the purine antimetabolites toxic to human monocytes at nanomolar concentrations in vitro (Table I), only CldAdo, dAdo, and 9-β-D-arabinofuranosylguanine caused DNA strand breaks.

Simultaneous addition of deoxycytidine 10 μM to the culture medium partially protected monocyte DNA from damage caused by CldAdo or dAdo treatment. In contrast, neither 3-aminobenzamide 1 mM, cycloheximide 5 μg/ml, nor the calcium chelator EGTA had any effect on early DNA strand break accumulation in monocytes exposed to the deoxynucleosides.

To determine whether DNA strand break accumulation in the presence of dAdo or substituted analogs was a general response of phagocytes, we compared the effects of dAdo plus deoxycoformycin on the DNA integrity of monocytes and neutrophils. The lability of neutrophils in vitro precluded comparison tests of deoxynucleoside toxicity. However, there was no decrease in either neutrophil or monocyte viability during the 4 h assay for DNA damage. Table II shows that human neutrophils were at least 100-fold more resistant than monocytes to the DNA-damaging effects of dAdo during short-term culture. At equivalent dAdo concentrations, monocytes accumulated four times more DNA strand breaks than did neutrophils.

Table II. dATP Formation and DNA Damage in Monocytes vs. Neutrophils after dAdo Treatment In Vitro

dAdo concentration*	Cell type	dATP formed‡	DNA strand breaks§
		pmol/10⁶ cells	*Gy equivalents*
1 μM	Monocyte	1.11	1.15
	Neutrophil	0.22	0.28
100 μM	Monocyte	19.62	2.50
	Neutrophil	2.65	0.52

* Cells were cultured for 4 h with dAdo plus deoxycoformycin 5 μM to inhibit ADA.

‡ dATP in methanol/perchloric acid cell extracts was measured by a modified DNA polymerase assay (22). Untreated monocytes and neutrophils contained < 0.1 pmol dATP/10⁶ cells.

§ DNA strand breaks were measured by the fluorescent assay for DNA unwinding in alkaline solution (17). Results are expressed in gray equivalents derived from a standard curve for radiation-induced DNA damage in monocytes.

Biochemical effects of CldAdo in human monocytes

NAD and ATP content. NAD consumption for poly(ADP-ribose) synthesis is a known consequence of severe DNA damage in eukaryotic cells (27). To determine the potential role of NAD depletion in the marked toxicity of CldAdo toward monocytes, we studied the temporal changes in oxidized NAD and ATP in cells exposed to CldAdo 1 μM. In contrast to measures of DNA strand breaks, monocyte NAD content remained relatively constant during the first 4 h (95% of control NAD), but declined progressively thereafter (Fig. 3). By 8 h, the NAD content of CldAdo-treated monocytes was significantly lower than that of control cells (319±104 vs. 384±93 pmol/10⁶ cells, mean±SD for 11 experiments, $P < 0.001$ by paired-sample t test). The fall in NAD preceded the concordant decreases in ATP and cell viability evident after a 16 h exposure to CldAdo.

To assess further the relationship of ADP-ribosylation to monocyte NAD content during CldAdo toxicity, monocytes were tested with the ADP-ribosylation inhibitor 3-aminobenzamide, alone and in combination with CldAdo. Simultaneous exposure to 3-aminobenzamide 5 mM prevented the NAD depletion observed with CldAdo 1 μM alone (111% vs. 34% of control NAD at 16 h, means of four experiments). In contrast, the ADP-ribosylation inhibitor had no effect on either the ATP decline or the loss of viability (84% of control) in monocytes treated for 16 h with CldAdo 1 μM. Interestingly, culture with 3-aminobenzamide alone caused a significant, sustained rise in monocyte NAD above control levels (675±93 vs. 392±26 pmol/10⁶ cells at 16 h, mean±SD for four experiments, $P = 0.004$ by paired-sample t test).

