Studies on the Effect of Deoxyadenosine on Deoxycoformycin-Treated Myeloid and Lymphoid Stem Cells

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Adenosine deaminase (ADA) deficiency has been reported in association with severe combined immunodeficiency disease (SCID). The mechanism by which ADA deficiency causes immune dysfunction has been investigated in model systems to which the ADA inhibitor deoxycoformycin (dCf) had been added. Previously, we demonstrated that dCf did not prevent proliferation and differentiation of myeloid and lymphoid stem cells. We have now shown that addition of deoxyadenosine to dCf-containing cultures inhibited proliferation of hemopoietic stem cells. This inhibition was, however, equally effective for both normal myeloid and lymphoid stem cells. These findings suggest that other differences may exist between SCID myeloid and lymphoid stem cells to account for the relative sparing of myelopoiesis in SCID patients.

THE FINDING by Giblett et al.¹ that some patients with severe combined immunodeficiency (SCID) have an associated genetic deficiency of adenosine deaminase (adenosine aminohydrolase, E.C.3.5.4.4.) (ADA) has aroused considerable interest in the role of ADA in immune function. ADA is an enzyme in the purine salvage pathway responsible for the deamination of adenosine and deoxyadenosine (dAdo) to inosine and deoxyinosine, respectively. Recent investigations have shown that in the absence of ADA activity, the excess deoxyadenosine is phosphorylated to its respective nucleotide.² It has been speculated that the resulting increase in deoxyATP (dATP) levels, by inhibiting ribonucleotide reductase and thereby DNA synthesis, may be responsible for interfering with cellular proliferation in lymphocyte populations.³ In support of this view, elevated levels of deoxyadenosine and dATP have been detected in cells from patients with SCID.⁴ ADA, however, is a ubiquitous enzyme present in all tissues and in ADA deficiency, although the enzyme is markedly reduced in all cells, the pathology is largely limited to the immune system.⁵ However, myeloid differentiation, a process dependent on cellular proliferation, is not affected by this enzyme deficiency, and in most instances, hemopoiesis proceeds normally.⁶

Similar differences in the behavior of ADA-deficient leukemic and normal myeloid cells have also been observed. Marked elevations of ADA have been detected in blast cells from patients with T-cell acute lymphoblastic leukemia, and a positive correlation has been demonstrated between elevated ADA and terminal deoxynucleotidyl transferase (Tdt) levels in these patients.⁷ The ADA inhibitor deoxycoformycin (Pentostatin) has in some cases been shown to be highly effective in eliminating leukemic blasts from the circulation;⁸ however, when tested in vitro, this drug was found unable to inhibit proliferation of normal myeloid and T-lymphocyte colony-forming cells.⁹ These findings indicate that the effects of ADA deficiency may vary from cell population to population and suggest the possibility that such deficiency may be lineage related. This discrepancy in the effect of ADA deficiency on different cell populations has prompted us to investigate the role of this enzyme in hemopoietic cell differentiation.

2'-Deoxycoformycin (dCf), an antibiotic nucleoside analog obtained from the culture broth of Streptomyces antibioticus, is a highly potent and specific inhibitor of ADA with a Kᵰ of approximately 2.5 x 10⁻¹² M. While we found that dCf had no effect on the ability of myeloid and T-lymphocyte colony-forming cells to proliferate in culture,⁹ others have reported that proliferation of ADA-inhibited lymphocytes or of lymphoid cell lines could be prevented by micromolar quantities of deoxyadenosine.¹¹ We report our studies on the effect of deoxyadenosine on ADA-inhibited myeloid and T-lymphocyte colony-forming cells. The results indicate that, under these conditions, deoxyadenosine is equally toxic for both populations of colony-forming cells.

MATERIALS AND METHODS

Marrow was aspirated from the sternum or iliac crest of patients undergoing routine hematologic investigations. Peripheral blood was obtained from normal volunteers. All samples were collected in heparin or ethylene bis (ethylene oxynitrito) tetraacetic acid (EGTA) and mononuclear cells obtained by centrifugation on a cushion of 60% Percoll (Pharmacia, Piscataway, N.J.) as previously described.¹²
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2′-Deoxycoformycin (Pentostatin) was donated by Dr. M. L. Black of Warner-Lambert, Parke Davis Company, Ann Arbor, Mich. It was dissolved in tissue culture medium at a concentration of 10 mM/liter and was greater than 95% pure as determined by ultraviolet spectrophotometry. Its activity was confirmed, using differential spectrophotometry, by demonstrating its ability to inhibit the ADA (partially purified from calf intestine, Boehringer Mannheim) catalyzed conversion of adenosine to inosine. Deoxyadenosine (Sigma, St. Louis, Mo.) was dissolved in tissue culture medium at a concentration of 10 mM and diluted to required concentrations before use.

