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Effect Of 2',3'-Dideoxynucleosides On Proliferation And Differentiation Of Human Progenitor Cells.

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EFFECT OF 2',3'-DIDEOXYNUCLEOSIDES ON PROLIFERATION AND DIFFERENTIATION OF HUMAN PROGENITOR CELLS

by

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2', 3'-dideoxynucleosides (ddNs) have demonstrated clinical benefits in inhibition of HIV progression in AIDS patients, but the accompanying toxicity often limits their use for long-term treatment. These toxicities have been investigated using a variety of animal models and cell lines in which the mechanism(s) of toxicity have been characterized as being multifactorial. The mechanism(s) of these toxicities need to be more clearly defined so that alternative strategies can be developed to alleviate the toxic effects and thereby improve the therapy for AIDS patients. The following studies are presented in this dissertation and focus on the effects of various anti-HIV ddNs active toward proliferation and differentiation of relevant human progenitor cells, and the potential for alternative strategies involving protection and/or rescue of hematopoietic toxicity by hemin and a recombinant human hemoglobin.

These studies demonstrated that the effect of AZT on erythroid gene expression appears to be specific to the globin gene as erythroid-inducible non-globin genes were not affected by exposure to AZT, nor were heme biosynthetic enzyme activities. Investigations using a liquid culture of relevant CD34+ human progenitors demonstrated normal hematopoietic development of myeloid and erythroid lineages in response to selected hematopoietic growth factors, and various ddN treatment resulted in unique and selective

effects on lineage-specific differentiation antigen expression, indicating the validity of this culture system as a sensitive predictor of ddN-induced toxicity. Studies of the potential for hemin protection and/or rescue of AZT-induced toxicity indicated the ability of hemin to protect cells from toxic effects toward proliferation and differentiation and to rescue hemoglobin-synthesizing cells, while failing to rescue toxic effects toward proliferation.

These studies further suggested that the mechanism of action of AZT does not involve commitment of cells to differentiate along the erythroid lineage, but appears to inhibit erythroid maturation and/or globin synthesis. Studies of the effect of recombinant human hemoglobin indicated no toxicity in hematopoietic development and suggested an ability to protect progenitor cells from the AZT-induced toxicity toward proliferation, but suggested an inability to rescue progenitor cells pre-exposed to AZT.

Abstract Approved by: **Committee Chairman** _____

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DEDICATION

To Rhoades Marsh Dayton

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TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

APPENDICES

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF FIGURES

LIST OF FIGURES (Contunued)

LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
ALA	aminolevulinic acid
ALAD	aminolevulinic acid dehydrase
ALAS	aminolevulinic acid synthase
ALAS-E	aminolevulinic acid synthase erythroid
AMT	3'-amino-3'-deoxythymidine
ARC	AIDS related complex
AzdU	3'-azido-2',3'-dideoxyuridine
AzddMeC	3'-azido-2',3'-dideoxy-5-methylcytosine
AZT	3'-azido-3'-deoxythymidine
AZT-MP	AZT-monophosphate
AZT-TP	AZT-triphosphate
BFU-E	burst forming unit-erythroid
CFU	colony forming unit
CFU-E	colony forming unit-erythroid
CFU-GEMM	colony forming unit-granulocyte, erythroid, monocyte,macrophage
CFU-GM	colony forming unit-granulocyte,macrophage
d4T	2',3'-didehydro-3'-dideoxythymidine
ddC	2',3'-dideoxycytidine
ddN	dideoxynucleoside
ddU	dideoxyuridine
DMSO	dimethylsulfonate

ABBREVIATIONS (Continued)

DNA	deoxyribnucleic acid
EPO	erythropoietin
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FDA	Food and Drug Administration
FITC	fluoroscein isothiocyanate
FLT	3'-fluoro-3'-deoxythymidine
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte-macrophage colony stimulating factor
Hb	hemoglobin
HBSS	Hank's buffered salt serum
HIV	human immunodeficiency virus type I
HIV-RT	human immunodeficiency virus reverse transcriptase
HPLC	high performance liquid chromatograph
IC_{50}	50% inhibitory concentration
IL-3	interleukin 3
MACS	magnetic activated cell sorting
MAIDS	murine acquired immunodeficiency syndrome
MCV	mean cell volume
MNC	mononuclear cells
mRNA	messenger RNA
mt	mitochondrial
mtDNA	mitochondrial deoxyribonucleic acid
PBG-D	porphobilinogen deaminase
PBGD-E	porphobilinogen deaminase erythroid

PBS	phosphate buffered saline
PE	phycoerythrin
Uro-D	uroporphyrin dehydrase
rHB	recombinant hemoglobin
RNA	ribonucleic acid
SCF	stem cell factor
SIV	semian immunodeficiency virus
TTP	thymidine triphosphate

INTRODUCTION

The acquired immunodeficiency syndrome (AIDS) epidemic continues to be one of the major health concerns facing the world, second only to cancer in research grant funding. The concern for AIDS has generated a social and economic impact of such magnitude that concerns will likely continue to impact the health behavior of the world community for many years. Although no cure for the virus responsible for the common cold has been found and influenza epidemics are common, the AIDS epidemic has generated concern for similar or more deadly viral epidemics occurring, such as the Hanta virus outbreak of 1992 and the Ebola virus threat of 1989 and 1995. Vaccines have been successfully developed for some viral strains such as hepatitis B, but have been largely unsuccessful for most. While the origin of the AIDS virus is unclear, the causative agent of AIDS, a lentivirus called the human immunodeficiency virus (HIV), has been detected in frozen blood samples from patients dating as far back as 1959. According to the Centers for Disease Control and Prevention, 401,749 cases of AIDS have been reported in the United States through June, 1994, and cases diagnosed during the last three years have increased (53). Through education and improved anti-HIV and AIDS therapies, the fatality rate has decreased from greater than 90% to a case-fatality rate of 60.7%, and while this reflects the prolonged life of current AIDS patients, the prognosis remains fatal (7, 50). The development of a safe and effective vaccine for HIV seems unlikely in the near future, given the rapid mutation rate of HIV and the lack of a clearly defined pathology. Therefore, the need for long-term therapy is likely to remain, with concern for the associated toxicities of antiviral agents and the development of resistance.

The development of potential drugs for antiviral therapy relies on information concerning and understanding of the viral life cycle and pathogenic mechanisms. Differences between the cellular and molecular biology of the virus and the host cell can be used to design drugs and drug therapies to maximize efficiency and reduce toxicity. The life cycle of HIV includes attachment to the host cell membrane, reverse transcription of viral RNA to DNA, integration of viral DNA into the host cell genome, transcription of viral DNA, translation of viral proteins, and assembly of viral RNA and proteins into virions for release from the host cell (11). Among the steps in the virus life cycle, HIV-encoded reverse transcriptase (HIV-RT) has proven to be one of the most successful targets for the development of antiviral agents. After viral entry into the host cell, HIV-RT uses viral RNA to make a complementary DNA copy. RNase H, an enzymatic activity of HIV-RT, then degrades the viral RNA from the RNA-DNA strand, and a complementary copy of the remaining DNA strand is synthesized. This double-stranded viral DNA is integrated into the host cell genome, allowing transcription of viral DNA, translation of viral proteins, and subsequent assembly of additional virions. Inhibition of HIV-RT would thus prevent additional virions from being formed. 2',3'-dideoxynucleosides (ddNs) were observed to inhibit HIV-RT and HIV replication in vitro, and this discovery has resulted in the development of these compounds as the only approved antiviral agents for first-line therapeutic use in the treatment of AIDS (42, 43). While other steps in the viral life cycle are potential targets for development of antiviral drugs and while many of these novel approaches seem promising, ddNs represent the only presently approved drugs for first-line therapy.

Endogenous nucleosides are anabolized to 5'-triphosphate derivatives by cellular kinases and are incorporated into DNA at the 3'-hydroxyl of an existing sugar moiety with the 5'-triphosphate of the nucleoside added by cellular DNA polymerases. ddNs are likewise phosphorylated to the 5'-triphosphate derivative by cellular kinases. However, they differ from endogenous nucleosides in that they have no hydroxyl group at the 3'

position of the sugar moiety, but instead may have an azide, fluorine, hydrogen, or the absence of a group. The substitution of the hydroxyl group does not allow DNA chain elongation, terminating DNA synthesis after incorporation of the analog into DNA. This chain termination is believed to be the major mechanism of action of ddNs active against HIV-RT (45), and ddNs generally exhibit a greater affinity for HIV-RT than for host cellular DNA polymerases (58, 36, 44), contributing to the efficacy of ddNs as antiviral agents. Different cell types display differing efficiencies in anabolizing these ddNs to their triphosphate derivatives and display differing accumulations of intermediates, which may affect other cellular components (31, 8). As a result, ddNs display varying efficiency in the inhibition of HIV-RT, and therefore varying potential as antiviral agents. For example, although a potent inhibitor of HIV-RT in vitro, 2',3'-dideoxyuridine (ddU) is inactive against HIV-RT in vivo because the triphosphate derivative is not accumulated intracellularly in sufficient levels to inhibit HIV-RT (32). In contrast, 3'-azido-3'-deoxythymidine (AZT) is efficiently phosphorylated to the triphosphate derivative and is a potent inhibitor of HIV replication, both in vivo and in vitro (26, 56). The rate of ddN phosphorylation to the triphosphate derivative varies considerably from one cell type to another because of varying enzymatic activity. Therefore, the unique and selective character of ddNs must be considered when investigating effects of ddN administration.

The need for long-term therapy of AIDS patients is of concern because of the toxicity associated with ddNs employed as antiviral agents. ddNs demonstrate unique toxicity profiles in patients as well as in vivo and in vitro investigations. Because of their proven efficacy in demonstrating long-term effectiveness against HIV, ddNs are included in most combination trials and are likely to continue to play a major role in AIDS chemotherapy, emphasizing the need to understand the mechanisms underlying ddN-associated toxicity. To ascertain the mechanism of action of a particular ddN in inducing toxicity, the target cell type and stage of development must be determined. Many cell types

may be affected by ddNs, but the toxic effect of concern may reside in a particular type of cell or in a particular stage of development of the cell.

AZT, licensed in 1987, was the first agent to demonstrate in vitro activity against HIV and was rapidly evaluated in clinical trials and found to decrease mortality and incidence of opportunistic infections in patients with AIDS and advanced AIDS-related complex (ARC) (60, 61, 24). While AZT demonstrates effectiveness as an anti-HIV agent, AZT also demonstrates hematopoietic toxicity. This toxic effect is observed in patients as anemia and neutropenia, and in advanced HIV-infected patients, the toxic effects are severe enough to warrant transfusions, dose reduction, or cessation of treatment altogether. This anemia is clinically characterized as macrocytic anemia with a megaloblastic bone marrow, with reduced hematocrit and reticulocyte counts. In some patients, red cell aplasia has been reported (16).

As the first antiviral agent approved for the treatment of AIDS, AZT has been the most investigated ddN in an effort to determine its mechanism of action in inducing hematopoietic toxicity. While previous studies have demonstrated a lower affinity of AZT triphosphate (AZT-TP) for human DNA polymerases compared to HIV-RT (18), substantial levels are incorporated into DNA in human bone marrow cells and various cell lines (54, 57, 6). Studies have shown AZT-TP to be an inhibitor of mitochondrial associated DNA polymerase and mitochondrial replication (52, 14), and exonuclease activities of DNA polymerases are inhibited by AZT monophosphate (AZT-MP) (44). AZT has also been shown to inhibit both burst-forming units-erythroid (BFU-E) and granulocyte-macrophage (CFU-GM) in semi-solid colony-forming assays of human bone marrow cells, and demonstrates toxicity to the proliferation and differentiation of human leukemia K-562 cells. A metabolite of AZT, 3'-amino-3'-deoxythymidine (AMT), is reported to be more toxic than AZT to human bone marrow cells in semi-solid colony-forming assays at pharmacologically relevant concentrations found in the plasma of patients undergoing AZT therapy. AZT and AMT, to a lesser extent, have also been reported to

inhibit hemoglobin synthesis through inhibition of globin gene expression in K-562 cells. While none of these individual observations is sufficient to fully explain the toxic effects of AZT at pharmacologically relevant doses, they indicate that the mechanism of action of AZT may be multifactorial and effects may be different in different cell types. Therefore, it is critical that the toxic effects of AZT and other ddNs be investigated in a physiologically relevant model system that would allow detailed investigation and proper control of variable parameters.

The toxic effects of anemia and neutropenia suggest that AZT may effect hematopoiesis, the process of the development of the cells of the blood from bone marrow. Current opinion of the process is that all cells of the blood and bone marrow are developed from the pluripotent stem cell, a cell capable of self-renewal and of differentiating into all of the blood cell lineages. There are at least seven lineages produced by the stem cell including red blood cells, monocytes and macrophages, T cells, B cells, granulocytes, eosinophils, and megakaryocytes. The process regulating the proliferation and differentiation of the pluripotent stem cell into cells of the lymphoid, myeloid, and erythroid lineage is not well understood. While it appears that early events regulating differentiation may be stochastic, it appears that later events may be regulated by a complex interplay between growth factors, hormones, cytokines, and the stromal environment (40). It has been demonstrated that the stem cell can be induced to develop primarily, though not exclusively, along a particular lineage with appropriate use of selected drugs. In particular, studies of erythropoiesis, the developmental process forming red blood cells, demonstrate that the stem cell produces a multipotent progenitor cell (CFU-GEMM) capable of producing colony-forming cells of the erythroid, monocyte, macrophage, and granulocyte lineage (22). This progenitor cell further differentiates along the erythroid lineage to produce the BFU-E, which produces the CFU-E, which matures in subsequent stages, extruding the nucleus to form the reticulocyte. Reticulocytes enter the blood stream from the bone marrow, maturing into red blood cells with an average life of 120 days (34).

While the stem cell and early progenitors of erythropoiesis cannot be recognized morphologically, they can be identified by expression of the CD34 antigen, which is expressed from the stem cell to BFU-E, but not on progeny of BFU-E. Progeny of BFU-E can be identified by expression of glycophorin A, expressed only on more mature erythroid cells, and production of hemoglobin, which can be detected by benzidine staining of more mature erythroid cells. BFU-E and CFU-E can be detected by their ability to form bursts and colonies, respectively, in semi-solid culture media.

Due to the anemia and neutropenia induced by AZT in patients, investigations of the toxicity of AZT have focused on its effect on hematopoiesis, and in particular, on erythropoiesis, as anemia is often the dose-limiting toxicity. Investigations using animal models in vivo reveal that AZT induces anemia in normal rats and mice, as well as in the immuno-suppressed murine AIDS model (MAIDS) (15) and in cats infected with feline leukemia virus (33). Investigations of human bone marrow cells in semi-solid culture media indicate a toxic effect of AZT on the number of BFU-E and CFU-E, as well as colony-forming units of other bone marrow lineages such as CFU-GM. In addition, these investigations suggest that AZT may act preferentially on the erythroid lineage, as the concentration required to inhibit CFU-GM is much higher than the concentration required to inhibit BFU-E. Investigations in a K-562 human leukemia cell line, induced to differentiate along the erythroid lineage, indicate that AZT inhibits both proliferation and differentiation, as well as globin gene expression. These investigations in K-562 cells indicate that AZT treatment results in an inhibition of the total number of cells and of the percentage of cells synthesizing hemoglobin, as compared to control cells. This reduction in the percentage of hemoglobin-synthesizing cells could be due to an inhibition of hemoglobin synthesis, an inhibition of erythroid cell maturation and/or proliferation, or an inhibition of the differentiation process of commitment of progenitor cells along the erythroid lineage. These investigations in colony-forming assays and K-562 cells indicate that AZT may inhibit the proliferation of cells, and suggest that AZT may effect the

differentiation of cells along the erythroid lineage, the maturation and/or proliferation of cells differentiating along the erythroid lineage, and/or the synthesis of hemoglobin.

The process of hemoglobin synthesis is complex and involves the regulation of the independent but coordinated pathways of globin and heme. The heme biosynthetic pathway consists of eight enzymes acting sequentially, beginning and ending in the mitochondria with intermediate steps in the cytosol (3). Heme then combines with globin in the cytosol, forming hemoglobin. In hemoglobin, heme constitutes the majority of the total heme in mammalian organisms, but carries out many other additional vital functions. It serves as the prosthetic group of hemoproteins which include mediation of oxygen transport and storage, generation of cellular energy, reduction reactions, and most detoxifications (9). It is clear that heme is involved in the regulation of hemoglobin, heme, and globin synthesis, but the mechanisms of regulation remain unclear. While the role of heme in regulating hepatic heme synthesis has been well characterized (51), its role in regulating erythroid-specific heme synthesis has been less characterized. Erythroid cells contain erythroid-specific isotypes of the enzymes aminolevulinic acid synthase (ALAS) and porphobilinogen deaminase (PBG-D), which are regulated differently than the ubiquitous isotypes. Previous investigations in rats implicate AZT in inhibiting the activity of the heme biosynthetic enzyme ALAS, but information is limited in studies performed on the erythroid specific isotype of the enzyme in human erythroid cells. Preliminary unpublished studies in our laboratory of K-562 cells suggest an AZT-induced inhibition of total hemoglobin synthesis, but suggest no accumulation of heme biosynthetic enzyme products such as occur with porphorias (37, 29). Therefore, further investigation of the effect of AZT on the biosynthesis of hemoglobin is indicated and necessary in determining the mechanism of action of AZT in inducing anemia.

The need for long-term therapy in AIDS patients has increased the need for effective alternative approaches to diminish the associated toxicity of the antiviral agents. One alternative approach is combination chemotherapy of anti-HIV agents with different major

sites of toxicity, as suggested for AZT and 2',3'-dideoxycytidine (ddC). Another approach is synergistic combination chemotherapy, such as concurrent administration of erythropoietin (EPO) and granulocyte, macrophage-colony stimulating factor (GM-CSF) with AZT to increase bone marrow cellularity and erythroid stimulation in an effort to reduce the AZT-induced hematopoietic toxicity. Administration of EPO has been observed to reduce the AZT-associated anemia in patients with EPO levels below 500 IU per liter while demonstrating no reduction in patients with higher levels of EPO (46). Evidence indicates that GM-CSF can increase bone marrow cellularity in some patients, and granulocyte-colony stimulating factor (G-CSF) has shown some efficiency in reducing neutropenia (41, 30, 39, 47). Perhaps the most appealing alternative approach is the protective or rescue approach such as that demonstrated with methotrexate in cancer therapy. This approach has also been successfully utilized against protozoan infections and in treatment of schistosomiasis (20). In addition, uridine has been demonstrated to protect human bone marrow CFU-GM from AZT-induced toxic effects in clonogenic assays. Hemin, a chloride analog of heme, has been used to successfully reduce the AZT-induced anemia in animal models, while not adversely affecting and possibly enhancing the antiviral activity of AZT (38, 1), and has demonstrated effectiveness in protection of human bone marrow BFU-E in semi-solid culture assays. Another heme analog, heme arginate, has been used successfully in reducing the anemia associated with myelodysplastic syndromes (55). These investigations suggest that heme analogs may have value in protecting against or rescuing from the AZT-induced erythropoietic toxicity. In addition, investigations of heme protection and rescue may aid in ascertaining the mechanism of toxicity of AZT.

The methods employed in investigation and the type of culture system are important in determining the mechanism of toxicity. The semi-solid colony-forming assay has proven beneficial in predicting toxicity of ddNs in human bone marrow cells, but is limited in the information that can be obtained. Colony-forming assays yield information specifically about the number of colony-forming cells that develop colonies in the assay. In

erythroid colony-forming assays, inhibition of the number of BFU-E could be the inhibition of the BFU-E itself or the inhibition of the ability of the BFU-E to subsequently produce progeny capable of forming colonies. Removal of intact cells from the semi-solid culture media in sufficient numbers for further analysis is difficult, if not impossible, given the low cloning efficiency of semi-solid cultures and the difficulty in removing intact cells from the media without damaging the cells. The advent of the liquid suspension culture system may have advantages over the semi-solid colony-forming assay in that the intact cells can be removed in sufficient numbers for further analysis, including monoclonal antibody labeling for phenotype analysis, hemoglobin staining, and detailed biochemical and molecular analysis.

The type of cells used in investigations of ddN toxicity is also important in that different cells metabolize nucleoside analogs differently. For example, the CEM cell line has been reported to extrude AZT-MP from the cell while this has not been observed in other cell lines (25). The use of immortalized cell lines has been of paramount importance in investigating mechanisms of drug action in that they can be cultured in large numbers and induced to develop along a particular lineage such as with K-562 cells, allowing detailed investigation of the biochemical and molecular mechanisms. The use of these transformed cells, because of their very transformed nature, is questioned by many scientists as to their applicability as a model. For example, the concentration of AZT required to inhibit K-562 cell proliferation by 50% is several orders of magnitude higher than the 50% inhibitory concentration required for bone marrow cells. The use of animal models is clouded because of the reduced control possible in whole organisms as well as the extrapolation, or perhaps generalization, from the rodent and mammalian models to the human being. The ideal investigation of the mechanisms producing effects of ddNs on hematopoiesis or erythropoiesis in the laboratory should involve the development of the targeted human cell from the stem cell to the cell exhibiting the effects of ddN toxicity because ddNs may affect cells only in a particular stage of development. It is therefore

necessary to be able to observe ddN effects on the various stages of development of the red blood cell as it develops from the stem cell. The cells involved should be cultured in sufficient numbers to allow for detailed biochemical and molecular analysis.

The use of CD34 positive human bone marrow cells in liquid suspension culture may allow investigation of the effects of ddNs on the proliferation, differentiation, and hemoglobin synthesis in a pertinent culture system. The determination of the effects may suggest possible target cells and allow detailed investigation into the biochemical and molecular mechanisms producing these effects in these cells.

