Inhibitory Effect of 2-Chlorodeoxyadenosine on Granulocytic, Erythroid, and T-Lymphocytic Colony Growth


Previous studies have shown that 2-chloro-2'-deoxyadenosine (CdA) is markedly toxic to normal and malignant human lymphocytes in vitro and in vivo. Recent clinical trials have shown that CdA is a very promising drug for the treatment of lymphoid malignancies. The present investigations were designed to test the effect of CdA on the in vitro clonal growth of both myeloid progenitors and T-lymphocyte colony-forming cells (CFU-TL) obtained from normal human bone marrow and peripheral blood. Cells were exposed to CdA in doses up to 1280 nmol/L. To reduce indirect effects of CdA mediated by accessory cells, monocyte- and T-lymphocyte-depleted bone marrow cells were used for our investigations. The results show a marked inhibition of myeloid progenitor and lymphocyte colony-forming cells in a dose-dependent manner, correlating with maturation stage in that the immature progenitor cells are more sensitive to this drug. Furthermore, our studies suggest that a sequence of metabolic events previously described for lymphocytes may be operative in myeloid progenitor cells because a minimal exposure time of 48 hours is required to obtain a marked inhibition. CdA toxicity was proposed to be linked with phosphorylation by deoxycytidinkinase (E.C. 2.7.1.74), the levels of which have been found to be high in lymphocytes, but low in granulocytes. However, the marked inhibition of myeloid progenitor cells shown in these studies suggests that other factors such as modulation of the effect of CdA by the ambient levels of other deoxynucleosides might influence the apparent sensitivity of myeloid cells.

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A GENETIC DEFICIENCY of adenosine deaminase (ADA; adenosine aminohydrolase, E.C. 3.5.4.4.) causes severe combined immunodeficiency disease. The profound lymphopenia observed in children with this disease has been attributed to the toxic effects of increased levels of deoxyadenosine; because the normal catabolism from deoxyadenosine to deoxynosine in the purine metabolism is blocked. This observation induced the search for substances that mimic ADA deficiency and might prove clinically useful as new lymphocytotoxic agents: drugs that inhibit ADA, like deoxycytidinofuranoside (dCF), and those that act as deoxyadenosine congeners, like 2-chloro-2'-deoxyadenosine (CdA).

CdA is a simple nucleoside derived from deoxyadenosine by substituting chloride for hydrogen in the 2'-position of the purine ring, which renders it resistant to degradation by adenosine deaminase. Promising efficiency in the treatment of chronic lymphoid malignancies has been reported. Hairy cell leukemia is notably responsive to CdA, with 90% of patients entering durable complete remissions after treatment with this drug.

Previous studies with lymphoblasts and lymphocytes have shown that the drug is actively phosphorylated by deoxycytidine kinase and accumulates in cells as the nucleoside triphosphate (cdATP). Incorporation of cdATP into the DNA of proliferating cells accounts for their cell death. The drug is therefore a candidate for use as a therapeutic agent in lymphoid malignancies, because lymphoid cells have especially high deoxycytidine kinase activity.

The increasing use of CdA in clinical trials for the treatment of lymphoid malignancies made it important to determine the effect of CdA on hematopoietic progenitor cells, especially since neutropenia and bone marrow (BM) suppression have been reported in patients treated with high doses of CdA.

We report here the effects of CdA on the clonal growth of a series of progenitor cells (colony-forming unit granulocyte-macrophage [CFU-GM], primitive burst-forming unit-erythroid [pBFU-E], mature BFU-E [mBFU-E], and CFU-E) and T-lymphocyte colony forming cells (CFU-TL) obtained from normal human BM and peripheral blood (PB). Our data show that CdA inhibits these progenitors as well as CFU-TL colony formation in a dose-dependent manner. A much higher dose of CdA was required for maximal inhibition of CFU-TL colony formation than the dose required for an inhibition of myeloid progenitor cell proliferation. These results have led us to suggest a new therapeutic potential for this drug in myeloid leukemia.

MATERIALS AND METHODS

2-CdA. 2-CdA was synthesized as previously published by Carson et al and Cardinauld and was supplied by Dr E. Beutler (Scripps Clinic, La Jolla, CA). The lyophilized substance (molecular weight [MW] 271) was dissolved in 0.9% sodium chloride. CdA was added to cell culture systems at increasing doses ranging from 5 nmol/L to 1,280 nmol/L final concentration.

Specimens. Human BM cells were obtained with informed consent from patients undergoing hematologic assessment who did not display any hematologic disorder. Approximately 10 to 20 mL of BM was aspirated from the iliac crest and collected into syringes containing preservative-free heparin. Normal PB was taken from healthy volunteers.

