

# A Combination of Hydroxyurea and Isobutyramide to Induce Fetal Hemoglobin in Transgenic Mice Is More Hematotoxic Than the Individual Agents

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**Abstract:** Pharmacologic agents such as hydroxyurea (HU), N, 3-4 trihydroxybenzamide (didox), and isobutyramide (ISB) can elevate  $\gamma$ -globin as a potential treatment for the  $\beta$ -hemoglobinopathies. In these experiments, transgenic mice with 5'HS2 from the human  $\beta$ -globin locus control region, the fetal ( $\gamma^A$ ), and adult ( $\beta^S$ ) globin genes were used. Mice were treated with HU, didox, or ISB individually, or with combinations of HU or didox with ISB. The aim was to determine whether these drugs have synergistic effects on the induction of fetal hemoglobin (HbF) and whether the combination regimens are more hematotoxic. In the combination regimens, injections of HU or didox for five weeks were concomitant with ISB treatment every other day for the final three weeks of treatment. The combination of HU+ISB was more hematotoxic than the individual drugs based on significantly increased percentages of reticulocytes and reduced hemoglobin, indicating that caution should be taken in treatments involving combinations of these types of drugs. The didox+ISB combination was not more hematotoxic than the individual drugs. HbF was not induced in the groups treated with the combinations of HU or didox with ISB compared to the individual agents. There was a negligible effect on the percentage of HbF and an unexpected negative effect on the percentage of F cells. The results also have implications for future testing of HbF-inducing drugs in mouse models. In control mice that were phlebotomized but not treated with any drugs, increased percentages of F cells were observed, indicating that blood sampling can cause this effect. In addition, increases in the percentage of F cells did not correlate with increases in the percentage of HbF, indicating that monitoring F cells alone is not a sufficient measure of HbF induction.

**Keywords:** HbF induction,  $\gamma$ -globin, hydroxyurea, isobutyramide, hematotoxicity, transgenic mice

## INTRODUCTION

Administration of certain drugs can increase the level of fetal hemoglobin (HbF), which contains  $\gamma$ -globin, ameliorating the clinical symptoms of individuals with  $\beta$ -hemoglobinopathies. The beneficial effects of  $\gamma$ -globin in sickle cell disease include its higher solubility and oxygen affinity, which reduce the likelihood of polymerization of hemoglobin tetramers containing  $\beta^S$ -globin (1). In  $\beta$ -thalassemia,  $\gamma$ -globin can substitute for  $\beta$ -globin chains without loss of function. The drugs which increase  $\gamma$ -globin levels include S-phase-specific compounds such as hydroxyurea and 5-azacytidine, short-chain fatty acids such as butyrate and its

metabolites, and growth factors such as erythropoietin (2). The mechanisms of action of these drugs are not fully understood. S-phase specific drugs lead to decreased hematopoiesis and create a "stress erythropoiesis" state in which new red blood cells are recruited from a pool of precursors that are more likely to be programmed (possibly as an effect of the drug) to produce  $\gamma$ -globin, although the reason for this is unclear (3). Hydroxyurea (HU) prevents DNA synthesis by inhibiting ribonucleotide reductase through the scavenging of free radicals and by chelating the iron component of the enzyme. A newer and more potent ribonucleotide reductase inhibitor, N,3-4 trihydroxybenzamide (didox), is a more effective

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free radical scavenger than HU and can induce HbF in cells and in animals (4-6). Although the benefit of HU treatment in sickle cell disease is primarily an increase of fetal hemoglobin, the agent also increases the hydration, deformability and survival of red blood cells as well as decreasing adherence to vascular endothelial cells, all of which reduce the pathological effects of  $\beta^s$ -globin (7-9). Butyrate may affect  $\gamma$ -globin gene expression, because it can alter chromatin structure by inhibiting histone deacetylase (10). Studies of the chicken fetal globin promoter have shown that butyrate may act through a specific response element (11). There is also evidence that butyrate induces  $\gamma$ -globin transcription by inhibiting the binding of a negative regulator (12). Although growth factors can enhance the production of red blood cells, this study focuses on ribonucleotide reductase inhibitors and a butyrate analog which are currently being tested clinically.

Thus far, hydroxyurea has been the most effective agent in increasing fetal hemoglobin levels. Several clinical studies have shown that hydroxyurea significantly improved the clinical conditions of sickle cell disease patients in those who responded with increased HbF levels. (13-16) In a multi-center study of 299 individuals with sickle cell anemia, the number of painful crises requiring hospitalization was a median of one per year for the HU group with an average two-fold increase in HbF-containing red blood cells (F cells), compared to the placebo group which experienced 2.4 crises and no substantial variation in F cells (13). It has been postulated that an increase to 15-20%  $\gamma$ -globin synthesis is necessary to ameliorate symptoms related to sickle cell anemia (17), but at least one study has indicated that virtually any measurable increase in HbF would result in a decrease in pain (18). Response to HU appears to be host-dependent since some patients require weeks or months to respond, the degree of response is variable, and some individuals do not respond at all (13-16). Although HU has been used to treat severe sickle cell anemia in pediatric patients (19,20), an obstacle to widespread HU therapy

especially in children is its potential toxicity, since it is teratogenic, clastogenic and cytotoxic/cytostatic (21). Toxicity occurs primarily through myelosuppression, which is readily reversible within 1-2 weeks upon adjustment of the dose of the drug (13,16). The consequences of long-term HU therapy for the treatment of  $\beta$ -hemoglobinopathies is unknown, although it has been used in extended treatment of polycythemia vera with relative safety (22,23). An intriguing possibility is that hydroxyurea in combination with another HbF-inducing drug may cause a synergistic effect due to different mechanisms of action (24). Perhaps the augmentation of HU therapy with another compound can improve the effects of HU in patients considered non-responders and provide a treatment regimen which would allow a lower dose of HU to avoid potential toxicity.