The objective of this dissertation is to evaluate the effects of ddNs on relevant human progenitor cell development in an effort to determine the ddN effects on proliferation, differentiation, and hemoglobin synthesis. While we have investigated both the myeloid and erythroid lineages because of the well-documented AZT-induced effects of neutropenia and anemia in patients, emphasis has been placed on the erythroid lineage as anemia is often the limiting toxic effect in patients treated with AZT. Chapter 1 describes the investigation of K-562 human leukemia cells induced to synthesize hemoglobin with chemical agents to determine if AZT-induced toxic effects on hemoglobin synthesis are a result of a general inhibition of the erythroid lineage or specific effects on the heme or globin pathways leading to an inhibition of hemoglobin synthesis. Chapter 2 examines the effect of various ddNs active against HIV on the hematopoietic development of CD34+ human progenitor cells in a liquid culture system by investigating the lineage-specific antigen expression of cultured cells with monoclonal antibodies by FACS analysis. Chapter 2 also investigates the effect of ddNs on mitochondrial DNA (mtDNA) synthesis. Chapter 3 examines the effects of exposure to hemin on the proliferation and hemoglobin synthesis in CD34+ human progenitor cell development in liquid culture and semi-solid culture of human bone marrow cells, and the potential of hemin for rescue and/or protection of AZT-induced toxic effects in both myeloid and erythroid lineage cells. Chapter 4 investigates the effects of a human recombinant hemoglobin preparation on hematopoiesis

by examining the lineage-specific antigen expression of both myeloid and erythroid lineages, as well as the potential for rescue and/or protection of AZT-induced toxic effects in the erythroid lineage. Appendix A contains additional unpublished data from semi-solid methycellulose cultures of human rHb, and Appendix B contains additional unpublished data from liquid culture of CD34+ human progenitor cells. These appendises contain supportive data from research in our lab that has not been published and which relates to specific parts of the chapters and may aid in the interpretation and understanding of the appropriate sections or which includes additonal data which may be useful in evaluating mechanisms.

SPECIFIC AIMS

To better understand the effects of ddNs on the proliferation and differentiation of human progenitor cells, the following specific aims were undertaken:

(1) to determine whether the effects of AZT on erythroid inducible gene expression is a general or specific effect .

(2) to assess ddN effects on proliferation, differentiation, and mitochondrial DNA synthesis in CD34+ human progenitor cells.

(3) to evaluate the effects of hemin on proliferation and differentiation of CD34+ human progenitor cells and appraise the potential for protection and rescue from AZT-induced toxicity.

(4) to determine the effects of a recombinant hemoglobin on lineage-specific proliferation and differentiation of CD34+ human progenitor cells and evaluate the potential for protection and rescue of AZT-induced toxicity.

EFFECTS OF 3'-AZIDO-3'-DEOXYTHYMIDINE ON ERYTHROID INDUCIBLE GENE EXPRESSION IN HUMAN K-562 LEUKEMIA CELLS

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ABSTRACT

We previously demonstrated that 3'-azido-3'-deoxythymidine (AZT) down regulates hemoglobin (Hb) synthesis and globin gene expression. That inhibition may therefore result either from a direct effect on globin gene transcription or an indirect effect through inhibition of K-562 cell induction, thereby leading to inhibition of other inducible genes of heme biosynthesis. The present results demonstrate an inhibition of globin gene expression by AZT but an absence of AZT effects on expression of three other erythroid inducible genes (ALAS-E, ALAD, and PBGD-E), suggesting a direct gene effect rather than a general inhibition of K-562 cell induction.

1. INTRODUCTION

AZT, the first clinically approved and most widely used anti-AIDS drug, is therapeutically limited by its toxic side-effects, including anemia requiring blood transfusions in 30-40% of AZT recipients [1]. Although the anemia in AZT recipients has been characterized as macrocytic [1], very few molecular studies have explored the mechanisms involved in this side-effect. In recent reports, we have studied mechanism(s) involved in AZT toxicity during erythroid cell differentiation using a human K-562 erythroleukemia cell model. In these studies, we demonstrated that AZT inhibited Hb synthesis during butyric acid-induction of these cells, an effect associated with a decrease in globin gene expression [2]. In contrast, 2',3'-dideoxycytidine [2] and several other dideoxynucleoside derivatives had no measurable effect on Hb synthesis[1]. The observed inhibition of globin gene expression by AZT was consistent with decreased rate of globin mRNA transcription, as demonstrated by nuclear run-on transcription analysis [2]. In contrast, no effect on globin mRNA stability was detected [3]. More recently, 3'-amino-3'-deoxythymidine, a toxic metabolite of AZT detected in AZT recipients [4], was also shown to inhibit globin mRNA transcription in these cells during Hb induction [3].

Production of Hb requires coordinated synthesis of both globin chains and heme molecules. Heme biosynthesis requires eight enzymes, each of which is coded by a

separate gene. Although genes of the heme biosynthetic pathway are expressed in all cells (heme being the prosthetic group of respiratory cytochromes), two of these enzymes have erythroid specific isozymes whose activity is predominant in erythroid cells. 5-aminolevulinic acid synthase (ALAS) and porphobilinogen deaminase (PBGD) have erythroid specific mRNAs which are distinct from the ubiquitous forms of ALAS and PBGD mRNA [5]. In murine Friend erythroleukemia cells, induction by DMSO is accompanied by increased enzymatic activity [6] as well as enhanced gene expression of several heme biosynthetic enzymes, including erythroid specific ALAS (ALAS-E), aminolevulinic acid dehydratase (ALAD), erythroid specific PBGD (PBGD-E), and uroporphyrinogen decarboxylase (UroD) [7]. The induction of these mRNAs occurs in a sequential manner in the same order as they appear in their biosynthetic sequence. Although heme pathway gene expression studies have not been reported for K-562 cells, enzyme activities of both ALAS and ALAD were shown to increase during K-562 cell induction by hemin or butyric acid. [8,9]. It is, therefore, very likely that the corresponding genes are also induced in K-562 cells as demonstrated herein.

In the present study, we evaluated whether AZT effects on globin transcription are specific to that gene or are associated with a general inhibition of inducible erythroid genes of the heme biosynthetic pathway. In that context, AZT effects were assessed on enzyme activities of ALAS, ALAD, and PBGD and their respective mRNA's during butyric acid induction of K-562 cells. These data should permit discrimination between a specific inhibition of globin gene expression by AZT from an indirect general inhibition of cell differentiation.

2. MATERIALS AND METHODS

2.1 Cells and Culture Conditions

K-562 human leukemia cells were maintained as described previously [3]. Cells in log growth were induced with 1.4 mM butyric acid at a density of 1.6×10^5 cells/ml. Cell

aliquots were removed at various time points for isolation of poly A+ mRNA's and ALAS, ALAD and PBGD enzyme activity assays.

2.2 Isolation of poly A+ mRNA and Northern blot analysis

Total RNA was isolated as described previously [3] by an acid-guanidinium-phenol-chloroform method [10]. Poly A+ mRNA was then purified as follows: The RNA pellet was dissolved in 500 µl H_2O, denatured by heating to 65°C for 5 minutes, to which was added 500 µl of 2 x binding buffer [1 x binding buffer = 20 mM Tris HCl (pH 7.6), 0.5 M NaCl, 1mM EDTA, 0.1% SDS]. Oligo-dT cellulose (30 mg) was then added, and the mixture was gently shaken for 15 minutes at room temperature. Unbound RNA was eliminated from the oligo dT-cellulose by five washes with 1 x binding buffer using a 0.22 µm cellulose acetate microcentrifuge filtration column. Poly A+ mRNA was eluted from the oligo dT-cellulose in low salt buffer (10mM Tris-Cl, pH 7.6, 1 mM EDTA, 0.05% SDS), and precipitated with ethanol. RNA was electrophoresed as described previously [2], and transferred to nylon membranes [3].

2.3 Probes

A. Probes for actin and globin genes were plasmid DNA fragments isolated by polyacrylamide gel.

1] Actin: a 735 b.p. Ava I fragment from the cDNA plasmid pRBA-1 [3].

2] γ. globin: 1.35 kilobase PvuII fragment of the human γ-globin cDNA plasmid pγ IVS(-) Sp3 containing the first exon and part of the second exon of the Gγ-globin gene [3].

DNA fragments were labeled by nick translation as described previously [2].

B. Probes for ALAS-E, ALAD, and PBGD-E were single stranded oligonucleotides purchased from Oligos Etc. (Wilsonville, OR).

[1] ALAS: 24 b.p. Oligonucleotide 5'CTGAGTAAATCTCGCACCCTGGCA, complementary to erythroid-specific ALAS bp 870-893 [11]

[2] ALAD oligonucleotide 24 b.p. oligonucleotide 5'GGGACACAGGCAGACATCA-CAGGC, complementary to human ALAD bp 352-375 [12]

[6] PBGD oligonucleotide: 24 b.p. oligonucleotide 5'GAAGCAGAGACTAGAGGGAGGCGA Complimentary to erythroid-specific PBGD [13,14]

Oligonucleotide probes were labeled at the 3'-termini using deoxynucleotidyl-transferase and alpha-^{32}P dATP [15]. A higher specific activity was obtained with this methodology as compared to 5'-labeling with T4 polynucleotide kinase, since more than one ^{32}P atom is added to the 3' ends of the oligonucleotides.

2.4 Hybridization

When DNA fragments were used as probes, hybridization was carried out at 40° overnight in 50% formamide buffer containing 5 X SSC (1 X SSC=0.15M NaCl, 0.015M sodium citrate), 0.1% SDS, 1 mM EDTA, 2.25 X Denhardt's solution (1 X Denhardt's = 0.02% polyvinylpyrollidone, Bovine serum albumin), 5mM sodium phosphate (pH 6.5) 50 µg/ml salmon sperm DNA (sonicated + denatured). The blot was then washed twice in 2 X SSC, 0.1% SDS at room temperature and three times in 0.1 X SSC, 0.1 SDS with the final wash at 40°C.

When oligonucleotides were used as probes, hybridization was carried out at 55°C overnight in 7% SDS, 0.25 M sodium phosphate (pH 7.2) 0.25 M NaCl, 10 µg/ml bovine serum albumin and 1 mM EDTA. The blot was washed four times with 2 X SSC, 0.1% SDS with a final wash at 55°C.

2.5 Assays of Heme biosynthetic enzymes

A. Aminolevulinic Acid Synthetase activity.

ALAS activity was determined essentially as described by Gardner, et al [16]. Dowex 50W X 8 resin was heated twice in 2M NaOH at 50°C and washed with distilled

water. The resin was then equilibrated in 0.05 M sodium acetate (pH 3.9). K-562 cells, induced to synthesize hemoglobin with 1.4 mM butyric acid for 96 hours with AZT added after 24 hours induction, were pelleted at 350 x g for 10 minutes and resuspended in an incubation mixture containing 130 mM NaCl, 5.5 mM KCl, 7.4 mM $MgSO_4$, buffered to pH 7.35 with 10 mM Na-HEPES containing 0.1% (w/v) D-glucose and 100 μM of L-amino acids. Cells were preincubated with AZT (100 μM) and succinyl acetone (1 mM) for 90 minutes in the incubation mixture at 37°C, and ^{14}C-glycine (100 μCi/mL) was subsequently added to a final concentration of 110 dpm/pmole for 90 minutes. The reaction was stopped by addition of ice-cold incubation buffer. Cell pellets were washed three times with cold phosphate buffer saline (pH 7.4) and lysed with 200 μL of 0.2% (w/v) aqueous sodium deoxycholate. Proteins were precipitated by trichloroacetic acid at a final concentration of 5% (w/v). Supernatants were applied to Dowex columns and washed with 30 mL of 0.05 M sodium acetate buffer (pH 3.9) to remove free labeled glycine and other radiolabeled impurities. ^{14}C-ALA was eluted with 0.25 M sodium phosphate buffer (pH 6.8), and two mL fractions were collected with radioactivity being determined in a liquid scintillation counter.

B. Aminolevulinic acid dehydrogenase assay.

ALAD activity was determined essentially as described by Kondo, et al [17]. Dowex columns were converted to acetate form by treatment with 3 M sodium acetate and washed three times with 10 volumes of distilled water. K-562 cells were induced as described above. After 24 hours of induction, either AZT or succinyl acetone was added at concentrations of 100 μM or 250 μM and 1 mM, respectively. After 96 hours of induction, cells were harvested by centrifugation at 350 x g for 10 minutes, washed twice in phosphate buffer saline (pH 7.4), and lysed with 5 volumes of ice cold deionized water. Isotonicity was restored by adding phosphate buffer saline, and the solution was centrifuged at 16,000 RCF for 30 minutes. An aliquot (30 μl) of the supernatant was added to the incubation mixture which contained 10 mM glutathione, 50 mM sodium

phosphate buffer at pH 6.2, and ^{14}C ALA (100 μCi/ml., 110 DPM/pM) in a final volume of 50 μL. This mixture was incubated for one hour at 37°C and terminated by addition of 1.0 mL of 0.2 M trichloroacetic acid. The precipitate was discarded after centrifugation at 735 RCF for 10 minutes, and the supernatant mixed with 0.2 mL of sodium carbonate (2.4 M). This portion was then applied to a Dowex column and washed with 30 mL of sodium carbonate (0.75 M) and 10 mL of distilled water. ^{14}C PBG was eluted from the Dowex column with 3.0 mL of 12 M acetic acid and analyzed by HPLC (Hewlett-Packard 1050, Avondale, PA), using a Partisil 10 SAX column (4 x 250 mm, Jones Chromatography, Littleton, CO) and isocratic elution with 15 mM potassium phosphate buffer at a flow rate of 0.5 mL/min.

C. Porphobilinogen deaminase activity.

The assay for PBGD activity was performed using the method of Sassa et al [18]. Cells (1 x 10^6) were pelleted and washed with phosphate buffered saline, then lysed in 40 μl of deionized water with three cycles of freezing and thawing. Forty μl of 0.5 mM porphobilinogen in 0.1 M phosphate buffer (pH 7.4) was then added and incubated in the dark at 37°C for one hour. A control reaction containing cell extract to which no porphobilinogen was added was run in parallel to determine fluorescence resulting from endogenous uroporphyrin I. Reactions were stopped by freezing, and free porphyrins were extracted with 500 μl of ethylacetate-acetic acid (2:1, v/v). The porphyrins were then separated from heme by addition of 500 μl of 0.5 N HCl, and fluorescence of the aqueous phase was determined in a Perkin-Elmer model 650-10S fluorescence spectrophotometer, using exciting and emission light of 400 nm and 658 nm, respectively. Uroporphyrin I content was determined from a standard curve obtained with uroporphyrin I standards (Porphyrin Products, Inc., Logan, UT).

2.6 Chemicals

Chemicals used for cell culturing and RNA analysis have been described previously [2, 3]. For enzyme assays, 2-^{14}C-glycine and ^{14}C-aminolevulinic acid (ALA) were

purchased from Dupont, NEN Division (Boston, MA). Dowex 50W X 8, (mesh size 200-400) ion exchange resin columns and Dowex 1-X8 (200-400 mesh) were obtained from Bio-Rad (Richmond, CA). Aminolevulinic acid, succinyl acetone, L-amino acids, and porphobilinogen were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade or higher.

2.7 Statistical Analysis

Statistical analyses were performed using Student's *t* test of unpaired independent data.

3. RESULTS

3.1 Effect of AZT on induction of mRNA's involved in heme biosynthesis

To assess the kinetics of induction of the various studied genes, RNA was isolated at 0, 48, and 72 hours after onset of induction. Due to low amount of several of the mRNAs assayed, isolation of poly A+ mRNA was necessary.

The ALAS isozymes are coded by two separate genes; the erythroid specific gene (ALAS-E) is located on the X-chromosome, while the nonspecific gene (ALAS-N) is located on chromosome 3 [11]. For detection of ALAS-E mRNA, we used a 24-mer synthetic oligonucleotide complementary to the erythroid specific gene [11]. As shown in Fig. 1, the level of ALAS-E mRNA increased rapidly during butyric acid induction of K-562 cells, reaching a 12-fold increase after 48 hr of induction, and 14-fold by 72 hr of induction. Globin mRNA reached about a 10-fold induction by 72 hr (Fig. 1). The PBGD gene, in contrast to ALAS, is composed of two overlapping transcription units: transcription initiated from the upstream promoter produces the ubiquitous PBGD mRNA, while transcription from the downstream promoter produces the erythroid specific mRNA (PBGD-E). Each type of PBGD mRNA differs exclusively at its 5' end as a result of differential splicing [13]. To detect PBGD-E mRNA, we used a 24-mer synthetic oligonucleotide complementary to a portion of the erythroid specific exon of PBGD-E (see experimental procedures). As shown in Fig. 1, PBGD-E mRNA was induced by butyric

acid, but at a lower level of induction than for ALAS-E or globin mRNAs, reaching about a 5-fold induction by 48 hr and increasing to 7-fold by 72 hr. Using the 24-mer oligonucleotide probe described in "Materials and Methods," a quantitable hybridization signal was not obtained for ALAD mRNA.

Cells were exposed to AZT 24 hours after onset of induction by butyric acid, allowing the early stages of the induction process to occur in the absence of drug. AZT (100 μM) substantially inhibited globin mRNA steady-state level by approximately 35% (Fig. 1, quantitated in Fig. 2), consistent with previous time course studies [2]. In contrast, no suppression of ALAS-E or PBGD-E mRNA steady-state level was observed in AZT treated cells at either 48 or 72 hr (Figs. 1 and 2).

3.2 Effect of AZT on ALAS, ALAD, and PBGD activity

In addition to its transcriptional regulation, ALAS-E gene expression is regulated post-transcriptionally via repression of ALAS-E mRNA translation by the iron responsive element-binding protein [11]. Possible effects of AZT on ALAS activity were determined using a radiochemical method [16]. In this assay, ^{14}C labeled glycine combines with succinyl-CoA to form ^{14}C labeled aminolevulinic acid. Subsequent conversion of aminolevulinic acid is blocked by succinyl acetone, an inhibitor of ALAD activity [19]. Table 1 shows that no difference is observed in ALAS activity after four days of induction in cells incubated with 100 μM AZT as compared to control. The enzyme activity of ALAS in control experiments was in agreement with previously published values [9,20].

ALAD activity in control cells, as assessed by radiochemical method [17], was consistent with previous data [8, 9]. No significant difference in ALAD activity was detected between control and cells treated with 100 and 250 μM of AZT after 4 days of induction (Table 1). In contrast, ALAD activity was inhibited by approximately 80% as compared to control in cells treated with 1 mM succinyl acetone.

AZT effects were also assessed on PBGD activity in K-562 cells under similar conditions. As shown in Table 1, PBGD activity (induced approximately 5-fold after 96 hr, data not shown) was not affected in AZT-treated cells as compared to control. These results are consistent with the mRNA PBGD analysis (Fig. 2), and indicate that AZT has no post-transcriptional effect on PBGD expression in K-562 cells during butyrate induction.

4. DISCUSSION

In this study, we have extended our previous findings on the effects of AZT on gene expression in K-562 erythroleukemia cells. We have previously shown that AZT inhibited induction of hemoglobin synthesis by butyric acid, and this effect was associated with decreased globin mRNA steady-state levels and rates of transcription [2,3]. Potential effects of AZT on other erythroid inducible genes, including ALAS-E, ALAD, and PBGD-E, were assessed to determine whether AZT inhibition is specific to globin genes or may represent a general effect on inducible gene expression. None of these inducible genes, which code for enzymes involved in heme biosynthesis, was suppressed by AZT, while globin gene expression was substantially inhibited, consistent with our previously published results [2,3]. Inhibition of globin gene expression by AZT in these cells is therefore not associated with a general effect on inducible gene expression, but appears to be a specific inhibition of globin RNA transcription.

ALAS-E mRNA was induced more rapidly than globin or PBGD-E mRNAs in these cells. ALAS-E mRNA induction rate plateaued between 48 and 72 hr, while PBGD mRNA levels were still increasing at 96 hr (data not shown). Our results are in agreement with data reported with murine friend erythroleukemia cells, in which a similar sequential induction of heme synthesis genes was observed [7]. Of importance, AZT at a concentration of 100 μM did not suppress ALAS-E or PBGD-E mRNA steady-state levels while globin mRNA expression was substantially inhibited. This further suggests that AZT does not cause a temporal inhibition of K-562 cell differentiation since neither

expression of a gene induced earlier (ALAS-E) nor one induced later (PBGD-E) than globin was inhibited by AZT. Three of the studied genes (i.e., globin, ALAS-E, and PBGD-E) have similar erythroid specific consensus sequences in their 5' upstream regulatory regions, including GATA-1, CACCC, and NFE-2 consensus sequences [11]. However, there are likely as yet undiscovered regulatory sequences inherent to each erythroid specific gene which are responsible for relative differences in expression of each gene, controlling both the timing of its onset of induction and its peak rate of synthesis. AZT may have different effects on expression or binding of these gene-specific DNA binding proteins resulting in differential effects on the various erythroid specific genes. Studies are currently in progress to pinpoint the specific regulatory sites involved in AZT-induced inhibition of globin gene transcription.

Since ALAS-E gene expression in erythroid cells is also controlled in part translationally, via binding of the iron responsive element binding protein to its consensus sequence on the mature ALAS-E mRNA [11,20], AZT may have effects on ALAS enzyme activity via effects on ALAS mRNA translation, which would not be detected in the Northern assay. However, using a radiochemical method, no effect of AZT was observed on ALAS enzyme activity. These data suggests that AZT does not affect ALAS-E gene expression, either transcriptionally or post-transcriptionally.

Lutton et al. have reported that ALAS activity was inhibited in bone marrow cells isolated from rats treated with AZT [21]. It is important to note that AZT anabolic phosphorylation is different in rodents as compared to human cells [22]. In addition, in the studies reported by Lutton et al., ALAS activity was determined on total bone marrow mononuclear cells with no separation of erythroid and myeloid cells. Therefore, a substantial portion of ALAS activity may have resulted from the ubiquitous isozyme as compared to the erythroid specific isozyme. The two forms of ALAS are controlled differently with the ubiquitous form being controlled primarily by negative feedback regulation of heme by destabilization of ALAS mRNA [23, 24], while the erythroid form is

controlled both transcriptionally and translationally, as described above, by erythroid specific factors.

The data presented herein demonstrate that AZT does not inhibit the erythroid gene induction process in general, since several inducible genes (ALAS-E, PBGD-E, and ALAD) were not suppressed by AZT exposure to K-562 cells. AZT inhibition of globin gene expression appears to involve direct mechanism(s) on that gene rather than a secondary effect resulting from a general inhibition of K-562 cell induction.