Preparation of cells. Human BM cells were layered over Lymphoprep (density 1.077 g/mL; Nyegaard, Oslo, Norway). Mononuclear cells were depleted of adherent cells and T lymphocytes by plastic adherence and erythrocyte-rosetting technique, respectively, as previously described. Briefly, cell suspensions were incubated in Petri dishes (100 × 20 mm; No. 1005, Falcon Labware, Oxnard, CA) at a concentration of 1 × 10^6 cells/mL for 30

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minutes. Nonadherent cells were gently decanted and incubated for a further 90 minutes. After the second incubation period, nonadherent cells were depleted of T lymphocytes by the erythrocyte-rosetting technique, using neuraminidase-treated sheep erythrocytes. Cells were incubated with equal volumes of 1% sheep red blood cells (RBCs) for 15 minutes at room temperature and centrifuged at 1,000 rpm for 10 minutes. The pellet was kept at 4°C overnight. After resuspension, the cells were gently layered over Lymphoprep and centrifuged at 1,600 rpm for 30 minutes. Interphase cells were washed three times in Hanks Balanced Salt Solution (HBSS) and were finally resuspended in Iscove’s medium containing 20% fetal calf serum (FCS). This cell population contained less than 1% monocytes and 3% T lymphocytes.

**Clonal progenitor assays.** Erythroproietic (CFU-E, BFU-E) and granulopoietic (CFU-GM) progenitors were assessed as previously described. Briefly, depleted BM mononuclear cells were plated at a final concentration of 2.5 x 10^5 cells/dish (1.1 mL culture medium per dish) in 0.8% methylcellulose in Iscove’s medium containing 30% FCS, 10% bovine serum albumin (BSA), 1% 2-mercaptoethanol (2-Me) (1 x 10^-4 mol/L) and 200 mmol/L L-glutamine, using 3 U/mL recombinant human erythropoietin (Epo; Cilag, Vienna, Austria) and 10% of agar-stimulated human leukocyte-conditioned medium as stimulants. CdA was added in increasing amounts ranging from 5 nmol/L to 1,280 nmol/L (final concentrations). Each assay was set up in duplicate.

Colonies derived from late-stage erythroid progenitor cells (1 to 2 clusters) (CFU-E) and small erythroid bursts thought to be derived from mature subclasses of BFU-E (3 to 8 clusters) (mBFU-E) were scored under an inverted microscope after 12 days of incubation. Colonies derived from primitive subclasses of BFU-E (320 nmol/L) were again less sensitive than the more immature progenitors (CFU-GM, pBFU-E) (160 nmol/L) as well as colonies derived from granulocytic/macrophagic progenitor cells (CFU-GM) containing more than 50 cells were scored in the same assay dishes after 18 days of incubation. The percentage of growth inhibition was determined by the formula: percent inhibition = (number of colonies of control group – number of colonies of test group) x 100/number of colonies of control group.

**CFU-TL assay.** The culture method used for CFU-TL was a microagar culture system described previously. PB mononuclear cells were suspended in Iscove’s medium containing 20% FCS and 0.3% agar. Subsequently 250-µL aliquots of this suspension containing 2 x 10^5 PB mononuclear cells were plated in multwell tissue culture plates. The agar was allowed to solidify at room temperature and was then overlaid with 250 µL medium containing 0.5% phytohemagglutinin (PHA) and 0.5% 2-Me (1 x 10^-4 mol/L) (final concentrations). The cultures were incubated at 37°C in a fully humidified atmosphere containing 5% CO₂, and colonies were scored using an inverted microscope after 7 days of incubation.

**Time course studies of mononuclear cells.** Depleted BM mononuclear cells (1 x 10^5/µL), suspended in Iscove’s medium containing 20% FCS, were incubated with increasing doses of CdA ranging from 5 nmol/L to 1,280 nmol/L in a fully humidified atmosphere at 37°C and 5% CO₂ for 24, 48, and 72 hours. After incubation, cells were washed three times in HBSS, resuspended in Iscove’s medium containing 20% FCS, and cell viability was determined by dye exclusion test. Viability of mononuclear cells with and without CdA was over 90%. Cells were then grown without CdA in methylcellulose cultures as described above.