The fatty acid, butyrate, was first detected in the plasma of diabetic mothers whose infants born at term expressed elevated  $\gamma$ -globin levels (25). There is evidence that butyrate, its derivatives and other short-chain fatty acids can induce fetal hemoglobin expression in patients with sickle cell anemia (26-28). In two small clinical studies, arginine butyrate administered by intravenous infusion for as little as two weeks was effective in increasing  $\gamma$ -globin mRNA and F reticulocyte levels by more than two-fold in some patients (29,30). Pulse butyrate therapy has recently proven successful in increasing mean HbF from 7.2% to 21.0% in 9 of 11 patients (31). One disadvantage of the use of butyrate salts (usually sodium or arginine) is that their plasma half-life is short (5-15 mins), which makes continuous intravenous infusion necessary. A butyrate analog, isobutyramide (ISB), was chosen for this study because it has a longer half-life (7.5-10 hours) and anemic baboons treated with the drug displayed dramatic increases in HbF synthesis (32). At doses of 500-600 mg/kg,  $\gamma$ -globin chain synthesis increased five- to nine-fold after as little as three days with no apparent toxicity. There has been very little testing of ISB in humans, however four patients treated with a dose of 150

mg/kg/day for three months exhibited only slight changes in HbF (33). It is not known whether this lack of response is due to the small number of patients, the low dose of ISB, or ineffectiveness of the drug.

Transgenic mice with the human  $\gamma$ -globin gene can be used as a model system to test pharmacologic agents to induce fetal hemoglobin. Constantoulakis et al. (34) used  $\mu$ LCR- $\gamma$  mice to examine the effects of several compounds including HU and butyrate. F reticulocytes increased to a maximum of approximately 2-fold with either of the drugs, and butyrate induced increases in  $\gamma$ -globin chain synthesis to a maximum of about 3-fold. Similar increases in F reticulocytes and  $\gamma$ -globin chains were observed in the same line of transgenic mice when treated with didox (6). In the present study, we have evaluated the effect of novel combinations of HbF-inducing drugs, compared to individual treatments, on fetal hemoglobin expression in transgenic mice. The mice have a construct consisting of DNaseI hypersensitive site 2 (5'HS2) from the human  $\beta$ -globin locus control region (LCR) linked to the fetal and adult  $\beta$ -like globin genes (35). Hemoglobin concentrations and the percentage of reticulocytes were measured to monitor the hematotoxicity of the treatments. Fetal hemoglobin expression was monitored through the use of F cell assays and ELISA, and  $\gamma$ -globin mRNA was quantitated using primer extension assays. The results demonstrate that treatments involving combinations of HbF-inducing drugs may lead to complications, such as increased hematotoxicity and adverse physical effects, and do not necessarily have a synergistic effect on HbF production.

## MATERIALS AND METHODS

### *Transgenic Mouse Line*

The transgenic mouse line used in this study was described previously (5'HS2 $\gamma\beta$ , line 2) (35). The HS2 site is contained in a 1.9-kb KpnI-PvuII

fragment. The  $\gamma$ -globin gene is contained within a 3.3-kb HindIII fragment (-1350 to +1950). A 4.5-kb ApaI-EcoRV fragment contains the  $\beta^s$ -globin gene with the adenine to thymine change in the sixth codon and includes the 3' enhancer (-1250 to +3291). There are three copies of the transgene per haploid genome in this line of FVB/N mice. The use of an inbred strain for the study eliminates differences in the genetic background of the individual mice. To maintain as close to physiological amounts of expression as possible, only hemizygotes are used in these experiments. The human  $\gamma$ -globin gene is expressed at about 2.4% per gene copy in the adult and both the  $\gamma$ - and  $\beta$ -globin genes are developmentally regulated (35).

### *Drug Preparation and Administration*

Hydroxyurea (Sigma) and didox (Molecules for Health, Inc., Richmond, VA) were prepared at a concentration of 40 mg/ml in 0.25X phosphate buffered saline (PBS). Didox was dissolved in 0.25X PBS at 37 C. Isobutyramide (Aldrich Chemicals) was prepared at a concentration of 100 mg/ml in PBS. All drugs were stored in aliquots at -20 C and administered by intraperitoneal injection upon thawing. HU was administered seven days a week for the first week and five days a week for the following four weeks. Didox was injected daily for five weeks. ISB was injected as an individual agent every other day for 19 days. In the combination treatments, ISB was administered every other day from day 15 through day 33. Injections of HU or didox were separated by at least five hours from those of ISB in the combination treatments. A dose of 400 mg/kg of didox in mice is approximately equal to 20% of the dose used in a 36-hour infusion in a human anti-tumor trial (36), when adjustments are made for differences in surface area between mice and humans (37). A dose of 400 mg/kg of HU in mice is approximately equal to the maximum prescribed daily dose (35 mg/kg) in humans for treatment of sickle cell anemia, with the same surface area adjustment (13). The control mice were

injected with approximately the same volume of PBS as the treated animals (0.01 ml/g body weight).

### *Statistical Analysis*

The statistical analyses were performed using SAS (Statistical Analysis System) software. Before performing analyses, the fold change was calculated by dividing all results by the average pretreatment value. This allowed the fold change from day 0 to be determined without regard to differences in the basal values for the parameter of interest. Dunnett's T test was used to compare the overall means of each of the individual treatments to the control mice injected with PBS. Duncan's multiple range test was used to analyze differences in the means of the fold change between all treatment regimens. All findings were judged to be significant at an  $\alpha$ -level of 0.05.

### *Monitoring Hematotoxicity by Hemoglobin Measurement and Reticulocyte Counts*

Approximately 15  $\mu$ l of blood was drawn from the tail vein for hemoglobin concentrations and reticulocyte counts. An aliquot of the blood was added to cyanmet reagent (Baxter) and the absorbance was determined at a wavelength of 540 nm on a spectrophotometer. To determine the hemoglobin concentration (g/dl), the absorbance was multiplied by the dilution factor and an empirically-derived constant (1.14). The percentage of reticulocytes in a count of 1000 red blood cells was determined by manual counting using brightfield microscopy. In these and all of the studies in which results are compared to a pretreatment value, the mice were tested 2-4 times to determine a mean pretreatment value.