RNA and protein synthesis. The effects of DNA strand breaks on the synthesis of RNA and protein were measured in monocytes treated with CldAdo 1 μM for 1–16 h. As shown in Fig. 3, RNA synthesis decreased during the first hours of culture and showed further decline during prolonged CldAdo exposure. Within 2 h, the pace of [³H]uridine incorporation by the CldAdo-treated cells had slowed significantly to 89% of control ($P = 0.004$ by t test on paired samples in six experiments). In contrast to RNA, protein synthesis remained at

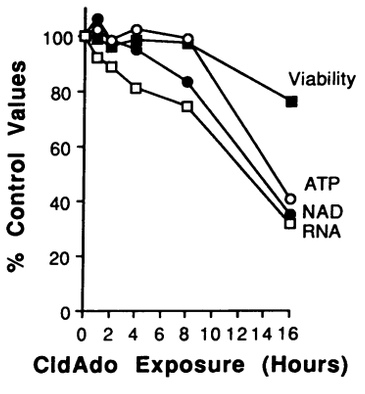

Figure 3. Biochemical consequences of DNA damage in CldAdo-treated monocytes. Monocytes were cultured up to 16 h with CldAdo 1 μM. At intervals, samples were assessed for viability (■), intracellular ATP (▲), NAD (●), and for the incorporation of [5-³H]-uridine into RNA (□). Results are expressed as the percentage of the values from control cultures without CldAdo, and represent the means of 3–11 experiments. Control monocyte NAD = 384 ± 93 pmol/10⁶ cells and ATP = 1,363 ± 121 pmol/10⁶ cells.

control levels until cell viability was impaired (results not shown).

Formation of dAdo and CldAdo nucleotides. Monocytes treated with dAdo 1 μM plus deoxycoformycin 5 μM accumulated far more dATP than did neutrophils under similar culture conditions (Table II). Surprisingly, raising neutrophil dATP in excess of 2 pmol/10^6 cells by treatment with dAdo 100 μM caused fewer strand breaks than occurred in monocytes containing half as much dATP. As expected, dATP formation by the ADA-inhibited monocytes was substantially prevented by deoxycytidine 100 μM (0.18 vs. 0.38 pmol dATP/10^6 cells at 1 h).

Monocytes rapidly accumulated CldAdo nucleotides when exposed to the deoxynucleoside in vitro. Intracellular CldAdo nucleotides were detectable after 1 h, and reached a stable level after 4 h (Table III). After exposure to CldAdo 1 μM for 8 h, monocytes contained 1.84 pmol CldATP per 10^6 cells, which was 15% of the total acid-soluble CldAdo nucleotide pool.

Effects of low-dose CldAdo on monocyte function

We sought to determine whether sublethal CldAdo concentrations affected monocyte function in vitro. Cells were cultured with CldAdo 5-20 nM for 3 d, after which phagocytosis and supernatant IL-6 activity were assayed. Fig. 4 shows that despite unchanged cell viability, the phagocytosis of antibody-coated erythrocyte targets was markedly suppressed.

The ability of dialyzed culture supernatants to promote proliferation of B9.9 hybridoma cells is a measure of IL-6 activity (25). Monocytes cultured in autologous plasma 20% for 3 d secreted ~ 22 U of IL-6 per ml of medium. Fig. 4 shows that subtoxic concentrations of CldAdo inhibited the spontaneous secretion of IL-6 by the cultured monocytes. However, at cytotoxic concentrations of CldAdo the supernatants contained high levels of IL-6 (results not shown), presumably from the release of intracellular cytokines after cell lysis.

Effects of CldAdo on circulating monocytes in vivo

The absolute leukocyte counts, hemoglobin, and platelet counts were analyzed from nine patients with cutaneous T cell lymphoma receiving CldAdo chemotherapy. Pretreatment leukocyte and platelet counts were normal in six patients, and were reduced in two patients owing to prior chemotherapy. Circulating lymphocytes were increased in one patient. CldAdo was administered at 0.1 mg/kg by continuous intravenous infusion for 7 d. During the CldAdo treatment the monocyte counts declined dramatically in all patients (Fig. 5; 94% mean decrease at day 7). Monocytes disappeared completely from the circulation in five patients. Lymphocyte counts were decreased ~ 35% at the end of the infusion period. In contrast, CldAdo caused little change in neutrophil and platelet counts, or in the patients' hemoglobin levels. 10 d after the end of the CldAdo infusion, the monocyte counts had returned to normal in all patients.

Table III. Formation of CldAdo Nucleotides by Human Monocytes In Vitro

[^{3}H]CldAdo 1 μM exposure	Total CldAXP	CldATP
h	*pmol/10^6 cells*	
1	9.1	0.84
4	12.9	1.69
8	12.2	1.84

Monocytes were cultured with [8-^{3}H]CldAdo 1 μM (6.5 Ci/mmol) for the indicated times, and the total acid-soluble intracellular CldAdo nucleotides (CldAXP) and CldATP were quantitated by anion exchange HPLC (21).

