Clinical Response to Deoxycoformycin in Chronic Lymphoid Neoplasms and Biochemical Changes in Circulating Malignant Cells In Vivo

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Deoxycoformycin (DCF), an adenosine deaminase (ADA) inhibitor, has been shown to be active in lymphoid neoplasms. The mechanism of cytotoxicity might involve accumulation of deoxyadenosine triphosphate (dATP), depletion of the nicotinamide adenine dinucleotide (NAD) and ATP pool, induction of double-stranded DNA strand breaks, or inhibition of S-adenosyl homocysteine hydrolase (SAH-hydrolase). We have investigated the biochemical changes in the circulating malignant cells of patients with chronic leukemia/lymphoma who were treated with DCF (4 mg/m² weekly). Blood samples were taken from 17 patients with 60% or more circulating leukemic cells before, 4, 24, and 48 hours and five days after the first administration of DCF. Leukemic cells were separated and studied for changes in ADA, dATP, ATP, NAD, and SAH-hydrolase levels and DNA strand breaks and the data analyzed according to clinical response. Inhibition of ADA activity was found in all except one patient at 4 to 24 hours after the first administration of DCF. dATP started to accumulate at four hours, reached a maximum level between 24 and 48 hours, and returned to base values on the fifth day. Intracellular ATP and NAD levels were transiently reduced in some of the patients. However, no correlation between these changes and a clinical response could be found. DNA strand breaks could be studied in 13 patients. A significant increase in DNA breaks at 24 to 48 hours was found in six of the seven responders but only in one of the six nonresponders. At 24 hours, SAH-hydrolase levels were reduced in all seven responders studied, but only in two of the seven nonresponders. The difference in inhibition of SAH-hydrolase was statistically significant (P = .0023). These results suggest that DNA strand breaks and inhibition of SAH-hydrolase correlate with clinical response.

The vital role of adenosine deaminase (ADA) in the differentiation of T and B lymphocytes has been shown by the discovery that the genetic absence of this enzyme leads to a fatal severe combined immunodeficiency disease. ADA catalyzes the deamination of adenosine and deoxyadenosine to inosine and deoxyinosine and is widely distributed in different tissues. Nevertheless, inherited deficiency of ADA leads to selective impairment of lymphoid development. This observation has stimulated interest in the pharmacologic inhibition of ADA as specific cytotoxic therapy for lymphoproliferative diseases.

2'-Deoxycoformycin (DCF) is a potent tight-binding inhibitor of ADA and has been shown in phase I and phase II studies to be active in lymphoid neoplasms. The mechanism of lymphocytotoxicity is still controversial. Inhibition of ADA leads to accumulation of 2'-deoxyadenosine, which is rapidly metabolized by the high activity of deoxyadenosine kinase of the lymphocytes into the corresponding triphosphate (dATP). In dividing lymphoid cells, cytotoxicity is believed to be mediated by dATP inhibition of ribonucleotide reductase, which results in the starvation of the other three DNA precursor deoxynucleotide triphosphates needed for DNA synthesis. The successful use of DCF in patients with acute T-cell lymphoblastic leukemia characterized by high ADA activity seems to support this hypothesis. However, DCF has been found to be active in chronic T- or B-cell lymphocytic leukemia (T-, B-CLL) and in hairy cell leukemia (HCL). The leukemic cells of these diseases typically have a low proliferative rate and concentrations of ADA that can be inhibited by small amounts of the drug. The cytotoxic mechanism of DCF in nonproliferating cells is not understood. Mechanisms proposed include ATP depletion, inhibition of RNA synthesis, interference with the synthesis and processing of polyadenylated RNA (poly-A), incorporation of DCF into DNA, induction of DNA strand breaks, depletion of cellular nicotinamide adenine dinucleotide (NAD), and inactivation of S-adenosyl-homocysteine hydrolase (SAH).

In a phase II trial of the European Organization for Research in Treatment of Cancer (EORTC), the activity of DCF in chronic T- or B-cell neoplasms is being investigated. This clinical trial offers an opportunity to investigate the biochemical changes in the circulating malignant cells from patients with high leukemic counts after treatment with DCF. Changes in intracellular concentrations of ADA, dATP, ATP, NAD, and SAH-hydrolase and DNA strand breaks were studied before and at defined intervals after the first administration of DCF. The data were then analyzed according to the clinical response to DCF therapy. Our aim was to investigate whether any of the studied parameters correlated with response and to define the mechanism of cytotoxicity.