### *F Cell Assay*

Red blood cells were fixed as described by Dover and Boyer (38). Fixed red blood cells were permeabilized and stained according to the Pharmingen 1997 catalog (San Diego, CA). Briefly, approximately  $10^6$  fixed cells were permeabilized

with buffer containing saponin followed by sonication. The sample was incubated with either an HbF monoclonal antibody (a gift from Dr. George Dover, Johns Hopkins University) or non-specific mouse ascites fluid (Sigma) followed by incubation with a fluorescein-conjugated secondary antibody (rabbit anti-mouse IgG F(ab<sub>2</sub>), Pierce Chemicals). The percentage of F cells and mean fluorescent intensity of five thousand cells was determined by flow cytometry (Coulter EPICS Elite ESP) followed by analysis with Immuno-4 software.

### *mRNA Preparation and Primer Extension Analysis*

Approximately 25  $\mu$ l of blood from the tail vein was drawn into a capillary tube before beginning the drug treatment of the mice, and then three times a week for six weeks on the days indicated in the figures. Total RNA was obtained as described by Chomczynski et al. (39). The primer extension assays were performed with end-labeled primers specific for the human  $\gamma$ - and  $\beta$ -globin mRNAs (35), using the protocol of Krakowsky et al. (40). The primer extension products were separated on a 5% denaturing urea-polyacrylamide sequencing gel. The products were visualized by autoradiography and the relative concentrations of human  $\gamma$ - and  $\beta$ -globin mRNA were quantitated using a Molecular Dynamics Phosphorimager. The product sizes were as follows: Human  $\gamma$ -globin, 105 bp; human  $\beta$ , 95 bases.

## RESULTS

Adult transgenic mice with the 5'HS2 $\gamma\beta$  construct were treated with HU or didox at a dose of 400 mg/kg for five or seven days a week, respectively, for five weeks. As an individual agent, ISB was administered at 1.25 g/kg every other day for three weeks. In the combination regimens, ISB was administered at a dose of 1.25 g/kg every other day for the last three weeks of treatment in mice treated with HU or didox. Erythropoietic effects of

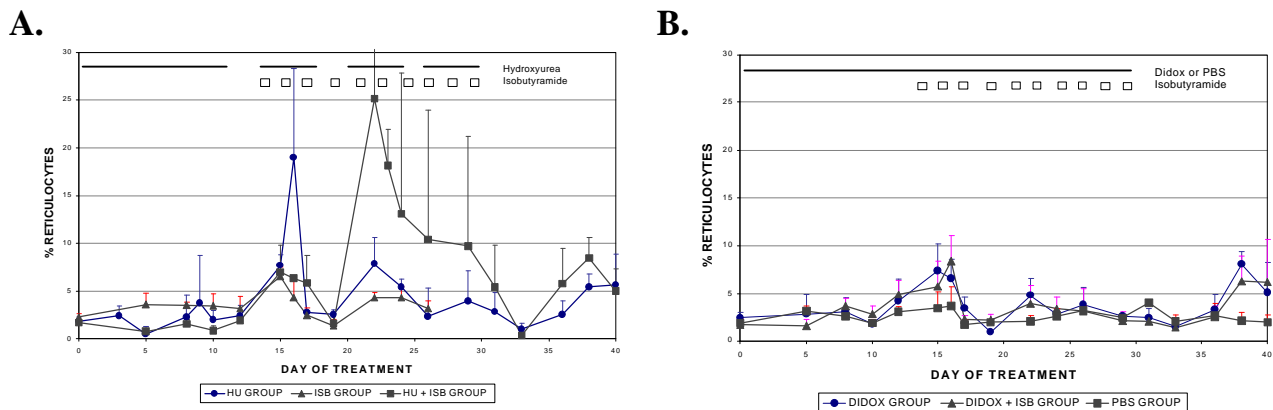
the drugs were monitored through measurements of hemoglobin and of the percentage of reticulocytes. The percentage of HbF-containing red blood cells (F cells) was quantitated by flow cytometry,  $\gamma$ -globin mRNA (as a percentage of human  $\beta$ -globin mRNA) was measured in selected samples using primer extension, and fetal hemoglobin (as a percentage of the total blood protein) was measured by ELISA.

### *The Combination of HU with ISB Is More Hematotoxic Than the Individual Agents*

One aspect of our study was to determine whether increased hematotoxicity occurred during combination therapy with HU or didox and ISB. Generally, increased numbers of reticulocytes are present during periods of decreased hemoglobin, which can be caused by anemia or hemolysis of red blood cells. A cytotoxic effect of the drugs could lead to stimulation of reticulocyte production in order to compensate for the loss of red blood cells. It has been postulated that a certain level of

hematotoxicity is necessary during treatment with HU to elicit the production of HbF, although it is not known whether excessive toxicity can lead to suppression (41). Furthermore, several studies have indicated that butyrates administered at elevated doses can lead to hematotoxicity and decreased HbF (42-44).

In the present study, the combination of HU and ISB induced the most significant increases in reticulocyte counts which varied from a baseline of 1-2% to a peak of 25% (Fig 1A). The percentage of reticulocytes ranged from 1-2% to 8% for mice in the other treatment groups (except one reading of 20% on day 15 for the HU-treated group). The overall mean of 6.3% for all days of treatment in the HU+ISB group was significantly higher than the other treatment groups (HU, 3.8%; didox, 3.7%; ISB, 3.3%; didox+ISB, 3.3%; PBS, 2.5%;  $p < 0.05$ ; Figs 1A-B). The highest percentages of reticulocytes were during the period in which both HU and ISB were administered, from days 15 to 33. Although treatment with HU alone decreased hemoglobin (-31% on average), hemoglobin



**Figure 1.** The percentages of reticulocytes in 5'HS2 $\gamma\beta$  transgenic mice treated with each of the agents and combinations of agents. Drugs were administered as described in the Materials and Methods. The data represents the group averages for the percentage of reticulocytes per 1000 RBCs. The days of treatment are indicated by the horizontal lines (HU, didox or PBS) and by the boxes (ISB) at the top of the graph. Vertical lines represent the standard deviation and are shown in one direction only. (A) Mice treated with HU, ISB, and HU+ISB. The averages and standard deviations for the pretreatment values of the groups are: HU,  $1.8 \pm 0.4\%$ ; ISB,  $2.2 \pm 0.4\%$ ; HU+ISB,  $1.7 \pm 0.2\%$ . The standard deviation for HU+ISB treatment on day 22 is  $\pm 10.2$ . (B) Mice treated with didox, didox+ISB and PBS. The averages and standard deviations of the pretreatment values for the groups are: didox,  $2.4 \pm 0.5\%$ ; didox+ISB,  $1.7 \pm 0.3\%$ ; PBS,  $1.9 \pm 0.5\%$ .