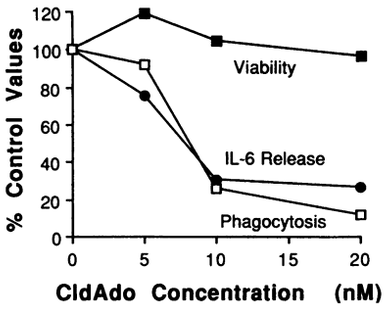

Figure 4. Effects of low-dose CldAdo on monocyte phagocytosis and IL-6 secretion in vitro. Monocytes were cultured 72 h with concentrations of CldAdo that did not affect cell viability (■). Phagocytosis of antibody-coated erythrocytes (□) was then measured by a spectrophotometric assay for hemoglobin (24), and IL-6 activity in dialyzed culture supernatants (●) was measured by specific bioassay (15). Results are expressed as the percentage of control values from cultures without CldAdo, which contained 22 U/ml of IL-6 activity.

CldAdo is currently under investigation for the treatment of rheumatoid arthritis, a disease in which monocyte-derived macrophages play a role in synovial inflammation and joint destruction. The effects of CldAdo on circulating monocytes from a representative patient is shown in Fig. 6. This 63-yr-old woman with seropositive rheumatoid arthritis received three treatments with CldAdo by continuous infusion for 5 d. The blood monocyte count fell to zero during each infusion, returning to normal within 1 wk after the treatments.

Discussion

Our results demonstrate a surprising sensitivity of human monocytes to dAdo and CldAdo that was not anticipated from studies of lymphoid depletion in congenital ADA deficiency. Despite their myeloid origin, monocytes exposed in vitro to CldAdo underwent biochemical events before cell lysis that paralleled those reported for nondividing lymphocytes (6). Toxicity of CldAdo or dAdo toward monocytes required

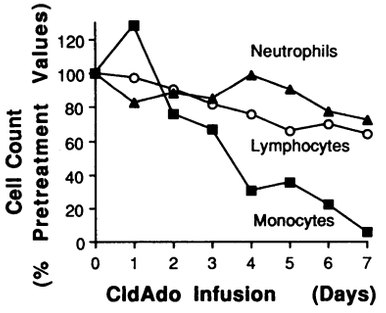

Figure 5. Effects of CldAdo infusion therapy on circulating leukocyte counts in patients with cutaneous T cell lymphoma. Daily neutrophil (▲), lymphocyte (○), and monocyte (■) counts were obtained from nine cutaneous lymphoma patients receiving CldAdo chemotherapy. CldAdo was administered at 0.1 mg/kg per day for 7 d by continuous intravenous infusion. Results represent the mean daily leukocyte counts, expressed as a percentage of the mean pretreatment counts.

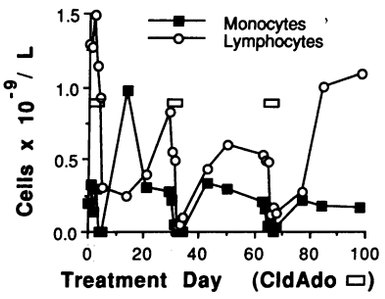

Figure 6. Effects of repeated CldAdo infusions on circulating monocytes and lymphocytes in rheumatoid arthritis. Serial monocyte (■) and lymphocyte (○) counts were performed on a patient with seropositive rheumatoid arthritis who received three courses of CldAdo infusion chemotherapy (□). CldAdo was administered at 0.1 mg/kg per day for 5 d by continuous intravenous infusion.

phosphorylation, presumably by deoxycytidine kinase, and was prevented by deoxycytidine. Accumulation of CldAdo nucleotides by cultured lymphoblasts can be prevented by deoxycytidine (3), and in monocytes the formation of dATP from dAdo was similarly inhibited. Substantial DNA damage occurred during the first hour that monocytes were exposed to CldAdo or to dAdo plus deoxycoformycin. A prompt, partial inhibition of monocyte RNA synthesis was the earliest consequence of these DNA strand breaks. Although NAD levels subsequently declined in CldAdo-treated monocytes, the addition of ADP-ribosylation inhibitors failed to avert toxicity. Ultimately, protein synthesis and cellular ATP content declined in concert with measures of cell viability. The dose- and time-dependent parameters of CldAdo toxicity we observed in vitro correlated well with the selective, often complete disappearance of peripheral blood monocytes in patients receiving CldAdo chemotherapy.