Footnotes:

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Abbreviations used are as follows: AIDS, acquired immunodeficiency syndrome; ALAD, aminolevulinic acid dehydrogenase; ALAS, aminolevulinic acid synthase; ALAS-E, erythroid specific aminolevulate synthase; AZT, 3'-azido-3'-deoxythymidine; Hb, hemoglobin; PBGD, porphobilinogen deaminase; PBGD-E, erythroid specific porphobilinogen deaminase; SDS, sodium dodecyl sulfate; SSC, standard saline citrate.

[1] D.A. Fowler, D.A. Weidner and J.P. Sommadossi, manuscript in preparation.

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TABLE 1. Effect of AZT (100 μM) on enzyme activity in K-562 cells at 96 hr after induction initiation -enzyme activity was determined as described in experimental procedures. Data expressed as mean ± SD.
ND - not determined.

Treatment	ALAS Activity pmole/10^6 cells/hr	ALAD Activity pmole/10^6 cells/hr	PBGD Activity nmole/10^6 cells/hr
Control	1.54 ± 0.47	90.4 ± 23.0	1.43 ± 0.04
AZT	1.48 ± 0.48	81.1 ± 13.0	1.61 ± 0.12
Succinyl Acetone	ND	17.8 ± 12.0	ND

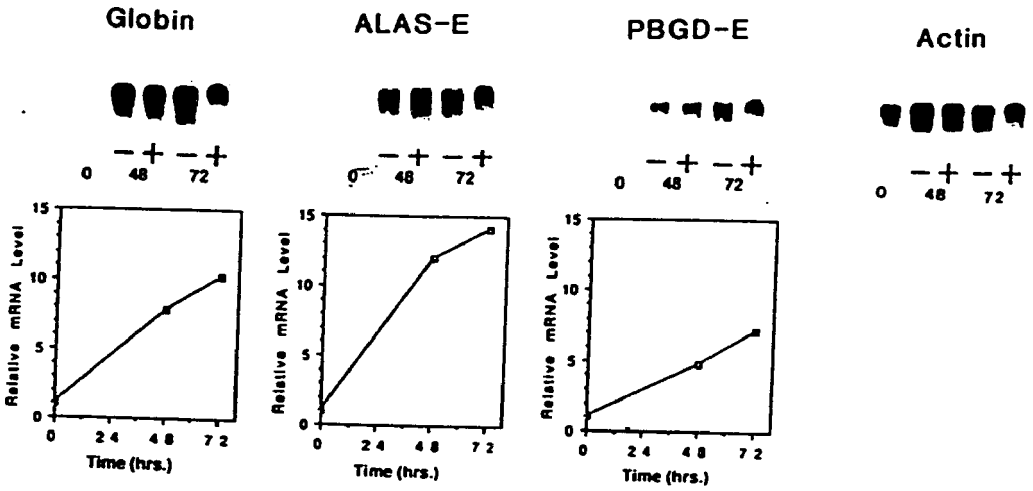

Figure 1. Time-course of mRNA inductions, and effect of AZT on mRNA levels. K-562 cells were induced with 1.4 mM butyric acid, and test cultures were exposed to 100 μM AZT at 25 hr. Cells were harvested at 0, 48, and 72 hr after induction initiation, and poly A+ mRNA from 5 x 10^6 cells was electrophoresed on a denaturing 2.2 M formaldehyde/ 1% agarose gel, transferred to nylon filters, and sequentially hybridized to ^{32}P labeled globin, ALAS-E, PBGD-E, and actin probes (see experimental procedures). Autoradiograms of Northern blots. -, mRNA from control culture; +, mRNA from culture exposed to 100 μM AZT. Relative mRNA levels were determined by densitometry and adjusted for minor differences in actin mRNA level used as a reference control.

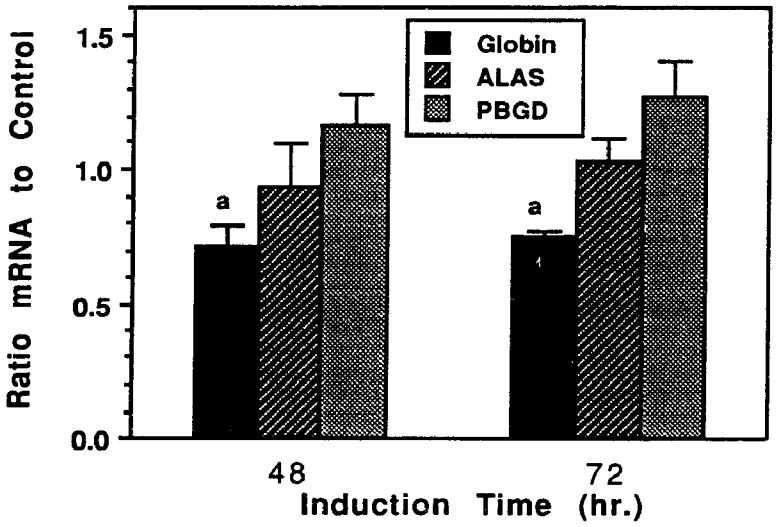

Figure 2. Effect of 100 μM AZT on globin, ALAS-E, and PBGD-E mRNA levels at 48 and 72 hr of induction. Data are mean of three experiments with error bars representing standard deviation. Experimental conditions are similar to those described in Figure 1.
a = $p < 0.01$ as compared to controls.

EFFECTS OF 2',3'-DIDEOXYNUCLEOSIDES ON THE PROLIFERATION AND DIFFERENTIATION OF HUMAN PLURIPOTENT PROGENITORS IN LIQUID CULTURE AND THEIR EFFECTS ON MITOCHONDRIAL DNA SYNTHESIS

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Running Title: Effects of ddNs on progenitor cells

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SUMMARY

2',3'-dideoxynucleosides (ddNs) including 3'-azido-3'-deoxythymidine (AZT), 3'-fluoro-3'-deoxythymidine (FLT), 3'-amino-3'-deoxythymidine (AMT), 2',3'-dideoxycytidine (ddC), and 2',3'-didehydro-3'-deoxythymidine (d4T) were tested for their effects on proliferation and differentiation of pluripotent progenitors ($CD34^+$) purified from human bone marrow cells grown in liquid cultures. These highly purified progenitors undergo extensive proliferation during 14 days with a marked differentiation during the last 7 days. These differentiated cells exhibit normal morphological features in response to specific hematopoietic growth factors of both erythroid and granulocyte-macrophage lineages as demonstrated by flow cytometry cell phenotyping. The potency of these ddNs in inhibiting proliferation of granulocyte-macrophage lineage cells was in the order of FLT>AMT=ddC>AZT>>d4T and FLT>AMT>AZT>ddC>>d4T for erythroid lineage cultures. Toxic effects of ddNs assessed in these liquid cultures were in agreement with data obtained using semi-solid cultures, demonstrating the consistency of these two in vitro hematopoietic systems toward ddN toxicity. ddC was toxic to $CD34^+$ progenitors and/or early stages of differentiation whereas the inhibitory effect of AZT on the erythroid lineage was predominately observed on a more mature population of erythroid progenitors during the differentiation process. Slot blot analysis of granulocyte-macrophage cultures demonstrated that exposure to ddC and to FLT was associated with a decrease in total mitochondrial (mt) DNA content suggesting that these two ddNs inhibit mtDNA synthesis. In contrast, no difference in the ratio of nuclear DNA to mtDNA was observed in cells exposed to toxic concentrations of AZT and AMT, demonstrating that bone marrow toxicity induced by AZT and its metabolite AMT is not associated with an inhibition of mtDNA synthesis. This human pluripotent progenitor liquid culture system should permit detailed investigations on the cellular and molecular events involved in ddN-induced hematological toxicity.

INTRODUCTION

The thymidine analogue, 3'-azido-3'-deoxythymidine (AZT), was the first drug approved for the treatment of acquired immunodeficiency syndrome (AIDS). AZT has been demonstrated to induce immunological improvement, decrease the incidence of opportunistic infections, and reduce AIDS mortality (15, 42). The major limitation to AZT therapy is bone marrow toxicity, manifested as anemia and neutropenia (30). Our laboratory was the first to demonstrate that direct exposure of human bone marrow cells to AZT in vitro effected a dose-dependent inhibition of granulocyte-macrophage colony-forming units (CFU-GM) and erythroid burst-forming units (BFU-E) (33). While these clonogenic assays using human bone marrow mononuclear cells in semi-solid media have been shown to be an adequate model for predicting the cytotoxicity of antiretroviral compounds to bone marrow cells (32), the semi-solid nature of the assay and the low cloning efficiency of progenitor cells make it difficult to ascertain the cellular and molecular mechanism(s) of drug-induced toxicity in a relatively pure and homogenous marrow cell population.

Recent studies have demonstrated that a population of pluripotent progenitor cells termed CD34+ exist in bone marrow that are capable of both self-renewal and differentiation into all lineages of mature hematopoietic cells (13, 31). This study describes the use of highly purified progenitor CD34+ cells for the culture of granulocyte-macrophage and erythroid cell lineages. The effects of AZT, 3'-fluoro-3'-deoxythymidine (FLT), 3'-amino-3'-deoxythymidine (AMT), 2',3'-dideoxycytidine (ddC), and 2',3'-didehydro-3'-deoxythymidine (d4T) were determined in these liquid culture systems and compared to soft-agar and methylcellulose clonogenic assays. The mechanism(s) of AZT-induced host toxicity is multifactorial (32) including inhibition of nuclear DNA synthesis following incorporation into host DNA (17, 34, 38), inhibition of polymerases and exonucleases (3, 7, 10, 23, 29), inhibition of gene expression (20, 39, 40) and others (14, 16, 22). In addition, some investigators have reported that inhibition of mitochondrial DNA (mtDNA)

synthesis, a potential mechanism involved in the peripheral neuropathy observed in patients treated with ddC, ddI and d4T (4, 6, 26, 32), may also be responsible for AZT hematological effects (24, 27). Using these newly developed liquid cultures of human pluripotent progenitor cells, mtDNA content of AZT-treated cells was assessed and compared to that of cells exposed to other ddNs including FLT, d4T, ddC and the myelotoxic metabolite of AZT, 3'-amino-3'-deoxythymidine (AMT).

MATERIALS AND METHODS

Materials. FLT was a gift from Dr. R.F. Schinazi (Emory University, Atlanta, GA). D4T was kindly provided by Bristol Myers Squibb, Co., and the AZT, AMT, ddC, Stem Cell Factor (SCF or c-kit ligand), IgG1-FITC, IgG2b-PE, and CD15-FITC were purchased from Sigma Chemical Co. (St. Louis, MO). Erythropoietin (EPO) and human recombinant GM-CSF were purchased from Connaught (Willowdale, Ontario, Canada) and Amgen Biologicals (Thousand Oaks, CA), respectively. IL-3 was purchased from Upstate Biotechnologies, Inc (Lake Placid, NY). CD34-FITC, Simultest CD3-FITC/CD19-PE, and CD11c-PE were purchased from Becton-Dickinson (Mountain View, CA). Glycophorin A-FITC was purchased from Amac, Inc (Westbrook, ME). Hank's balanced salt solution, fetal bovine serum (FBS), and McCoy's 5A media were purchased from Gibco/BRL (Grand Island, NY). All other chemicals and reagents were of the highest analytical grade available.

Mononuclear cell isolation. Human bone marrow cells were collected by aspiration from the posterior iliac crest of normal healthy volunteers, according to a protocol approved by the Institutional Review Board Committee at the University of Alabama at Birmingham. Alternatively, cells were flushed from a rib obtained following thoracic surgery as previously described (31).The mononuclear cells (MNCs) were isolated by gradient centrifugation on Ficoll-histopaque of heparinized bone marrow samples. The isolated MNC were washed in Hank's balanced salt solution (HBSS) supplemented with 10% FBS.

Progenitor purification. Approximately 4.0 x 10^7 MNC were incubated in a T-25 flask for at least two hours in minimum media (McCoy's 5A and nutrients) at 37°C with 5% CO_2 to adhere the monocytes and macrophages. For enrichment of hematopoietic progenitor (CD34+) cells, mononuclear nonadherent cells were pelleted, resuspended in 1 ml of cold phosphate buffered saline (PBS) with 2% FBS and 0.1% sodium azide and subsequently 200 µl of anti-CD34 monoclonal antibody was added. After incubation of 1 hour on ice and under dark conditions, the cells were washed twice with cold PBS to eliminate excess of antibodies and resuspended in 3 mL of McCoy's 5A plus nutrients containing 5 ng/mL of propidium iodide. CD34+ cells were then positively selected by cell sorting using FACStar instrument (Becton-Dickinson). The purity of the CD34+ cells was more than 99% with a viability of more than 96% as measured by trypan blue staining.

Liquid suspension cultures. Cell cultures were performed in supplemented McCoy's 5A medium enriched with 15% FBS and alpha-mercaptoethanol (0.1 mM). For granulocyte-macrophage differentiation, purified CD34+ cells were cultured in the presence of human recombinant GM-CSF (125 U/mL), and human recombinant IL-3 (200 U/mL). For erythroid differentiation, purified CD34+ cells were cultured in the presence of human recombinant erythropoietin (2 U/ml), SCF (7 ng/mL), and human recombinant IL-3 (100 U/mL). CD34+ cells (1.7-2.0 x 10^4 cell/mL) were incubated in 24-well tissue culture dishes in media containing cytokines, with or without drugs. After 7 and 14 days of incubation, viable cells from individual wells were counted and phenotyped. All experiments were performed at least in triplicate. The viability of the 14-day cultured cells was greater than 95% as measured by trypan blue staining.

Cell phenotyping. Primary isolated nonadherant MNCs and cultured cells following 7 and 14 days of incubation were analyzed by flow cytometry using six different monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE): CD3-FITC (T-cell marker), CD19-PE (B-cell marker), CD15-FITC (granulocyte

marker), CD11c-PE (monocyte marker), CD34-FITC (pluripotent progenitor marker), and Glycophorin A-FITC (erythrocyte marker). IgG1-FITC and IgG2b-PE were used as negative controls to insure accurate analysis. Cells (10^5) were incubated in 50ml of cold phosphate buffered saline (PBS) with 2% FBS and 0.1% sodium azide at 4°C in the dark with 5 µl of monoclonal antibodies. After 60 min of incubation, the cells were washed twice with PBS. The cells were then resuspended in PBS containing 1% paraformaldehyde and analyzed by FACStar (Becton-Dickinson) using the lysis program for analysis of double fluorescence.

Mitochondrial DNA content analysis. After 14 days of incubation, in the absence and presence of drugs, approximately 1-2 x 10^5 cells were heated under alkaline conditions and blotted onto a nylon membrane (Zeta-Probe GT, Bio-Rad, Richmond, CA) for Slot-Blot analysis (Bio-Rad Apparatus). The mtDNA was detected using a specific human oligonucleotide mitochondrial probe, encompassing nucleotide positions 4212-4242 (1). A human beta-actin probe was used as a control (40). The actin probe was a gel-purified 625-base pair fragment of a human beta-actin cDNA plasmid. DNA was fixed to the filters by heating for 10 min at 100°C, and slot blots were hybridized to oligonucleotide mitochondrial or beta-actin probes according to the manufacturer procedures. Autoradiograms were scanned using a Shimadzu CS 9000 U flying-spot densitometer.

RESULTS

Progenitor cell proliferation and differentiation. The isolated nonadherent mononuclear cells contained mostly erythrocytes (glycophorin A+), B-cells (CD19+) and T-cells (CD3+) while only 1 to 4% of the cell population was identified being pluripotent progenitor cells as determined by CD34 binding. A yield approximating 80% was obtained after positive selection by cell sorting.

After 14 days in liquid cultures, CD34+ cells showed a pronounced growth with granulocyte-macrophage and erythroid progenitors proliferating approximately 35- and 25-fold, respectively (Figure 1). Flow cytometry analysis of purified progenitors incubated

for 14 days in the presence of GM-CSF and IL-3 is shown in Figure 2 (control conditions). More than 70% monocytes, granulocytes, and macrophages (CD15+, CD11c+) were expressed in these granulocyte-macrophage cultures. Of note, no T-cells, B-cells, erythroid cells or pluripotent progenitor cells were detected. Figure 3 (control conditions) shows the analysis of a 14-day culture of pluripotent progenitor cells in the presence of EPO, SCF and IL-3. These erythrocytic cultures were characterized as being 80% erythroid (glycophorin A+), with no detection of T-cells, B-cells, or pluripotent progenitor cells, and low levels of granulocyte and monocyte as assessed by associated cell surface markers. Phenotyping of erythrocytic cultures demonstrated that the percentage of expressed erythroid cell glycophorin A+ had increased from 30% at day 7 to more than 80% at day 14 (Figure 3, panel A and C versus B and D). Monocytes (CD11c+) and granulocytes (CD15+) accounted for less than 4% in erythroid cultures.

Effects of 2',3'-dideoxynucleosides on human hematopoietic progenitors. The effects of various ddNs on proliferation of CD34+ progenitors in liquid cultures of human CFU-GM and BFU-E lineages are shown in Table 1. Potency in inhibiting proliferation of human hematopoietic progenitors in liquid cultures is in the order of FLT>AMT= ddC>AZT>>D4T for granulocyte-macrophage cultures and FLT>AMT>AZT>ddC>>D4T for erythroid lineage cultures. FLT was the most cytotoxic compound among ddNs tested with an IC_{50} of 0.14 μM in granulocyte-macrophage cultures and 0.05 μM in erythroid cultures, whereas D4T was found to have no significant cytotoxicity at concentrations up to 10 μM. In liquid cultures of human BFU-E, AZT strongly inhibited cell proliferation with an IC_{50} of 0.18 μM and was less inhibitory to CFU-GM with an IC_{50} of approximately 5 μM. ddC inhibited to a similar extent erythroid and granulocyte-macrophage cell growth with an IC_{50} of 0.5 μM and 0.6 μM, respectively. Toxicity data of ddNs in these liquid cultures were compared to IC_{50} values obtained using semi-solid cultures of human bone marrow progenitor cells previously published by our group (Table 1). Of note, IC_{50}

values of ddNs obtained either in liquid or semi-solid cultures were in the same order of magnitude, demonstrating the consistency of these two in vitro hematopoietic systems toward ddN toxicity.

Figure 2 illustrates myeloid-associated markers of AZT and ddC-treated cells compared to control of granulocyte-macrophage lineage cultures after 14 days. AZT inhibited granulocyte progenitors (CD15+), whereas the monocyte lineage (CD11c+) increased in a dose dependent manner as compared to control. In contrast, both myeloid cell lineages (CD11c+) and (CD15+) decreased as a function of ddC concentration.

Figure 3 shows the expression of phenotypic markers of AZT treated cells compared to control after 7 days (panel A) and 14 days (panel B) of erythroid culture. No effect was observed on the erythroid population (glycophorin A+) after 7 days of culture, whereas a pronounced inhibitory effect by AZT was detected during the second week of culture. In contrast, ddC inhibited the erythroid cell lineage by 7 days of culture (Figure 3, panel C), and that effect did not increase during the second week of culture (Figure 3, panel D).

Effects of 2',3'-dideoxynucleosides on mitochondrial DNA content in CFU-GM liquid culture. ddC was the most potent inhibitor of mtDNA content in human CFU-GM liquid cultures in Table 2. At 1μM, only a weak mtDNA signal could be detected accounting for approximately 90% inhibition as compared to control, clearly demonstrating the preferential effect of ddC on mtDNA synthesis. Of interest, FLT also substantially inhibited the mtDNA content of cells in human CFU-GM liquid cultures, with a 33% inhibition compared to control at an IC_{50} value of 0.1 μM increasing to a 60% inhibition at a concentration of 1 μM. In contrast, neither AZT nor its catabolite AMT had any inhibitory effect on mtDNA synthesis at concentrations inhibitory to cell growth proliferation and differentiation. Also, AZT and AMT had no effect on mtDNA content in erythroid progenitors (data not shown). D4T exhibited a minor inhibitory effect on mtDNA content a 10 μM, however it should be noted that D4T was not toxic to these cells.

DISCUSSION

Bone marrow is a heterogeneous population of cells representing at least seven lineages of hematopoietic cells in various stages of maturation. Approximately 1 to 5% of gradient centrifuged mononuclear cells from bone marrow are CD34+ cells. The CD34 antigen is expressed on myeloid precursors of the granulocyte, monocyte, erythroid, and megakaryocytic lineages. In this study, pluripotent progenitor CD34+ cells were isolated from human bone marrow using an anti-CD34 monoclonal antibody conjugated to FITC by cell sorting (FACStar) with a degree of purity greater than 99%. Isolation of highly purified progenitors was more rapid than the usual immunomagnetic affinity technique which requires a double selection procedure (13, 31). In addition the yield of CD34+ progenitor cells was approximately 10 to 15 fold higher using the present methodology as compared to the commonly used immunomagnetic bead method.

The purified CD34+ progenitors undergo extensive proliferation during 14 days of liquid culture, with a marked increase in differentiation for the last 7 days as demonstrated by flow cytometry. The in vitro differentiated cells exhibit normal morphological features in response to specific hematopoietic growth factors. Human bone marrow progenitors, triggered into cycling by hematopoietic growth factors in liquid suspension culture, undergo extensive proliferation coupled with expression of lineage specific differentiation antigens along either the granulocyte-macrophage pathway (upon treatment with IL-3 and GM-CSF) or the erythroid pathway (upon addition of IL-3, SCF and EPO). Thus, these liquid cultures appear to be a potential in vitro system to assess bone marrow toxicity of drugs and also permit study of their mechanism(s) of cytotoxicity by biochemical and molecular biological techniques. In contrast, while the semi-solid agar system requires less sophisticated techniques and is still highly predictive of in vivo bone marrow toxicity of antiviral drugs (32), the limited number of progenitor cells available with the agar system does not allow investigative pharmalogical techniques in an homogeneous cell population.

Among the tested ddNs, FLT was the most toxic compound, inhibiting proliferation of CD34+ cells induced towards both lineages. This severe inhibition of human progenitors is consistent with the pronounced hematotoxicity observed in patients (18) which has led to discontinuation of clinical studies with this anti-HIV drug.

