Pergamon

Biochemical Pharmacology, Vol. 50, No. 3, pp. 413–419, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)00134-4

INHIBITORY EFFECTS OF ZIDOVUDINE IN ERYTHROID PROGENITOR CELLS

REVERSAL WITH A COMBINATION OF ERYTHROPOIETIN AND INTERLEUKIN-3

SUDHIR R. GOGU,* BARBARA S. BECKMAN,* RUSSELL B. WILSON† and KRISHNA C. AGRAWAL*‡

*Department of Pharmacology and †Department of Pathology and Laboratory Medicine, Tulane University School of Medicine, New Orleans, LA 70112, U.S.A.

(*Received* 19 *July* 1994; *accepted* 2 *February* 1995)

Abstract—To investigate the mechanisms that may be involved in zidovudine (AZT)-induced hematopoietic toxicity, spleen cells isolated from phenylhydrazine-treated anemic mice or murine bone marrow erythroid progenitor cells were treated with AZT (1–10 μM) for 24 hr. A concentration-dependent inhibition of the binding of ^{125}I-labeled erythropoietin (Epo) was observed, suggesting down-regulation of Epo receptors. To determine if this effect is due to modulation of the levels of Epo receptor mRNA and to assess the effect of AZT on the expression of protooncogenes, mRNA levels were monitored by the slot blot hybridization technique. AZT caused a concentration-dependent inhibition in the levels of the mRNA of Epo receptors and c-*fos*, whereas the level of c-*myc* mRNA was unaffected. AZT also inhibited protein kinase C (PKC) activity in a concentration- and time-dependent manner, causing 50% inhibition at 10 μM within 3 hr. Simultaneous addition of Epo or interleukin-3 (IL-3) partially reversed the inhibitory effects of AZT on the levels of the mRNAs and on PKC activity; however, a combination of Epo and IL-3 was significantly more effective. These studies demonstrate that (i) AZT-induced down-regulation of Epo receptors and c-*fos* expression coupled with inhibition of Epo receptor-mediated signal transduction through PKC are significant contributory factors to AZT-induced erythroid toxicity, and (ii) these inhibitory effects can be overcome by treatment with a combination of Epo and IL-3.

Key words: zidovudine; erythropoietin receptor; interleukin-3; bone marrow cells; protooncogenes; protein kinase C

AZT§ is the first drug in the 2′,3′-dideoxynucleoside class that has been approved by the FDA for the treatement of AIDS. Although treatment with AZT has decreased the mortality and frequency of opportunistic infections in patients with AIDS or AIDS-related complex, significant dose-related toxicity, primarily anemia, remains a limiting factor in the clinical management of AIDS [1, 2]. Recombinant human Epo has been approved by the FDA for the treatment of AZT-induced anemia in AIDS patients. However, the beneficial response of Epo was observed only in those patients whose serum Epo levels were less than 500 IU/L [3]. Since the serum Epo levels in some AIDS patients receiving AZT therapy are significantly higher (3000 IU/L) [3, 4], it suggested a relative resistance to the proliferation and differentiating action of Epo on bone marrow progenitor cells. Indeed, AZT was shown to cause a concentration-dependent down-regulation of Epo receptor expression as monitored by the number of Epo receptors and the levels of Epo receptor mRNA in bone marrow progenitor cells [5]. In a preliminary communication, we described the quantitation of Epo receptors with the biotinylated-Epo/streptavidin-R phycoerythrin method, using flow cytometry [5].

The interaction of Epo with Epo receptors of committed but undifferentiated late erythroid progenitor cells initiates a series of predictable biochemical events leading to terminal differentiation [6]. Several investigators have studied the transmembrane signalling by the Epo ligand–receptor complex, which undergoes rapid endocytosis [7] associated with activation of PKC [8]. Furthermore, Epo has been shown to induce an early increase in the expression of the protooncogenes c-*fos* and c-*myc*, which is followed by an increase in the expression of the globin gene. In this report, we have extended the preliminary studies of down-regulation of Epo receptors with AZT by utilizing ^{125}I-labeled Epo and have further investigated the effects of AZT on PKC activity and on the expression of the protooncogenes c-*myc* and c-*fos*. Since the hematopoietic growth

‡ Corresponding author: Dr. Krishna C. Agrawal, Tulane University School of Medicine, 1430 Tulane Ave., New Orleans, LA 70112. Tel. (504) 588-5444; FAX (504) 588-5283.

§ Abbreviations: AZT, zidovudine; AIDS, acquired immunodeficiency syndrome; Epo, erythropoietin; FDA, Food and Drug Administration; IL-3, interleukin-3; and PKC, protein kinase C.

factor IL-3 induces proliferation and differentiation of erythroid progenitor cells in the initial stages [9], we have investigated the possible use of a combination of Epo and IL-3 in reversing AZT-induced biochemical events.

