

Tetrahydrouridine, Cytidine Analogues, and Hemoglobin F

Joseph DeSimone, Paul Heller, Robert E. Molokie, Lemuel Hall, and David Zwiers

Veterans Administration West Side Medical Center and The Department of Medicine, University of Illinois College of Medicine, Chicago

5-Azacytidine (azaC) has previously been shown to raise Hb F levels in the repeatedly phlebotomized baboon (PCV: around 20%). The administration of tetrahydrouridine (THU), an inhibitor of the enzymatic conversion of azaC to 5-azauridine, made it possible to reduce the amount of azaC and also of 2-deoxy-5-azacytidine (d-azaC) by more than 90% and still achieve maximal Hb F elevations. However, the granulocytopenia, usually occurring after 5-azaC, was not altered by the lowering of the dosages in the presence of THU. Thus, the granulocytopenia is not due to 5-azauridine or other catabolic products resulting from deamination. It is also unlikely that it is caused by a direct influence of azaC on RNA since d-azaC also causes granulocytopenia. The persistence of reticulocytosis throughout the treatment with azaC or d-azaC makes it appear likely that the observed increase in Hb F levels to more than 60% of total hemoglobin is not due to a cytotoxic effect on erythropoiesis resulting in a shift of cell populations toward greater immaturity, but to a direct influence of the drug on the regulation of γ globin chain production.

Key words: cytidine analogues, tetrahydrouridine, hemoglobin F, cytidine deaminase inhibition

INTRODUCTION

5-Azacytidine (azaC) has been shown to increase the production of fetal hemoglobin in nonhuman primates [1] and man [2,3], presumably because its incorporation as deoxyazaC into DNA is associated with DNA hypomethylation [2-4], although other mechanisms may also play a role [5,6]. The use of azaC may be therapeutic since the increase in the synthesis of γ chains by the drug is accompanied by a reciprocal decrease in that of β chains [1,2]. Indeed, several reports [2-4] have suggested that the parenterally administered drug has a beneficial effect in patients with sickle cell anemia and β -thalassemia, but its potential short- and long-term toxicity is cause for concern and the parenteral route is impractical for long-term administration.

5-Azacytidine is rapidly deaminated to azauridine in vivo by cytidine deaminase, which is widely distributed in various tissues, including plasma and granulocytes. Therefore, only a fraction of the administered amount is available for incorporation into DNA and RNA. It has been previously shown [7,8] that tetrahydrouridine (THU), an otherwise apparently nontoxic agent, effectively inhibits this enzyme by competitive binding and its administration should make it possible to reduce the dosage of

Received for publication May 17, 1984; accepted July 19, 1984.

Address reprint requests to Joseph DeSimone, Dept. of Medicine, University of Illinois College of Medicine, Chicago, IL 60612.

azaC and achieve the same levels of Hb F as with larger amounts of azaC alone. Because the gastrointestinal mucosa is rich in this enzyme, orally administered azaC together with THU is absorbed with minimal inactivation [8] and should lead to Hb F elevations. The present study in baboons, parts of which were previously presented and published as abstract [9], was undertaken to explore these possibilities and to determine if the smaller dosages of azaC have reduced toxicity, mainly with regard to granulocytopenia. In addition, we also tested 2'-deoxy-5-azacytidine (d-azaC), which is also deaminated by cytidine deaminase, for its effect on Hb F production without and with THU. The amount of this analogue which is left after the enzymatic deamination has reached its plateau is only incorporated into DNA.

MATERIALS AND METHODS

An estimate of the *in vivo* inhibition of cytidine deaminase (identical with cytosine deaminase) activity by THU was obtained in peripheral blood granulocytes isolated by density gradient centrifugation, which yields an approximately 90% pure population. These granulocytes were lysed by freezing and thawing, and the lysate of 2.5×10^7 cells was adjusted to 1 ml. Cytidine deaminase activity was measured spectrophotometrically [10] as a decrease in absorbance at 282 nm when cytidine (1.67×10^{-4} M) is converted to uridine. Activity was expressed as Δ absorbance/ 10^8 granulocytes/minute. Hb F levels were determined by alkali denaturation [11]. The THU and azaC were gifts of the National Cancer Institute, and d-azaC was purchased from Sigma Chemical Company, St. Louis, MO.

Baboons 1.5–6 years old, were phlebotomized to reduce the packed red cell volume (PCV) to 20% in 5 days. This reduction was maintained for the remainder of the experimental period. As in our previous experiments with azaC in baboons [1], the drug was given every morning during two 5-day periods separated by the weekend during which the phlebotomies were also omitted, and THU was given subcutaneously or orally in two 10-mg/kg doses for the same duration. In the initial experiments in four baboons THU was only given subcutaneously and the dose of azaC varied between 0.1 mg/kg and 4 mg/kg per day. In preparation for the oral administration the animals had no access to food, but to water, during the preceding night. The two agents were given through a nasogastric tube. The d-azaC was given only parenterally for the same duration as azaC at dose levels indicated in Table 2. Since this agent does not require enzymatic reduction prior to incorporation into DNA and is not incorporated into RNA, we assumed that a lower dose than of azaC would be effective. The THU was administered parenterally. All animals had been previously tested with azaC, two of them also had received THU.