**RESULTS**

**Effect of CdA on BM myeloid progenitors.** To determine whether CdA could inhibit CSF- and Epo-stimulated proliferation of hematopoietic progenitor cells, monocyte- and T-lymphocyte-depleted normal human BM mononuclear cells were cultured with agar-stimulated human leukocyte-conditioned medium, Epo, and varying concentrations of CdA. As shown in Fig 1, growth factor-induced colony formation derived from granulo-macrophagic (CFU-GM) and erythroid progenitors (pBFU-E, mBFU-E, CFU-E) were inhibited by CdA in a dose-dependent manner. However, there was some difference in the sensitivity of the various progenitor cells to CdA. Thus, both CFU-GM and pBFU-E colony formation were already inhibited up to 40% at a CdA concentration of 10 nmol/L whereas for mBFU-E and CFU-E an inhibition of about 12% was noted. If the inhibitory effect of CdA was expressed as the IC50 value (a concentration required to inhibit 50% of colony growth) a significant difference in the sensitivity to CdA between pBFU-E and late-stage erythroid (CFU-E) was found (P < 0.05), indicating that the pBFU-E were the most sensitive cells to the action of CdA. The IC50 of CFU-GM was nearly identical to that of pBFU-E. An indirect effect mediated by residual monocytes (1%) and T lymphocytes (3%) present in our target cell population cannot be completely excluded. However, this seems unlikely because identical results were obtained whether unseparated or monocyte- and T-lymphocyte-depleted BM cells were used (data not shown).

When the dose necessary for 100% inhibition of colony formation by progenitor cells was compared, it was found that the CFU-E (320 nmol/L) were again less sensitive than the more immature progenitors (CFU-GM, pBFU-E) (160 nmol/L). These results suggest that the sensitivity of myeloid progenitors to CdA decreases with increasing maturation stage.

**Time course studies on BM myeloid progenitors.** Previous studies have shown that incubation of normal and malignant lymphocytes with CdA causes cell death after at least 48 hours as a consequence of various metabolic changes. Therefore, to determine whether this is the case with CdA-treated myelopoietic progenitor cells, time course studies were performed. T-lymphocyte- and monocyte-depleted BM mononuclear cells were incubated in liquid cultures with varying doses of CdA for 24, 48, and 72 hours. CdA was then removed by careful washing and the cells were seeded in the clonogenic assays. Our results show that a minimal precultivation period of 48 hours is required to show an inhibition of different subclasses of erythroid progenitors similar to that found in experiments in which CdA is continuously present in the culture (Fig 2).

**Effect of CdA on lymphoid colony formation (CFU-TL).** Numerous previous studies have shown that the viability of normal and malignant lymphocytes is affected by CdA in a dose-dependent manner. However, little is known about the ability of CdA to interfere with lymphocyte colony formation. Therefore, PB mononuclear cells were cultured with PHA and varying concentrations of CdA for 7 days.

As shown in Fig 3, the PHA-induced lymphocyte proliferation is inhibited by CdA in a dose-dependent manner. Moreover, our results show that for complete inhibition of CFU-TL colony growth a much higher CdA concentration (1,280 nmol/L) is required than for complete inhibition of immature progenitor cell (CFU-GM, pBFU-E) proliferation.
EFFECT OF 2-CHLORODEOXYADENOSINE 2585

Fig 1. Inhibitory effect of varying doses of CdA on colony formation derived from monocyte- and T-lymphocyte-depleted BM mononuclear cells (2.5 x 10^6 cells/dish). The results for CFU-GM, pBFU-E, mBFU-E, and CFU-E are expressed as the mean ± SEM of percent inhibition obtained from four experiments. The percent of growth inhibition was determined by the formula: percent inhibition = (number of colonies of control group - number of colonies of test group) x 100/number of colonies of control group.

DISCUSSION

We report here for the first time that CdA has a dose-dependent effect on all stages of erythroid progenitor cells (Fig 1). Moreover, our results show that the sensitivity of erythroid progenitors to CdA decreases with increasing maturation stage. Thus, as shown by box plots (Fig 4), the IC50s for pBFU-E, mBFU-E, and CFU-E were 19, 38, and 56 nmol/L, respectively. This different sensitivity to CdA between immature- and late-stage erythroid progenitors is also observed when colony growth of cells treated with the concentrations required for complete inhibition of colony formation is compared. Thus, in the continuous presence of CdA, pBFU-E and mBFU-E proliferation were completely suppressed at 160 nmol/L, whereas CFU-E required a two times higher concentration (320 nmol/L) of CdA for complete suppression (Fig 1).

A dose-dependent inhibitory effect was also found on CFU-GM colony growth (Fig 1). In contrast to a previous preliminary report, 5 our results show a marked inhibition of myeloid progenitor cells even at low concentrations of CdA (10 nmol/L) and compared with the data by Carson et al,4 about half of the CdA dose was required for a 50% and 100% inhibition. This discrepancy in the results may lie in the fact that because of a longer incubation time (18 days v 10 days) more immature CFU-GM are detected in our system. However, both data support our conclusion that more mature progenitor cells are less sensitive to CdA.