MATERIALS AND METHODS

Bone marrow cells. Bone marrow cells were obtained from femurs of 8-week-old normal CD-1 mice as described previously [10]. The femurs were removed aseptically, bone marrow cells were flushed with RPMI-1640 medium using a 21-gauge needle, and a single cell suspension was made by repeated pipetting. The cells were plated on 35-mm petri dishes for 2 hr at 37° to remove monocytes and macrophages, and the supernatant cell suspension consisting of enriched erythroid progenitor cells was collected. The cells were then counted using a hemocytometer, and viability was assessed by the trypan blue exclusion method.

Spleen cells from anemic mice. CD-1 mice were made anemic by injecting them intraperitoneally with a solution of phenylhydrazine hydrochloride in saline (60 mg/kg) for 2 consecutive days. At day 5, the mice were killed, spleens were removed aseptically, and single cell suspensions were prepared in α-Minimum Essential Medium as described earlier [11].

^{125}I-Epo binding studies. Human recombinant Epo was used in all the described studies. ^{125}I-Epo was obtained as a gift from Dr. J. W. Fisher (Department of Pharmacology, Tulane University School of Medicine, New Orleans, LA). The murine bone marrow cells or the spleen cells isolated from the phenylhydrazine-treated mice (1×10^6 cells/mL) were incubated with various concentrations of AZT (0–10 μM) for 24 hr in RPMI-1640 medium. The cells were centrifuged, washed with fresh medium, and incubated in 100 μL of binding buffer (RPMI-1640 medium containing 25 mM HEPES, pH 7.4, and 1% bovine serum albumin) in the presence of 100 mU/mL ^{125}I-Epo and with or without 100-fold excess of unlabeled Epo for 3 hr at 10° [12]. The cells were then transferred to Eppendorf tubes containing 0.5 mL of dibutyl phthalate oil and centrifuged (350 *g* for 2 min). The tips of the tubes containing cell pellets were cut off after freezing and were counted in a gamma counter (Packard Auto-Gamma 5650). Specific binding was calculated by subtracting non-specific binding in the presence of a large excess of unlabeled Epo from the total binding.

Determination of the mRNA expression. The enriched erythroid progenitor cells obtained from murine bone marrow (1×10^6 cells/mL) in RPMI 1640 medium were treated with AZT (0–10 μM) for 24 hr in the presence and absence of Epo (200 mU/mL) or a combination of Epo and IL-3 (100 U/mL). Total RNA was extracted using the RNAzol method [13, 14]. The mRNA levels of c-*myc*, c-*fos* and Epo receptors were monitored using 5 μg of total RNA by slot blot analysis. The cDNA (40-mer) for c-*fos* and c-*myc* was obtained from Oncogene Science, Inc. (Uniondale, NY). The Epo receptor cDNA probe, a 39-mer oligonucleotide, was synthesized as described previously [5]. A β-actin probe was used to monitor the actin mRNA by hybridization of the same blots after stripping. The probes were end-labeled with [α-^{32}P]dCTP with terminal deoxynucleotide transferase. The density of the hybridization signals after autoradiography was measured with a GS300 transmittance/reflective scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA).

Protein kinase C assay. PKC activity was determined by assaying phosphorylation of endogenous substrates using a PKC assay kit (GIBCO BRL). Cell lysates of enriched erythroid progenitor cells treated with AZT (0–10 μM) in the presence and absence of Epo and/or IL-3 for various time intervals were made in a Dounce homogenizer using the extraction buffer from the PKC assay kit as described [15]. The cell lysates were incubated at room temperature for 30 min, followed by centrifugation for 2 min at 14,000 *g* to remove cellular debris. Five microlitres of cell supernatant was added to the lipid preparation with and without PKC inhibitor peptide (PKC 1931), and the mixture was incubated at room temperature for 20 min to allow binding of the inhibitor to the substrate. At the end of 20 min, 10 μL of [γ-^{32}P]ATP was added to each tube and incubated at 30° in a water bath for 5 min. At the end of 5 min, 25 μL of the reaction sample was collected on GFC filter paper and washed two times with 1% phosphoric acid followed by water. The radioactivity was determined by liquid scintillation counting (Packard 1500 Tri-carb, Downers Grove, IL). PKC activity was determined by the difference in the amount of [^{32}P]ATP incorporation in the absence and in the presence of the inhibitor. Each determination was performed in duplicate in at least three different experiments.