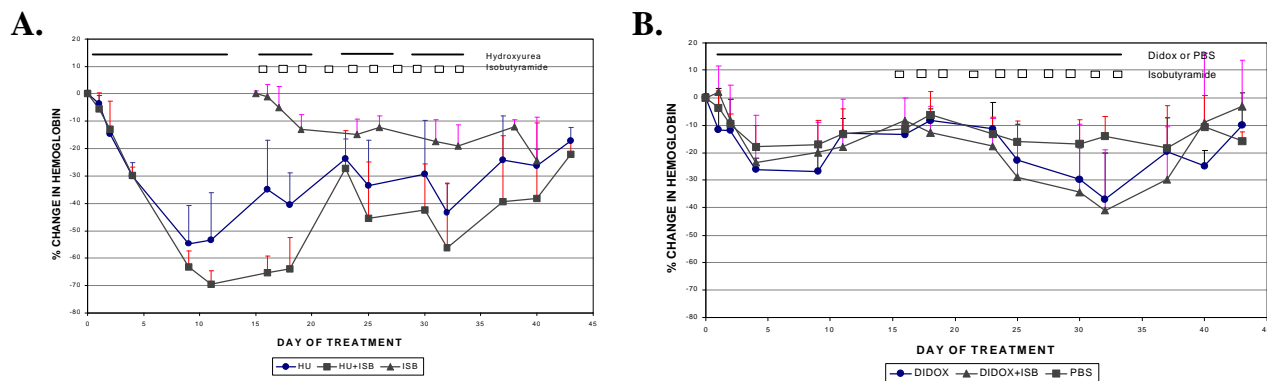
concentrations in HU+ISB-treated mice were significantly lower (-42%) than the other treatment groups (didox, -19%; ISB, -13%; didox+ISB, -18%; PBS, -13%;  $p < 0.05$ ) during the same time period (Fig 2A-B). The increases in reticulocytes and decreases in hemoglobin are more pronounced in the mice treated with HU and ISB, suggesting that this combination of drugs can have an adverse effect on erythropoiesis. The results indicate that although HU has a hematotoxic effect on erythropoiesis, the effect is exacerbated with HU+ISB.

Physical effects were also observed in mice treated with combinations of agents, HU+ISB or didox+ISB, which were not observed when the agents were given individually. Administration of ISB with HU or didox in concurrent injections in a pre-trial led to significant lethargy in the mice which began 3-5 minutes after the injections and lasted up to 30 minutes. The effect ranged from reduced alertness to immobility. This problem was partially eliminated by spacing the injections at least five

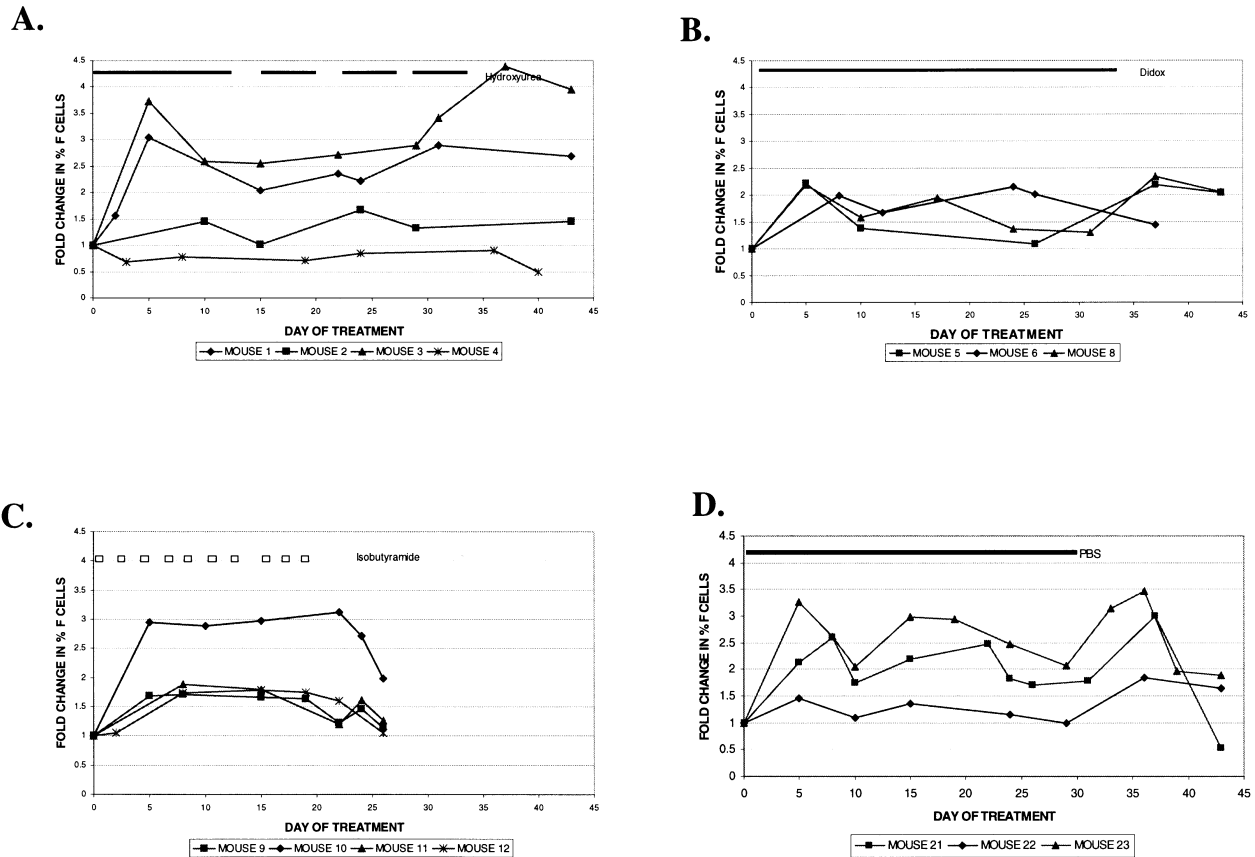
hours apart, although a minor reduction in alertness was still observed. Since only the mice administered the combination treatments demonstrated this finding, the adverse physical effects suggest a toxic effect of the combination of HU or didox with ISB. This data corroborates the hematotoxic effect of the combination of HU and ISB as observed in the increases in reticulocytes and decreases in hemoglobin. The cumulative results suggest that combining cytotoxic agents, such as HU, with butyrate derivatives, such as ISB, is more toxic than either of the agents individually.