As previously reported in clonogenic assays (32), D4T did not exhibit any cytotoxicity at concentrations up to 10 μM in human CFU-GM liquid cultures. AMT, a metabolite of AZT detected both in vitro (11) and in vivo (36), was shown to be more toxic than AZT to either granulocyte-macrophage or erythroid lineages using liquid cultures. These results are in agreement with data obtained with semi-solid cultures of human CFU-GM and BFU-E clonogenic assays (Table 1) and confirm the validity of this novel assay . In particular, inhibition of erythroid phenotype by AZT occurred during the second week of culture as determined by decreased glycophorin A expression. Therefore these data would suggest that AZT inhibits differentiation of more mature BFU-E progenitors. Consistent with these data, our group has previously demonstrated that AZT inhibits hemoglobin synthesis through down-regulation of globin gene transcription (39, 40) and in vivo studies have reported a blockage in the maturation of erythroid cells in a MAIDS model after AZT administration (8). Therefore, a blockage of erythroid differentiation may also play a potential role in AZT-induced anemia observed in patients.

The morphological features of human CFU-GM after 14 days of continuous exposure to AZT showed a perturbation in the ratio of granulocyte to monocyte lineages (Figure 2). AZT markedly decreased the granulocyte population consistent with AZT-induced neutropenia observed in patients (15, 30, 42). In contrast, the monocyte population increased in a dose dependent manner and this effect was not detected with ddC (Figure 2) or the other ddNs tested (data not shown). Although the CD11c antibody (predominantly monocyte phenotype) has also an overlapping specificity for granulocyte lineage cells recognized by the CD15 antibody (predominantly granulocyte phenotype) the

significance of these data is unclear and further studies are needed to understand the underlying mechanism(s) and their consequences.

ddC was toxic to the same extent to erythroid and granulocyte-macrophage lineages with a prominent inhibitory effect during the first week of culture. In contrast, when CD34+ cells were also incubated for seven days in the presence of AZT, FLT or AMT, concentrations as high as 10 μM were required in order to achieve a 50% inhibition of cell growth (data not shown). In addition, when these drugs are added at day 8 (when no CD34+ progenitor cells are detected) the IC_{50} value of ddC increases several fold whereas no substantial changes in the IC_{50} values were detected with the thymidine derivative. This data suggests that AZT, FLT, and AMT may interfere at a later stage in the proliferation/differentiation processes of progenitor cells as compared to ddC, with the latter being more toxic to progenitors CD34+ and/or to the early stage erythroid and granulocyte-macrophage progenitors.

Large variability by several orders of magnitude in IC_{50} values has been reported with ddC between different groups (19, 12, 25, 9) including our own laboratory (21, 32, 35). These apparent discrepancies may possibly result from a wide variation in the percentage of CD34+ pluripotent cells present in human bone marrow mononuclear cells which are used in semi-solid clonogenic assays.

Hematological toxicity of ddC is not usually a limiting factor in the treatment of AIDS with this antiretroviral agent, probably due to administration of low ddC dosages which result in exposure to concentrations below those necessary to affect hematopoietic cell lineages. Consistent with this hypothesis, early clinical trials of ddC at higher doses had led to the development of hematotoxic side-effects in 40% of patients (41), in agreement with in vitro studies in animals (37), and our present study also demonstrates the sensitivity of human CD34+ pluripotent cells to ddC.

Previous studies have suggested that inhibition of mtDNA synthesis is a possible mechanism of AZT-induced hematopoietic toxicity (24, 27). In contrast, Cheng's group has reported that high concentrations of AZT well above the IC_{50} values were required to affect mitochondrial DNA content (6). This same group has demonstrated that ddC, and to a lesser degree D4T, preferentially inhibit mitochondrial DNA synthesis in a human lymphoblastoid cell line and that the degree of inhibition correlates with drug-induced toxicity (4, 6). To further elucidate the role of inhibition of mitochondrial DNA synthesis in ddN-induced toxicity, we investigated the potential effects of these ddNs on mitochondrial DNA content of human CFU-GM after 14 days of continuous exposure to drugs. Consistent with previous reports (4, 6), ddC preferentially decreased mtDNA content. In contrast, AZT and AMT, at concentrations inhibiting more than 75% of cell growth, had no detectable effect of mitochondrial DNA content in liquid cultures of CFU-GM (Table 2) or BFU-E (data not shown) suggesting that this cellular event does not play a major role in AZT or AMT-induced bone marrow suppression. The possible role of inhibition of mtDNA synthesis in D4T-induced host toxicity could not be ascertained in this in vitro model, since D4T at a concentration up to 10μM was not affecting cell growth of human CFU-GM liquid cultures. Of particular interest, was the substantial inhibition of mitochondrial DNA content by FLT, a close analog of AZT which has shown pronounced hematotoxicity in preclinical (2) and clinical studies (18). As previously suggested for ddC (5), FLT is probably not a substrate for the relevant mitochondrial phosphorylating enzyme which will lead to formation of its 5'-triphosphate derivatives within mitochondria (28). This study further raises the question of the cellular mechanism(s) leading to that selective toxicity for some ddNs.

In conclusion, this is the first report of a simple methodology to isolate homogeneous human erythroid and granulocyte-macrophage lineage cells in primary liquid cultures. This report further supports previous studies demonstrating the unique and multifactorial mechanism(s) of ddN-induced toxicities on various stages of proliferation and

differentiation of human BFU-E and CFU-GM lineages. This human primary cell liquid culture system provides an adequate model for investigating the toxicity of antiviral compounds on human bone marrow function and should allow detailed examination of cellular and molecular events involved in ddN-induced hematological toxicity .

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TABLE 1. Effect of ddNs on proliferation of CD34+ progenitor cells in liquid cultures of human CFU-GM and BFU-E.

Compound	Mean ± SD IC50 (μM)[a]			
	CFU-GM culture		BFU-E culture	
	Liquid	Semisolid	Liquid	Semisolid
AZT	4.90 ± 0.35	1.90 ± 1.20[b]	0.18 ± 0.03	0.60 ± 0.50[b]
ddC	0.60 ± 0.05	0.70 ± 0.20[c]	0.50 ± 0.05	0.05 ± 0.04[c]
FLT	0.14 ± 0.02	0.55 ± 0.37[d]	0.05 ± 0.02	0.04 ± 0.01[d]
AMT	0.64 ± 0.04	0.40 ± 0.20[b]	0.06 ± 0.01	0.06 ± 0.05[b]
d4T	>10	20-100[e]	>10	10[e]

[a] Each set of data represents the arithmetic mean value ± standard deviaton of at least three independent experiments performed in triplicate. IC_{50}s were obtained by using least-squares linear regression analysis of the logarithm of drug concentration versus CFU-GM or BFU-E survival fractions.
[b] Taken from reference 11.
[c] Taken from reference 21.
[d] Taken from reference 35.
[e] Taken from reference 32.
nd: not determined

TABLE 2. Effect of ddNs on mt DNA levels of human CFU-GM in liquid cultures.

Compound (conc μM)	ratio mtDNA/actin	% decrease in mt DNA
Control	1.50 ± 0.20	0
ddC (0.1)	0.80 ± 0.05	47
ddC (1.0)	0.15 ± 0.05	90
FLT (0.1)	1.00 ± 0.10	33
FLT (1.0)	0.60 ± 0.05	60
AZT (1.0)	1.65 ± 0.20	NM[b]
AZT (10.0)	1.55 ± 0.25	NM
AMT (0.1)	1.70 ± 0.15	NM
AMT (1.0)	1.60 ± 0.15	NM
d4T (1.0)	1.45 ± 0.10	NM
d4T (10.0)	1.28 ± 0.05	15

[a] Each set of data represents the arithmetic mean value ± standard deviation of at least three independent experiments.
[b] NM, not measurable.

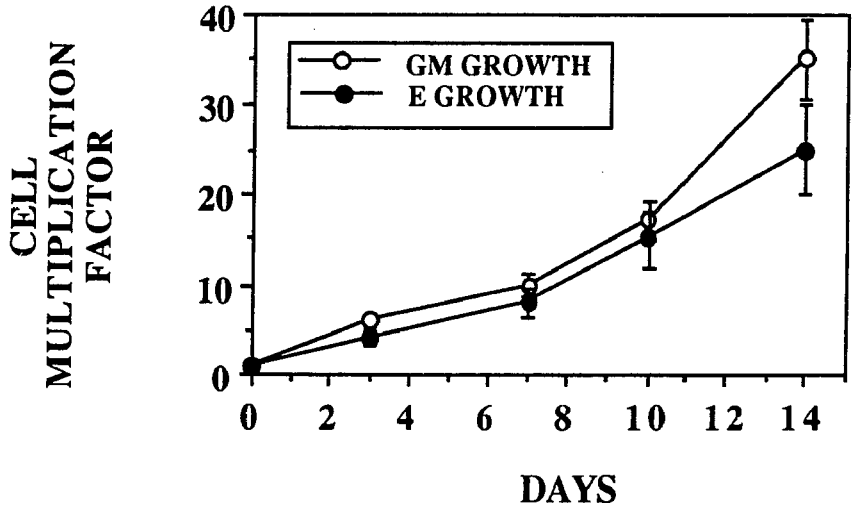

FIG. 1. Proliferation of CD34+ pluripotent progenitor cells in the presence of GM-CSF and IL-3 (GM culture, O) or EPO, stem cell factor, and IL-3 (for erythroid culture, ●) over 14 days in liquid cultures.

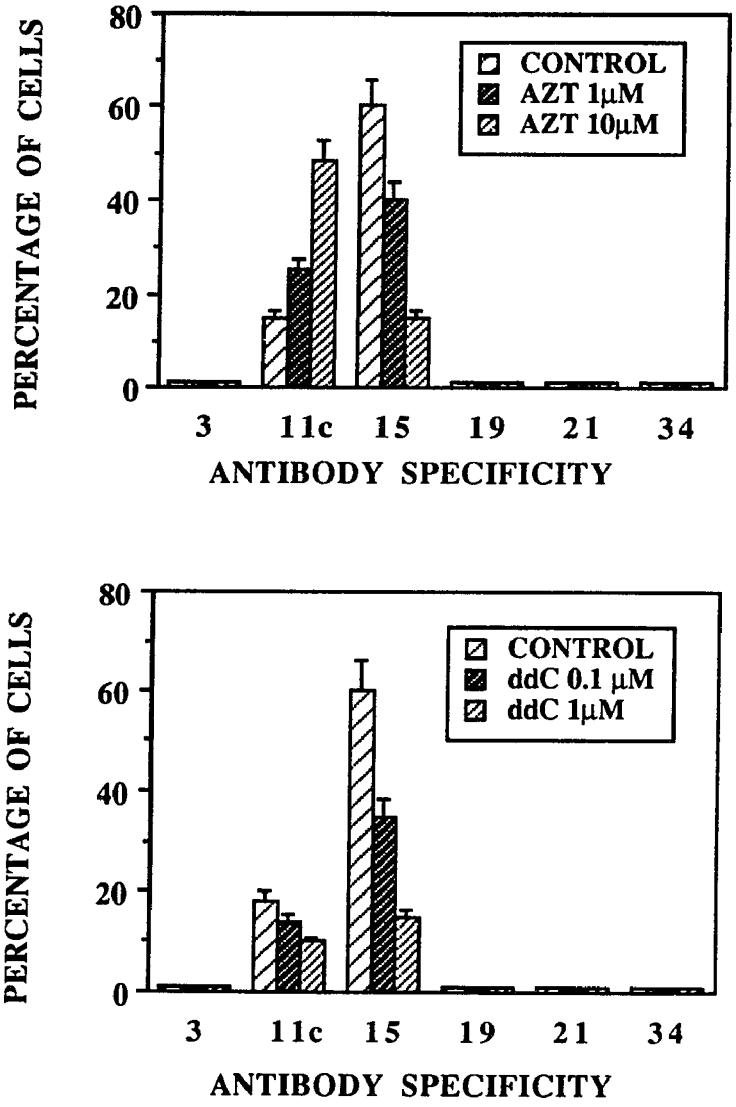

FIG. 2. Effects of AZT (A) and ddC (B) on CFU-GM liquid cultures. Columns represent the mean percentage of cells expressing different myeloid-and lymphoid-associated cell surface markers assessed after 14 days of culture of pluripotent CD34+ progenitor cells with and without drugs in the presence of GM-CSF and IL-3 in at least three separate experiments performed in triplicate; bars represent standard deviations. The antibody specificity is given as CD-cell numbers with the exception of glycophorin A (21): 3 for T cells, 11c for monocytes, 15 for granulocytes, 19 for B cells, and 34 for pluripotent progenitor cells.

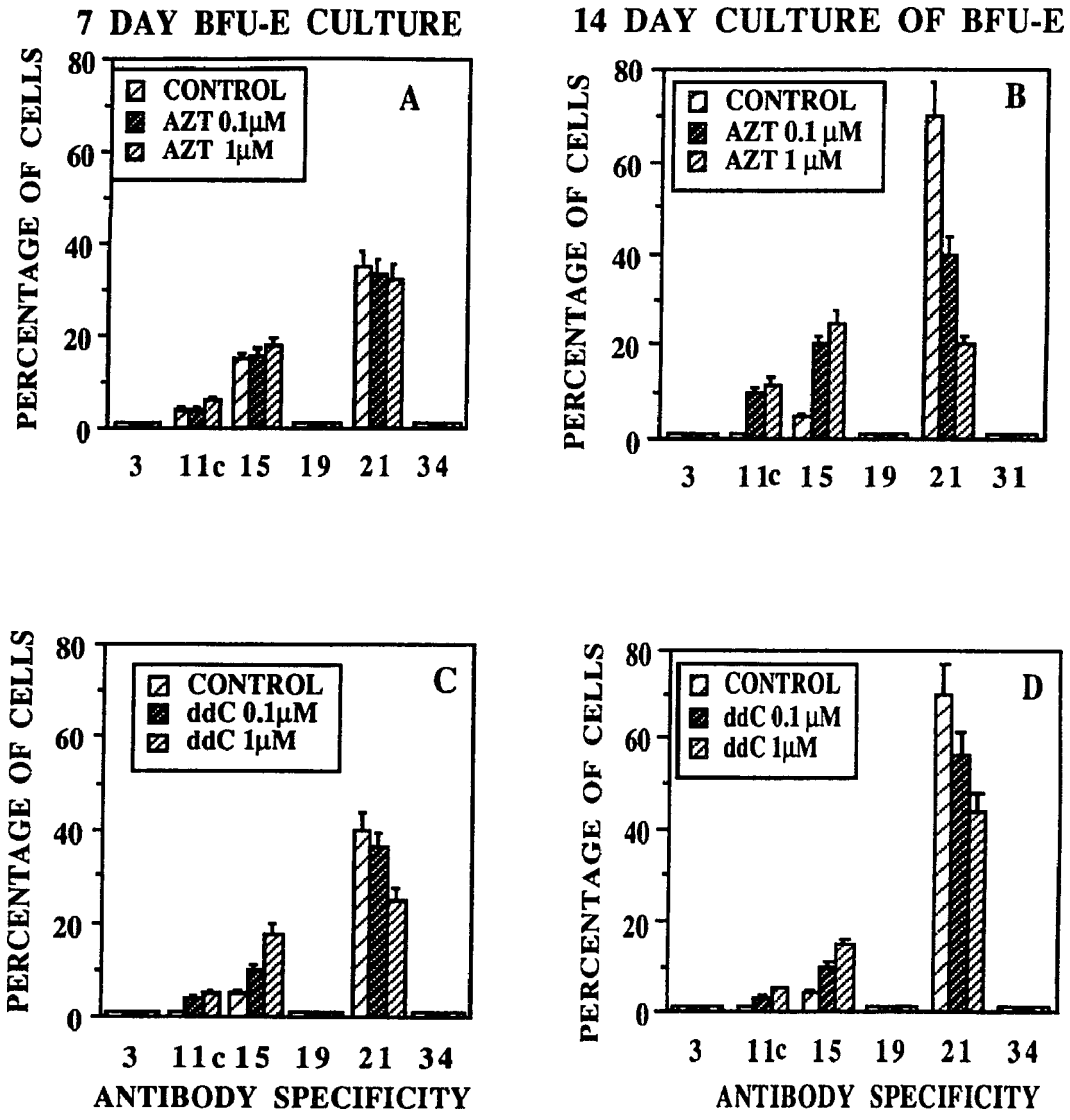

FIG. 3. Effect of AZT (A and B) and ddC (C and D) on BFU-E liquid cultures. Columns represent the mean percentage of cells expressing different myeloid- and lymphoid-associated surface markers after 7 and 14 days of culture of pluripotent CD34+ progenitor cells with and without drugs in the presence of EPO, SCF, and IL-3 in at least three separate experiments performed in triplicate; bars represent standard deviations. Cell surface markers are as described in the legend to Fig. 2.

PROTECTION AND RESCUE OF 2',3'-DIDEOXYPYRIMIDINE NUCLEOSIDE ANALOG TOXICITY BY HEMIN IN HUMAN BONE MARROW PROGENITOR CELLS

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ABSTRACT

Long-term therapy of AIDS patients with 3'-azido-3'-deoxythymidine (AZT) remains of concern due to resulting hematopoietic toxicity. While the mechanism(s) of this toxicity remain elusive, alternative strategies are being developed to reduce these toxic effects including combination therapy with non-myelotoxic anti-HIV drugs and/or administration of protective or rescue agents which include cytokines and growth factors. Using a particularly relevant human CD34+ liquid culture system, the unique toxicity profiles of dideoxynucleosides (ddNs) to both proliferation and differentiation was demonstrated with potencies in the order of 3'-fluoro-3'-deoxythymidine (FLT) = 3'-amino-3'-deoxythymidine (AMT) = 2',3'-dideoxycytidine (ddC) > AZT for inhibition of proliferation and in the order of FLT=AMT>AZT>>ddC for inhibition of hemoglobin synthesis. Hemin selectively protected erythroid lineage human BFU-E from AZT and AMT-induced inhibition but had no effect on FLT toxicity under similar conditions. Myeloid lineage human CFU-GM were also not protected by hemin against all three ddN analogs. Simultaneous exposure of cells to hemin and AZT resulted in a complete protection of both cell proliferation and hemoglobin synthesis. In contrast, in reversal studies, only the inhibition of the percentage of hemoglobin-synthesizing cells returned to control levels, but inhibition of proliferation of cells previously exposed to AZT was not reversed by hemin. These studies further define the unique and multifactorial mechanism(s) of ddN-induced toxic effects during hematopoietic development of pluripotent stem cells and suggest the beneficial use of hemin in possibly alleviating the toxicity of certain ddN analogs.

INTRODUCTION

3'-azido-3'-deoxythymidine (AZT) remains the first line therapy for the management of AIDS patients but the accompanying hematopoietic toxicity remains an obstacle for continued long-term treatment (24, 25). Because the development of a safe and effective vaccine appears unlikely in the near future, reduction of the toxic side effects

of chemotherapy becomes even more important (6). The mechanisms of AZT-induced hematopoietic toxicity are multifactorial (26), and several strategies have been devised in attempts to reduce the hematopoietic toxicity of that drug. These approaches include combination chemotherapy with other anti-HIV agents effecting different toxicities (30) and association with modulating agents protecting or reversing the toxic effects. In that context, we have previously reported the protective and selective effect of uridine against AZT-induced toxicity in human bone marrow CFU-granulocyte, macrophage (CFU-GM) clonogenic assays (28).

To further explore possible pharmacological modulation of dideoxynucleosides (ddNs) with agents possibly affording protection or reversal of this toxicity against human bone marrow cells, the effect of hemin on the proliferation and differentiation of CD34+ human bone marrow cells treated with ddNs was studied. Hemin has been implicated in a variety of cellular events, including growth promotion (22), regulation of hemoglobin synthesis (3), and acceleration of erythroid maturation (19). In addition, other investigations have suggested that hemin may act preferentially on early erythroid progenitors such as CFU-granulocyte, erythrocyte, monocyte, macrophage (CFU-GEMM) and burst forming units-erythroid (BFU-E) with less influence on non-erythroid progenitors, such as CFU-GM (23). The role of hemin in erythropoiesis has led to the question of whether hemin may alter AZT-induced hematopoietic toxicity. Previous studies have suggested that exposure of human colony-forming cells obtained from peripheral blood and bone marrow to hemin leads to an increase in colony growth compared to normal and AZT-treated cells (4). Decreased cellularity induced by AZT was partially recovered in vitro by a mixture of hemin and erythropoietin (EPO) (2, 9) as well as in vivo either with hemin alone (1), in combination with EPO (18) or stem cell factor (SCF) (17).

The toxic effects of anti-HIV ddNs have been ascertained by our group on human bone marrow cells in semi-solid colony-forming assays (10, 16, 29) and in liquid

suspension cultures of the highly purified CD34+ cells (11). The CD34+ fraction of human bone marrow cells represents the pluripotent stem cell and early progenitors (31) and can be induced with EPO, interleukin-3 (IL-3), and SCF to differentiate along the erythroid lineage in liquid suspension culture (11). It should be noted that the liquid suspension culture may have an advantage over the semi-solid cultures, in that cells can be removed in sufficient numbers for further analysis (7). We have therefore extended our previous investigations to determine the protective effect of hemin on the proliferation and differentiation of marrow cells toward various ddN toxicity by using both in semi-solid colony-forming assays and CD34+ liquid cultures. Further motivation for these investigations was stimulated by our demonstration that AZT and 3'-amino-3'-deoxythymidine (AMT), a toxic metabolite of AZT known to inhibit BFU-E in semi-solid cultures (27, 32), inhibited hemoglobin synthesis through a down-regulation of globin gene expression (34, 33), raising the question as to whether hemin could also alleviate the negative interaction of the drug and its metabolite with this erythropoietic function. The present study reports on the effects of hemin on various human bone marrow toxicities induced by AZT, AMT, 2',3'-dideoxycytidine (ddC), and 3'-fluoro-3'-deoxythymidine (FLT), a potent anti-HIV drug with major hematopoietic toxicity (13). Photofrin, a porphyrin derivative currently investigated in clinical trials (14), was also evaluated for possible modulating activities of ddNs and to ascertain whether hemin effects were a characteristic of heme analogs, in general.