CFU-E assay. The proliferative capacity of the bone marrow erythroid progenitor cells treated with AZT (0–20 μM), AZT plus Epo, and AZT plus Epo and IL-3 was measured by the CFU-E assay [10]. Briefly, a methylcellulose mixture (1.3%) was prepared in RPMI 1640 medium containing L-glutamine (2.0 mM), heat-inactivated fetal bovine serum (20%), 2-mercaptoethanol (0.1 mM), penicillin and streptomycin (100 U/mL and 100 μg/mL, respectively) and Epo (200 mU/mL). IL-3 (100 U/mL) was also added in those samples that were used to determine the effect of a combination of Epo and IL-3. The bone marrow cells were diluted in the methylcellulose mixture to give a cell suspension of 1×10^5 cells/mL. One milliliter of this cell suspension was plated in duplicate in 35-mm petri dishes and incubated in 5% CO_2 and 95% humidified air at 37° for 48 hr. The plates were stained with 3, 3′-diaminobenzidine, and colonies containing 8 or more benzidine-positive cells were counted under an inverted microscope.

RESULTS

Effect of AZT on the expression of Epo receptors. Detection and quantitation of Epo receptors has been carried out previously upon binding with either ^{125}I-labeled ligand [12] or biotinylated-ligand conjugate followed by reaction with a dye such as streptavidin-R phycoerythrin for flow cytometric

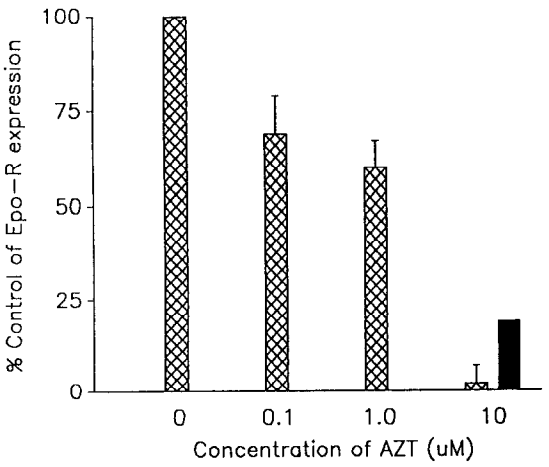

Fig. 1. Effect of AZT on Epo receptor (Epo-R) expression as determined by the binding of ^{125}I-Epo to spleen cells isolated from phenylhydrazine-treated anemic mice (hatched bars) and in erythroid progenitor cells isolated from murine bone marrow (solid bar). The cell suspensions (1×10^6 cells/mL) were incubated with or without AZT for 24 hr and then assayed for binding with ^{125}I-Epo. The specific binding of ^{125}I-Epo with untreated spleen cells was 16,180 cpm and with erythroid progenitor cells was 3,340 cpm. Each value is the mean ± SEM of values obtained from at least two experiments carried out in duplicate.

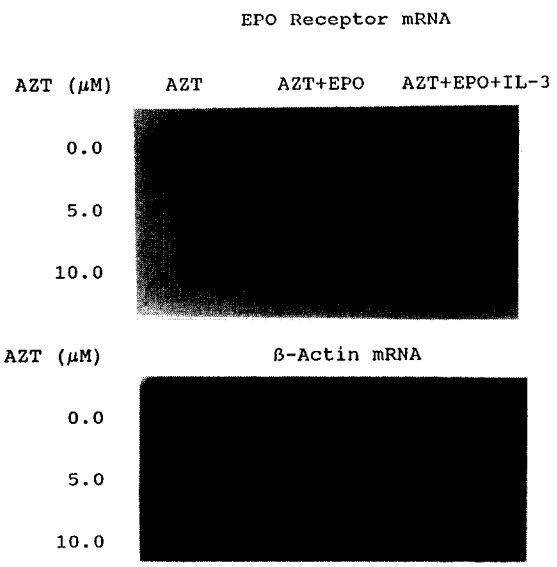

Fig. 2. Levels of Epo receptor mRNA in murine erythroid progenitor cells (1×10^6 cells/mL) treated with various concentrations of AZT for 24 hr in the absence and presence of Epo (250 mU/mL) or a combination of Epo and IL-3 (100 U/mL). Slot blot analysis was carried out with 5 μg of cellular RNA, as described in Materials and Methods. A β-actin probe was used to monitor the actin mRNA after stripping the same blot.

analysis [5]. In this report, we have used the specific binding of ^{125}I-labeled Epo to its receptors as an index for monitoring the effect of increasing concentrations of AZT on Epo receptor expression in spleen cells obtained from mice made anemic with phenylhydrazine. These spleen cells have been reported to have enhanced specific binding of ^{125}I-Epo due to a significant increase in Epo receptor expression [16]. Enriched erythroid progenitor cells isolated from murine bone marrow were also used for comparative purposes in these studies. Indeed, the specific binding of ^{125}I-Epo to the spleen cells was approximately 5-fold higher than to the bone marrow erythroid progenitor cells (see legend of Fig. 1). The data shown in Fig. 1 demonstrate that treatment of the splenic erythroblast cells with AZT for 24 hr caused a concentration-dependent inhibition of the binding of ^{125}I-Epo, resulting in a 95% decrease at 10 μM. AZT (10 μM) also inhibited binding of ^{125}I-Epo to the bone marrow erythroid progenitor cells by approximately 80% (Fig. 1). Under these conditions, AZT was not cytotoxic to the cells, as determined by the trypan blue exclusion method.