The 40% inhibition of CFU-GM and pBFU-E observed at 10 nmol/L of CdA (Fig 1) may be clinically important, because reported blood concentrations in patients treated with CdA vary between 10 and 20 nmol/L.56 Our results agree with the clinically observed decrease in the neutrophil count found during CdA treatment.7 The lack of a clinical effect on the RBC parameters such as hematocrit and hemoglobin level can possibly be explained by the fact that the late-stage erythroid progenitors (CFU-E) are relatively insensitive to the toxic action of CdA. The mature erythrocytes may even be less sensitive to CdA and their long lifetime may compensate for the transient inhibitory effect of CdA on BFU-E.

Our time course experiments on myeloid progenitors show that a minimal exposure time of 48 hours is required for a maximal inhibition of pBFU-E and CFU-GM colony formation. These results suggest, therefore, that, as in lymphocytes, a series of metabolic events takes place when myeloid progenitors are treated with CdA.

While it is well established that CdA affects lymphocyte viability,5,8,18 there has been no information available on the effects of CdA on CFU-TL. In this report we show that CdA also inhibits CFU-TL colony formation in a dose-dependent manner. It is noteworthy that the optimal doses required to inhibit CFU-TL colony formation were even higher than that to inhibit the mature erythroid progenitor cells. We therefore conclude that the more mature the colony-forming cells are, the higher is the dose of CdA required for inhibition. However, the discrepancy between previously reported viability studies for lymphocytes and the results on CFU-TL presented here require an explanation. A similar discrepancy has also been reported by Aye and Dunne in CFU-TL studies with dCF, a high-affinity
Fig 2. Inhibition of proliferation of monocytedand T-lymphocyte-depleted BM mononuclear cells as a function of time. Cells were incubated with varying doses of CdA for 24 (□), 48 (○), or 72 (■) hours, then washed and plated without CdA in methylcellulose at a concentration of $2.5 \times 10^6$ cells/dish (1.1 mL). Results are the mean ± SEM of percent inhibition obtained from five experiments.

Fig 3. Percent inhibition of T-lymphocyte colony formation evaluated after 7 days of incubation. Mononuclear cells from PB were seeded at a concentration of $2 \times 10^6$ in the presence and absence of varying doses of CdA. Results are the mean ± SEM of percent inhibition obtained from five experiments.

Fig 4. Comparison of the IC50 (concentration required to inhibit 50% of colony growth) of CFU-GM, pBFU-E, mBFU-E, and CFU-E. IC50s were deduced graphically from interpolated dose-response curves obtained from four independent experiments each performed in duplicate. IC50s of CFU-GM and pBFU-E (*) are significantly different compared with the IC50 of CFU-E ($P < .05$, Wilcoxon-Mann-Whitney test).
adenoSine deaminase inhibitor. The most likely reason for these differences may well lie in the fact that the response of lymphocytes to CDA and dCF may vary according to the proliferative status. Thus, because the CFU-TL assay probably does not detect the true progenitor cell for T lymphocytes, this assay may not accurately reflect the proliferation-regulatory mechanisms prevailing in vivo.

These studies also raise some interesting questions concerning the mechanism of the CDA action in vivo. Active phosphorlation of CDA, mediated by deoxycytidine kinase, has been reported to be essential for CDA toxicity in lymphocytes, because T and B lymphoblasts deficient of this enzyme are resistant to the cytotoxic action of CDA. Because levels of deoxycytidine kinase are high in lymphocytes but low in granulocytes it was suggested that lymphoid cells are particularly sensitive to the action of CDA. We have found that myeloid progenitors are also markedly inhibited by CDA, suggesting that there is a high level of deoxycytidine kinase in these cells as well. However, this is not supported by the observation that only lymphopenia occurs in the genetic ADA deficiency disease. Moreover, in vitro studies have shown that the action of dCF, which mimics an ADA deficiency, does not affect myeloid colony growth even at concentrations at least 2 magnitudes higher than that necessary to totally ablate intracellular ADA levels.

However, as shown in our studies, the deoxyadenosine analog CDA, resistant to ADA activity, does produce a marked inhibition of proliferation of immature progenitor cells, even at low concentrations (10 to 20 nmol/L), commonly detected in the plasma of CDA-treated patients. If levels of deoxycytidine kinase are not only low in granulocytes but also in myeloid progenitors, treatment with CDA would result in low accumulation of cytotoxic cdATP and would therefore not completely explain the marked cytotoxic effects observed. Other factors such as modulation of the effect of CDA by the ambient levels of other deoxynucleosides might influence the apparent sensitivity of myeloid cells, while this might not be the case for ADA deficiency.

Further studies will be necessary to show whether CDA could be considered for therapy not only in lymphoid but also in myeloid malignancies such as chronic myeloid leukemia and acute myeloid leukemia. This possibility is strengthened by a previous observation that a patient with acute granulocytic leukemia resistant to all chemotherapy showed a marked cytoreduction and gradual recovery after treatment with CDA.

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