RESULTS

In vitro, 10^{-4} M THU caused almost complete loss (98%) of granulocyte cytosine deaminase activity. For determining the parenteral and oral dose of THU we assumed that THU is completely absorbed and distributed uniformly in the body and a 20-mg/kg dose would result in the approximate body solute concentration of 10^{-4} M. Figure 1 demonstrates the change in granulocyte cytosine deaminase activity following treatment with THU (20/mg/kg/day). Maximal inhibition ($\sim 70\%$) occurred after 2 days of treatment and normal activity returned at the earliest 5 days after discontin-

TABLE I. Maximal Hb F Level Attained in Baboons by Oral Treatment With 5-azaC and THU (Compared to Parenteral Administration)

Baboon	Mode of administration	Dose mg/kg		Maximal Hb F Level (%)
		5-azaC	THU ^a	
3950	Oral	8	20	42.6
	Subcutaneous	8		47.2
4625	Oral	4	20	62.0
	Subcutaneous	4		49.8
4626	Oral	8	20	69.0
	Oral	2	20	68.5
	Intravenous	4		68.0
4755	Oral	0.5	20	57.8
	Oral	0.25	20	34.2
	Intravenous	4		26.0

^aTHU was given orally in two 10-mg/kg doses. The first dose was given alone and the second together with 5-azaC 30 minutes later.

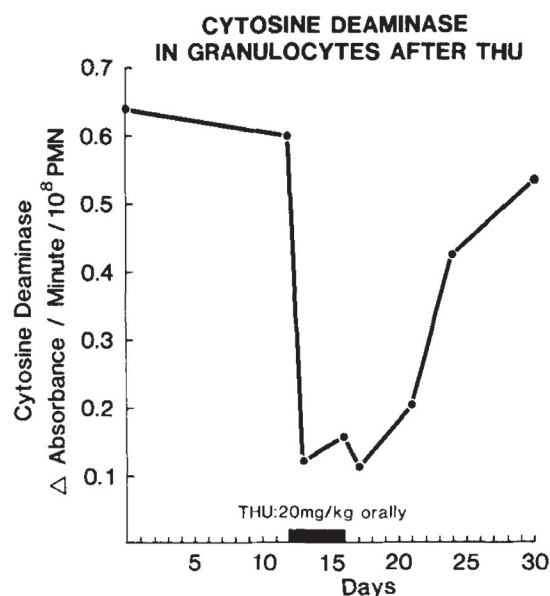


Fig. 1. Change in baboon granulocyte deaminase activity after oral administration of 20 mg of THU/kg/day for 5 days.

uation of THU. In eight THU-treated animals, the deaminase activity fell from 0.49 ± 0.08 before treatment to 0.15 ± 0.03 after 2 days of treatment, an average decrease of 72%.

Initially, four baboons received THU only subcutaneously. In its presence, 0.25 mg/kg/day of azaC was as effective as 4 mg/kg/day without it, and in either case the resulting Hb F levels were 59–60.3%. It was found that 0.1 mg/kg/day of azaC with THU increased the Hb F level only to 35.1%.

The effectiveness of orally administered azaC with regard to Hb F elevations is demonstrated in Table I. Although Hb F production is not increased as a result of orally administered azaC (8 mg/kg) in the absence of THU (data not shown), maximal Hb F production can be achieved when it is given with THU. As with subcutaneous

TABLE II. Maximal Hb F Levels, Nadir Numbers of Granulocytes, and Reticulocyte Percentages in Baboons Treated Parenterally With 2'-deoxy-5-azaC Without or With THU* (Compared to azaC)

Baboon	Drug	Dose (mg/kg)	THU	Granulocyte numbers		Maximal Hb F level (5)	Reticulocyte percentages ^a
				Before treatment	Nadir		
4756	d-azaC	0.06	+	3,000	1,000	64.4	13-14
	azaC	0.25	+	3,000	1,000	69.4	16-19
4612	d-azaC	0.04	+	2,800	2,400	31.3	20-30
	azaC	0.25	+	2,100	1,600	67.8	13-15
4625	d-azaC	1.5	-	3,000	500	61.5	14-13
	azaC	4.0	-	3,200	1,000	49.8	11-14
4624	d-azaC	0.75	-	3,200	800	43.4	9-10
	azaC	4.0	-	4,200	1,600	39.0	12-10
4626	d-azaC	0.3	-	3,000	1,200	39.2	11-11
	azaC	4.0	-	3,500	1,300	68.0	15-12

*THU was given in two 10-mg/kg doses, the first one alone intravenously and the second 5 minutes later subcutaneously with d-azaC.