The effect of AZT on the levels of Epo receptor mRNA in enriched erythroid progenitor cells, as monitored by slot blot hybridization, is shown in Fig. 2. The quantitation data, expressed as a percent of control in Table 1, demonstrate that AZT caused a concentration-dependent decrease in 24 hr in the levels of Epo receptor mRNA producing 35 and 59% inhibition at 5 and 10 μM, respectively. The treatment of the erythroid progenitor cells with AZT in the presence of Epo partially reversed the AZT-induced inhibition to 38% at 10 μM. However, simultaneous treatment with a combination of Epo and IL-3 caused a complete reversal in the AZT-induced inhibition of Epo receptor mRNA levels at 5 μM, and an unexpected significant stimulation was observed at the high concentration (10 μM) of AZT.

Table 1. Reversal of AZT-induced down-regulation of Epo receptor mRNA levels in murine erythroid progenitor cells with Epo and a combination of Epo and IL-3

AZT concentration (μM)	Epo receptor mRNA levels (% of control)		
	AZT	AZT + Epo	AZT + Epo + IL-3
0	100	100	100
5	65	62	100
10	41	62	165

The enriched erythroid progenitor cell suspension (1×10^6 cells/mL) isolated from the murine bone marrow was treated with various drug combinations for 24 hr in the presence or absence of Epo (200 mU/mL) ± IL-3 (100 U/mL). Total RNA was isolated, and slot blot analysis was carried out with 5 μg of cellular RNA as described in Materials and Methods. A β-actin probe was used to monitor the actin mRNA after stripping the same blot. Data are presented as percent of control (untreated cells) of the density of the hybridization signals.

Table 2. Effect of AZT on the levels of c-*fos* mRNA in murine erythroid progenitor cells in the presence and absence of Epo and a combination of Epo and IL-3

AZT concentration (μM)	c-*fos* mRNA levels (% of control)		
	AZT	AZT + Epo	AZT + Epo + IL-3
0	100	100	100
5	80	82	110
10	50	97	125

The enriched erythroid progenitor cell suspension (1×10^6 cells/mL) isolated from the murine bone marrow was treated with various drug combinations for 24 hr in the presence and absence of Epo (200 mU/mL) ± IL-3 (100 U/mL). Total RNA was isolated, and slot blot analysis was carried out with 5 μg of cellular RNA as described in Materials and Methods. A β-actin probe was used to monitor the actin mRNA after stripping the same blot. Data are presented as percent of control (untreated cells) of the density of the hybridization signals.

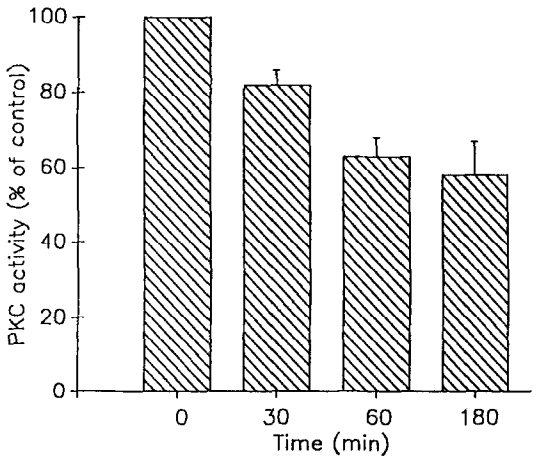

Fig. 3. Effect of AZT (10 μM) on protein kinase C activity in lysates of enriched erythroid progenitor cells (1×10^6 cells/mL) as a function of time. Values are the means ± SEM from at least three different experiments carried out in duplicate. Control protein kinase C activity was 18.7×10^3 cpm/10^6 cells.

Effect of AZT on c-myc *and* c-fos *expression.* Since expression of protooncogenes is an early Epo-induced response for proliferation and differentiation of erythroid progenitor cells [17], we determined the expression of c-*myc* and c-*fos* protooncogenes in erythroid progenitor cells after treatment with various concentrations of AZT for 24 hr. The levels of c-*myc* mRNA remained unaltered up to a 10 μM concentration of AZT (data not shown); however, the c-*fos* transcripts were reduced to 50% of control at 10 μM AZT (Table 2). Simultaneous treatment of erythroid progenitor cells with Epo or a combination of Epo and IL-3 reversed the AZT-induced inhibition of c-*fos* protooncogene transcription. The effect of the combination of Epo and IL-3 in reversing the inhibition of AZT at 10 μM was found to be stimulatory (125% of control), similar to the effect observed in the case of the Epo receptors.