The selective toxicity of CldAdo or dAdo towards monocytes and lymphocytes depends on inherent biochemical features that determine (*a*) the net intracellular formation of CldATP or dATP and (*b*) the effect of CldAdo or dAdo nucleotides on DNA integrity. The formation of purine deoxyribonucleotides in nondividing cells most likely reflects a balance between the activities of deoxycytidine kinase and cytosolic 5′-nucleotidases (4, 28). Although monocytes and lymphocytes are of disparate lineage, both cell types form equivalent amounts of CldAdo nucleotides (Table III, (4)), and the toxicity curves are nearly identical (Fig. 1). However, sensitivity to dAdo or congener drugs is not a property of all nondividing cells. Confluent, normal fibroblasts were impervious to CldAdo. Moreover, neutrophils not only accumulated dATP less efficiently than monocytes, but also developed fewer DNA strand breaks at equivalent levels of intracellular dATP. Accordingly, the neutrophils of patients receiving CldAdo chemotherapy are resistant to the drug compared to their monocytes and lymphocytes (Fig. 5). Because uridine failed to prevent CldAdo toxicity to monocytes in vitro, it is unlikely that CldAdo nucleotide synthesis causes the same "pyrimidine starvation" found in rodent cell lines treated with adenosine (29).

Several biochemical changes associated with monocyte differentiation into macrophages are mimicked during short-term culture in vitro (30). Fischer et al. (31) documented a nine-fold rise in ADA activity in cultured human monocytes, and found that ADA inhibitors suppressed both the morphologic and enzymatic changes that characterize monocyte maturation. Interestingly, these authors also noted that inhibiting ADA caused a decrease in monocyte viability. Similarly, Kaplan et al. (32) showed that ADA inhibition blocked monocyte cytolytic functions in vitro. These suppressive effects of ADA inhibitors were attributed to an undefined role of ADA activity in monocyte differentiation and function. Alternatively, our results suggest that small amounts of endogenous dAdo released in vitro causes DNA damage in ADA-inhibited monocytes, suppressing RNA synthesis and other metabolic functions. Chan et al. (33) showed that murine macrophages treated with deoxycoformycin in vitro excreted substantial amounts of dAdo as a result of nucleic acid catabolism. DNA from cells dying during culture (50% loss is commonly reported [31, 34, 35]) may provide sufficient dAdo to produce the reported effects of ADA inhibitors on monocyte survival and function. These observations were perhaps early clues to the selective toxicity of dAdo and congener drugs towards human monocytes.

The precise mechanism of dAdo and CldAdo toxicity toward human monocytes is incompletely understood. DNA strand breaks appear to be requisite for toxicity, and deoxycytidine protection experiments suggest that dAdo or CldAdo nucleotides cause the DNA damage through effects on enzyme(s) involved in DNA repair or synthesis. Both CldAdo and combinations of dAdo plus ADA inhibitors produced DNA strand breaks in proliferating cell lines (36–38), attributed to mis-incorporation during semiconservative DNA replication or to unbalanced deoxynucleotide synthesis. Nanomolar concentrations of CldATP are sufficient to inhibit ribonucleotide reductase (39). By contrast, the level of DNA breaks or apurinic sites in quiescent cells likely reflects a balance between spontaneous formation and the ongoing repair of such lesions (40). CldAdo nucleotides may disturb this dynamic process in monocytes, resulting in the accumulation of DNA strand breaks. Both dAdo plus deoxycoformycin and CldAdo inhibited the repair of DNA lesions in resting lymphocytes treated with γ-irradiation or alkylating drugs (41–43). Parker et al. (39) recently showed that CldATP competitively inhibited the incorporation of dATP into DNA by all three mammalian DNA polymerases. Moreover, CldATP was itself a poor substrate for DNA polymerase α, and the incorporation of two consecutive CldAMP residues resulted in chain termination. It is relevant that both DNA polymerases α and β were inhibited by CldATP (K_i = 3–5 μM) at concentrations that in the present study caused prompt accumulation of DNA strand breaks in situ (Table III; CldATP 1 pmol/10^6 monocytes is ~ 3 μM, if 3×10^9 monocytes is taken to equal 1 ml [44]). In other studies, the combination of dATP and deoxycoformycin inhibited the activity of DNA ligase purified from T lymphoblasts (45).