^aFirst number indicates reticulocyte percentage after bleeding and prior to use of drug, the second at the time of maximal Hb F response. Corresponding erythrocyte counts varied between 2.0 and $2.4 \times 10^6/\mu\text{l}$.

administration, the dose can be reduced to 0.25 mg/kg to obtain maximal Hb F elevations.

During the two experimental periods the animals ate normally and no weight loss occurred. At the dose level which led to maximal Hb F elevations the reticulocyte percentage stayed between 10 and 20 (average reticulocyte percentage before treatment was 13.4 and at the time of the lowest granulocyte count it was 13.8) but the granulocyte count decreased in all animals to a variable degree (Table II) 5-10 days after discontinuation of the drug. This was also the time at which maximal elevations of Hb F were observed. The lowest granulocyte count was $500/\mu\text{l}$. These decreases were always reversible. They occurred regardless of the mode of administration of the cytidine analogue and the use of THU. The number of circulating lymphocytes was not affected.

DISCUSSION

Neil et al [8] determined blood levels of azaC in mice after intraperitoneal injection of the drug with and without THU and found that higher blood levels were achieved with THU than without it. The same was true after oral administration of the two agents, although the blood levels were lower than with the parenteral administration. These observations persuaded us to use THU in association with 5-azaC in order to determine whether the prevention of inactivation of azaC might permit the lowering of the parenteral dosage or the oral administration of the drug without a decrease in its effect on the stimulation of Hb F production. In addition, inhibition of deamination might also prevent the formation of toxic catabolic products, which might be responsible for undesirable effects, especially granulocytopenia. The same question was raised with regard to d-azaC, which can only be incorporated into DNA; therefore, all desirable and undesirable effects could only be due to this mode of action and not due to incorporation into RNA. The results of the study with regard to Hb F stimulation clearly indicate that the lower doses in the presence of THU are

equally effective and, moreover, that THU permits the oral administration of the drug. Can one expect less toxicity from the smaller dosages? All regimens, azaC and d-azaC alone or with THU, which lead to maximal levels of Hb F were associated with a decrease of the absolute granulocyte count. Thus the granulocytopenia is not prevented by the lower dosage of the analogue in the presence of THU. It should be emphasized that d-azaC also produced granulocytopenia, a fact which makes it appear likely that this toxic effect of both analogues is due to their incorporation into DNA.

The mechanism of the elevation of Hb F as the result of treatment with azaC or d-azaC has not yet been unequivocally clarified. We have postulated [1] that it is chiefly due to DNA hypomethylation and to the subsequent enhancement of the expression of γ genes as suggested by a) the demonstration of hypomethylation of DNA of bone marrow cells, 90% of which were erythropoietic, after treatment of the baboons with azaC [12] and b) the persistence of reticulocytosis in the treated animals and the marked erythroid hyperplasia despite the administration of a cytotoxic agent, seemingly speaking against a shift in erythroid progenitor populations in the recovery phase from cytotoxicity favoring the synthesis of Hb F. Since then, hydroxyurea [6] and cytosine arabinoside [13,14] have been shown to increase fetal hemoglobin levels in nonhuman primates. Hydroxyurea has also been found to elevate Hb F in patients with sickle cell anemia [15]. The findings in nonhuman primates [6,14] suggest that the mean corpuscular content of Hb F and the proportion of Hb F in hemolysates was in the range of that found in the chronically bled animals with an approximate hematocrit of 20% [16,17]. Thus, the marked increase of Hb F beyond that level after the administration of azaC which is associated with high values for Hb F per F-cell is likely to be chiefly due to DNA hypomethylation.

It has been postulated that DNA hypomethylation is implicated in carcinogenesis [18], but the experimental data are still ambiguous. A small degree of hypomethylation has been detected in DNA extracted from hyperplastic erythroid bone marrow of baboons [12] which were chronically bled and had Hb F elevations around 20%, suggesting that some DNA hypomethylation may occur as a consequence of increased cell proliferation. Nevertheless, global hypomethylation by azaC may result in activation of oncogenes and this possibility, theoretical at this time, demands the exercise of caution in the long-term therapeutic use of azaC. If azaC and d-azaC with THU should become acceptable in the treatment of sickle cell anemia and β -thalassemia, the possibility of oral use will facilitate the long-term administration of the drug. The usefulness of hydroxyurea and of cytosine arabinoside in the treatment of these diseases also remains to be evaluated.