Effect of AZT on the activity of PKC. Since transmembrane signalling by the Epo ligand–receptor complex in erythroid progenitor cells may be associated with activation of PKC [8], we initially monitored the activity of PKC in the presence of 10 μM AZT as a function of time (Fig. 3). The data indicate that AZT inhibited the PKC enzymatic activity in a time-dependent manner, reaching approximately 50% within 180 min. The inhibition of PKC activity by AZT was also concentration dependent, as shown in Fig. 4. The modulation of AZT-induced inhibition of PKC activity with Epo, IL-3 or a combination of Epo and IL-3 is shown in Fig. 5. Although both Epo and IL-3 as single agents reversed the AZT-induced inhibition to some extent, a combination of Epo and IL-3 was significantly superior to either drug alone in reversing the AZT-induced inhibition.

Amelioration of AZT-induced proliferative blockade of erythroid progenitor cells. Since the simultaneous addition of Epo did not prevent the AZT-induced proliferative blockade of the formation

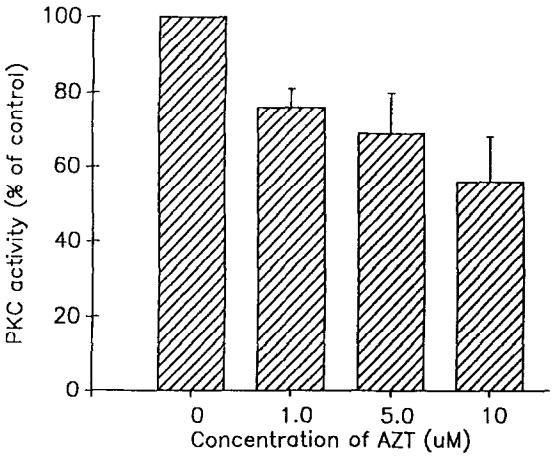

Fig. 4. Effect of AZT on protein kinase C activity in lysates of enriched erythroid progenitor cells (1×10^6 cells/mL) treated with various concentrations (0–10 μM) of AZT for 3 hr. Values are the means ± SEM obtained from at least three different experiments carried out in duplicate. Control protein kinase C activity was 16.1×10^3 cpm/10^6 cells.

of CFU-E derived colonies, it was important to determine whether a combination of Epo and IL-3 would be more beneficial than either growth factor alone in reversing the AZT-induced toxicity. The data in Fig. 6 demonstrate that the combination of Epo and IL-3 caused a significant increase in the CFU-E derived colonies at each of the lower (up to 5 μM) concentrations of AZT. The stimulative effect of the combination of Epo and IL-3 at the higher concentrations of 10 and 20 μM AZT was not significant, since AZT was extremely toxic *per se* at these levels. Thus, at clinically achievable

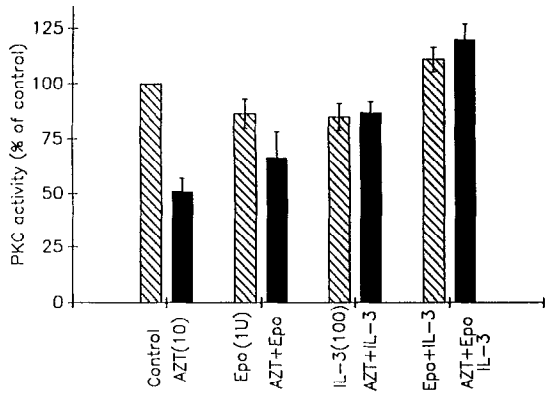

Fig. 5. Effects of Epo (1 U/mL), IL-3 (100 U/mL) and Epo plus IL-3 on AZT-induced (10 μM) inhibition of protein kinase C activity in lysates of enriched erythroid progenitor cells (1×10^6 cells/mL) treated with various drug combinations for 3 hr. Values are the means ± SEM obtained from at least two different experiments carried out in duplicate. Control protein kinase C activity was 12.8×10^3 cpm/10^6 cells.

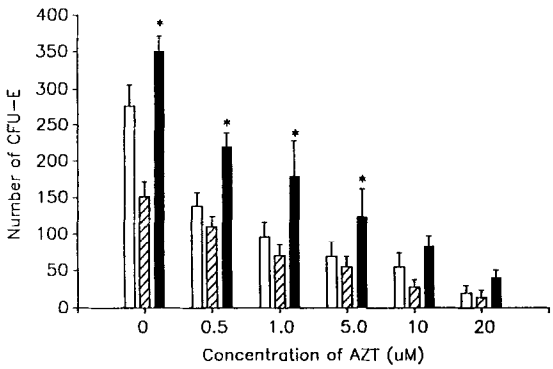

Fig. 6. Amelioration of AZT-induced proliferative blockade with Epo (200 mU/mL) (□), IL-3 (100 U/mL) (▨) and a combination of Epo and IL-3 (■). Enriched erythroid progenitor cells (2×10^6 cells/mL) were treated with AZT (0–20 μM) for 24 hr in the presence and absence of the growth factors. The cells were then diluted in a methyl cellulose mixture and plated to determine their colony-forming ability as described under the CFU-E assay. The control value for the CFU-E-derived colonies was 276 ± 30. These experiments were carried out in duplicate, and each value is the mean ± SEM of values obtained from at least three experiments. Key: (*) significantly different ($P < 0.05$) from the Epo or IL-3 group, as determined by Student's *t*-test.

concentrations, a combination of Epo and IL-3 resulted in significant protection of the bone marrow cells.