A decrease in RNA synthesis was the earliest biochemical event associated with DNA damage in monocytes exposed to CldAdo (Fig. 3). A partial block in [5-^{3}H]uridine incorporation also occurred in lymphocytes treated with dAdo plus deoxycoformycin (6, 46). The inhibition of RNA synthesis was presumably a result of template DNA fragmentation, but direct effects of CldATP on RNA polymerase activity have not been examined. Experiments with 6-thiopurines showed that short-term inhibition of RNA synthesis was not lethal to quiescent lymphocytes (6). By comparison, monocytes are metabolically active cells, quite sensitive to 6-thioguanine by mecha-

nisms independent of DNA damage (Table I). Thus, the partial inhibition of RNA synthesis caused by CldAdo may result in the selective depletion of critical, short-lived proteins necessary for monocyte survival.

Activation of the nuclear enzyme poly(ADP-ribose) polymerase does not appear to determine the toxicity of dAdo or CldAdo towards monocytes. Poly(ADP-ribose) polymerase is stimulated by DNA strand breaks to synthesize ADP-ribose homopolymers from substrate NAD (47, 48). NAD turnover increases in nondividing lymphocytes treated with dAdo plus deoxycoformycin as a consequence of DNA damage (49). Depletion of lymphocyte NAD occurs because the synthesis of pyridine nucleotides from nicotinamide cannot keep pace with NAD consumption in these metabolically quiescent cells (6). Both the NAD decrease in CldAdo-treated monocytes (Fig. 3) and the preservation of cellular NAD by 3-aminobenzamide are findings consistent with the activation of monocyte poly(ADP-ribose) polymerase. However, inhibitors of ADP-ribosylation failed either to preserve monocyte viability during CldAdo exposure, or even to avert the penultimate fall in ATP. Although the accumulation of DNA strand breaks in the presence of CldAdo nucleotides seems critical to the mechanism of CldAdo action, it is thus unlikely that consumption of NAD for poly(ADP-ribose) synthesis contributes to the toxic effects of the drug towards human monocytes in vitro or in vivo.

Both deoxycoformycin and CldAdo are effective drugs in the treatment of chronic lymphocytic leukemia and hairy cell leukemia (11, 12, 50, 51). However, there are important differences between the effects of CldAdo and deoxycoformycin on the circulating monocyte count. Blood monocytes were found to be decreased in patients with hairy cell leukemia at presentation (52, 53). The monocyte counts declined further at the initiation of deoxycoformycin therapy, but subsequently rose to normal levels as the leukemia responded to continued treatment. Thus, deoxycoformycin does not appear to be toxic towards monocytes in vivo, despite the potent toxicity of dAdo plus deoxycoformycin in vitro. Deoxycoformycin therapy at high doses does result in elevated plasma dAdo (54). However, the intermittent, low-dose deoxycoformycin regimens used to treat chronic leukemia may either fail to completely inhibit monocyte ADA, or may not produce sufficient elevation of plasma dAdo to impair monocyte survival.

In contrast with deoxycoformycin, CldAdo infusion consistently reduces the absolute monocyte count, often to zero (Figs. 5 and 6). Circulating monocytes also decrease rapidly during corticosteroid therapy, but return to normal levels by 12 h despite continuation of the drug (55). Thus, CldAdo appears to be the most selective antimonocyte agent yet described that has been extensively tested in vivo, affecting monocyte function and viability at concentrations below those that cause general myelosuppression. Also, CldAdo continuous infusion therapy appears to be significantly less toxic at clinically useful dosages than deoxycoformycin given by intermittent bolus infusion (11, 50). Recently, the dAdo analog 9-β-D-arabinofuranosyl-2-fluoroadenine 5'-monophosphate was shown in a Phase I clinical trial to lower circulating $OKM1^+$ mononuclear cells, presumably monocytes, in addition to lowering patient lymphocyte counts (56).

Phagocytosis, secretion of inflammatory mediators, and local tissue destruction are mononuclear phagocyte functions implicated in the pathogenesis of diverse syndromes such as immune cytopenias, arthritis, and sarcoidosis (7). Phagocytosis of immune complexes by monocyte-derived synoviocytes may foster inflammation and joint damage in rheumatoid arthritis (57). More recently, the levels of IL-6 in synovial fluid and serum from rheumatoid arthritis patients have been found to correlate with certain indices of disease activity (58). Because IL-6 appears to stimulate megakaryocyte development and platelet production (59), the supression of monocyte IL-6 secretion by CldAdo may explain the thromocytopenia despite adequate marrow megakaryocytes occasionally observed in patients receiving the drug. In conclusion, the potent toxicity of CldAdo towards monocytes in vitro and in vivo, together with the inhibition of phagocytosis and IL-6 secretion at very low drug concentrations in vitro, provides a rationale for exploring further the use of CldAdo in the management of rheumatoid arthritis and other chronic inflammatory disorders.

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