ACKNOWLEDGMENTS

This study was supported by Veterans Administration Research Funds and NIH Grant No. HL 20920-06.

REFERENCES

1. DeSimone J, Heller P, Hall L, Zwiers D: 5-azacytidine stimulates fetal hemoglobin synthesis in anemic baboons. *Proc Natl Acad Sci USA* 79:4428, 1982.
2. Ley TJ, DeSimone J, Anagnou NP, Keller GH, Humphries RK, Turner PH, Young NS, Heller P, Nienhuis AW: 5-azacytidine selectively increases γ -globin synthesis in a patient with β^+ thalassemia. *N Engl J Med* 307: 1469, 1982.

3. Ley TJ, DeSimone J, Noguchi CT, Turner PH, Schechter AN, Heller P, Nienhuis AW: 5-azacytidine increases γ -globin synthesis and reduces the proportion of dense cells in patients with sickle cell anemia. *Blood* 62:370, 1983.
4. Charache S, Dover G, Smith K, Talbot CC, Moyer M, Boyer S: Treatment of sickle cell anemia with 5-azacytidine results in increased fetal hemoglobin production and is associated with nonrandom hypomethylation of DNA around the γ - δ - β globin gene complex. *Proc Natl Acad Sci USA* 80:4842, 1983.
5. Torrealba-de-Ron AT, Papayannopoulou T, Knapp MS, Feng-Ruen Fu M, Knitter G, Stamatoyannopoulos G: Perturbations in the erythroid marrow progenitor cell pools may play a role in the augmentation of Hb F by 5-azacytidine. *Blood* 63:201, 1984.
6. Letvin NL, Linch DC, Beardsley P, McIntyre KW, Nathan DG: Augmentation of fetal-hemoglobin production in anemic monkeys by hydroxyurea. *N Engl J Med* 310:809, 1984.
7. Neil GL, Moxley TE, Manak RC: Enhancement by tetrahydrouridine of 1- β -D-arabinofuranosylcytosine (cytarabine) oral activity in L 1210 leukemic mice. *Cancer Res* 30:2166, 1970.
8. Neil GL, Moxley TE, Kuentzel SL, Manak RC, Hanka W: Enhancement by tetrahydrouridine (NSC-112907) of the oral activity of 5-azacytidine (NSG-102816) in L 1210 leukemic mice. *Cancer Chemother Rep* 59:459, 1975.
9. DeSimone J, Heller P, Hall L and Zwiers D: The effectiveness of 5-azacytidine (5-azaC) in increasing Hb F levels is enhanced by inhibition of cytosine deaminase. *Blood* 62:73A, 1983.
10. Cohen RH, Wolfenden R: Cytidine deaminase from *Escherichia coli*: Purification, properties, and inhibition by the potential transition state analog 3,4,5,6-tetrahydrouridine. *J Biol Chem* 246:7561, 1983.
11. Singer K, Chernoff AL, Singer L: Studies on abnormal hemoglobin I. Their demonstration in sickle cell anemia and other hematologic disorders by means of alkali denaturation. *Blood* 6:413, 1951.
12. DeSimone J, Heller P, Schimenti JC, Duncan CH: Fetal hemoglobin production in adult baboons by 5-azacytidine or by phenylhydrazine-induced hemolysis is associated with hypomethylation of globin gene DNA. In Stamatoyannopoulos G, Nienhuis AW (eds): "Globin Gene Expression and Hematopoietic Differentiation." New York: Alan R. Liss, 1983, p 489.
13. Veith R, Torrealba de Ron AT, Papayannopoulou T, Fu M, Knapp M, Knitter G, Stamatoyannopoulos G: On the mechanism of 5-azacytidine induced Hb F in baboons: comparisons of data obtained from cytosine-arabinoside and 5-azacytidine treatments. *Blood* 62:76A, 1983.
14. Papayannopoulou T, Torrealba de Ron A, Veith R, Knitter G, Stamatoyannopoulos G: Arabinosylcytosine induces fetal hemoglobin in baboons by perturbing erythroid cell differentiation kinetics. *Science* 224:617, 1984.
15. Dover G, Miller B, Orkin S, Beardsley P, Linch D, Levin N, Nathan D: Hydroxyurea increases fetal hemoglobin production in sickle cell anemia. *Clin Res* 32:552A, 1983.
16. DeSimone J, Heller P, Biel M, Zwiers D: Genetic relationship between fetal Hb levels in normal and erythropoietically stressed baboons. *Brit J Haematol* 49:175, 1981.
17. DeSimone J, Biel M, Heller P: Maintenance of fetal hemoglobin (HbF) elevations in the baboon by prolonged erythropoietic stress. *Blood* 60:519, 1982.
18. Riggs AD, Jones PA: 5-methylcytosine, gene regulation and cancer. *Advanc Canc Res* 40:1, 1983.