MATERIALS AND METHODS

Patients. The patients included in this study were treated with DCF according to an EORTC protocol. Patients with Sézary syndrome (SS), T-CLL, and prolymphocytic leukemia (PLL) were eligible for this protocol as initial or salvage therapy after failure of...
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one chemotherapy. Patients with B-CLL and HCL were eligible after the failure of one to three trials of conventional therapy. They should have a World Health Organization performance status of <2, normal renal and hepatic function, and no signs of active infection. Informed consent was obtained from all patients for the clinical trial and for the biochemical studies of their leukemic cells. Among the patients entered for the clinical trial, only 17 patients with high WBC counts and high percentages of circulating malignant cells (>60%) who were treated at Heidelberg were selected for this study of cellular biochemical changes. There were four patients with B-CLL, four with PLL (two T cell and two B cell), four with SS, four with HCL, and one with T-CLL.

**DCF treatment.** The clinical study protocol has been approved by the Protocol Review Committees of the EORTC and the National Cancer Institute (NCI). DCF was supplied by the Investigational Drug Branch, NCI, Bethesda, MD. The drug was administered at a dosage of 4 mg/m² as an intravenous (IV) push once every week for the first 3 weeks and then at the same dosage once every other week for the next 6 weeks. Those patients achieving at least a partial response (PR) were administered monthly injections of DCF as maintenance therapy. Clinical response to DCF was evaluated at the end of the tenth week. Complete remission (CR) was defined as the complete disappearance of all evidence of disease, (b) normal phenotypic findings of peripheral lymphocytes, (c) hemoglobin level > 12 g/dL, and (d) platelet count >100 x 10⁹/L. PR was defined as a reduction in the leukemic lymphocyte count by 50% or more in the peripheral blood and in the bone marrow for at least 4 weeks. In addition, the following criteria should be met (if corresponding pathologic findings were present before treatment): >50% reduction in the number or size of skin lesions, reduction by >50% of the sum of products of the perpendicular diameter of enlarged nodes, regression of hepatosplenomegaly by >50%, and an increase in hemoglobin concentration, platelet count, or granulocyte count by >50% of the deviation from normal or pretreatment values. Progression is defined as an increase by more than 25% in any of the following: leukemic lymphocyte count, the sum of the products of the diameters of enlarged nodes, hepatosplenomegaly, and the severity of symptoms. For analysis of the biochemical data, patients with CR and PR at the end of 10 weeks were classified as responders and those with less than PR and with progressive disease as nonresponders.

**Cell samples.** In each patient, blood samples were taken before, 4, 24, and 48 hours, and 5 days after the first administration of DCF. The mononuclear cells were separated by means of a Ficoll-Isoaque density gradient. After separation, only cell samples with more than 80% malignant leukemic cells (as indicated by morphology, monoclonal surface membrane immunoglobulin (SMIg), or expression of the corresponding monoclonal differentiation antigens) were used for the biochemical analysis. The WBC and differential counts of most patients did not change significantly during the first three days. Viability of the cell preparations was always more than 95% as revealed by the trypan blue (0.2%) dye exclusion test. The leukemic cells were then studied for changes in ADA, dATP, ATP, NAD, and SAH-hydrolase levels and DNA strand breaks.