Erythroid and granulocytic colony growth was obtained using methods previously described.15 T-lymphocyte colony-forming cells in marrow and blood were assayed using a method recently developed in this laboratory and to be described in a forthcoming paper.14 Briefly, mononuclear cells were prepared for plating in 3-ml aliquots, each containing 0.4 ml human plasma, 0.3 ml of a 10% bovine serum albumin solution, 1 ml of 1% agar (Difco, Detroit, Mich.) made up in medium, 0.3 ml of appropriately diluted cells, and the balance made up with medium. The cultures also contained 5% leukocyte conditioned medium (LCM), 0.1% phytohemagglutinin (PHA; Burrough Wellcome, HA15), and 100 ng/ml 12-0-tetradecanoyl phorbol 13-acetate (TPA; Consolidated Midland Chemicals, Brewster, N.Y.). The tissue culture medium used in these experiments was modified Dulbecco’s Minimal Essential Medium (GIBCO, H21, Grand Island, N.Y.) prepared according to a method described by Guilbert and Iscove.15 LCM was prepared using 1% PHA as previously described.

After 7 days of culture at 37°C in a 5% CO2 humidified incubator, colonies containing in excess of 50 cells were counted. The numbers of colonies in these cultures were found to be linearly related to the numbers of cells cultured, thus allowing it to be used as a quantitative assay. Using G6PD isoenzymes to assess clonality, Singar et al.16 have shown that T-lymphocyte colonies obtained in the presence of TPA and PHA are of unicellular origin. The T-lymphocyte nature of the cells in our colonies have been confirmed by rosetting with sheep red blood cells and by surface membrane immunofluorescence with the monoclonal antibodies OKT3, OKT4, and OKT8.

Adenosine deaminase was measured by the spectrophotometric method of Kalckar,17 in which the decrease in absorbance at 265 nm is used to measure the conversion of adenosine to inosine. It was also assayed by measuring the conversion of 14C-adenosine to 14C-inosine (using descending chromatography on DE-81 paper to separate the two nucleosides) as described by Coleman and Hutton.18

RESULTS

To determine the effects of dCf and deoxyadenosine on myeloid colony formation, Percoll-separated marrow cells were culture at 2 x 10^5/ml with epo 1 U/ml, 5% LCM, and varying concentration of these compounds. The results are shown in Fig. 1 and 2. It will be seen that, as previously described, dCf by itself, even at a concentration of 1 mM, had little or no effect on erythroid or granulocytic growth. Deoxyadenosine, however, gave different results. While at up to 10 μM there was little or no effect on myeloid colony growth, at concentrations beyond 100 μM there was a definite decrease in colony numbers such that at 1 mM colony growth was consistently less than 10% of controls in all marrow samples tested. When the dose–response curve of deoxyadenosine was repeated in the presence of a fixed concentration of dCf (1 or 5 μM), a marked increase in the sensitivity of the cells to deoxyadenosine was observed. While there was some variation from marrow to marrow, in most instances, 20 μM deoxyadenosine, in the presence of 1 μM dCf, was sufficient to reduce colony counts to less than 20% of controls, and in all instances, 50 μM deoxyadenosine totally suppressed growth. Results obtained using 5 μM dCf were not significantly different from those obtained with 1 μM dCf. These results are representative of 10 different experiments with human marrow.

In similar experiments, the effects of dCf and deoxyadenosine on T-lymphocyte colony formation was determined by culturing cells in the presence of TPA, PHA, and LCM (see Materials and Methods).
Results, shown in Fig. 3, are not markedly different from that observed for myeloid colony formation. It can be seen that inhibition of ADA with 1 μM dCf resulted in a marked increase in the sensitivity of the cells to deoxyadenosine, and complete abrogation of colony growth was obtained with 1 μM dCf and 50 μM deoxycytidine. Table 1 gives a comparison of the effects of deoxyadenosine on ADA-inhibited myeloid and T-lymphocyte colony-forming cells. These results, representative of 3 different experiments, show no significant differences in the response of myeloid and T-lymphocyte colony-forming cells to deoxyadenosine.

Confirmation of the absence of ADA activity in dCf-treated cultures was obtained by measuring its level in pooled colonies obtained from cells grown in the presence or absence of dCf. The results are shown in Table 2. The level of ADA in cultures containing dCf were undetectable, even with the radioisotopic technique. Table 2 also shows that cell extracts from dCf-containing cultures were not able to inhibit normal ADA activity, thus discounting the possibility that lack of activity was due to carry-over of dCf from the cultures.