MATERIALS AND METHODS

Materials. FLT was a gift from Dr. R.F. Schinazi (Emory University, Atlanta, GA). AMT, AZT, ddC, SCF, anti-IgG1-FITC, anti-IgG2a-PE, anti-IgG2b-FITC, anti-IgM-PE, CD15-PE, and hemin were purchased from Sigma, St. Louis, MO. Anti-CD34-PE (HPCA-2), Simultest CD3-FITC/CD19-PE, and anti-CD11c-PE were purchased from Becton Dickinson, Bedford, MS. Glycophorin A-FITC was purchased from Amac, Inc., Westbrook, ME. IL-3 was purchased from Upstate Biotechnologies, Lake Placid, NY.

EPO was purchased from Connaught Labs, Ontario, Canada. Hank's balanced salt solution, fetal bovine serum, and McCoy's 5A medium was obtained from Gibco/BRL, Grand Island, NY. MACS antibodies (IgG and anti-CD34 QBend/10), magnetic beads, and magnetic separation columns were purchased from Miltenyi Biotech, Inc., Sunnyvale, CA. All other chemicals and reagents were of the highest analytical grade available.

Mononuclear cell isolation. Heparinized bone marrow samples were obtained from the posterior iliac crest in normal human volunteers after informed consent of a protocol approved by the Institutional Review Board Committee at the University of Alabama at Birmingham. The mononuclear cells were separated by Ficoll-Histopaque gradient centrifugation, and adherent cells were removed as previously described (11). Non-adherent cells were cultured in methylcellulose semi-solid cultures or further purified for liquid cultures.

Colony-forming assay. Methylcellulose colony-forming culture assays were performed as previously described (27). Briefly, culture plates were set up in triplicate for each treatment for at least three different bone marrow donors. In separate experiments, BFU-E or CFU-GM colonies were scored after 15 days incubation at 37°C in a humidified atmosphere with 5% CO_2.

Progenitor enrichment and purification. CD34+ cells were enriched according to a protocol using MiniMACS magnetic separation columns, essentially as previously described by Lansdorp et al. (20). Briefly, non-adherent cells from the mononuclear fraction were incubated with IgG blocking antibody and a modified anti-CD34 antibody (QBend/10). Magnetic beads were attached as recommended by the manufacturer. The CD34+ cells were then retained on MiniMACS magnetic separation columns, eluted with buffer, and incubated with anti-CD34-PE (HPCA-2) monoclonal antibody before being subsequently sorted by Fluorescence Activated Cell Sorting (FACS), as previously described (11). FACS analysis was performed on a Becton Dickinson FACStar instrument using the lysis program for analysis of double fluorescence. Dead cells were gated out

using propidium iodide staining, and remaining cells were gated according to side and forward light scatter and analyzed for fluorescence, with phycoerythrin (PE) positive cells collected for liquid culture. The purity of the CD34+ cells was greater than 99% with a viability of more than 96%, as measured by trypan blue staining.

Liquid suspension cultures. FACS sorted CD34+ cells (2.0 X 10^4 cells/ml) were cultured in supplemented McCoy's 5A media enriched with 15% FBS and 2-mercaptoethanol (0.1 mM). Human recombinant IL-3 (100 U/ml), SCF (7 ng/ml), and recombinant human EPO (2 U/ml) were added in 24-well tissue culture dishes to stimulate erythropoiesis in the 7 or 14 day cultures. Hemin alone, ddNs alone, or hemin plus ddNs were incubated at selected concentrations. At least triplicate wells were cultured for each treatment from at least three different bone marrow donors. In toxicity determinations, drugs were added to wells on Day 1 and cultured for 14 days, counted, and then analyzed for proliferation and glycophorin A expression. In the protection investigations, AZT (0.1 or 1 μM) and either medium (control) or 10 μM hemin were added simultaneously on Day 1 and cultured for 14 days, counted, and analyzed for proliferation and percentage of benzidine positive cells. In the reversal investigations, cells were exposed to either no drug (control), 0.1 or 1 μM AZT for 7 days and washed with fresh incubation medium to wash out the AZT. Cells were subsequently cultured for another 7 days with AZT (0.1 or 1 μM) and either medium (control) or 10 μM hemin. Cells were then counted and analyzed for proliferation and percentage of benzidine positive cells. Exposure of 10 μM hemin alone was also investigated both in the protection and reversal studies.

Flow Cytometry Analysis. To insure positive antibody controls, primary isolated nonadherent mononuclear cells (MNCs) and cultured cells following 14 days of incubation were analyzed by flow cytometry using six different monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC) or (PE): CD3-FITC (T-cell marker), CD19-PE (B-cell marker), CD15-FITC (granulocyte marker), CD11c-PE (monocyte marker), CD34-FITC (pluripotent progenitor marker), and Glycophorin A-FITC (erythrocyte marker).

IgG1, IgG2b, IgG2a, and IgM were used as negative controls. The cells (1.5×10^5) were incubated at 4°C in the dark in the presence of 10 uL of monoclonal antibodies at the appropriate dilution. After 30 min of incubation, the cells were washed twice with phosphate-buffered saline (PBS). The cells were then resuspended in PBS and immediately analyzed by a FACStar instrument (Becton-Dickinson) using the lysis program for analysis of double fluorescence. Glycophorin-A expression was quantitated for analysis of erythroid differentiation.

Cell proliferation and viability were assessed by counting viable cells in a hemocytometer and by a trypan blue exclusion method, respectively. The viability of the 14-day cultured cells was greater than 95%. Benzidine staining allowed measurement of cells synthesizing hemoglobin as previously described (15).

Statistical analysis was performed using Student's *t* test for independent non-paired samples.

RESULTS

Protection from ddN toxicity by hemin in erythroid and myeloid semi-solid cultures. Hemin, at concentrations of 50, 100, and 250 μM stimulated BFU-E colony formation by 20 to 40% in methylcellulose clonogenic assays. The effects of adding hemin to human hematopoietic progenitor cells in the presence of toxic concentrations approximating the 40 to 60% inhibitory concentration (IC_{40} - IC_{60}) of AZT, AMT, and FLT are shown in Table 1. Non-toxic concentrations of hemin effected a significant and dose-dependent protection of AZT and AMT toxicity. Essentially, complete protection of AZT and AMT was achieved with 50 μM hemin, and only a minimal difference was detected in the presence of higher concentrations of 100 and 250 μM of hemin. In contrast, hemin up to a concentration as high as 250 μM did not reverse the toxic effects of FLT on BFU-E colony formation. As illustrated in Table 1, Photofrin, another porphyrin derivative, exerted a dose-dependent toxicity on human BFU-E colony growth with an IC_{50} value of 19.0 ± 5.4 μM. Of particular note, Photofrin, at non-toxic or moderately toxic

concentrations of 1 and 10 μM, did not protect human BFU-E from toxic effects induced by 1 μM AZT or 0.1 μM AMT (50% inhibitory concentration).

Clonal growth of human CFU-GM was also enhanced by a concentration of 50 or 100 μM of hemin, whereas a higher concentration of 250 μM of hemin led to a 40% inhibition of colony number. No protection was detected in myeloid methylcellulose assays when human bone marrow cells were exposed simultaneously to a toxic concentration of 1 μM of AZT, AMT, or FLT and non-toxic concentrations of 50 or 100 μM of hemin (Table 2).

Effect of ddNs on the proliferation and differentiation of CD34+ progenitor cells in liquid cultures of human BFU-E lineage. Cell proliferation and differentiation, as assessed by glycophorin A phenotyping, of CD34+ progenitor cells in liquid cultures of human BFU-E lineage were inhibited by AZT to the same extent with IC_{50} values of 0.17 ± 0.02 and 0.19 ± 0.01 μM, respectively. Similarly to AZT, AMT and FLT demonstrated equivalent toxicities for proliferation and differentiation with IC_{50} values of 0.07 ± 0.04 and 0.04 ± 0.01 μM, respectively. In contrast, ddC exhibited an IC_{50} value of 3.0 ± 1.7 μM for differentiation which was at least 40 fold higher than its IC_{50} value of 0.07 ± 0.01 μM for proliferation. No difference was observed when the differentiation process was assessed by measuring benzidine positive cells, indicative of hemoglobin synthesis.

Protection from AZT toxicity by hemin on the proliferation and differentiation of CD34+ progenitor cells in liquid cultures of BFU-E lineage. Simultaneous exposure to hemin and AZT was also investigated in our studies to assess whether hemin could protect cell growth and hemoglobin synthesis of human erythrocytic cultures from the inhibitory effect of AZT. Figure 1 demonstrates that 10 μM hemin completely protected erythroid cell growth as well as hemoglobin synthesis from the inhibitory effects of 0.1 or 1 μM AZT. Of note, exposure of cells for 14 days to a concentration of 10 μM of hemin resulted in an increased stimulatory effect on erythroid cell growth as compared to hemoglobin synthesis (Figure 1).

Ability of hemin to reverse the toxicity of AZT in human CD34+ progenitor cells in liquid cultures of BFU-E lineage. The effects of adding 10 μM of hemin in the presence or absence of AZT following a 7-day exposure to a 50% inhibitory concentration of 0.1 μM AZT are shown in Figure 2.

Essentially complete reversal of AZT inhibitory effects on hemoglobin synthesis was achieved with 10 μM of hemin, whereas reversal of AZT toxicity on cell growth was not detected under any investigated conditions. These data suggest that hemin cannot overcome cell growth inhibition mediated by pre-exposure of cells to AZT, further confirming our previous data which suggested that AZT toxicity occurs at early stages in human bone marrow progenitor cells (11).

DISCUSSION

The chronic and long term therapy of patients with AIDS may lead to a dose-limiting drug toxicity which could be minimized by the development of reversal and/or protection strategies. The latter has been demonstrated to be a highly selective and viable alternative in other disease states (5, 8). The present investigations report the effects of hemin on the protection and rescue of the hematopoietic toxicity induced by ddNs in a physiologically relevant liquid culture system of CD34+ human bone marrow cells. This liquid culture system allows purified hematopoietic progenitor cells to be induced to develop along a particular lineage in response to hematopoietic growth factors. The cells can be further analyzed to determine the effects of drugs added at various stages of development.

Among the tested ddNs, FLT was the most inhibitory toward proliferation and differentiation of hematopoietic progenitor cells, consistent with the hematotoxicity which has led to its discontinuation in clinical studies (12). AZT and its toxic metabolite, AMT, inhibited both proliferation and hemoglobin-synthesizing cells to the same extent as FLT. These data thus confirm our previous studies with K-562 erythroleukemia cells, suggesting an inhibition of erythroid differentiation by AZT and AMT through decreased

hemoglobin production. Of particular interest, the IC_{50}s for proliferation and hemoglobin-synthesizing cells were equivalent for AZT, AMT, and FLT, whereas despite a similar profound toxic effect towards proliferation, ddC had a minimal effect on hemoglobin-synthesizing cells, as previously demonstrated in butyric-acid induced human K-562 cells (34). These data demonstrate that proliferation and differentiation of the erythroid lineage may be affected differently by ddNs at pharmacologically relevant concentrations.

Hemin had a stimulatory effect on the proliferation of both human BFU-E and CFU-GM in semi-solid cultures, but failed to significantly protect CFU-GM from ddN toxicity while significantly protecting BFU-E from AZT and AMT-induced toxicity. Therefore, hemin exerts a selective and protective effect on cells of the erythroid lineage, consistent with previous reports (21). The absence of hemin protection of FLT-treated cells may also indicate that hemin acts at a later stage in the development of hematopoietic progenitor cells than FLT. Investigations using the liquid culture system suggest indeed that hemin may effect early progenitor cells after commitment of cells to the erythroid lineage, as treatment of hemin alone resulted in a stimulation of cell proliferation without enhancement of hemoglobin-synthesizing cells as compared to control. Simultaneous incubation of AZT and hemin resulted in complete protection from AZT-induced inhibition of both cell proliferation and hemoglobin synthesis, suggesting that AZT may affect later progenitor cells than hemin in erythroid development. Of particular interest, inhibition of proliferation of cells previously exposed to AZT was not reversed by hemin, while the inhibition of the percentage of hemoglobin-synthesizing cells returned to control levels under similar experimental conditions. These data indicate that AZT-induced effects on hemoglobin synthesis occur after cells are committed to differentiation along the erythroid lineage, consistent with the observed increase in bone marrow cellularity of AZT-treated mice receiving hemin treatment after a two-week recovery period as compared to an immediate treatment (2). In addition, these findings may suggest that hemin and AZT did not directly affect the differentiation of erythroid progenitor cells, but rather the maturation

of committed erythroid cells or the synthesis of hemoglobin. Independent of its effect on cell proliferation, hemin may have a direct effect on hemoglobin synthesis, as previously suggested (19), and therefore may overcome the inhibition of hemoglobin synthesis induced by AZT in human erythroid cells. Further studies are necessary to detail the interactive mechanism(s) between hemin and AZT and its toxic metabolite, AMT, but it is clear that human erythroid progenitor cells are protected by hemin against the inhibition of cell proliferation and hemoglobin synthesis induced by these two ddNs.

The present studies detail some basic mechanism(s) which may have been responsible for the observed protection of hemin against AZT-induced anemia in mice (2). The absence of protecting effects of hemin against FLT-induced toxicity in human erythroid cells provides further evidence that ddN myelotoxicity may not only be related to a general and unique mechanism(s) of toxicity, but also to multifactorial events involving both cell proliferation and differentiation.

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TABLE 1. Effect of AZT, AMT and FLT on the survival (% of untreated control)[a] of human BFU-E in the presence or absence of Hemin or Photofrin.

Compound Conc (μM)	Alone	With AZT (1 μM)	With AMT (0.1 μM)	With FLT (0.1 μM)
Hemin				
0	100	55.2 ± 9.2	54.7 ± 6.6	33.5 ± 1.0
50	118.3 ± 11.0[b]	82.3 ± 26.5[b]	86.0 ± 15.6[b]	36.6 ± 1.0
100	126.3 ± 20.8	93.7 ± 21.0[b]	92.5 ± 14.8[b]	37.0 ± 7.1
250	137.7 ± 25.5[b]	108.7 ± 19.0[b]	99.5 ± 6.4[b]	37.0 ± 9.9
Photofrin				
0	93.5 ± 2.1	59.4 ± 1.0	54.9 ± 3.3	ND
50	76.5 ± 6.4	62.4 ± 3.2	51.4 ± 9.8	ND
100	13.0 ± 4.2	56.0 ± 3.4	55.1 ± 2.2	ND

[a] Each value represents the mean ± standard deviation in at least three experiments with at least three different marrow donors after 14 days culture.

[b] $p < 0.05$ as compared with the control.

ND not determined.

TABLE 2. Effect of AZT, AMT and FLT on the survival (% of untreated control)[a] of human CFU-GM in the presence or absence of Hemin.

Compound Conc (μM)	Alone	With AZT (1 μM)	With AMT (1 μM)	With FLT (1 μM)
Hemin				
0	100	60.5 ± 4.2	33.0 ± 3.0	38.3 ± 10.0
50	134.3 ± 14.4	75.0 ± 15.5	49.3 ± 6.0	59.0 ± 16.1
100	123.7 ± 6.3	68.0 ± 5.6	44.0 ± 10.6	48.0 ± 14.5
250	55.7 ± 15.3	ND	ND	ND

[a] Each value represents the mean ± standard deviation in at least three experiments with at least three different marrow donors.

ND not determined

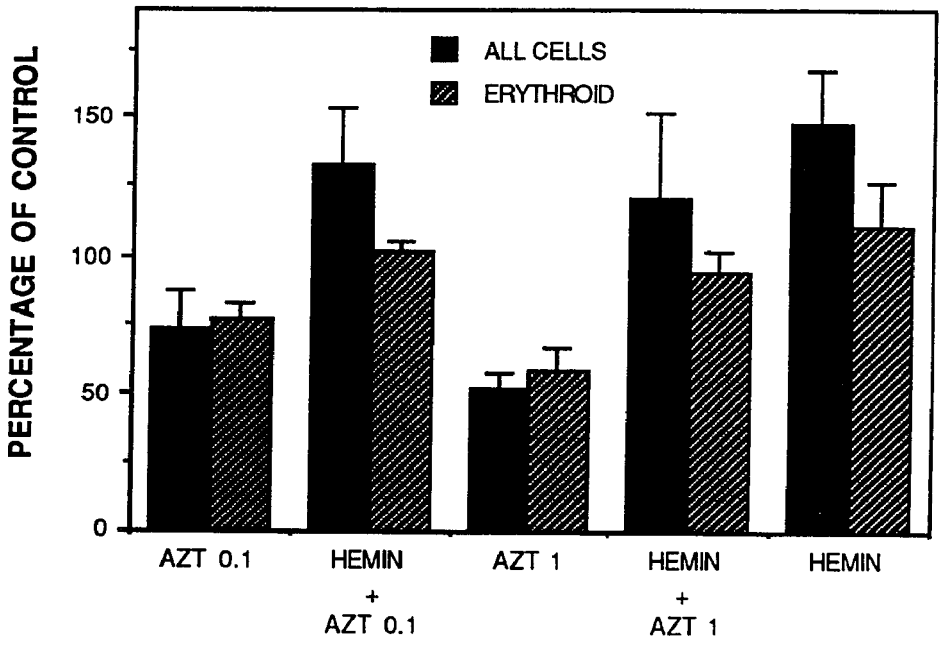

FIG. 1. Protection of AZT toxicity by hemin in human CD34+ progenitor cells in liquid cultures of BFU-E lineage. Cells were incubated 14 days with 0.1 or 1 μM in the presence or absence of 10 μM hemin. Hemin alone (10 μM) was also used as control. Columns represent the mean percentage of the proliferation of all cells and erythroid cells as compared to controls in three separate experiments; bars represent the standard deviation.

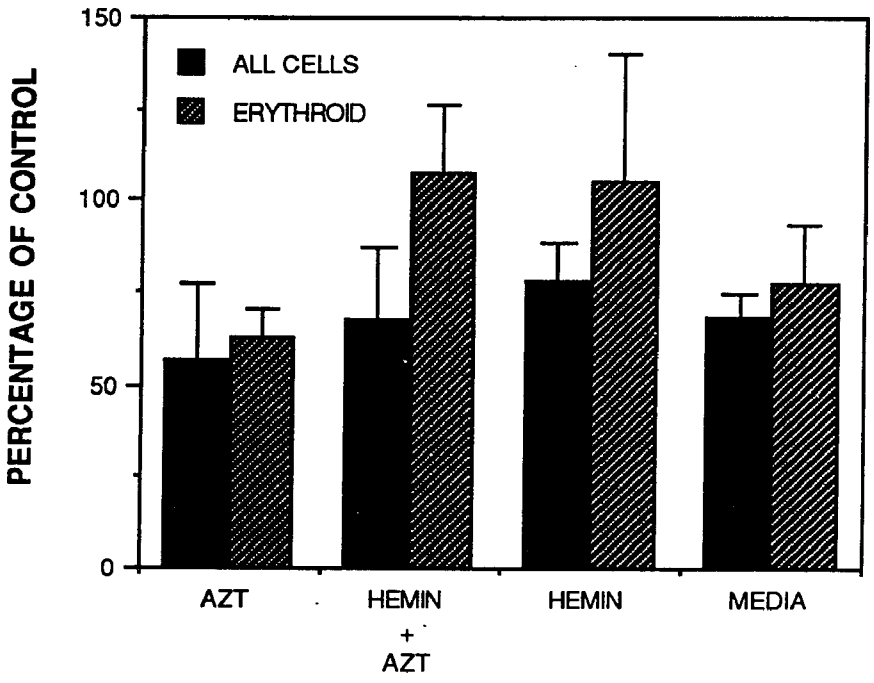

FIG. 2. Reversal of AZT toxicity by hemin in human CD34+ progenitor cells in liquid cultures of BFU-E lineage. Cells were incubated with 0.1 μM AZT for 7 days, washed twice, and recultured for 7 more days either in medium alone, with the same concentration of AZT, with the same concentration of AZT in the presence of 10 μM hemin, or in the presence of 10 μM hemin alone. Columns represent the mean percentage of the proliferation of all cells or erythroid cells as compared to controls in three separate experiments; bars represent the standard deviations.

EFFECT OF RECOMBINANT HUMAN HEMOGLOBIN ON HUMAN BONE MARROW PROGENITOR CELLS: PROTECTION AND REVERSAL OF 3'-AZIDO-3'-DEOXYTHYMIDINE-INDUCED TOXICITY

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SUMMARY

Long-term therapy of AIDS patients with 3'-azido-3'-deoxythymidine (AZT) remains limited due to resulting hematopoietic toxicity. While the mechanism(s) of this toxicity remain elusive, alternative strategies are being developed to reduce these toxic effects including combination therapy with non-myelotoxic anti-HIV drugs and/or administration of protective or rescue agents, including cytokines and growth factors. Using a physiologically relevant human CD34+ bone marrow cell liquid culture system, a crosslinked human recombinant hemoglobin (rHb), currently in Phase II clinical trials, was investigated for effects on hematopoiesis and evaluated for its potential in protecting or reversing AZT-induced hematopoietic toxicity. These investigations demonstrated the absence of toxic effects of 0.01, 0.1, or 1 μM of human rHb at concentrations up to 1 μM toward proliferation of erythroid or myeloid lineage cells. Proliferation of the erythroid lineage was protected against toxic concentrations (0.1 and 1 μM) of AZT by 1 μM human rHb. Inhibition of proliferation of cells previously exposed to AZT was not reversed at this concentration. These data suggest that human rHb may be of benefit in reducing the toxic effects of AZT toward anemia in transfusion-dependent AIDS patients.

INTRODUCTION

The management of AIDS patients with 3'-azido-3'-deoxythymidine (AZT) therapy has resulted in delayed progression of the disease, but treatment is limited by bone marrow toxicity, leading to anemia and neutropenia (21, 23). This hematopoietic toxicity is of concern as long term therapy is indicated and the development of a safe and effective vaccine appears unlikely in the near future (4). While the mechanism(s) of this toxicity remain elusive, alternative strategies are being evaluated, including association with modulating agents protecting or reversing the AZT toxic effects (27). In that context, we have previously reported the protective and selective effect of uridine against AZT-induced toxicity in human bone marrow CFU-granulocyte, macrophage (CFU-GM) clonogenic assays (26), and have recently described the selective effect of hemin in protecting and

reversing the erythropoietic toxicity induced by AZT in both clonogenic and CD34+ liquid cultures of human bone marrow cells (10).