**Enzyme assays.** The enzymes ADA and SAH-hydrolase were assayed in cell extracts. The ADA assay has been described in detail elsewhere.⁹⁰ The ADA concentration was measured in a mixture containing ³¹H-adenosine (1 mmol/L, 0.6 μCi) and 10 μL of cell extract (in 5 mmol/L potassium phosphate buffer with 1 mmol/L β-mercaptoethanol, pH 7.4) in a final volume of 50 μL. The conversion of adenosine to inosine and hypoxanthine was measured by thin-layer chromatography. ADA activity was expressed in units per 10⁹ cells, where 1 unit = 1 μmol substrate converted per hour. SAH-hydrolase activity was measured in the direction of synthesis of adenosyl-homocysteine (AdoHcy) by using a modification of the method described by Kredich and Hershfield.⁹¹ L-Homocysteine was prepared before use by the alkaline hydrolysis of l-homocysteine thiolactone (Sigma Chemical Co, St Louis). The reaction mixture consisted of 25 mmol/L potassium phosphate, pH 7.0, 1 mmol/L Na₂EDTA, 1 mmol/L dithiothreitol, 0.01 mmol/L DCF, 100 to 200 μmol/L 8⁴⁻C-adenosine (15 to 20 μCi/mmol), and 5 mmol/L L-homocysteine. Ten microliters of cell extract was added to 40 μL of reaction mixture and incubated for 15 minutes. The reaction was stopped by adding 10 μL of 2 N formic acid. Ten-microliter aliquots were pipetted onto cellulose thin-layer plates to which unlabeled markers (adenosine, inosine, and AdoHcy) had been applied at the origin and dried under a stream of cold air. Chromatograms were developed in butanol-1-methanol/H₂O/NH₄OH (60:20:20:1 vol/vol) and dried. The spots were identified under UV light, cut out, and counted in 10 mL of toluene-based scintillation fluid. One unit of homocysteine is defined as that converting 1 pmol of adenosine to AdoHcy per minute. Values represent the mean of duplicate assays.

**Assays of dATP, ATP, and NAD.** The intracellular concentration of dATP was measured by using a DNA polymerase assay as previously described.⁹² After separation, the leukemic cells were washed in phosphate-buffered saline (pH 7.4), and 1 mL of 60% methanol was added to the cell pellet, which was then stored at −20°C overnight to extract the nucleotides. The supernatant was freeze-dried and reconstituted in appropriate volumes of water. Assays were carried out for 35 minutes at 37°C by using polydeoxynucleotide-thymidylicate (Poly-d(A-T)) as the template and H-thymidine triphosphate (dTTP) as the radiolabeled nucleotide, Micrococcus luteus DNA polymerase, and known amounts of dATP for standard curves. Results were expressed as picomoles dATP per 10⁹ cells.

NAD and ATP were extracted from the cell pellet with 0.5 mol/L perchloric acid; this was followed by neutralization with 0.5 mol/L potassium hydroxide. NAD concentrations were measured by using the enzymatic assay of Jacobson and Jacobson.⁹³ To a 1-mL reaction mixture, 0.2 mL of either NAD standards or cell extract and 0.1 mL alcohol dehydrogenase (16 units) was added and incubated in the dark for 20 minutes at 37°C. The reaction was stopped by adding 0.5 mL of 12 mol/L β-methiodate. Aliquots of 0.2 mL of each tube were transferred into a 96-well microtiter plate and the absorbance measured in an automatic scanner (Titertek Multiscan, Liebyen, Norway). Standards and extracts were assayed in duplicate with suitable dilutions whenever the NAD levels were expected to be high. ATP measurements were performed by Dr R. Hutton (Royal Free Hospital, London) using an LKB (Stockholm) assay kit on neutralized perchloric acid cell extracts.

**DNA strand breaks.** A rapid fluorometric method for analysis of DNA unwinding in alkaline solution (FAU) was used as described by Birnboim and Javcak.⁹⁴ The leukemic cell samples were washed with phosphate-buffered saline and resuspended in a buffer containing 0.25 mol/L meso-inositol, 10 mmol/L sodium phosphate, and 1 mmol/L MgCl₂ (pH 7.2). Cell lysis and chromatin disruption were achieved by a solution with 9 mol/L urea, 10 mmol/L NaOH, 2.5 mol/L cyclohexane-diaminetetraacetic acid, and 0.1% sodium dodecyl sulphate; this was followed by exposure to an alkaline urea detergent (final pH, 11.8) for one hour at 15°C to allow partial unwinding of the DNA. Denaturation was stopped by chilling to 0°C and adding 0.4 mL of 1 mol/L glucose plus 14 mmol/L mercaptoethanol. Ethidium bromide, 6.7 μg/mL in 13.3 mmol/L NaOH, was added to each sample. The relative fluorescence intensity was measured in a spectrofluorometer (excitation, 520 nm; analyzer, 590 nm). Damaged DNA unwinds more rapidly under these conditions, and binding of ethidium bromide reflects the amount of double-stranded DNA (ds-DNA) in the sample. Each point was based on three sets of measurements.