### Percentages of F Cells in Mice Treated with Single Agents

In order to determine the effect of treatment with individual drugs on the percentage of F cells and HbF in the mice, both parameters were measured at several points during the study. Substantial increases of 2-3 fold in the percentage of F cells relative to pretreatment values were



**Figure 2.** The percentage change in hemoglobin in 5<sup>+</sup>HS2γβ transgenic mice treated with each of the agents and combinations of agents. The data represent the percentage change in hemoglobin values (g/dl) relative to the average pretreatment value as measured by spectrophotometric analysis (O.D. = 540 nm) using cyanmet reagent. For example, if the average pretreatment value is 19.0 g/dl and the mean hemoglobin reading for a sample day is 17.0 g/dl, then the percentage change is calculated as follows:  $((17.0/19.0)-1)*100 = -10.5\%$ . Vertical lines represent the standard deviation and are in one direction only. The drugs were administered as described in the Materials and Methods. The days of treatment are indicated by the horizontal lines (HU, didox or PBS) and by the boxes (ISB) at the top of the graph. (A) Mice treated with HU, HU+ISB, and ISB. The averages and standard deviations of the pretreatment Hb values (in g/dl) for the groups are: HU,  $19.6 \pm 0.9$ ; HU+ISB,  $20.4 \pm 0.9$ ; ISB,  $19.9 \pm 1.0$ . (B) Mice treated with didox, didox+ISB, and PBS. The averages and standard deviations of the pretreatment Hb values (in g/dl) for the groups are: didox,  $19.1 \pm 1.1$ ; didox+ISB,  $19.3 \pm 0.9$ ; PBS,  $20.3 \pm 1.3$ .

