5-Azacytidine increases the synthesis of embryonic hemoglobin (E2) in murine erythroleukemic cells

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The addition of 5-azacytidine to erythroleukemic cells which were induced to differentiate with DMSO or BA altered the expression of the hemoglobins. After the addition of 5-azacytidine there was an increase in hemoglobin synthesis especially in the embryonic E2 band. The β-globin increased in synthesis after 5-azacytidine treatment. The level of hemoglobin synthesis in DMSO-induced cells is less than BA-induced cells while the effect of the 5-azacytidine stimulation was greater with DMSO induction than with BA induction.

Hemoglobin *Globin* *Murine* *Erythroleukemic cell* *Electrophoresis* *Differentiation* *5-Azacytidine*

1. INTRODUCTION

In humans, fetal hemoglobin genes are undermethylated and expressed in fetal life while they are methylated and unexpressed in adult life [1]. It has been shown with tissue culture cells that the addition of 5-azacytidine to cells growing in tissue culture can undermethylate newly-synthesized DNA resulting in altered gene expression [2–5]. In vivo studies on anemic baboons showed that 5-azacytidine increased the production of fetal hemoglobin [6], while studies on human patients suffering from β-thalassemia or sickle cell anemia showed an increase in the synthesis of γ-globin and ϵ-globin after the administration of 5-azacytidine [7].

In murine erythroleukemic cells, 5-azacytidine has been shown to be a weak inducer of hemoglobin synthesis [8]. Here, the addition of 5-azacytidine to DMSO- or butyric acid-induced cells increased the amount of hemoglobin translated. By adding hemin the quantity of hemoglobin and globin was increased in 5-azacytidine-treated cells suggesting that there was additional globin and messenger RNA in the cell. The findings suggested, but not conclusively, that the 5-azacytidine is demethylating the DNA resulting in an increased embryonic globin gene expression.

2. MATERIALS AND METHODS

2.1. *Growth and induction of Friend-erythroleukemic cells*

Friend-erythroleukemic cells (T3C12) derived from a DDD mouse were obtained from the Mammalian Genetics Mutant Cell Repository at the Institute of Medical Research (Camden NJ; classified as GM979). The cells are grown in suspension in a humidified tissue culture incubator containing 5% CO_2 at 37°C. Alpha-medium supplemented with 10% fetal calf serum and antibiotics–antimycotics is used to support cell growth. The cells are maintained in continuous log phase culture between 5×10^4 cells/ml and 1×10^6 cells/ml. Optimum conditions for the induction of differentiation in the MELC system occur with the addition of 1.5% DMSO or 1 mM butyric acid (BA). To prepare a sufficient quantity of hemoglobin or globin, 20 mM HEPES is added to α-medium containing 10% fetal calf serum, and the cells are grown in

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spinner culture flasks. The cells are washed 3-times and lysed as follows.

Hemoglobin was obtained by washing the erythrocytes 3-times in phosphate-buffered saline followed by lysing the cells by adding 3 volumes of lysing medium for 15 min at 4°C (0.5% Triton X-100, 25 mM Tris–HCl, 25 mM KCl, 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 20 mM KCN) [9]. The KCN was freshly made and added to lysing buffer prior to use. Cell debris was then removed by centrifugation at 20000 × *g* for 20 min and the supernatant solution containing hemoglobin was stored at −70°C [9].

2.2. *Cell volume and cell growth*

The electronic cell volume is measured on a Coulter H_4 Channelyzer and the electronic cell count is made simultaneously on a Coulter Counter, Model ZBI equipped with an aperture tube containing a 70-μm diameter and 84-μm length orifice on the aperture tube (Coulter Electronics, Hialeah FL). To obtain reproducible results for electronic cell volume analysis and counting, the tissue culture cells are removed from culture, dispersed, and immediately fixed with an equal volume of filtered glutaraldehyde in phosphate-buffered saline resulting in a final fixation concentration of 3% glutaraldehyde. Due to cell shape factors, mouse fetal blood cells can be counted and sized without prior fixing.

2.3. *Chemicals*

A 10-mM solution of hemin is made by dissolving 13 mg in 0.2 ml 0.5 M NaOH buffered with 0.25 ml 1 M Tris–HCl (pH 7.8) and subsequently diluting the solution to 2 ml with H_2O. The stock solution is then sterilized by filtration, stored at −70°C and used at 10^{-4} for experiments [10].

DMSO (Pierce) is added to cultures directly. Butyric acid (100 mM) is dissolved in H_2O, sterilized by filtration, stored at 4°C, and used at a concentration of 1 mM.