**Statistical analysis.** The differences in changes in DNA strand breaks and SAH-hydrolase activity on DCF treatment were ana-
RESULTS

Clinical responses. For the purpose of this study, clinical response was evaluated at the end of the tenth week of treatment according to the criteria mentioned in Materials and Methods. Nine of the 17 patients achieved a partial remission (PR): two of the four with B-CLL, two of the four with PLL, two of the four with SS, and three of the four with HCL. The other patients had progressive disease with the exception of one (♀) who achieved stable disease. One (♂) of the three patients with HCL who achieved a PR went subsequently into complete remission upon further treatment with the maintenance therapy. In Figs 1 through 5, the same symbol was used throughout for each individual patient.

ADA activity. The pretreatment ADA activities in the leukemic cells ranged from 0.5 to 105.4 µmol/10^8 cells (Fig 1). B-CLL and HCL had the lowest activities, thus confirming our previous studies.36,37 After DCF treatment, a decrease in intracellular ADA activity was found at 4 to 24 hours. In most cases, the enzyme activity remained suppressed up to the fifth day. In one of the nonresponders with T-CLL whose pretreatment activity was the highest of all at 105.4 µmol/10^8 cells, ADA activity was reduced at four hours to 27.5 µmol/10^8 cells, but increased again to 63.2 µmol/10^8 cells at 24 hours. Thus, the ADA activity in this patient was not sufficiently inhibited. Apart from this patient, no difference in the suppression of ADA activity between responders and nonresponders was evident.

dATP levels. The dATP levels before DCF therapy ranged from 0.35 to 8.0 pmol/10^6 cells (Fig 2). Parallel to the suppression of ADA activity, an increase in intracellular dATP was found as early as four hours. This reached a maximum at 24 to 48 hours and returned to base values on the fifth day in most patients. Again no significant difference between responders and nonresponders could be established. It was interesting to note that the patient whose ADA concentration was not adequately inhibited by DCF showed no accumulation of dATP in the malignant cells.

NAD levels. The effect of DCF on the intracellular NAD levels was variable (Fig 3). The NAD levels ranged from 35 to 170 pmol/10^6 cells before treatment. Except in four patients with posttreatment levels of <10 pmol/10^6 cells, the decrease in NAD levels after therapy was not striking. Among the responders, the NAD levels decreased by more than 50% within 24 to 48 hours in five of the nine patients. However, a reduction of this order was also found in four of the seven nonresponders, and no difference in NAD depletion between responders and nonresponders was evident.
**ATP levels.** The pretreatment levels of ATP ranged from 2 to 98 nmol/10^6 cells. The variations in intracellular concentrations of ATP after DCF therapy could be studied in 13 patients. A decrease in ATP concentrations within four to 24 hours was observed in four of the seven responders and three of the six nonresponders, whereas an increase was found in two responders with HCL and no significant change in the other patients. Again no difference was found between responders and nonresponders.

**SAH-hydrolase activity.** The SAH-hydrolase activity of the leukemic cells ranged from 4.1 to 49 pmol/min/10^6 cells. As shown in Fig 4, higher base values of this enzyme were found in the nonresponders, although the differences were not significant. After the administration of DCF, SAH-hydrolase activity was suppressed at 24 hours in all the responders. Among the nonresponders, SAH-hydrolase activity was suppressed transiently at four hours in one patient, but increased to the base value at 24 hours. Suppression of SAH-hydrolase at 24 to 48 hours could be demonstrated in only two of the six nonresponders. In one nonresponding patient SAH-hydrolase activity increased from the untreated value at 4, 24, and 48 hours and returned to base concentration on the fifth day. The difference in inhibition of SAH-hydrolase activity at 24 hours was statistically significant (P = .0023).