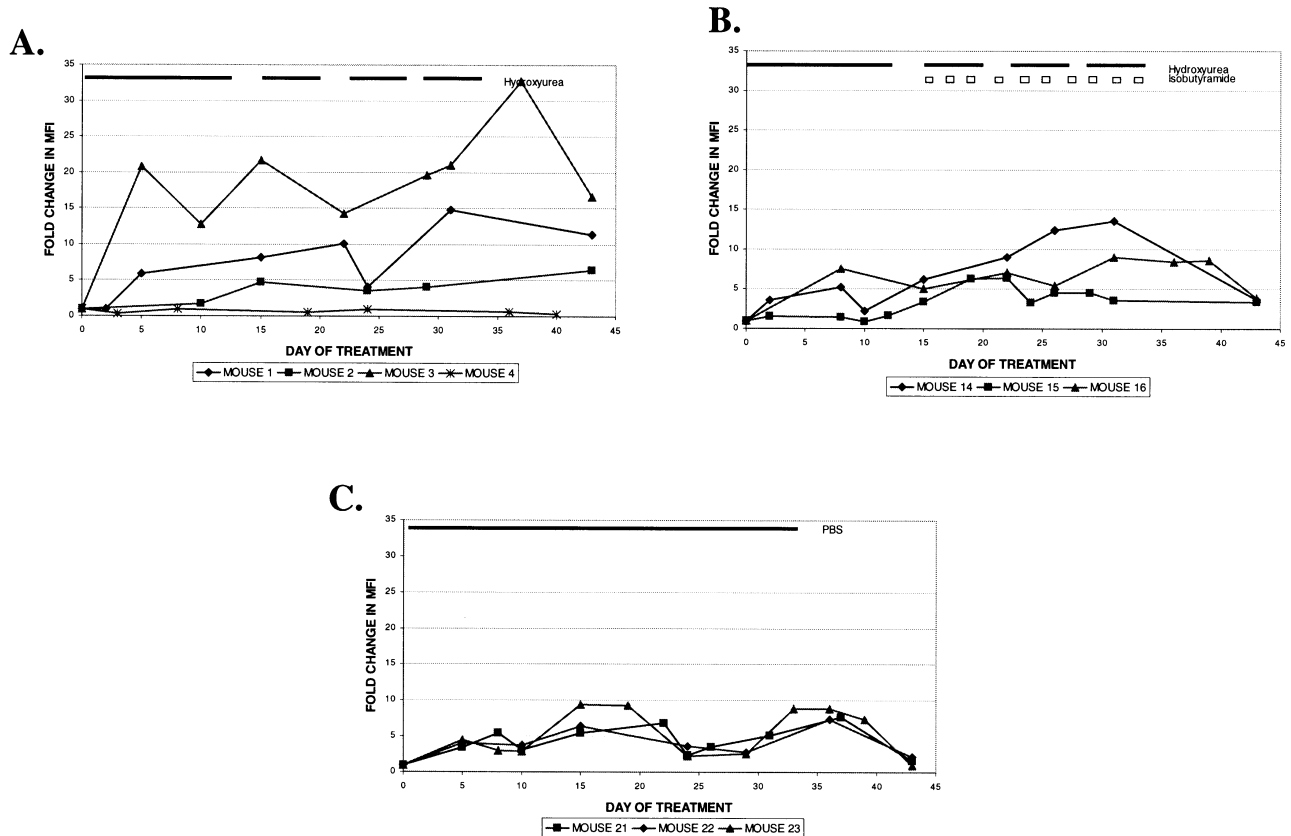


**Figure 3.** Fold change in the percentage of F cells in 5'HS2 $\gamma$  $\beta$  transgenic mice treated with single agents. For Figs. 3A-D, the percentage of F cells was determined as described in the Materials and Methods. The percentage of F cells for each day was divided by the average pretreatment value for each mouse to determine the fold change in F cells, in order to allow comparisons between mice. The days of drug treatment are as indicated by the horizontal lines and the boxes at the top of each graph. The percentages of F cells were determined on the days indicated on the graph. (A) Mice treated with HU. The averages and standard deviations of the pretreatment values for percentage of F cells for these mice are: Mouse 1,  $27.6 \pm 1.7\%$ ; Mouse 2,  $39.7 \pm 20.6\%$ ; Mouse 3,  $21.3\%$  (one value); Mouse 4,  $90.7 \pm 1.3\%$ . (B) Mice treated with didox. The averages and standard deviations of the pretreatment values for these mice are: Mouse 5,  $43.0 \pm 4.5\%$ ; Mouse 6,  $37.0 \pm 3.9\%$ ; Mouse 8,  $41.8 \pm 24.8\%$ . (C) Mice treated with isobutyramide. The averages and standard deviations of the pretreatment values for these mice are: Mouse 9,  $58.1 \pm 0.9\%$ ; Mouse 10,  $28.7\%$  (one value); Mouse 11,  $50.9 \pm 10.5\%$ ; Mouse 12,  $47.9 \pm 12.4\%$ . (D) Mice treated with PBS. The averages and standard deviations of the pretreatment values for these mice are: Mouse 21,  $31.7 \pm 11.1\%$ ; Mouse 22,  $51.7 \pm 0.4\%$ ; Mouse 23,  $28.4 \pm 9.2\%$ .

recorded in most mice administered HU, didox, and ISB by day 5 of treatment (Fig 3A-C, respectively). The mean fold change in F cells over all days tested was significantly higher in the HU group (2.4-fold without Mouse 4,  $p < 0.05$ ) compared to the didox (1.8-fold) and ISB (1.8-fold) groups. Note that the magnitude of the increase in the percentage of F cells which can occur is dependent on the percentage observed on the pretreatment days, that is, a mouse with an initial value of 50% could

experience no more than a 2-fold increase. For this reason, data for Mouse 4 was excluded from the F cell analyses since pretreatment values were approximately 90%. Most of the mice had initial F cell levels of 27-45%; the initial levels for each mouse are indicated in the Fig. 3 legend.

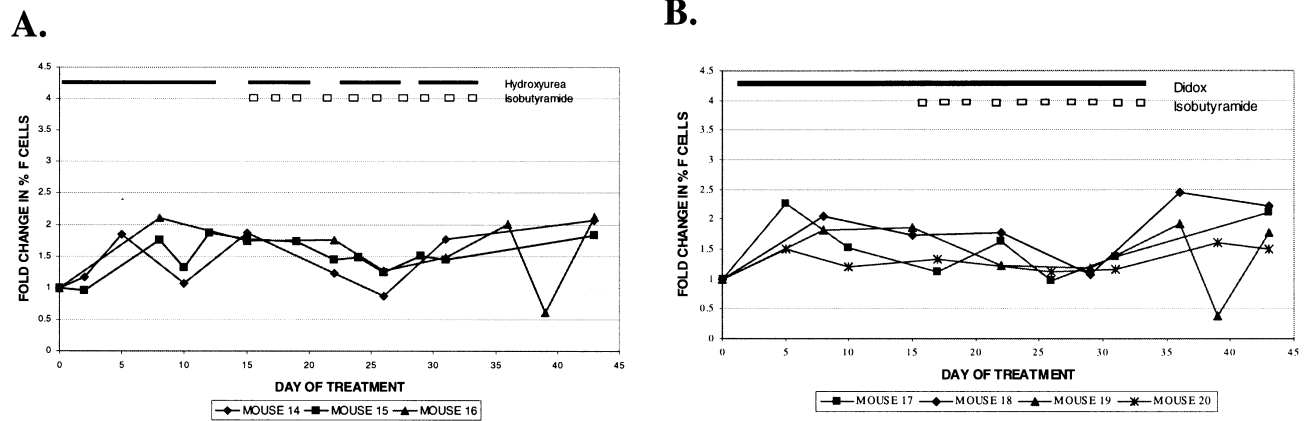
Unexpected increases in the percentage of F cells were also observed in the control group injected with PBS, such that the PBS group was not significantly different from HU-treated mice



**Figure 4.** Fold change in the mean fluorescence intensity (MFI) in individual 5<sup>+</sup>HS2γβ transgenic mice treated with HU, HU+ISB, or PBS. The MFI in F cells was monitored to obtain a relative estimate of the amount of HbF per red blood cell. The MFI was determined as described in the Materials and Methods. The MFI value for each day was divided by the average pretreatment value to determine the fold change in order to allow comparisons between mice. The days of drug treatment are indicated by the horizontal lines and boxes at the tops of the graphs. The days on which the MFI were determined are indicated by points on the graph. (A) Mice treated with HU. The averages and standard deviations of the pretreatment values (in fluorescence intensity units) for these mice are: Mouse 1, 4.3 ± 0.4; Mouse 2, 6.1 ± 1.4; Mouse 3, 2.3 (one value); Mouse 4, 40.6 ± 11.3. (B) Mice treated with both HU and isobutyramide. The averages and standard deviations of the pretreatment values (in fluorescence intensity units) for these mice are: Mouse 14, 3.85 ± 0.2; Mouse 15, 5.8 ± 0.3; Mouse 16, 5.9 ± 0.9. (C) Mice injected with PBS. The averages and standard deviations of the pretreatment values (in fluorescence intensity units) for these mice are: Mouse 21, 6.2 ± 1.5; Mouse 22, 5.0 ± 0.5; Mouse 23, 4.4 ± 2.3.

(2.1-fold, Fig 3D). Increases in F cells in the control group of 1.5-3.3 fold above pretreatment values occurred by day 5 and remained in the range of approximately 1-3 fold until the end of treatment. This result indicates that phlebotomy alone can stimulate F cell production (see Discussion). In support of this, the percentage of F cells decreased upon cessation of phlebotomy (day 40) and returned to near baseline concentrations in 12 out of 14 mice at one month post-treatment (data not shown).