DISCUSSION

The toxicity induced by AZT in bone marrow progenitor cells has been well documented [18], but the exact underlying biochemical mechanisms have not been elucidated. Several reports have demonstrated in both the cell-free system [19] and in intact cells [20] that AZT anabolite is incorporated into cellular DNA. The extent of AZT incorporation into the DNA correlated directly with toxicity of the human bone marrow progenitor cells [21]. Inhibition of purified cellular DNA polymerase by AZT triphosphate has also been identified as one of the inhibitory steps [22, 23]. Alternatively, inhibition of mitochondrial DNA replication was reported to be the primary cellular target of AZT in a Friend murine erythroleukemic cell model [24]. Inhibition of mitochondrial DNA replication has also been shown to play an important role in 2′,3′-dideoxycytidine-induced peripheral neuropathy [25]. Recently, it was suggested that AZT monophosphate may contribute to cytotoxicity since it inhibited the replication of simian virus 40 origin-dependent double-stranded DNA in extracts of human HeLa cells at concentrations reported to accumulate intracellularly [26]. Other mechanisms reported to have a role in AZT-induced anemia include inhibition of the rate-limiting heme synthetic enzyme δ-aminolevulinic acid synthase [27] and inhibition of globin gene expression [28]. However, the inhibition of globin mRNA accumulation in these experiments required at least 25 μM or higher concentrations of AZT, suggesting that AZT-induced toxicity to erythroid progenitor cells at lower concentration levels may involve other mechanisms. Our data also demonstrate that although AZT is an antiproliferative agent in the CFU-E assay at low concentrations (IC_{50} 1.5 μM), it was not cytotoxic to the erythroid progenitor cells up to 10 μM, as determined by the dye exclusion method.

The anomalous finding of elevated endogenous serum Epo levels in AZT-treated patients [3, 4] and a relatively limited beneficial effect of exogenously administered Epo suggests target organ resistance. To examine this hypothesis, we initially investigated Epo receptor expression and function in murine bone marrow cells treated with the 2′,3′-dideoxynucleosides AZT and ddI [5]. The biotinylated-Epo/streptavidin-R phycoerythrin method using fluorescence cytometric analysis demonstrated that AZT decreased the cell-surface expression of Epo receptors on murine bone marrow progenitor cells in a concentration- and time-dependent manner. In contrast, ddI, which does not produce significant anemia in AIDS patients, had no significant effect on Epo receptor expression up to a concentration level of 20 μM, which caused proliferative blockade of erythroid progenitor cells [5]. In the present study, specific binding of ^{125}I-Epo to Epo receptors in splenic erythroblast cells obtained from phenylhydrazine-treated mice was utilized to demonstrate that AZT induces significant down-regulation of Epo receptor expression at therapeutic concentration levels of less than 10 μM (Fig. 1). It is likely that this blockade may precede the eventual inhibition of the globin gene expression [28]. The splenic erythroblast cells provide an important model to investigate the Epo receptor mRNA expression since there is an abundance of Epo receptors and Epo receptor

mRNA levels in these cells similar to that reported for the Friend virus-infected mouse splenic erythroblast cells [14]. These cells are therefore significantly more sensitive to AZT than the bone marrow cells. The data on down-regulation of Epo receptors suggest a cause-and-effect relationship between Epo receptor expression and inhibition of AZT-induced erythroid colony formation.

Studies were undertaken to determine if the down-regulation of Epo receptor expression results from decreased Epo receptor mRNA levels by monitoring the levels of the Epo receptor mRNA. A concentration-dependent inhibition of the Epo receptor mRNA levels by AZT (Table 1) demonstrates a direct relationship between the levels of Epo receptor mRNA and the number of Epo receptors (Fig. 1). In addition, the levels of mRNA of the protooncogenes c-*myc* and c-*fos* were also measured, since they have been shown to be linked directly to Epo-induced proliferation and differentiation of erythroid progenitor cells [18]. These studies were carried out after exposing the cells to AZT for 24 hr based upon our earlier time–course studies of the effect of AZT on Epo receptor mRNA levels [5]. The data in Table 2 indicate that AZT caused a concentration-dependent inhibition of the expression of the c-*fos* gene in erythroid progenitor cells. Under these conditions, the levels of the mRNA of c-*myc* were unaltered (data not included), suggesting that the inhibitory effect of AZT on the mRNA levels is not indiscriminatory but is selective.