Blood substitutes have recently received renewed interest as a consequence of contamination of the blood supply by human immunodeficiency virus (HIV) and hepatitis B virus (HBV). Cell-free hemoglobin solutions have been considered potential blood substitutes for more than 50 years following observations of increased reticulocytes and hematocrit consistant with erythropoiesis stimulation (1, 2). However, early investigations of crude hemoglobin preparations were not successful due to accompanying toxic effects including renal damage and anaphylaxis, and may have contributed to death (5, 17, 3). The recent development of a crosslinked recombinant human hemoglobin (12), which imparts stability to the hemoglobin tetramer and prevents its dissociation into aß subunits with subsequent renal damage, has been reported as a safe replacement of whole blood (16). Clinical investigations involving large doses of hemoglobin have shown stimulation of erythropoiesis. After introduction into plasma, hemoglobin binds to serum haptoglobin (13), an a_2-globin that forms a stable complex with hemoglobin and is removed from the circulation by histiocytes of the reticuloendothelial system in the spleen, liver, and bone marrow (11). Unbound hemoglobin can be demonstrated in plasma only after the binding capacity of haptoglobin has been exceeded. Therefore, it was of interest to investigate the effect of this recombinant cell-free hemoglobin in human bone marrow cells to determine its hematopoietic effects, especially in light of reports of cell-free hemoglobin stimulation of erythropoiesis.

In this report, we have investigated the effect of low doses of this crosslinked recombinant human hemoglobin on the development of erythroid and myeloid lineage cells from the pluripotent stem cell by using a liquid culture system of CD34+ human bone marrow cells and appropriate hematopoietic factors. This in vitro model system has been previously demonstrated by us to be an accurate and sensitive indicator of the development of human blood lineage cells and as a predictor of specific toxic effects of 2', 3'-

dideoxynucleosides, as well as protection and/or recovery effects of modulating agents on AZT toxicity (8, 10). Therefore, the potential protection and reversal of AZT-induced hematopoietic toxicity by recombinant human hemoglobin was assessed.

MATERIALS AND METHODS

Materials. Recombinant human hemoglobin (rHb) was provided by Somatogen, Inc., Boulder, CO. AZT, human recombinant stem cell factor (SCF), anti-IgG1-fluorescein isothiocyanate (FITC), anti-IgG2a-phycoerythrin (PE), anti-IgG2b-FITC, anti-IgM-PE, anti-CD14-FITC, and anti-CD15-PE were purchased from Sigma, St. Louis, MO. Anti-CD34-PE (HPCA-2), Simultest CD3-FITC/CD19-PE, anti-CD11c-PE, and human recombinant interleukin-3 (IL-3) were purchased from Becton Dickinson, Bedford, MS. Glycophorin A-FITC was purchased from Amac, Inc (Westbrook, ME). Human recombinant erythropoietin (EPO) and granulocyte, macrophage-colony stimulating factor (GM-CSF) were purchased from Connaught Labs, Ontario, Canada. Hank's balanced salt solution, fetal bovine serum, and McCoy's 5A medium was obtained from Gibco/BRL, Grand Island, NY. MiniMACS magnetic separation system, MACS antibodies (IgG and anti-CD34 QBend/10), magnetic beads, and magnetic separation columns were purchased from Miltenyi Biotech, Inc., Sunnyvale, CA. All other chemicals and reagents were of the highest analytical grade available.

Mononuclear cell isolation. Heparinized bone marrow samples were obtained from the posterior iliac crest in normal human volunteers after informed consent of a protocol approved by the Institutional Review Board Committee at the University of Alabama at Birmingham. The mononuclear cells were separated by Ficoll-Histopaque gradient centrifugation and adherent cells were removed as previously described (8). Non-adherent cells were further purified for liquid cultures.

Progenitor enrichment and purification. CD34+ cells were enriched according to a protocol using MiniMACS magnetic separation columns, essentially as previously described by Lansdorp et al. (15). Briefly, non-adherent cells from the mononuclear

fraction were incubated with IgG blocking antibody and a modified anti-CD34 antibody (QBend/10). Magnetic beads were attached as recommended by the manufacturer. The CD34+ cells were then retained on MiniMACS magnetic separation columns, eluted with buffer, and incubated with anti-CD34-PE (HPCA-2) monoclonal antibody before being subsequently sorted by Fluorescence Activated Cell Sorting (FACS), as previously described (8). FACS analysis was performed on a Becton Dickinson FACStar instrument using the lysis program for analysis of double fluorescence. Dead cells were gated out using propidium iodide staining and remaining cells were gated according to side and forward light scatter and analyzed for fluorescence, with PE positive cells collected for liquid culture. The purity of the CD34+ cells was greater than 99% with a viability of more than 96%, as measured by trypan blue staining.

Liquid suspension cultures. FACS sorted CD34+ cells (2.0 X 10^4 cells/ml) were cultured in supplemented McCoy's 5A media enriched with 15% FBS and 2-mercaptoethanol (0.1 mM). Human recombinant IL-3 (100 U/ml), SCF (7 ng/ml), and human recombinant EPO (2 U/ml) were added in 24-well tissue culture dishes to stimulate erythropoiesis in the 7 or 14 day erythroid cultures. Human recombinant IL-3 (200 U/ml) was used with human recombinant GM-CSF (125 U/ml) to stimulate myeloid cultures. Human rHb alone (0.01, 0.1, or 1 μM), AZT alone (0.1 or 1 μM), or human rHb in combination with AZT were incubated at selected concentrations. At least triplicate wells were cultured for each treatment from at least three different bone marrow donors. Either compound was added to wells on Day 1 and cells were cultured for 14 days, counted and analyzed for proliferation with phenotypic marker expression studied by flow cytometry as described below. In the protection investigations, AZT (0.1 or 1 μM) and either medium (control) or 1 μM rHb were added simultaneously on Day 1 and cells were cultured for 14 days, counted, and analyzed for proliferation. In the reversal investigations, cells were exposed to either no drug (control), 0.1 or 1 μM AZT for 7 days and washed with fresh incubation medium to wash out the AZT. Cells were subsequently cultured for another 7

days with AZT (0.1 or 1 μM) and either medium (control) or 1 μM rHb. Cells were then counted and analyzed for proliferation. Exposure of 1 μM rHb alone was also investigated both in the protection and reversal studies.

Flow Cytometry Analysis. To insure positive antibody controls, primary isolated nonadherent MNCs and cultured cells following 14 days of incubation were phenotyped by flow cytometry analysis using six different monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE): CD3-FITC (T-cell marker), CD19-PE (B-cell marker), CD14-FITC (monocyte marker), CD15-FITC (granulocyte marker), CD11c-PE (monocyte marker), CD34-PE (pluripotent progenitor marker), and Glycophorin A-FITC (erythrocyte marker). IgG1, IgG2b, IgG2a, and IgM were used as negative controls. The cells (1.5×10^5) were incubated at 4°C in the dark in the presence of 10 μL of monoclonal antibodies at the appropriate dilution. After 30 minutes of incubation, the cells were washed twice with phosphate-buffered saline (PBS). The cells were then resuspended in PBS and immediately analyzed by a FACStar instrument (Becton-Dickinson) using the lysis program for analysis of double fluorescence. Glycophorin-A expression was quantitated for analysis of erythroid differentiation.

Cell proliferation and viability were assessed by counting viable cells in a hemocytometer, and by a trypan blue exclusion method, respectively. The viability of the 14-day cultured cells was greater than 95%.

Statistical analysis was performed using Student's *t* test for independent, non-paired samples.

RESULTS

Effect of human rHb and AZT on the proliferation and lineage-specific antigen expression of human CD34+ progenitor cells in liquid cultures of erythroid and myeloid lineage. Cell proliferation of CD34+ human progenitor cells of either erythroid and myeloid lineage was not stimulated or inhibited by exposure of cultures to human rHb at concentrations ranging between 0.01 to 1 μM while AZT (0.1 and 1 μM) significantly

inhibited proliferation of erythroid cultures by 30 and 54%, respectively (Table 1). In myeloid cultures, AZT (1 and 10 μM) inhibited proliferation by 29 and 57%, respectively (Table 1). AZT effects were consistent with previously published results (8).

Lineage-specific antigen expression in erythroid cultures suggested a minor but non-statistically significant stimulation of erythroid lineage cells (glycophorin A) and monocyte-macrophage lineage cells (CD11c and CD14) by human rHb (except for glycophorin A expression with 0.1 μM rHb) as compared to control cell antigen expression (Table 2). The granulocyte lineage (CD15), T (CD3) and B (CD19) cell lineage, and progenitor cell lineage (CD34) expression was < 1% of total cells, either control or drug-treated (Table 2). AZT (0.1 and 1 μM) inhibited glycophorin A expression by 35 and 48%, respectively, but demonstrated no effect on other lineage expressions as compared to control (Table 2).

Lineage-specific antigen expression in myeloid cultures exposed to human rHb (0.01, 0.1, and 1 μM) demonstrated a slight dose dependent increase in granulocyte lineage cells (CD15) (99 ± 11, 107 ± 7, and 129 ± 16 as a percentage of control cultures, respectively), and a corresponding dose dependent decrease in monocyte-macrophage lineage cells (CD11c) (152 ± 50, 123 ± 74, and 48 ± 45 as a percentage of control, respectively). Erythroid cell lineage (glycophorin A), T (CD3) and B (CD19) cell lineage, and progenitor cell lineage (CD34) expression represented < 1%, either in control or drug-treated experiments.

Protection from AZT toxicity by human rHb on the proliferation of CD34+ progenitor cells in liquid cultures of erythroid lineage. Simultaneous exposure to human rHb and AZT was also investigated in our studies to assess whether human rHb could protect cell growth of human erythroid cultures from the AZT inhibitory effects. Figure 1 demonstrates that 1 μM human rHb protected erythroid cell growth from the inhibitory effects of 0.1 or 1 μM AZT (IC_{50} = 0.17 μM). A statistically significant increase from 55 to 79% and from 45 to 66% as compared to control was observed in cultures exposed to either 0.1 μM or 1 μM AZT with simultaneous exposure of 1 μM human rHb.

Ability of human rHb to reverse the toxicity of AZT in human CD34+ progenitor cells in liquid cultures of erythroid lineage. The effects of adding 1 μM human rHb in the presence or absence of AZT following a 7-day exposure to 0.1 or 1 μM of AZT are shown in Figure 2. Reversal of AZT toxicity on cell growth was not detected under any investigated conditions, suggesting that human rHb at that concentration cannot overcome cell growth inhibition mediated by pre-exposure of cells to AZT, further confirming our previous data which suggested that AZT toxicity occurred at early stages in human bone marrow progenitor cells (10).

DISCUSSION

The chronic and long term therapy of patients with AIDS may lead to a dose-limiting drug toxicity, often requiring transfusions, which could be minimized by the development of protection and/or reversal strategies. Protection strategies have been demonstrated to be highly selective and viable alternatives in other disease states (6). The present investigations report the effects of a crosslinked recombinant human hemoglobin on erythroid and myeloid lineage cell development, as well as possible protective and/or reversal effects of the hematopoietic toxicity induced by AZT. A physiologically relevant liquid culture system of CD34+ human bone marrow cells which allows purified hematopoietic progenitor cells to be induced to develop along a particular lineage in response to hematopoietic growth factors was used. In addition, these cells can be further analyzed to determine the effects of drugs added at various stages of development. Previously reported results of toxicity evaluations of ddNs using this in vitro system were shown to be predictive of the toxic effects observed in patients (8). For example, FLT was extremely toxic to these hematopoietic progenitors consistent with its recent withdrawal from further clinical evaluation as a consequence of potent hematopoietic toxicity (9).

Over 200 human subjects have received hemoglobin solutions either as therapy for anemia, blood loss, or investigations on the metabolic fate of hemoglobin after hemolysis (28). An array of side effects were observed, but toxicity due to hemoglobin itself was

difficult to characterize due to stroma contamination and degradation of hemoglobin into dimers (22, 24). The recombinant human hemoglobin investigated in the present in vitro study, has been evaluated in rats and dogs and was not found to induce renal or liver toxicity and did not adversely affect cardiac function (16). While some clinical studies of stabilized hemoglobins have still indicated toxicity, recent clinical investigations of human rHb have indicated a very safe clinical profile, and studies are currently in Phase II clinical trials (25).

We have investigated the effects of this human rHb on the development of hematopoietic progenitor cells in vitro and no toxic effects were discerned both on erythroid or myeloid proliferation and lineage-specific antigen expression, although we did not observe the erythropoietic stimulation indicated in early reports (1, 2). While that erythropoietic stimulation may have been due to either stroma contamination or hemoglobin catabolism, the absence of stimulation by human rHb in this liquid culture system may suggest an earlier involvement of stroma or a catabolic metabolite of hemoglobin stimulation of cells of the reticuloendothelial system. Cell-free hemoglobin is taken up and degraded by proteolysis in a variety of cells, releasing heme which can be further catabolized in the liver and excreted in bile (28). It is likely that hemoglobin may be transported by serum proteins to bone marrow (and other organs of the reticuloendothelial system) in vivo where it is catabolized and may stimulate release of growth promoting factors from histiocytes. The heme released in this process may thus be responsible for the erythropoietic stimulation observed in earlier reports. Of importance, a protective effect of human rHb was demonstrated on AZT-induced toxicity toward proliferation of hematopoietic progenitor cells of erythroid lineage (Figure 1). The mechanism of this protection may be similar to that by which hemin protects human progenitor cells from AZT-induced toxicity, as previously reported by us (10). Catabolism of human rHb may result in heme release in human bone marrow progenitor cells, leading to protection from AZT-induced toxic effects. Reversal studies of human rHb were consistent with the above

hypothesis, in that neither hemin nor human rHb was able to reverse AZT-induced toxicity toward cell proliferation in cultures pre-exposed to AZT. These studies suggest that human rHb, similar to hemin, may also affect an earlier progenitor cell than that affected by AZT.

As the scientific community continues the search for a blood substitute (14, 20, 7), the present data demonstrate the absence of toxic effects in human progenitor cell development and further confirms the potential of this human rHb as a viable blood substitute. In addition, protection and reversal studies suggest that human rHb may exert effects on an earlier hematopoietic progenitor than AZT, as suggested previously for hemin by our group and others (10, 18, 19). Protective effects of human rHb against AZT-induced toxicity may benefit AIDS patients who are intolerant to AZT therapy and require transfusions.

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TABLE 1. Effect of different concentrations of human recombinant hemoglobin and AZT on cell proliferation of erythroid and myeloid lineage CD34+ human bone marrow cells. Data expressed as the mean ± standard deviation of the percentage of control for at least three different bone marrow donors.

	rHb [μM]			AZT [μM]		
Lineage	0.01	0.1	1	0.1	1	10
Erythroid	96 ± 20	113 ± 17	96 ± 7	70 ± 4[a]	46 ± 9[a]	ND
Myeloid	114 ± 10	93 ± 4	106 ± 33	ND	71 ± 6[a]	43 ± 8[a]

[a] $p < 0.05$
ND not determined

TABLE 2. Effect of different concentrations of recombinant human hemoglobin and AZT on hematopoietic and lymphopoietic-associated cell surface markers in human BFU-E liquid cultures. Data expressed as the mean ± standard deviation of the percentage of control for at least three different bone marrow donors.

	rHb [μM]			AZT [μM]	
Antibody[a]	0.01	0.1	1.0	0.1	1.0
GPA	102 ± 16	78 ± 16	114 ± 21	65 ± 25	52 ± 10[b]
CD11c	104 ± 27	101 ± 25	110 ± 38	97 ± 29	88 ± 25
CD14	107 ± 17	113 ± 3	109 ± 10	104 ± 12	NA

[a] CD3 (T cell), CD19 (B cell), CD34 (progenitors), and CD15 (granulocytes) represented less than 1%.

[b] $p < 0.05$

NA not available

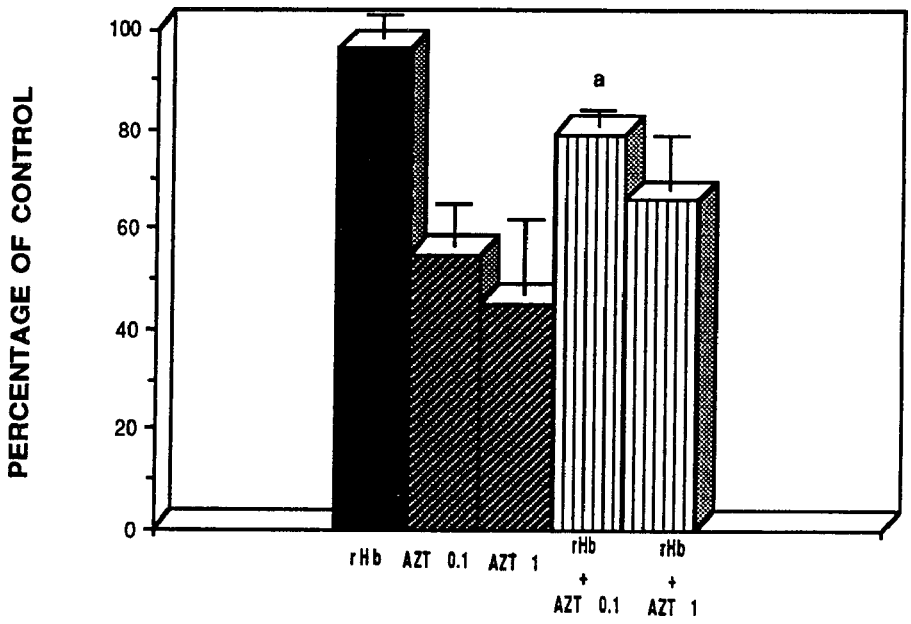

FIG. 1. Protective effect of 1 μM recombinant human hemoglobin on 0.1 and 1 μM AZT-induced toxicity in human CD34+ erythroid progeny in liquid cultures. Columns represent the mean of the percentage of control; bars represent standard deviation.
a $p < 0.06$ as compared to AZT 0.1.

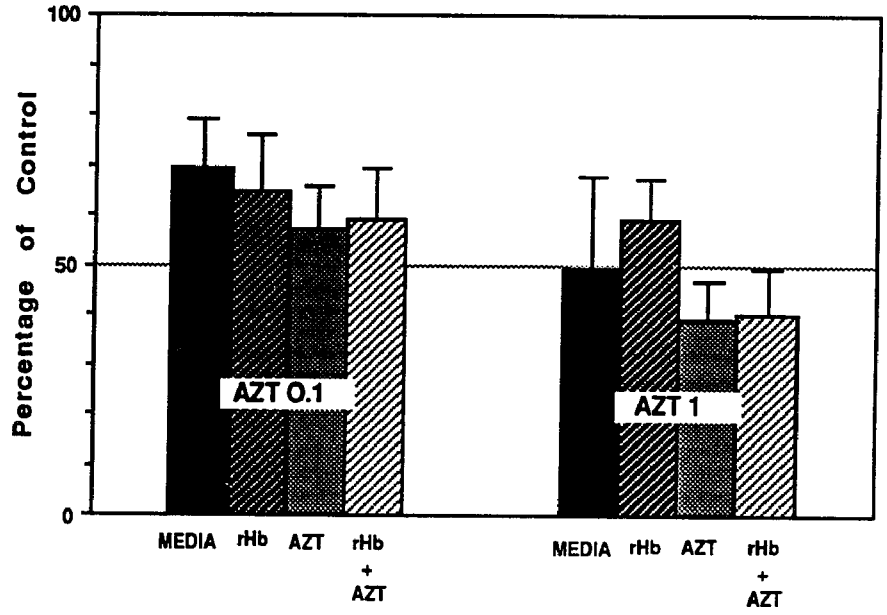

FIG. 2. Reversal effect of 1 μM recombinant human hemoglobin for the final 7 days of a 14 day liquid erythroid culture of CD34+ human bone marrow cells previously exposed to 0.1 or 1 μM AZT for 7 days. Columns represent the mean of the percentage of untreated control value of three different bone marrow donors; bars represent the standard deviation.

GENERAL CONCLUSIONS

Alternative therapeutic approaches to treatment of AIDS patients are under investigation. ddNs have proven clinical effectiveness against HIV replication and may likely continue to represent first line therapy, playing a major role in the treatment of AIDS in the future. Therefore, elucidation of the mechanisms underlying the ddN-induced toxicities should prove beneficial in developing novel anti-HIV pharmacological agents and in developing therapeutic agents with potential to reverse or protect from toxic effects. The objective of this dissertation is to evaluate the effects of ddNs on relevant human progenitor cell development by examining their effects on proliferation, differentiation, and hemoglobin synthesis. The relevance of these in vitro studies to our understanding of the in vivo toxic effects of ddNs is essential due to the multifactorial nature of the mechanisms of the toxic effects demonstrated in vitro. The results presented in this dissertation reflect the unique and diverse in vitro actions of this family of nucleoside analogs and demonstrate the potential for a beneficial therapeutic strategy in ameliorating AZT-induced hematopoietic toxicity by protection or rescue.

The toxicity of AZT to patients is evidenced by the associated anemia and neutropenia. In vivo investigations of AZT-induced toxicity using animal models have also resulted in anemia and neutropenia, and in vitro investigations of AZT-induced toxicity have suggested inhibitory effects on proliferation and differentiation of hematopoietic progenitor cells. The process of differentiation is not well understood, and cells are not morphologically distinguishable in the early stages of development, but differentiation can be observed in erythroid lineage cells by the production of hemoglobin or by expression of the glycophorin A surface antigen, both unique and specific to erythroid lineage cells. An

in vitro effect of AZT on differentiation has been suggested because of the reduction in the percentage of hemoglobin-synthesizing cells in K-562 cells induced to produce hemoglobin by butyric acid or hemin. In human and animal model bone marrow cells, the inhibition of differentiation by AZT has been suggested because of the reduction in BFU-E and CFU-E in semi-solid colony-forming assays. In addition, AZT has been reported to reduce globin gene expression in K-562 cells induced to produce hemoglobin with butyric acid. While these results indicate an effect on differentiation, they do not discern whether the effect is on the stem cell commitment to erythroid differentiation (thereby resulting in less hemoglobin-synthesizing cells), an effect on heme synthesis (as heme is reported to regulate globin synthesis and necessary for hemoglobin formation), or an effect specifically on globin synthesis. Any of the above would result in the observation of a decrease in the percentage of hemoglobin-synthesizing cells.