HU induced the most HbF per red blood cell based on the mean fluorescent intensity, which is an indicator of the relative level of HbF per red blood cell (HU, 10.9-fold mean MFI for the treatment period; didox, 3.4-fold; ISB, 2.8-fold; or PBS, 4.7-fold; data shown for HU and PBS, Fig. 4A and C). However, an ELISA assay to determine the percentage of HbF detected no differences between any of the treatment groups (data not shown). The results of the F cell assays, in which the percentages of F cells increased, compared to the results of the



**Figure 5.** Fold change in the percentage of F cells in 5'HS2 $\gamma$  $\beta$  transgenic mice treated with combinations of drugs. Fold change was calculated as described in Fig. 3. The days of drug treatment are indicated by the boxes and lines at the tops of the graphs, and the treatment regimen was as described in the Materials and Methods. (A) Mice treated with HU and isobutyramide. The averages and standard deviations of the pretreatment values for the mice are: Mouse 14, 42.3  $\pm$  5.0%; Mouse 15, 43.6  $\pm$  21.3%; Mouse 16, 45.3  $\pm$  9.1%. (B) Mice treated with didox and isobutyramide. The averages and the standard deviations of the pretreatment values for these mice are: Mouse 17, 40.0  $\pm$  15.5%; Mouse 18, 37.4  $\pm$  24.3%; Mouse 19, 50.4  $\pm$  6.7%; Mouse 20, 57.5  $\pm$  5.2%.

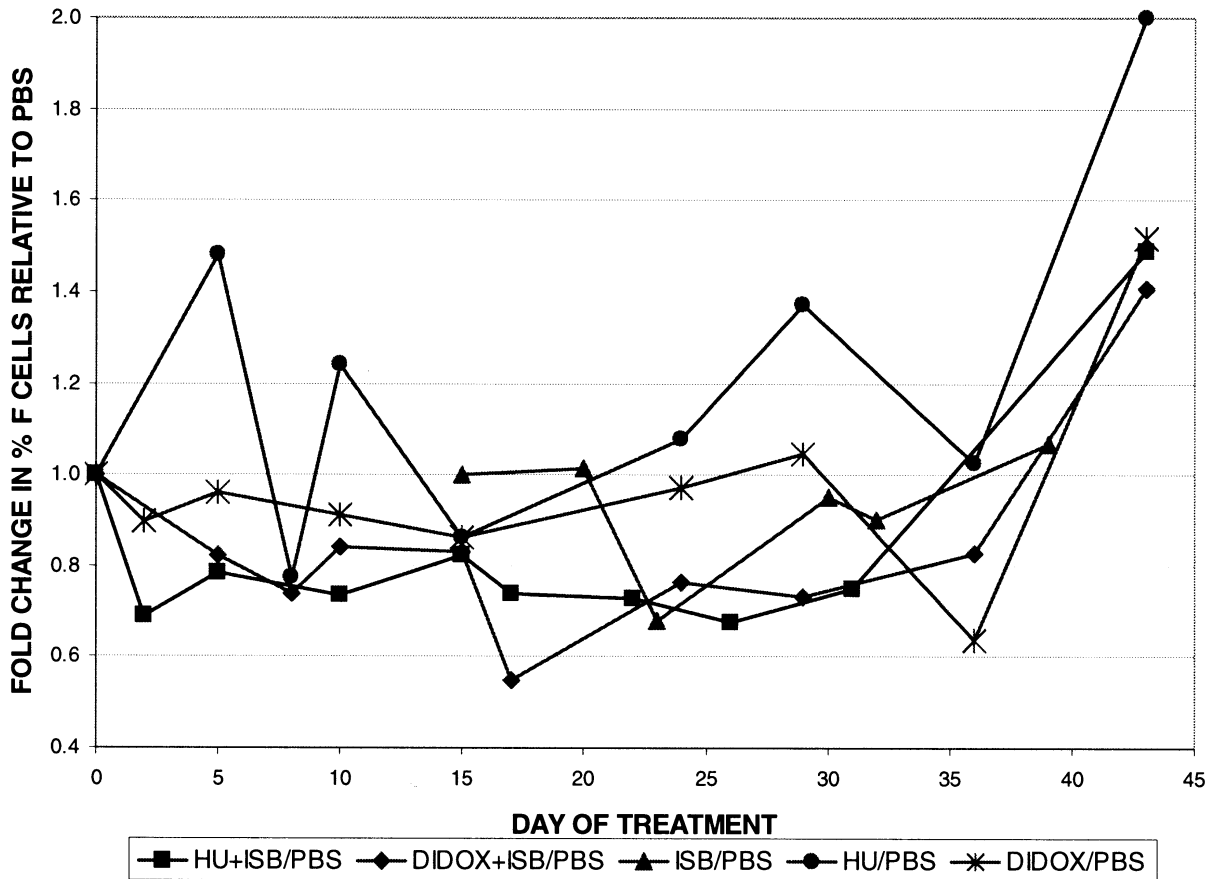
ELISA assay, in which no significant changes were observed, suggests that the increased amount of HbF was below the sensitivity of the ELISA assay but was within the detectable range of the F cell assay. The cumulative results of the F cell and MFI assays indicate that HU treatment induced the largest increase in F cells, with significantly more HbF per cell than the other treatments.

#### *Fetal Hemoglobin in Mice Treated with Combination Therapy*

In order to determine whether ISB treatment of mice previously administered HU or didox improved the HbF response over each agent alone, the percentage of F cells, percentage of HbF and the amount of  $\gamma$ -globin mRNA were measured. As in the treatment with the individual agents, the percentage of F cells increased 1.5-2.2 fold by day 5 of treatment in most HU+ISB and didox+ISB-treated mice (Fig 5A and B). Soon after the start of ISB treatment on day 15 and maximally apparent by day 26, a decrease in the percentage of F cells to almost baseline levels occurred in both groups. Statistical analysis demonstrated that both combination groups (average increases of 1.5-fold

for both HU+ISB and didox+ISB) had significantly lower mean fold increases in the percentage of F cells over all days than the groups treated with HU, didox, ISB, or PBS (2.4-, 1.8-, 1.8-, and 2.1-fold, respectively; Fig 3A-D). The relative amount of HbF per cell as determined by the mean fluorescence intensity (MFI) was significantly reduced in mice treated with HU+ISB (mean increase in MFI over all days tested of 5.1-fold; Fig 4B) compared to HU alone (mean 10.9-fold;  $p < 0.05$ ; Fig 4A). The cumulative results of the percentage of F cells and the MFI assays suggest that the combination of HU or didox with ISB actually had a negative effect on the percentage of F cells (HU+ISB and didox+ISB) and the amount of HbF per cell (HU+ISB).