**DISCUSSION**

While the association of ADA deficiency and SCID is clear, the exact mechanism by which this enzymatic defect causes immunodeficiency is not known. The demonstration by Green and Chan16 that addition of adenosine to established cell lines of fibroblastic or lymphoid origin results in accumulation of intracellular adenine nucleotides, pyrimidine starvation, and cell death led to speculations that a similar mechanism may be responsible for the immunodeficiency in SCID patients. Further investigations, however, revealed that these patients have markedly elevated levels of deoxyadenosine in their serum and urine and of dATP in their erythrocytes, marrow cells, and lymphocytes. These metabolic consequences of ADA deficiency are thought to be due to increased salvage of deoxyadenosine and its subsequent phosphorylation to dATP, a known feedback inhibitor of ribonucleotide reductase.3 These findings have therefore led to the hypothesis that immunodeficiency in SCID is probably due to dATP-mediated inhibition of ribonucleotide reductase, resulting in deoxypurine starvation (particularly deoxycytidine) and cell death.

The data in this article show that proliferation of ADA-inhibited myeloid and T-lymphocyte colony-forming cells can be inhibited by micromolar quantities of deoxyadenosine. Since the cultures for both
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myeloid and lymphoid colony-forming cells contained LCM (i.e., culture supernatants from PHA-stimulated peripheral blood mononuclear cells), it seems unlikely that the combined effects of dCf and dAdo were mediated by inhibition of T-cell function. Further, we have reported that T cells are not required for myeloid colony formation. However, it is of interest that the combined effects of dCf and dAdo were not sufficient to inhibit proliferation. Our results are in agreement with those reported by others showing that inhibition of growth in cultures of lymphoid cell lines or mitogen-stimulated lymphocytes required not only dCf but deoxyadenosine as well. These data indicate that while ADA deficiency per se is without any effect on cellular proliferation, the provision of excess substrate in the form of deoxyadenosine proves lethal for such enzyme-inhibited cells. Since our dCf-treated colony assays are exquisitely sensitive to added deoxyadenosine, it is possible that, in the cultures containing dCf alone, there was insufficient accumulation of endogenously produced deoxyadenosine, either in the culture medium or intracellularly, to inhibit cellular proliferation.

Our results showing that deoxyadenosine is equally toxic for ADA-inhibited marrow and lymphoid cells are, however, more difficult to explain. We had expected that, based on clinical findings in SCID, ADA-inhibited lymphoid cells would have been more susceptible than myeloid cells to growth inhibition by deoxyadenosine. It has been shown that deoxyadenosine-mediated killing of ADA-inhibited cells is accompanied by marked elevations of intracellular dATP. Thus, if dATP is the toxic metabolite, our results would suggest that normal populations of myeloid and lymphoid cells are equally susceptible to growth inhibition by this nucleotide. Others have shown that incubation of PHA-stimulated lymphocytes in dCF lead to intracellular accumulation of dATP. However, although it is possible to measure dATP in short-term liquid cultures of cells, we have found it impossible to measure it in our colony assays, which had been initiated with dCf and dAdo, since no viable cells could be detected in these plates (only cellular debris remain). Of significance, our data also show that myeloid and lymphoid proliferation and differentiation can occur in the absence of detectable intracellular ADA activity. Further, since the enzyme levels were measured in cells several generations removed from the colony-forming cell, our data provide evidence that the dCF added at the start is still active at the end of the culture period (7 days for T-lymphocyte cultures and 14 days for myeloid cultures).

In genetic ADA deficiency, although both myeloid and lymphoid cells have been shown to have high levels of dATP, myelopoiesis remains apparently normal while lymphopoiesis is not. This suggests a difference in the effect of dATP on SCID myeloid and lymphoid cell proliferation. If indeed the dATP levels detected in SCID lymphoid and myeloid cells are sufficient to inhibit proliferation, then our findings would indicate that myeloid cells in these patients are protected from the toxic effects of dATP by unknown mechanisms. An alternative possibility is that the observed dATP levels although not sufficient to inhibit normal cellular proliferation, are toxic for SCID lymphocytes either due to increased sensitivity of their ribonucleotide reductase or to effects of dAPT on cellular processes other than proliferation.

In conclusion, our findings that normal myeloid and lymphoid stem cells treated with dCF were equally susceptible to the inhibitory effects of deoxyadenosine suggest that the differential effects of genetic ADA deficiency on myeloid and lymphoid cells are not lineage related but may in fact be a reflection of the disease process itself. They further indicate that other metabolic defects, not occurring in other regenerative cell systems or in dCF-treated normal cells, may be additionally responsible for the lymphocyte abnormalities seen in genetic ADA deficiency.

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