Another parameter examined in this report was the effect of AZT on Epo-Epo receptor-mediated signal transduction at the level of PKC. Since activation of PKC has been implicated as a necessary step in proliferation and differentiation of erythroid progenitor cells [15, 29], we monitored the effect of AZT on PKC activity. The data in Fig. 4 demonstrate that inhibition of PKC activity may also contribute to AZT-induced toxicity. However, it is uncertain whether the inhibition of PKC enzymatic activity is the result of a direct or an indirect effect of AZT.

The down-regulation of Epo receptor and c-*fos* mRNA levels and inhibition of PKC activity by AZT led us to investigate the role of protecting agents in overcoming AZT-induced anemia. Since a synergism between Epo and IL-3 in the induction of hematopoietic stem cell proliferation and erythroid burst colony formation has been demonstrated [30], we investigated the role of IL-3 in overcoming the AZT-induced biochemical events. The data in Tables 1 and 2 and in Figs. 2 and 5 demonstrate that a combination of Epo and IL-3-induced proliferation led to overcoming the AZT-induced inhibitory events. A protective effect demonstrated by Epo alone in restoring the c-*fos* mRNA levels, despite the down-regulation of Epo receptors by AZT, is perhaps related to the proliferative and differentiating effect of Epo bound to the remaining receptors. The data in Fig. 6 also provide evidence that a combination of Epo and IL-3 ameliorated and AZT-induced proliferative blockade in erythroid progenitor cells. This combination led to a significantly higher number of CFU-E-derived colonies formed than those obtained with Epo or IL-3 alone, suggesting a protective role for IL-3 [31]. The data in this report further delineate the specific sites of activation of IL-3 resulting in an increase in the levels of mRNA of Epo receptor and c-*fos* and in the activity of PKC.

In conclusion, these results provide evidence for specific biochemical mechanisms that may contribute significantly to the AZT-induced toxicity, and the data further illustrate that these inhibitory events can be overcome by simultaneous treatment with a combination of Epo and IL-3.

Acknowledgements—This work was supported by a grant from the National Institute of Allergy and Infectious Diseases (AI–32893).

REFERENCES

1. Fischl MM, Richman DD, Grieco MH, Gottlieb MS, Volberding PA, Laskin OL, Leedom JM, Groopman JE, Mildvan D, Schooley RT, Jackson GG, Durack DT and King D, The efficacy of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. *N Engl J Med* **317**: 185–191, 1987.
2. Richman DD, Fischl MM, Grieco MH, Gottlieb MS, Volberding PA, Laskin OL, Leedom JM, Groopman JE, Mildvan D, Hirsch D, Jackson GG, Durack DT and Nusinoff-Lehrman S, The toxicity of azidothymidine (AZT) in the treatment of patients with AIDS and AIDS-related complex. *N Engl J Med* **317**: 192–197, 1987.
3. Fischl M, Galpin JE, Levine JD, Groopman JE, Henry DH, Kennedy P, Miles S, Robbins W, Starrett B, Zaluskey R, Abels R, Tasi HC and Rudnick SA, Recombinant human erythropoietin for patients with AIDS treated with zidovudine. *N Engl J Med* **322**: 1488–1493, 1989.
4. Spivak JL, Barnes DC, Fuchs E, and Quinn TC, Serum immunoreactive erythropoietin in HIV-infected patients. *J Am Med Assoc* **210**: 3104–3107, 1989.
5. Gogu SR, Malter JS and Agrawal KC, Zidovudine induced blockade of the expression and function of the erythropoietin receptor. *Biochem Pharmacol* **44**: 1009–1012, 1992.
6. Nijhof W, Wierenga PK, Sahr K, Beru N and Goldwasser E, Induction of globin mRNA transcription by erythropoietin in differentiating erythroid precursor cells. *Exp Hematol* **15**: 779–783, 1987.
7. Mufson RA and Gesner TG, Binding internalization of recombinant human erythropoietin in murine erythroid precursor cells. *Blood* **69**: 1485–1490, 1987.
8. Mason-Garcia M, Weill CL and Beckman BS, Rapid activation by erythropoietin of protein kinase C in nuclei of erythroid progenitor cells. *Biochem Biophys Res Commun* **168**: 490–497, 1990.
9. Ottman OG, Ganser A, Seipalt G, Eder M, Schultz G and Hoelzer D, Effects of recombinant human interleukin-3 on human hematopoietic progenitor and precursor cells *in vivo*. *Blood* **76**: 1494–1502, 1990.
10. Gogu SR, Beckman BS and Agrawal KC, Comparative toxicities in murine fetal liver and bone marrow erythroid progenitor cells. *Life Sci* **45**: iii–vii, 1989.
11. Krystal G, A simple microassay for erythropoietin based on ^{3}H-thymidine incorporation into spleen cells from phenylhydrazine treated mice. *Exp Hematol* **11**: 649–660, 1983.
12. Broudy VC, Nakamoto B, Lin N and Papayannopoulou T, Dynamics of erythropoietin receptor expression on erythropoietin-responsive murine cell lines. *Blood* **75**: 1622–1626, 1990.