**DNA strand breaks.** In the mononuclear cells of peripheral blood from normal controls (n = 13), the proportion of ds-DNA was 65% to 82%, with a median of 76%. In our hands, the percentage of ds-DNA in the same normal individual over a period of 2 months did not vary more than 10%. The cells of 13 patients with chronic leukemia tested tended to have more strand breaks than controls, the proportion of ds-DNA before DCF therapy ranging from 26% to 76%. There was a consistent decrease in ds-DNA in all the cells from the seven responders that was detectable in six of seven cases at 24 hours and in one only 48 hours after DCF therapy (Fig 5). A decrease by >10% in ds-DNA was found, albeit at 48 hours, only in one of the six nonresponders available for study, three showing no change at all at 24 or 48 hours and two very slight increases in breakage at 24 to 48 hours. The difference in percent increase of strand breaks between responders and nonresponders was significant (P < .0047).

When comparing the results from each individual patient, no correlation between the changes with DCF therapy in cellular concentrations of dATP, ATP, and NAD, the frequency of DNA strand breaks, and SAH-hydrolase activity was apparent.

#### DISCUSSION

Early experimental and clinical studies suggested that DCF was more active in cells of T lineage with high intracellular concentrations of ADA. However, at the dose levels used in the early clinical trials, DCF was associated in some patients with life-threatening side effects such as CNS disturbances; impairment of hepatic, renal, and respiratory functions; and acute hemolysis. The drug was then administered at dose levels of 0.1 to 1.0 mg/kg/d or 5 to 30 mg/m^2/d for one to five consecutive days. Safe dose schedules have been established in recent years. DCF has been shown to be active at low dose ranges (2 to 5 mg/m^2 once weekly or every other week) in chronic lymphoid neoplasms characterized by low concentrations of ADA and a low proliferative rate such as HCL.

Many studies have been performed to investigate the biochemical perturbations in the leukemic cells of acute lymphoblastic leukemia after treatment with DCF at high doses. It has been presumed that the cytotoxicity of DCF is mediated through the intracellular accumulation of deoxyadenosine and adenosine following ADA inhibition. Deoxyadenosine may be converted rapidly in lymphoid cells of dATP, which inhibits ribonucleotide reductase and DNA synthesis. The response of acute lymphoblastic leukemia indeed seemed to correlate with the accumulation of dATP. However, nondividing cells do not have detectable ribonucleotide reductase activity or replicative DNA synthesis. The cytotoxicity in the chronic leukemias is likely to involve different mechanisms.

Few in vivo data are available on the biochemical sequelae after the administration of DCF at the present dose range that is effective in nonreplicating leukemic cells. In this project, we have systematically studied the biochemical...
perturbations in the leukemic cells of patients with chronic T- or B-cell leukemia and have evaluated their relationship to clinical response within an EORTC phase II trial.

The present study has shown no correlation between suppression of ADA activity, dATP accumulation, ATP depletion, or NAD reduction with the subsequent clinical response to DCF in patients with slowly proliferating lymphoid malignancies. Following the first administration of 4 mg/m² of DCF, ADA was reduced in all patients except one. This one patient had an exceptionally high activity of ADA. No accumulation of dATP, inhibition of SAH-hydrolase, or DNA strand breaks could be found in her leukemic cells, and she did not respond to DCF. Therefore, “titration” of the drug might be necessary in some patients with high base concentrations of ADA, as indicated in some phase I studies.5,6 Other than in this patient, neither the extent nor the duration of ADA inhibition correlated with clinical response. The same was true for dATP accumulation. Based on observations in four patients with B-CLL, Begleiter et al22 also could not establish any correlations between changes in ADA or dATP and clinical response.

Profound depletion of ATP associated with dATP accumulation has been reported after in vitro or in vivo treatment with DCF.13,14 Bagnara and Hershfield suggested that deoxyadenosine formed by ADA inhibition would generate adenosine diphosphate (ADP) and adenosine monophosphate (AMP) by ATP-dependent phosphorylation.16 The accumulation of dATP, on the other hand, stimulates AMP deaminase and channels the AMP formed into inosine monophosphate (IMP), which in turn would be dephosphorylated by 5'-nucleotidase into inosine. This overall effect of coupling the degradation of AMP with the utilization of ATP leads to net ATP pool depletion and cell death. In our study, a transient decrease in ATP levels from four to 24 hours was found in four of seven responders and three of the six nonresponders with sufficient material for ATP investigation. Thus, reduction of ATP levels did occur in some of the patients with chronic leukemias in vivo but was probably not the mechanism leading to cell death. Johnston et al41 also showed that ATP depletion by leukemic cells in vivo after DCF treatment is not consistent.