Preliminary results of investigations in our lab of the effect of AZT on hemoglobin synthesis in K-562 cells indicated a decrease in hemoglobin content in populations of cells treated with AZT compared to control cell populations. To investigate the effect of AZT on heme synthesis, we developed a novel HPLC method to separate products of the eight heme biosynthetic pathway enzymes. Abnormalities of heme synthesis in humans results in accumulation of these enzyme products, a condition known as a porphyria. However, we determined no accumulation of heme enzyme products in these investigations. We hypothesized that the levels of accumulation might be too low for detection using this method, so we investigated the effect of AZT on mRNA levels and enzyme activities of the three erythroid inducible heme pathway enzymes most implicated in regulation of heme synthesis: ALAS-E, ALAD, and PBGD-E. Chapter 1 reports the investigations undertaken to determine whether AZT inhibition of hemoglobin synthesis in K-562 erythroleukemia cells is specific to globin genes or may represent a general effect on erythroid-inducible gene expression. We examined the effect of AZT on the erythroid inducible genes for globin, ALAS-E, ALAD, and PBGD-E, and the ubiquitous gene for

actin as control. None of the non-globin inducible genes, which code for enzymes involved in heme biosynthesis, was suppressed by AZT, while globin gene expression was substantially diminished, consistent with our previously published results. Of importance, AZT at a concentration of 100 μM did not suppress ALAS-E or PBGD-E mRNA steady-state levels while globin mRNA steady-state levels were substantially suppressed. This further suggests that AZT does not cause a temporal inhibition of K-562 differentiation since neither expression of a gene induced earlier (ALAS-E) nor one induced later (PBGD-E) than globin was suppressed by AZT.

Because ALAS-E gene expression in erythroid cells is also controlled in part translationally, via binding of the iron responsive element binding protein to its consensus sequence on the mature ALAS-E mRNA, AZT may have effects on ALAS enzyme activity via effects on ALAS mRNA translation, which would not be detected in the Northern assay. However, no effect of AZT was observed on ALAS enzyme activity. These data suggest that AZT does not effect ALAS-E gene expression, either transcriptionally or post-transcriptionally. In summary, these data demonstrate that AZT does not inhibit the erythroid gene induction process in general, since several non-globin inducible genes for heme pathway enzymes (ALAS-E, ALAD, and PBGD-E) were not inhibited by AZT exposure to K-562 cells nor were enzyme activities altered. AZT inhibition of globin gene expression may involve direct mechanism(s) on that gene rather than a secondary effect resulting form a general inhibition of K-562 cell induction.

The relevance of the use of immortalized cell lines, such as the K-562 cell line, in evaluation of ddN-induced toxic effects has been questioned because of its transformed nature and the inhibitory concentrations of ddNs required are sometimes orders of magnitude greater than pharmacologically relevant concentrations. Clonogenic assays of human and animal bone marrow cells have proven to be reliable predictors of ddN-induced cytotoxic effects on colony-forming progenitors, but the semi-solid nature of these assays and the low cloning efficiency make the removal of intact cells in sufficient quantity and

quality for further analysis difficult, if not impossible. The advent of the liquid suspension culture system and the discovery of the expression of the CD34 antigen on the pluripotent stem cell and early progenitors provide a culture method that will allow the growth and development of sufficient numbers of cells that can be easily removed without damage for further analysis, including cellular and molecular analysis. In this model system, human CD34+ progenitor cells can be induced to proliferate and differentiate along any of the blood cell lineages with appropriate use of selected cytokines and growth factors. The effects of ddN administration can then be observed on the development of lineage-specific cells.

Chapter 2 reports the results of investigations of the effects of ddNs on the proliferation and differentiation of pluripotent progenitors (CD34+) purified from human bone marrow cells grown in this liquid culture system and comparison of the results with the semi-solid culture system. These highly purified CD34+ progenitors undergo extensive proliferation during 14 days of culture with a marked expression of differentiation antigens during the last 7 days. The in vitro differentiated cells exhibit normal morphological features in response to specific hematopoietic growth factors of both the erythroid and granulocyte-macrophage lineages as demonstrated by flow cytometry cell phenotyping. Cultures were analyzed by FACS for lineage-specific antigen expression using monoclonal antibodies and demonstrated lineage specificity in response to appropriate hematopoietic growth factors.

The results of investigations of ddN-induced effects on proliferation were in general agreement with previous studies using semi-solid cultures, with AZT, AMT, and FLT exhibiting a more potent inhibition of erythroid lineage proliferation versus myeloid lineage proliferation. The effect of AZT on erythroid differentiation demonstrated a significant inhibition of erythroid-specific glycophorin A expression in the second week of culture, consistent with previous reports of inhibition of BFU-E in AZT-treated human bone marrow cells in semi-solid culture. The potency of these ddNs in inhibiting proliferation of

granulocyte-macrophage lineage cells was in the order of FLT>AMT=ddC>AZT>>d4T and FLT>AMT=AZT>ddC>>d4T for erythroid lineage cultures. AZT was found to be less inhibitory to the proliferation of myeloid lineage cells than other ddNs, with FLT the most toxic. In contrast, ddC had less effect on erythroid proliferation than AZT or the other ddNs at concentrations inhibitory to cell growth, consistent with clinical reports indicating the absence of hematopoietic toxicity as the major limiting toxicity at these concentrations.

Of note was the observation that AZT-treated myeloid lineage cultures demonstrated a perturbation in the ratio of granulocyte lineage antigen expression to monocyte lineage antigen expression, suggesting that AZT inhibits granulocyte lineage cells, consistent with clinical observations of neutropenia in AIDS patients receiving long-term AZT therapy. This perturbation was observed only with AZT and none of the other ddNs tested. These results reflect the unique and specific effects of ddNs on the proliferation of erythroid and myeloid lineage progeny of CD34+ human bone marrow cells and indicate that liquid culture of CD34+ human progenitor progeny may be a more preferred method, compared to semi-solid colony-forming cultures, as an indictor of toxic effects due to the additional information and specificity of information obtained by this culture and analytical method.

The ddNs were additionally assessed for effects on mtDNA synthesis, as some reports have suggested inhibition of mtDNA synthesis as a potential mechanism of action involved in the peripheral neuropathy of patients treated with ddC and d4T, and further suggested as possibly responsible for the hematological effects of AZT. Slot blot analysis of granulocyte-macrophage cultures demonstrated that exposure to ddC and to a lesser extent FLT and d4T, was associated with a decrease in total mtDNA content, suggesting that these ddNs inhibit mtDNA synthesis. In contrast, no difference in the ratio of nuclear DNA to mtDNA was observed in cells exposed to toxic concentrations of AZT and AMT. These data indicate that bone marrow toxicity induced by AZT and its in vivo catabolite, AMT, is not be associated with an inhibition of mtDNA synthesis.

While the mechanism(s) of AZT-induced hematopoietic toxicity remains elusive, investigations of hematopoietic stimulation have shown some success using endogenous agents to stimulate hematopoiesis by G-CSF and erythropoiesis using EPO. Chapter 3 describes investigations of the protective and rescue effect of hemin. Hemin was observed to protect human bone marrow cells from the ddN-induced inhibition of BFU-E colony formation, with the exception of FLT, although not protecting from the inhibition of CFU-GM colony formation in semi-solid culture for any of the ddNs tested. In liquid culture of CD34+ human bone marrow cells, hemin addition stimulated proliferation and coadministration with AZT protected cells from the AZT-induced inhibition of proliferation and percentage of hemoglobin-synthesizing cells. In cultures pre-exposed to AZT for 7 days, addition of hemin or coadministration of hemin and AZT resulted in recovery of the percentage of hemoglobin-synthesizing cells from AZT-induced inhibition, while not rescuing cultures from the AZT-induced inhibition of proliferation.

These results suggest several important conclusions. The hemin protective effect on proliferation occurs only when coadministered with AZT at the beginning of culture, while not occurring if cultures are pre-exposed to AZT, indicating a protective effect of hemin but an inability to rescue from AZT-induced inhibition of proliferation. In contrast, hemin was able to both protect and rescue cultures from the AZT-induced inhibition of the percentage of hemoglobin-synthesizing cells. The protection and rescue of the percentage of hemoglobin-synthesizing cells did not exceed control culture levels, suggesting that the mechanism of action producing these effects by hemin in this regard occurred after commitment of cells to differentiate along the erythroid lineage. This indicates that the mechanism of action toward protection and rescue of hemoglobin-synthesizing cells may be independent of its mechanism of action toward protection of proliferation. These results further suggest that commitment of cells to erythroid differentiation, at least in the earliest stages, may be a stochastic process, in that the stimulation of proliferation by hemin alone did not result in an increase in the percentage of hemoglobin-synthesizing cells above

control culture levels. These results may also aid in the explanation of the observation that mice treated with AZT demonstrated an enhanced recovery if hemin was administered after a two-week period following cessation of AZT treatment (2). Chapter 3 suggests that hemin may be a potential candidate for use in the amelioration of AZT-induced erythropoietic toxicity, consistent with published clinical reports of the successful use of a formulation of hemin (heme arginate) in alleviating the anemia in patients with myelodysplastic syndromes.

While additional investigations are required to ascertain the mechanism of action of hemin, these data are consistent with published reports on the effects of hemin on cell proliferation and globin synthesis. The apparent independent effects of hemin toward proliferation and hemoglobin synthesis observed in this investigation are consistent with previously published reports from this lab suggesting that AZT inhibits globin synthesis, and AZT inhibition of proliferation is well documented. Therefore, this chapter further supports the multifactorial nature of AZT hematopoietic toxicity and suggests that one mechanism of action may be directly on globin synthesis and indicates no effect of AZT on commitment of cells to differentiate along the erythroid lineage. This suggests that the effect of AZT on the inhibition of hemoglobin-synthesizing cells occurs after cells are commited to differentiate along the erythroid lineage and may be independent of AZT-induced effects on the inhibition of proliferation.

Investigations reported in Chapter 3 and additional, unpublished investigations in our lab (Appendix B, Table 1) also indicate the unique and selective character of ddNs and may indicate actions on different progenitor cells. FLT was found to be the most potent ddN towards proliferation and erythroid differentiation in liquid culture, with equivalent IC_{50}s, and in semi-solid culture of BFU-E. Hemin was unable to protect BFU-E or CFU-GM from the inhibitory effects of FLT. In contrast, hemin was able to protect BFU-E from the inhibitory effects of AZT and AMT, but was unable to protect CFU-GM from these toxic effects. AMT also exhibited approximately 3 times more potency toward inhibition of

proliferation and erythroid differentiation in liquid culture than AZT. The IC_{50}s of FLT, AZT, and AMT toward both proliferation and erythroid differentiation were equivalent, although the potency was in the order FLT>AMT>AZT. In contrast, the IC_{50} of ddC towards proliferation was much less than its IC_{50} towards erythroid differentiation. Other ddNs, 2',3'-didehydro-3'-deoxythymidine (d4T), 3'-azido-2',3'-dideoxyuridine (AzdU), and 3'-azido-2',3'-dideoxy-5-methylcytosine (AzddMeC), were investigated and not found to exhibit hematopoietic toxicity at pharmacologically relevant concentrations. From these limited investigations, it appears that the 3' substitution of the sugar moiety is important in determining potency of hematopoietic toxicity, but the base may be more important in determining selectivity toward hematopoietic toxicity. These differences may indicate a selective effect on different progenitor cells. For example, AZT exhibits greater potency toward BFU-E than CFU-GM in colony-forming assays and greater potency toward liquid culture than semi-solid culture, suggesting an effect on an earlier progenitor as liquid cultures contain a more homogeneous population of earlier progenitors than semi-solid culture of more heterogeneous bone marrow cells.

Other agents have been investigated in an effort to rescue patients from anemia. Cell-free hemoglobin preparations have been investigated as potential blood substitutes for over 50 years, and much progress has been made in preparations capable of oxygen delivery with reduced toxicity (4, 5). Previous problems such as stroma-induced toxicity and the breakdown of the hemoglobin tetramer have been overcome by the development of stroma-free preparations and crosslinked hemoglobin tetramers (13, 35). The need for a safe hemoglobin preparation is increasing in light of the poor quality of the blood supply and the enhanced need for alleviation of anemia in third world countries (23). In the United States, the blood supply is too often contaminated with virions such as Hepatitis and HIV. In Chapter 4, we investigated a cell-free human recombinant hemoglobin preparation, stroma-free and crosslinked between the two alpha subunits and currently in Phase II clinical trials, for its effect on hematopoiesis and its ability to protect and rescue the development of

human CD34+ progenitor cells from the toxicity of AZT. Cultures treated with this human rHb demonstrated normal expression of lineage-specific antigens when induced to develop along either the myeloid or erythroid lineage, with the exception that myeloid lineage cultures demonstrated a dose dependent increase in granulocyte antigen expression and a dose dependent decrease in monocyte antigen expression. The reason for this perturbation is unknown, but may aid in the alleviation of AZT-induced neutropenia. This human rHb demonstrated no effect on inhibition or stimulation of proliferation, consistant with unpublished results (Appendix A, Table 1) which demonstrated no effect on BFU-E or CFU-GM colony formation in semi-solid methylcellulose cultures. Protection studies demonstrated an ability to protect cultures from the AZT-induced inhibition of proliferation, but recovery studies demonstrated no ability to rescue cultures from AZT-induced toxic effects. These results suggest the potential for the use of this recombinant hemoglobin in AIDS patients suffering from AZT-induced anemia and neutropenia by providing efficient oxygen delivery with the additional benefits of protecting progenitor cells from the AZT-induced inhibition of proliferation while not evoking toxic effects in normal hematopoiesis. This human rHb may prolong the ability of the AIDS patient requiring transfusion to tolerate long-term AZT therapy.

Previous investigations of AZT-induced toxicity have been performed in various cell lines and animal models, and together with clinical reports of AIDS patients receiving AZT therapy indicate that the associated anemia may be due to the following effects of AZT on hematopoiesis: (1) inhibition of the proliferation of cells, (2) inhibition of the commitment of cells to differentiate, (3) inhibition of the proliferation or maturation of differentiating cells, and/or (4) inhibition of hemoglobin synthesis. These four possible hypotheses as to the mechanism of action of AZT have been explored in this dissertation, and while each hypothesis could be supported by data in the previous chapters, specific information has been obtained from these investigations that give greater insight into the mechanism of action of AZT. All investigations support the hypothesis that AZT inhibits the proliferation

of cells, and it is generally accepted in the scientific community that this occurs via the chain-terminating effect of AZT due to the azido group at the 3' position of the sugar moiety. The degree to which chain termination affects cell proliferation may be influenced by other factors such as the inhibition of repair mechanisms and anabolic kinases, whose activity and specificity may vary depending on species, cell type, and stage of development. However, inhibition of cell proliferation does not explain all of the observed effects of AZT, such as specific effects on the erythroid lineage cells and hemoglobin synthesis. Therefore, other mechanisms must exist to explain why the percentage of hemoglobin-synthesizing cells is reduced by AZT and not just the number of hemoglobin-synthesizing cells, as one might expect if the effect was just on proliferation of cells.

It has been suggested that AZT interferes with the differentiation of cells, which could explain the reduction in the percentage of hemoglobin-synthesizing cells, but data obtained from investigations in Chapter 3 disproves this hypothesis. The ability of hemin to rescue the percentage of cells synthesizing hemoglobin to control cell levels in cultures previously exposed to AZT, while failing to rescue the AZT-induced inhibition of proliferation, suggests that the action of AZT on these cells occurs after commitment of cells to the erythroid lineage. When hemin alone is added to cell cultures, a stimulation of proliferation occurs, but the percentage of hemoglobin-synthesizing cells is equal to control cell populations, indicating that hemin does not interfere with the commitment of cells to the erythroid lineage. Because the rescue of hemoglobin-synthesizing cells by hemin in cultures previously treated with AZT does not exceed control culture levels, this suggests that the action of AZT on hemoglobin-synthesizing cells occurs after cells are committed to the erythroid lineage, thereby indicating no effect on the commitment of cells to differentiate along the erythroid lineage. Therefore, the specific effects of AZT on the erythroid lineage may occur on the maturation or proliferation of differentiating cells and/or on hemoglobin synthesis itself.

Investigations of the effect of AZT on hemoglobin synthesis have indicated a reduction in the number of hemoglobin-synthesizing cells by benzidine staining in K-562 cells, and benzidine staining and glycophorin A antigen expression in progeny of CD34+ human bone marrow cells. AZT has also been reported to reduce BFU-E and CFU-E in semi-solid culture of human bone marrow cells. Investigations of globin synthesis have indicated a reduction in the rate of transcription and steady-state levels in K-562 cells exposed to AZT, although these studies did not correlate the number of hemoglobin-synthesizing cells to globin synthesis. In Chapter 1, we investigated the effect of AZT on erythroid-inducible gene expression in K-562 cells and determined that the effect of AZT on hemoglobin synthesis was not a general effect on erythroid gene expression, but was specific to the globin gene. In addition, in Chapter 3, hemin rescued the percentage of hemoglobin-synthesizing cells from the inhibition of AZT in cultures previously exposed to AZT. Previous investigations have reported specific effects of hemin on globin synthesis in erythroid maturation, which suggests that the action of hemin in rescuing AZT-induced inhibition of the percentage of hemoglobin-synthesizing cells may have been directed toward globin synthesis, as hemin failed to rescue AZT-induced inhibition of proliferation in the same cultures. Therefore, while there is a good indication that AZT may effect globin gene expression, additional investigations need to be performed.

The remaining hypothesis involves the effect of AZT on the proliferation or maturation of differentiated cells. Recent in vivo studies have demonstrated the exposure of AZT to cats infected with feline leukemia virus and to a murine model of AIDS is associated with progressive anemia. The percentage of immature erythroid cells in the bone marrow of cats treated with AZT was increased and abnormalities in their maturation was reported. Other investigations have also suggested a blockage in the differentiation of marrow cells with some suggesting a blockage in the differentiation from BFU-E to CFU-E (27). These observations are consistent with clinical reports characterizing AZT-induced anemia as macrocytic with megaloblastic bone marrow and reduced hematocrit and

reticulocytes. In addition, recent investigations have reported an AZT-induced inhibition of the expression of EPO receptors (28), which are expressed increasingly as erythroid cells mature from BFU-E to CFU-E (59). In this regard, clinical observations have reported that exogenous EPO can ameliorate AZT-induced anemia in patients with low endogenous EPO levels, while patients with high endogenous EPO levels do not respond to exogenous EPO. These investigations suggest that AZT may interfere with the maturation process of developing cells. Unpublished investigations in our lab (Appendix B, Figure 1) indicate that the inhibitory effect of AZT on cell proliferation is most pronounced when AZT is administered on the eighth day of culture of CD34+ human bone marrow progenitors. This is about the time that primitive BFU-E colony formation is evident in semi-solid culture, indicating that the inhibitory effect of AZT may be more pronounced on BFU-E and progeny than earlier progenitors. Although this effect may be due to the clonal expansion occuring from BFU-E, this seems unlikely as inhibition is reduced when AZT is administered on the twelfth day of culture. Our investigations in this dissertation support this hypothesis in that an inhibition of maturation or proliferation of erythroid differentiated cells could result in a decrease in the number and percentage of hemoglobin-synthesizing cells, reflected in a decrease in the benzidine positive percentage, the percentage expressing glycophorin A, and globin gene expression. However, as we find no reduction in mRNA expression of heme biosynthetic enzymes with AZT treatment of K-562 cells, nor with enzyme activities with these gene products, the interpretation of these results is unclear.

We have performed additional analyses to verify the relevance of the liquid culture system which in turn supports this hypothesis. Lactate dehydrogenase assays were conducted as a measure of general cytotoxicity and results are reported in Appendix B, Figure 2. Results demonstrate that AZT treatment does not result in additional cell death as compared to control and indicates that less cell death may occur with AZT treatment than control. Measurements of mean cell volume (MCV) were also performed and indicate that

AZT treatment results in a higher MCV than control cells (Appendix B, Figure 3), consistent with clinical reports of higher MCV in patients treated with AZT (46). In addition, protein analysis was performed and supports an increase in MCV as the protein content of cells treated with AZT was higher than control cells (Appendix B, Figure 4). These results support the hypothesis of a blockage of maturation or proliferation of differentiated cells in that lengthening of the cell cycle or inhibition of cell division could result in a larger cell if protein synthesis was not also inhibited. Previous investigations have reported the ability of nucleoside analogs to inhibit the cell cycle, resulting in increased MCV while not inhibiting RNA or protein synthesis (17, 48). In addition, AZT has been implicated in delaying cell division (49). Therefore, it may be possible that AZT delays cell division or blocks cell maturation by some other mechanism, resulting in less mature cells and less cells that synthesize hemoglobin and then resulting in clinical observations of macrocytic anemia with megaloblastic bone marrow and reduced hematocrit and reticulocytes. These conclusions are also consistent with studies of differential anemia indicating that mechanisms which interfere with hemoglobin synthesis result in a microcytic anemia, while mechanisms which interfere with nuclear maturation result in a macrocytic anemia (34), also consistant with clinical reports of macrocytic anemia in AIDS patients receiving AZT therapy. In addition, we have reported an AZT-induced inhibition of the percentage of benzidine positive cells and glycophorin A expression. If the AZT-induced inhibition of the percentage of hemoglobin-synthesizing cells were a result of only an inhibition in the ability of the cell to synthesize hemoglobin, this would not account for the observed inhibition of glycophorin A expression. The inhibition of the percentage of cells expressing glycophorin A suggests an inhibition in erythroid cell maturation or proliferation, which could also account for the inhibition of the percentage of hemoglobin-synthesizing cells, but not for the inhibition of globin gene expression while not inhibiting non-globin erythroid inducible genes (Chapter 1). Therefore, these data may indicate

additional mechanisms of AZT-induced effects on developing erythroid lineage cells and further confirm the multifactorial nature of AZT.