2.4. *Isoelectric focusing*

Analytical and preparative isoelectric focusing (IEF) is performed in 3 mm (inside diameter) tubes using 4% polyacrylamide gels containing 2% ampholines of the following ranges: 6–8 (50%), 8–9.5 (33%), 9.5–11 (17%) [11,12]. The tube gels are then prefocused at 0.2 W/gel for 0.5 h after which the samples are layered on the gel. Isoelectric focusing conditions consist of a constant 0.2 W/gel at a temperature of 4°C for 2.5–3 h. The bands are sliced out, eluted with H_2O and the eluent lyophilized for globin analysis [12]. To improve the analytical IEF resolution, we use an LKB multiphore unit with thin slab-type gels (0.4 mM) to separate hemoglobins (the gel is prepared using 3 sheets of parafilm gasketing to obtain a 0.4 mM spacing and a Gelbond plastic molding sheet, LKB). The slab gels are prefocused for 0.5 h at 10 W, after which the samples (0.5 μl–2 μl) are applied as a drop to the gel surface and then electrophoresed for and run for 1.5 h at 4°C.

The standard procedure in preparing hemoglobin for tube gel IEF is to add between 10–50 μl of this solution to the 3 mm polyacrylamide disc gels to achieve a hemoglobin concentration suitable for globin analysis of liquid scintillation counting. For analytical IEF (slab gels) 2 μl of hemoglobin are added to the cathode region of the gel. If there is low intensity in the hemoglobin bands the analytical gels are stained with 0.1% 3–3 dimethoxybenzidine hydrochloride dissolved in 1% acetic acid. About 10 μl 30% H_2O_2/10 ml are added to stain the hemoglobin bands. This is followed by photography and 10 W are applied for 1.5 h after which the gel is fixed in 5% trichloroacetic acid, 5% sulfosalicylic acid, 20% MeOH with 0.1% filtered Coomassie blue. The gels are processed for fluorography with Enhance (New England Nuclear), dried down and exposed to Kodak X-ray film XAR-5 for 1–3 days depending on the level of [^{3}H]leucine incorporation [13].

2.5. *Gel electrophoresis of globins*

Globin chains are separated by 0.75 mm thick slab gel electrophoresis (Bio Rad Model 220) on 9% polyacrylamide containing 6 M urea, 2% Triton X-100 in 5% acetic acid as in [14] and as modified in [15]. The gel is pre-electrophoresed for 30 min at 200 V at 4°C. Then 50 μl of 1 M β-mercaptoethylamine is added to each sample well, and a second pre-electrophoresis is made at 150 V for 30 min at 4°C. The β-mercaptoethylamine is removed and the lyophilized hemoglobins eluted from IEF gels or the unseparated hemoglobins are suspended into 20 μl of sample buffer (5 ml of freshly deionized 8 M urea, 0.5 ml glacial acetic acid, 0.5 ml 2-mercaptoethanol) and layered in

each well. The samples are electrophoresed for 3.5 h at 20 mA. The gel is fixed in a solution containing 3.5% sulfosalicylic acid, 11.4% trichloroacetic acid in H_2O for 0.5 h. The defixing, staining and destaining steps are carried out in 30% ETOH and 10% acetic acid. The gel is defixed for 0.5 h, stained with 0.1% Coomassie blue overnight and destained until the bands can be photographed. The gel is prepared for fluorography by treatment with Enhance (New England Nuclear), followed by drying the gel onto a filter paper. The gels are exposed to Kodak XAR-5 X-ray film for 3–7 days depending on the level of ^3H-incorporation and developed using Kodak X-ray developer for 5 min [13].

3. RESULTS AND DISCUSSION

T3C12 murine erythroleukemic cells have been shown to express both embryonic, adult hemoglobins [6,17]. The addition of 5-azacytidine (0.5 mm) to BA (1 mm)- or DMSO (1.5%)-induced cells resulted in altered hemoglobin gene expression. The T3C12 cells were induced to differentiate with BA or DMSO and at day 1 and/or day 2, 5-azacytidine was added to cultures. 5-Azacytidine had little effect on the growth of DMSO-induced cells while it reduced the growth of BA-induced cells by 50% (fig.1). The hemoglobins obtained from the induced cells between 3 and 5 days were separated and analyzed by 3 electrophoresis techniques: (i) isoelectric focusing slab gels (0.4 mm) with fluorography; (ii) isoelectric focusing tube gels (3 mm) for scintillation counts; (iii) acid–urea Triton X-100 slab gels (75 mm) for globin analysis. Analytical isoelectric focusing of hemoglobins derived from BA- or DMSO-treated cells showed that 5-azacytidine increased the synthesis of E_2 and A in DMSO-treated cells (fig.2A) and increased the synthesis of band 1, E_2 and A in BA-induced cells. The globin consistency of these bands has been established to be the following: band 6 ($\alpha_2\beta_2$), band 4 ($\alpha_2\gamma_2$), band 1 ($\alpha_2\gamma_2$). Previous data from our laboratory have shown an increased hemoglobin stimulation when hemin is added to MELC, which were induced to differentiate. In both BA- and DMSO-induced cells treated with 5-azacytidine, the synthesis of A and E_2 bands were greatly increased after hemin was added to the cultures. The data suggest that the level of untranslated mRNA was greater in DMSO-induced cells which were treated with 5-azacytidine than the level in DMSO cells which were not treated with 5-azacytidine.