Seto et al found a progressive and profound drop in intracellular NAD pools in normal peripheral lymphocytes incubated with DCF after detection of DNA strand breaks and before cell death.21 They proposed that an increase in DNA strand breaks may not only kill but also be the ultimate cause of cell death. In a previous study,42 we have shown that a decrease in NAD levels in treated cells could indeed be found in chronic leukemia cells incubated with DCF in vitro and ranged from 0% to 79% of untreated control values. However, no difference was observed between HCL and other chronic T or B leukemias even though HCL is considerably more sensitive to DCF treatment. The lack of correlation between NAD depletion and response to DCF was confirmed in the present in vivo study. Reduction in NAD levels was not striking and could be demonstrated equally in a few responders as well as in nonresponders.

The only differences between responders and nonresponders found were in the increase in DNA strand breaks and in the inhibition of SAH-hydrolase activity. The combination of DCF and deoxyadenosine has been shown in vitro to cause a significant increase of DNA strand breaks in mature human lymphocytes.20,23,43 Cohen and Thompson44 proposed that the DNA damage caused by DCF was the result of inhibition of repair rather than direct induction of strand breaks. Resting peripheral lymphocytes have a large number of spontaneous single strand breaks.44,45 After DCF treatment, the accumulation of large amounts of dATP might create an imbalance in the deoxynucleotide triphosphates, which then impairs the repair of DNA. With FADU in an alkaline medium, we have observed an increased background frequency of DNA strand breaks in CLL cells as compared with mononuclear preparations from normal controls. We also found a correlation, albeit in this small group of patients, between clinical response and the increase in DNA breaks in vivo after DCF administration. The exact mechanisms leading to the increase in strand breaks and the significance of these in causing cell death is unknown and clearly needs further study.

Inhibition of SAH-hydrolase was invariably found in the leukemic cells of the responders at 24 to 48 hours after DCF therapy. Hershfield, Kredich, and Johnston22,26,31 first suggested that inactivation of SAH-hydrolase might be a cytoxic mechanism induced by ADA inhibition. In a series of experiments performed on a WI-L2 lymphoblastoid cell line with adenosine kinase deficiency and unable to accumulate dATP, they showed that deoxyadenosine or adenosine caused “suicide-like” inactivation of SAH-hydrolase. In a few patients with T-acute lymphoblastic leukemia, inactivation of intracellular SAH-hydrolase in vivo has been shown to occur during treatment with DCF.27 Our study suggests that a correlation may exist between the extent of SAH inhibition and clinical response to low-dose DCF in the chronic leukemias. In addition, suppression of SAH-hydrolase preceded the detection of an increase in DNA breaks in two of the responders. Inhibitions of SAH-hydrolase could result in accumulation of SAH, a potent inhibitor of S-adenosylmethionine (SAM)-dependent methylation reactions affecting the synthesis and function of DNA and RNA. In vitro experiments suggest that interferon-α, which like DCF is effective in HCL and indolent T- or B-cell lymphomas, may also exert its effects by inhibition of methylation.46 Treatment of HeLa cells with α- or β-interferon resulted in a significant decrease in the SAM/SAH ratio. Thus, it is possible that the decrease in SAM/SAH ratio and the subsequent inhibition of methylation might be the common cytotoxic pathways of both interferon and DCF.

In a recent study of DCF therapy in mature T-cell malignancies, response was reported to correlate with a CD4+, CD8− phenotype (seven of ten patients), with no response in patients with a CD4+, CD8+ or CD4−, CD8+ phenotype.45 This observation has not, however, been confirmed by our present EORTC trial.48 Further studies are
clearly needed to establish the significance of different phenotypes or biochemical profiles in clinical response to DCF.

In conclusion, our study suggests that all the biochemical parameters studied the inhibition of SAH-hydrolase correlates best with clinical response to DCF. The subsequent suppression of methylation and repair of DNA strand breaks might be the ultimate cause of cytotoxicity in non-proliferating cells. Investigations of changes in the SAM/SAH ratio and methylation of RNA and DNA after DCF administration may give further evidence for this hypothesis and are underway.

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