In summary, the studies presented in this dissertation have focused on the cellular mechanisms by which pyrimidine nucleoside analogs active against HIV may exert toxic effects on the development of human bone marrow cells from pluripotent progenitor cells. ddNs have been implicated in exerting toxic effects towards proliferation, differentiation, maturation, and hemoglobin synthesis in a variety of animal models and cell lines. Biochemical pharmacological studies have demonstrated the incorporation of AZT into DNA and its potential role in cytotoxicity, and we have demonstrated the specific effects of AZT on globin gene expression versus erythroid-inducible, non-globin gene expression (Chapter 1). Using a liquid culture system, a more physiologically relevant cell model demonstrated potentially unique and selective mechanisms of action on the proliferation and differentiation of lineage-specific hematopoietic cells by various ddNs (Chapter 2). The mechanisms of AZT-induced toxicity were further clarified by investigations of the potential of hemin and a human rHb for protection and reversal of AZT-induced toxicity during the development of lineage specific progeny from human pluripotent stem cells (Chapters 3 and 4). These investigations demonstrate the validity and relevance of this liquid culture system as a predictor of cytotoxicity and alternative strategies to alleviate specific toxicities. In addition, these studies demonstrate an inhibitory effect of AZT on proliferation and hemoglobin synthesis, but indicate that AZT has no effect on commitment of cells to differentiate along the erythroid lineage.

FUTURE STUDIES

ddNs have proven clinical effectiveness and are likely to be involved in long-term therapy of AIDS patients, whether in monotherapy, combination therapy, or other alternative therapies. Therefore, elucidation of the mechanisms underlying ddN-induced toxicity are important in the development of novel agents and/or therapeutic strategies to ameliorate drug-induced toxicity. Selection of the biological system to be investigated should be relevant to the application of the drug under investigation and provide conditions suitable for detailed cellular, biochemical, and molecular analysis. We have demonstrated in this dissertation an in vitro culture system which is relevant and provides the capacity for detailed analysis of these parameters. The fund of knowledge continues to advance in the area of hematopoietic development and control of proliferation and differentiation. Advances in analytical technique and software in FACS analysis suggest this area as one of the most important analytical techniques of the future.

Future studies indicated by the investigations reported in this dissertation would include phenotype analysis of all potential ddNs active against HIV to be considered for AIDS therapy and, most importantly, a detailed investigation of the effect of AZT on the process of hemoglobin synthesis and maturation using the CD34+ liquid culture system. One of the problems we have encountered is a lack of a homogeneous population of cells on which to conduct analyses. Cell populations induced to develop along the erythroid lineage result in less than 100% benzidine and glycophorin A positive control cell populations, with treated populations being less than control. Recent advances in FACS software would allow a sample population of cultured cells to be analyzed for glycophorin A, and the physical parameters of the glycophorin A population identified and utilized for

sorting of the total population, resulting in collection of a homogeneous population of glycophorin A positive cells. This population could then be analyzed for specific biochemical and molecular parameters. This technique would of course extend to other types of lineage-specific antigen expressions. This would enable analysis to be conducted on pure populations of lineage-specific cell types, and in addition, eliminate the problem of overlapping antigen expression between different cell types, utilizing the difference in physical parameters.

A critical question to be investigated is the effect of AZT on the quantitative synthesis of hemoglobin. We have attempted to develop a competitive ELISA for sensitive and quantitative analysis of hemoglobin in our lab but have encountered problems with the sensitivity of the analysis and the biological variability of cultured cells. A homogeneous population of hemoglobin-synthesizing cells should reduce this problem. Preliminary results (Appendix B, Figure 5) indicate than AZT-treated cells may contain a higher concentration of hemoglobin, which would be consistent with other observations of increased MCV and protein content, and lend support to the hypothesis that AZT interferes with cell cycle or erythroid maturation. This question needs to be investigated in the future. Concurrent with the above suggestions, the effect of AZT on globin gene expression and erythroid-induced non-globin gene expression needs to be investigated in a more relevant model than K-562 cells, and additional parameters concurrently analyzed and correlated to globin synthesis such as protein, benzidine staining, and glycophorin A expression.

In addition, the effect of AZT on cell cycle should be further investigated in this culture system as well as the effect of hemin on cell cycle. These investigations should indicate if there is an inhibition in the cell cycle and where inhibition may occur, enabling further investigations of factors controling the cell cycle in those stages.

Finally, additional investigations need to be performed on the effect of ddNs on hematopoiesis by examining lineage-specific antigen expression in an effort to improve our understanding of the importance of the base and substitutions to the nucleoside, particularly

substitutions at the 3' position of the sugar moiety. Additional investigations with these ddNs need to be performed on cellular targets which may account for the unique and selective effects of these ddNs, such as affinity for DNA polymerases and anabolic kinases and their expression in progenitor cells.

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APPENDIX A

EFFECT OF RECOMBINANT HUMAN HEMOGLOBIN ON METHYLCELLULOSE SEMISOLID CULTURE OF HUMAN BONE MARROW CELLS

Methylcellulose semi-solid culture of human bone marrow cells. Human bone marrow cells were collected and assayed as previously described (32). Briefly, human bone marrow cells were collected from normal volunteers and the mononuclear fraction used as control, with or without AZT (0.1 or 1 μM for BFU-E cultures, and 1 or 10 μM for CFU-GM), with or without rHb (0.01, 0.1, or 1 μM), and each concentration of rHb co-administered with each concentration of AZT. All treatments were conducted in triplicate. Colonies were counted after 14 days culture.

TABLE 1. Effect of AZT and recombinant hemoglobin on colony formation in methylcellulose colony-forming assay. Data is expressed as percent of control, mean ± SD for at least four different bone marrow donors.

AZT [μM]	rHb [μM]	BFU-E	CFU-GM
0	0	100	100
	1.0	99 ± 4	99 ± 3
	0.1	96 ± 3	100 ± 3
	0.01	96 ± 5	98 ± 4
0.1	0	42 ± 8	ND[a]
	1.0	38 ± 6	ND
	0.1	37 ± 6	ND
	0.01	38 ± 8	ND
1.0	0	34 ± 6	52 ± 5
	1.0	31 ± 5	54 ± 4
	0.1	31 ± 7	56 ± 1
	0.01	32 ± 6	54 ± 3
10.0	0	ND	29 ± 8
	1.0	ND	29 ± 8
	0.1	ND	30 ± 8
	0.01	ND	29 ± 8

[a] Not Determined

APPENDIX B

EFFECTS OF AZT ON TEMPORAL INHIBITION, LACTATE DEHYDROGENASE ACTIVITY, MEAN CELL VOLUME, PROTEIN CONTENT, AND HEMOGLOBIN CONCENTRATION IN LIQUID CULTURE OF CD34+ PROGENITOR CELLS

Temporal Effect of AZT. The temporal effect of AZT in CD 34+ human bone marrow cells was investigated by using the previously described liquid suspension culture method (Chapter 2), with cells induced to develop along the erythroid lineage, as described. AZT was added at various time points (Day 1, Day 8, Day 12) to determine the effect on proliferation. Non-AZT treated cultures were used as positive controls.

Lactate Dehydrogenase Assay. The lactate dehydrogenase (LDH) assay was performed essentially as described by Cabaud and Wroblewski (12), measuring the reduction of pyruvate to lactate at a rate proportional to the amount of LDH. Culture of CD34+ human bone marrow cells was as previously described (21). Briefly, pyruvate substrate (Sigma 500L-1) and NADH (1 mg/ml, Sigma 340-101) were mixed and incubated for 5 minutes at 37°C. Samples of culture media were taken and appropriate dilutions were prepared and placed in the incubation vails containing pyruvate substrate and NADH, mixed and incubated in a 37°C water bath for 30 minutes. Vials were removed from the water bath, Sigma color reagent (505-2, 2,4-dinitrophenylhydrazine) added and allowed to incubate for 20 minutes at room temperature. Reaction was stopped with 0.4 N NaOH and after 5 minutes, aliquots were transferred to microtiter plate wells in triplicate. Calibration standards were prepared simultaneously with samples and blanks. Water was used as reference and optical density measured at 450 nm using a Molecular Dynamics microplate reader. Activity of LDH was calculated from the standard calibration curve prepared for each experiment.

Mean Cell Volume Assay. The assay was performed essentially as described by Cybulski et al. (19). Culture suspensions were incubated with 80 µl of [^{14}C]-Inulin (0.005 mCi/ml) per 6 ml cell suspension for 15 min. at 37°C. Triplicate assays were performed on each sample. Samples of the incubation mixture were then transferred to empty eppendorf tubes or eppendorf tubes containing silicon oil. All tubes were centrifuged and an aliquot of the supernatant removed for scintillation counting. The

remaining supernatant was discarded and tubes were weighed and dried overnight at 70°C. Tubes were weighed and the pellet was digested with 1 N KOH for 2 hours at 70°C. Digestion solution was neutralized and radioactivity counted in a scintillation counter. Calculations were performed as follows:

wet weight - empty weight = wet pellet weight

dry weight - empty weight = dry pellet weight

extracellular volume: DPM of Inulin pellet/DPM Inulin supernatant

Intracellular volume: wet pellet weight - extracellular volume

$$\text{Cell volume} = \frac{\text{Intracellular volume}}{\text{Number of Cells}}$$

Protein Assay. The Bio-Rad Protein Assay was used to assay protein and is based on the method of Bradford (10). Three to five dilutions of protein standard (bovine serum albumin) were prepared for each microtiter plate and triplicates were prepared for each sample dilution. The linear range of the microtiter plate assay performed was 0.05 mg/ml to approximately 0.5 mg/ml. Optical density was measured at 595 nm in a 96-well Molecular Dynamics microplate reader and concentrations calculated from the standard curve.

ELISA. A competitive ELISA was performed using adult human hemoglobin as the standard, rabbit anti-human hemoglobin anti-serum as primary antibody, and alkaline phosphatase-conjugated goat anti-rabbit antibody as secondary antibody. The general procedure is as follows: 96-well polystyrene flat-bottom microtitration plates were coated with 100 ul per well of 0.8 ug/ml hemoglobin (Hb) in borate saline buffer (BS), pH 8.4-8.5. The plates were incubated at 37°C. for one hour or overnight at 4°C. After the coating incubation time, the hemoglobin was flicked out of the wells and 200 ul/well borate saline - bovine serum albumin (BS-BSA) added to block the remaining binding sites in the wells. The plates were incubated for one hour at room temperature in a humidified atmosphere, and BS-BSA then flicked out of the wells.

A hemoglobin stock solution of 2 mg/ml was prepared by dissolving Hb in PBS, and dilutions for appropriate standards were prepared by dilution with BS-BSA. 75 ul of each dilution was added to triplicate wells. Cells were lysed in 200 ul 10 mM Tris (pH 7.5). Appropriate dilutions of sample were made and diluted with BS-BSA. Triplicate wells of each sample dilution were prepared.

Rabbit anti-human Hb antiserum (Sigma) was diluted in BS-BSA and 75 ul added to all wells, except the blank wells, which received BS-BSA, and mixed well with the multi-channel pipetor. The plates were then incubated for 2 hours at room temperature in a humidified atmosphere, flicked out, and washed 3 times with washing solution (8.5 g/l NaCl + 100 ul/l Tween-20).

Alkaline phosphatase-conjugated goat anti-rabbit antibody (Southern Biotech) was diluted with BS-BSA and 100 ul/well added to all wells. The plate was incubated overnight at 4^0C., flicked out the next morning, and washed six times with washing solution. Alkaline phosphatase substrate tablets (Sigma) were dissolved in alkaline phosphatase substrate buffer (one tablet/5 ml solution), 200 ul/well added to all wells, and incubated at room temperature for 50-70 minutes. The O.D. was read at 405 nm. Concentrations were calculated from the standard curve.

TABLE 1. Effect of ddNs on the inhibition of proliferation and erythroid differentiation of CD34+ human bone marrow cells after 14 days culture. Data is expressed as the mean ± standard deviation of the 50% inhibitory concentration (μM).

	IC_{50} (μM)	
Compound	Growth	GPA
AZT	0.17 ± 0.02	0.19 ± 0.01
AMT	0.06 ± 0.01	0.08 ± 0.03
FLT	0.05 ± 0.01	0.04 ± 0.01
ddC	0.07 ± 0.01	3. ± 1.7
d4T	10 ± 1.7	8.0 ± 0.25
AzdU	>10	> 5
AzMeC	14	10.

IC_{50} 50% inhibitory concentration calculated by least squares linear regression analysis.

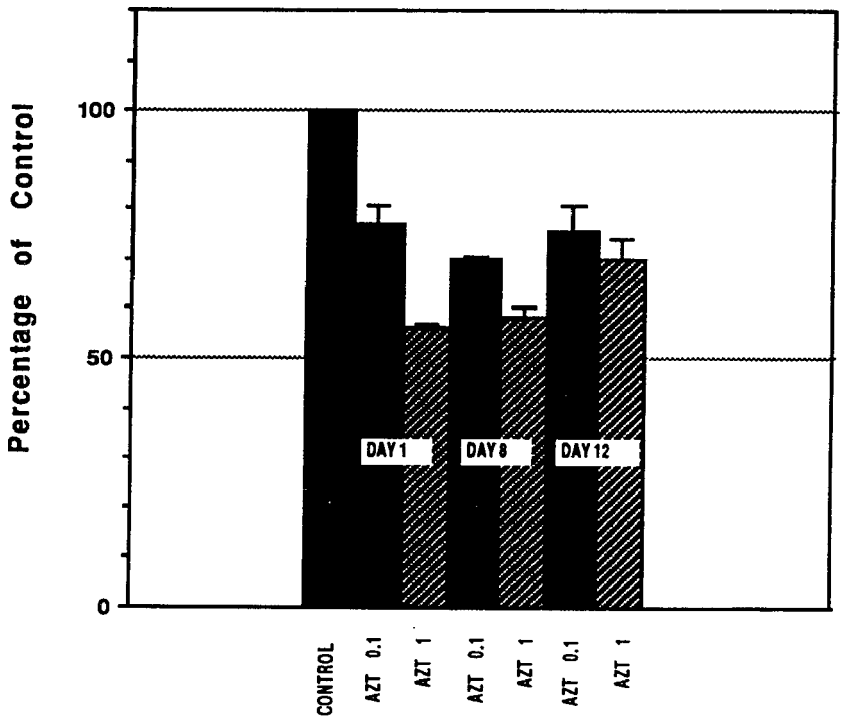

FIG. 1. Temporal effect of AZT on CD34+ human progenitor cell proliferation. AZT (0.1 or 1 μM) was added to erythroid cultures on days 1, 8, or 12. Columns represent the mean ± standard deviation of the percentage of control of three different bone marrow donors; bars represent standard deviation.

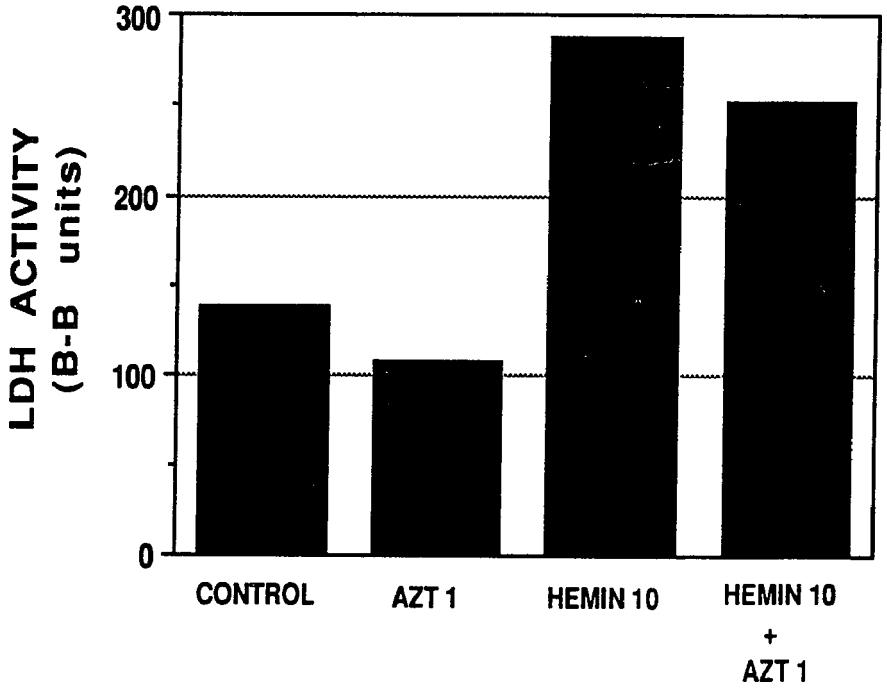

FIG. 2. Effect of AZT and hemin on lactate dehydrogenase activity in CD34+ progenitor cells. All concentrations are in μM. Columns represent activity after 12 days culture.

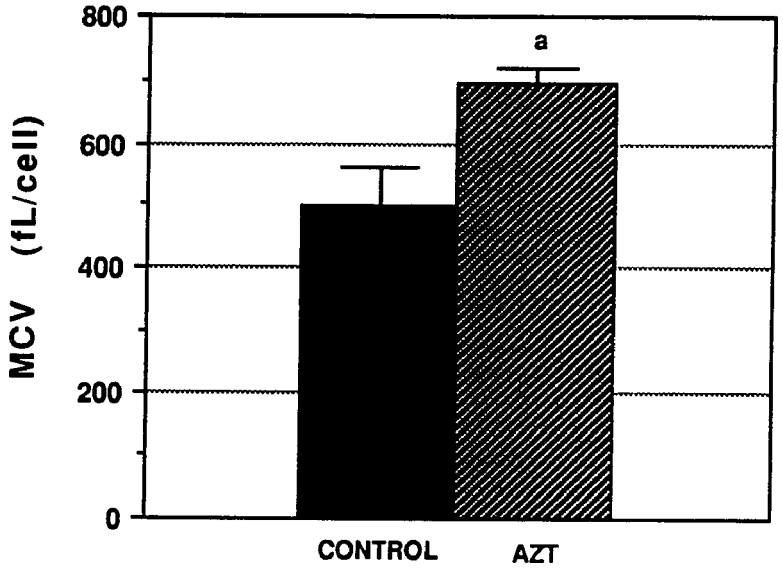

FIG. 3. Effect of 0.1 μM AZT on mean cell volume (MCV) of erythroid lineage CD34+ progeny. Columns represent mean ± standard deviation for three different bone marrow donors after 14 days culture of CD34+ human bone marrow cells induced to develop along the erythroid lineage.
[a] $p < 0.05$

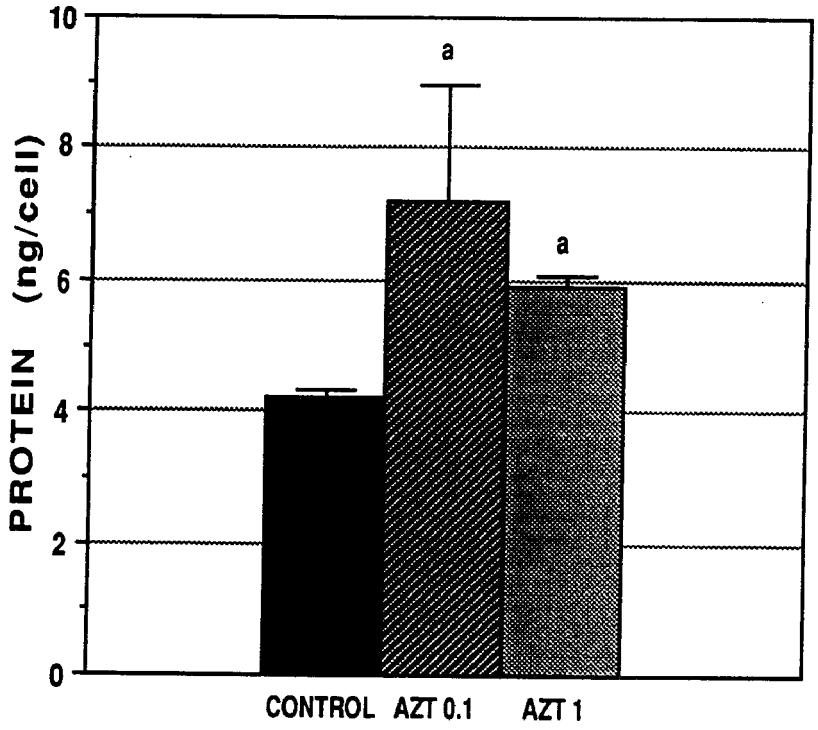

FIG. 4. Effect of AZT (0.1 and 1 μM) on protein content after 14 days culture of CD34+ human bone marrow cells induced to develop along the erythroid lineage. Columns represent mean ± standard deviation for at least three different bone marrow donors.
[a] $p < 0.05$

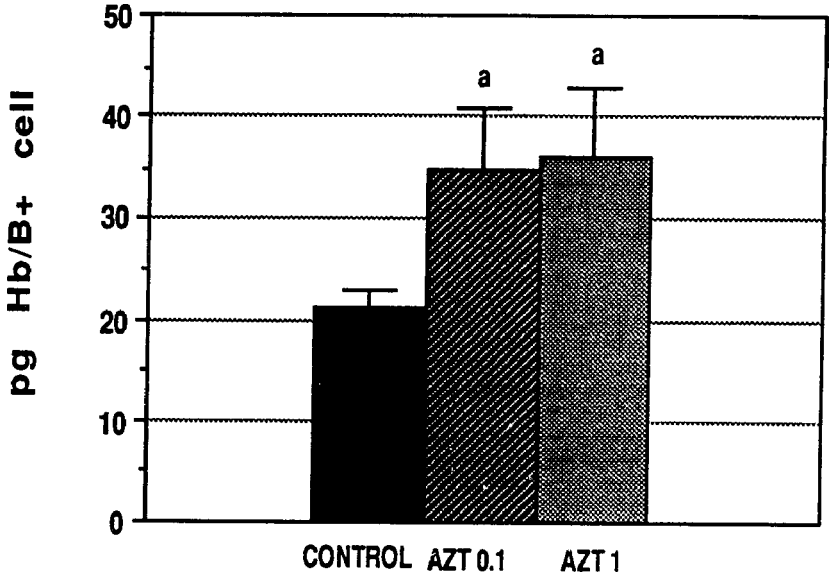

FIG. 5. Effect of AZT (0.1 and 1 μM) on hemoglobin concentration in erythroid-induced progeny of CD34+ human bone marrow cells after 14 days culture. Columns represent mean ± standard deviation for at least three different bone marrow donors. Values reflect the total hemoglobin content adjusted for the number of benzidine positive cells.
[a] $p < 0.05$

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