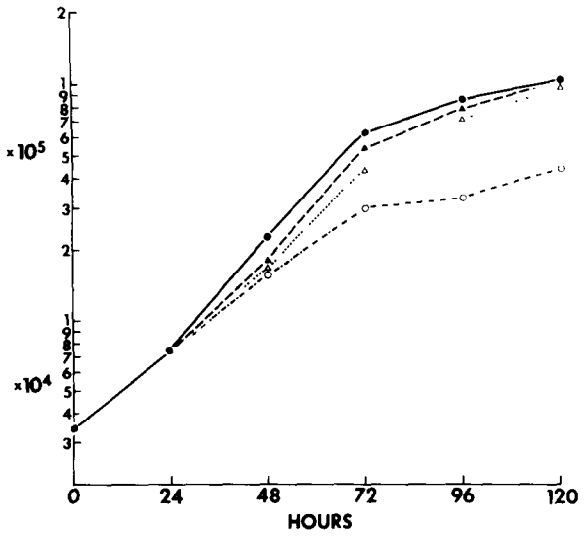

Fig.1. Growth of BA- and DMSO-induced cells in the presence of 5-azacytidine. MEL were induced with BA (1 mM) or DMSO (1.5%) and on days 1 and 2, 0.5 μM 5-azacytidine was added. The cells were monitored for growth and cell volume using a Coulter channelizer. The cell volume of the 5-azacytidine-treated cells was larger than the control cells. (●—●) DMSO; (▲---▲) DMSO + 5-Az; (Δ—Δ) BA; (○---○) BA + 5-Az.

In order to determine the relative rate of synthesis of the tissue culture-derived hemoglobins, 3-mm tube gels were used with the isoelectric focusing method to separate the hemoglobins. The hemoglobins of band 6 (A) and band 4 (E_2), which were visible were cut and eluted overnight from the gels in 100 μl H_2O. Band 1 was not obtained as it was not usually visible unless the gels were stained with benzidine. As shown in table 1, the addition of 5-azacytidine increased the synthesis of hemoglobin in both DMSO- and BA-induced samples. The addition of hemin also increased the synthesis of the A and E2 bands in both control and 5-azacytidine-treated cells. The addition of 5-azacytidine to 48-h DMSO-treated cells resulted in an increase in globin synthesis when assayed with hemin at 96 h (fig.3). The data correlate with fig.2A, showing that in addition to more hemoglobin, hemin synthesized more globin.