13. Chomcyzynski P and Sacchi N, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**: 156–159, 1987.
14. Wickrema A, Bondurant MC and Krantz SB, Abundance and stability of erythropoietin receptor mRNA in mouse erythroid progenitor cells. *Blood* **78**: 2269–2275, 1991.
15. Beckman BS, Mason-Garcia M, Martinez MI and Fermin C, Enhanced expression of the β II subspecies of protein kinase C in differentiating erythroleukemia cells. *Exp Cell Res* **191**: 319–322, 1990.
16. Sawyer ST, Krantz SB and Goldwasser E, Binding and receptor-mediated endocytosis of erythropoietin in Friend virus-infected erythroid cells. *J Biol Chem* **262**: 5554–5562, 1987.
17. Umemura T, Umene K, Takahira H, Takeichi N, Katsuno M, Fukumaki Y, Nishimura J, Sakaki Y and Ibayashi H, Hematopoietic growth factors (BPA and Epo) induce the expression of c-*myc* and c-*fos* proto-oncogenes in normal human erythroid progenitors. *Leuk Res* **12**: 187–194, 1988.
18. Sommadossi J-P and Carlisle R, Toxicity of 3′-azido-3′-deoxythymidine and 9-(1, 3-dihydroxy-2-propoxy-methyl)guanine for normal human hematopoietic progenitor cells *in vitro*. *Antimicrob Agents Chemother* **31**: 452–454, 1987.
19. St. Clair MH, Richards CA, Spector T, Weinhold KJ, Miller WH, Langois AJ and Furman PA, 3′-Azido-3′-deoxythymidine triphosphate as an inhibitor and substrate of purified human immunodeficiency virus reverse transcriptase. *Antimicrob Agents Chemother* **31**: 1972–1977, 1987.
20. Frick LW, Nelson DJ, St. Clair MH, Furman PA and Krenitsky TA, Effects of 3′-azido-3′-deoxythymidine on the deoxynucleotide triphosphate pools of cultured human cells. *Biochem Biophys Res Commun* **54**: 124–129, 1988.
21. Sommadossi J-P, Carlisle R and Zhou Z, Cellular pharmacology of 3′-azido-3′-deoxythymidine with evidence of incorporation into DNA of human bone marrow cells. *Mol Pharmacol* **36**: 9–14, 1989.
22. Huang P, Farquhar D and Plunkett W, Selective action of 3′-azido-3′-deoxythymidine 5′-triphosphate on viral reverse transcriptases and human DNA polymerases. *J Biol Chem* **265**: 11914–11918, 1990.
23. Nickel W, Austermann S, Bialek G and Grosse F, Interactions of azidothymidine triphosphate with the cellular DNA polymerases α, δ and ε and with DNA primase. *J Biol Chem* **267**: 848–854, 1992.
24. Hobbs GA, Keilbaugh SA and Simpson MV, The Friend murine erythroleukemia cell, a model system for studying the association between bone marrow toxicity induced by 3′-azido-3′-dideoxythymidine and dideoxynucleoside inhibition of mtDNA replication. *Biochem Pharmacol* **43**: 1397–1400, 1992.
25. Chen C-H, Vazquez-Padua M and Cheng Y-C, Effect of anti-human immunodeficiency virus nucleoside analogs on mitochondrial DNA and its implication for delayed toxicity. *Mol Pharmacol* **39**: 625–628, 1991.
26. Bebenek K, Thomas DC, Roberts JD, Eckstein F and Kunkel TA, Effects of 3′-azido-3′-deoxythymidine metabolites on simian virus 40 origin-dependent replication and heteroduplex repair in HeLa cell extracts. *Mol Pharmacol* **43**: 57–63, 1993.
27. Lutton JD, Matthew A, Levere RD and Abraham NG, Role of heme metabolism in AZT-induced bone marrow toxicity. *Am J Hematol* **35**: 1–5, 1990.
28. Weidner DA and Sommadossi JP, 3′-Azido-3′-deoxythymidine inhibits globin gene transcription in butyric acid-induced K-562 human leukemic cells. *Mol Pharmacol* **38**: 797–804, 1990.
29. Spangler R and Sytkowski AJ, c-*myc* Is an erythropoietin early response gene in normal erythroid cells: Evidence for a protein kinase C-mediated signal. *Blood* **79**: 52–57, 1992.
30. Migliaccio G, Migliaccio AR and Visser JWM, Synergism between erythropoietin stem cell proliferation and erythroid burst colony formation. *Blood* **72**: 944–951, 1988.
31. Gogu SR, Malter JS and Agrawal KC, Zidovudine blocks the function and expression of erythropoietin-receptor (Ep-R): Reversal with erythropoietin (Ep) and interleukin-3 (IL-3). *Blood* **78**: 398a, 1991.