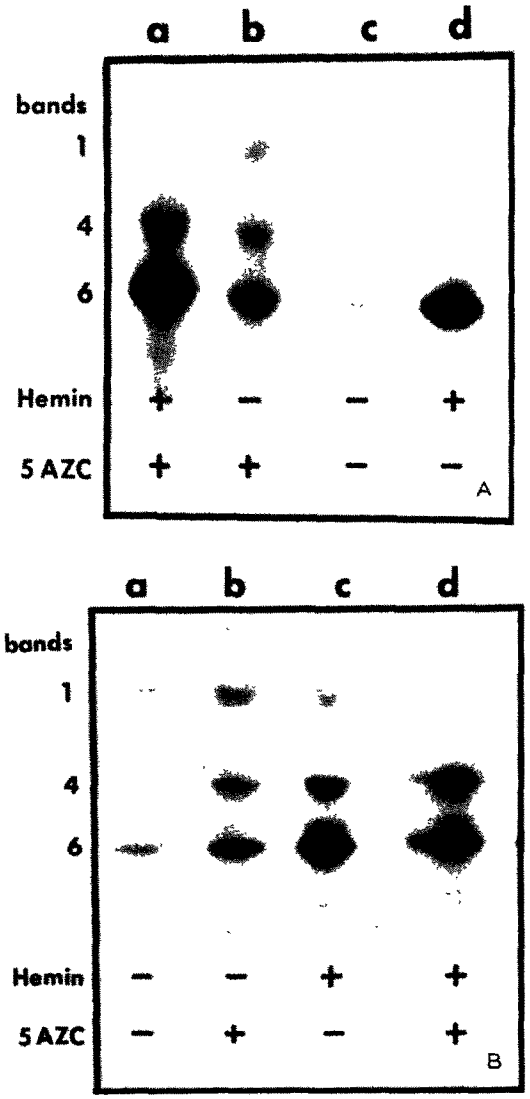

Fig.2. Fluorogram of hemoglobins derived from DMSO-stimulated (A) or BA-stimulated (B) cells. The cells were washed and lysed with a fixed volume of lysing solution and 2 μl were layered on 0.4 mM thin gel. 5-Azacytidine (5-Az, 0.5 μM) was added on days 1 and 2 of culture and hemin (100 μM) was added on day 5 of culture at the time of labeling. Fluorograms were incubated for 3 days at −70°C. In (A) all the samples have the same quantity of hemoglobin while in (B) the 5-azacytidine samples had half the amount of cells of control cells (A,C).

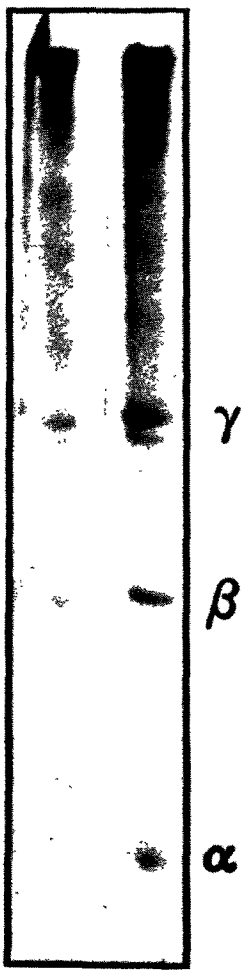

Fig.3. Fluorogram of globins derived from the DMSO-stimulated cells treated with 5-azacytidine (1 μM) at 48 h and hemin 100 μM for 4 h at the time of labeling (96 h). The data show the increase of α-, β- and γ-globins in the 5-azacytidine sample relative to the control. The addition of hemin will increase the synthesis of the α- and β-globins if the messenger is present. The hemoglobin lysate (20 μl) was mixed with 20 μl denaturing buffer and layered onto an acid–urea Triton X-100 gel. Electrophoresis lasted for 3.5 h at 20 MA, after which the gel was fixed, stained, enhanced and incubated at −70°C for 10 days.

The effect of 5-azacytidine on the differentiation of MEL cells can occur at the level of globin transcription or heme synthesis. Previous studies have shown that 5-azacytidine is an inducer of MELC and selectively stimulates embryonic globins in human β-thalassemia and sickle cell anemia patients [7]. The addition of hemin to 5-azacytidine-treated cells showed a greater synthesis of hemoglobins and globins than in control cells. This suggests that 5-azacytidine increases the

Table 1

Experiment	Band 6	Band 4	DMSO + 5-Az / DMSO		DMSO + 5-Az + hemin / DMSO + hemin		DMSO + 5-Az + hemin / DMSO + 5-Az	
	10^3 cells (cpm)	10^3 cells (cpm)	Band 6	Band 4	Band 6	Band 4	Band 6	Band 4
DMSO	7.1	3.4						
+ 5-Az	11.0	7.2	1.54	2.11	0.5	0.86	2.09	1.65
+ hemin	46	14						
+ 5-Az + hemin	23	12						
BA	3.1	6.3						
+ 5-Az	6.6	13.2	2.13	2.09	4.03	1.52	12.86	2.64
+ hemin	21.0	23.0						
+ 5-Az + hemin	84.0	35.0						
DMSO	5.2	1.9						
+ 5-Az	16.0	8.0	3.09	4.27	1.42	2.71	7.52	2.56
+ hemin	28.9	4.8						
+ 5-Az + hemin	41.0	13.0						
BA	3.4	2.1						
+ 5-Az	5.2	4.6	1.53	2.25	1.23	1.29	3.91	1.21
+ hemin	16.6	4.4						
+ 5-Az + hemin	20.5	5.6						

quantity of mRNA available for translation at the time of assay (5 days after differentiation). Thus, the addition of 5-azacytidine and a strong inducer (DMSO or BA) of MEL cells increases the level of hemoglobin synthesis occurring in the cell.

The T3C12 cells appear to be a good model system to investigate the effects on 5-azacytidine-altered gene expression of globin genes [16]. The model system can be easily manipulated, better defined and can facilitate and supplement the in vivo studies of 5-azacytidine on man.

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