To correct for F cells induced by phlebotomy, the percentages of F cells in the combination versus individual treatments were also standardized to the percentages of F cells for the PBS control group. As observed in Figure 6, both combination groups exhibited a decrease in the percentage of F cells (as a fraction of F cells in the PBS group for the same day) relative to the individual agents, which was evident during ISB administration, especially on days 15 through 30. During this time period, all of

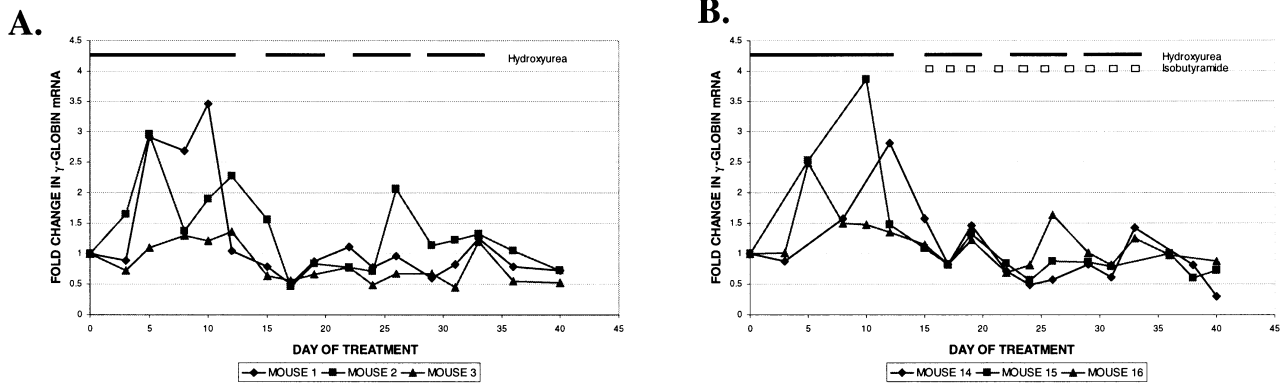


**Figure 6.** Fold change in the percentage of F cells in the treatment groups compared to the PBS group. Considering the PBS group as baseline F cell production, the average fold change in the percentage of F cells of each treatment group for each possible day was compared to the average fold change for the same day in the PBS group. Note that a value less than 1 indicates that the percentage of F cells for the group was lower than that for the PBS control group. The averages and standard deviations of the pretreatment values for each treatment group are as follows: HU,  $29.5 \pm 9.3\%$  (excluding Mouse 4); didox,  $40.6 \pm 3.1\%$ ; ISB,  $46.4 \pm 12.6\%$ ; HU+ISB,  $43.7 \pm 1.5\%$ ; didox+ISB,  $46.3 \pm 9.3\%$ ; PBS,  $37.2 \pm 12.6\%$ .

the HU+ISB and didox+ISB readings are lower than all of those for the single agents (except for one low point on the ISB curve at day 23). This confirms that the percentage of F cells in mice treated with combinations of HU or didox with ISB was lower than for mice treated with the individual drugs.

To determine whether a decrease in  $\gamma$ -globin gene expression occurred at the mRNA level in mice treated with HU+ISB, the amounts of  $\gamma$ -globin mRNA were measured in three mice each from the HU and HU+ISB treated groups. In both groups, increases in  $\gamma$ -globin mRNA to peaks of 3-4 fold

above the pretreatment value were observed between days 5 and 12 (Fig 7A-B). The concentrations of  $\gamma$ -globin mRNA declined by day 15 in both groups with no values above 1.5-fold observed for the duration of the treatments. The pattern of  $\gamma$ -globin mRNA expression in both groups is very similar even after the initiation of ISB treatment, indicating that the combination regimen did not decrease the percentage of  $\gamma$ -globin mRNA in the mice. Therefore, treatment with HU+ISB does not result in a measurable change in the steady-state level of  $\gamma$ -globin mRNA, even though the percentage of F cells decreases.



**Figure 7.** Fold change in  $\gamma$ -globin mRNA in 5'HS2 $\gamma\beta$  transgenic mice treated with HU and HU+ISB. The data in the graphs represent the fold change in the amount of  $\gamma$ -globin mRNA, expressed as a percentage of human  $\beta$ -globin mRNA. The fold changes in  $\gamma$ -globin mRNA were standardized by dividing the value on each day by the average of 3 pretreatment values. The days of drug treatment are indicated by the lines and boxes over the graph. Blood was drawn for preparation of RNA for the primer extension assays on the days indicated as points on the graph. (A) Mice treated with HU. The average and standard deviations for the pretreatment values for these mice are: Mouse 1,  $15.3 \pm 3.3\%$ ; Mouse 2,  $12.4 \pm 1.4\%$ ; Mouse 3,  $13.9 \pm 2.9\%$ . (B) Mice treated with HU+ISB. The averages and standard deviations for the pretreatment values for these mice are: Mouse 14,  $18.7 \pm 4.6\%$ ; Mouse 15,  $16.8 \pm 2.5\%$ ; Mouse 16,  $10.2 \pm 0.3\%$ .

## DISCUSSION

Transgenic mice with the 5'HS2 $\gamma\beta$  construct were treated with hydroxyurea, didox, and isobutyramide individually and with combinations of HU or didox with ISB. The study was designed to determine if combination drug therapy is more hematotoxic, and whether it is more effective in increasing HbF than the individual agents. The data have implications for the future use of transgenic mice to study HbF induction.

### *The Combination of HU+ISB Is More Hematotoxic Than HU Alone and Does Not Induce F Cells*

The combinations of HU or didox with ISB did not induce synergistic increases in HbF compared to mice treated with the single agents. On the contrary, the percentages of F cells were significantly decreased in the combination groups compared to mice treated with a single agent. Although hematologic toxicity may be a necessary prerequisite for the HbF-inducing effect of HU (41), there may be a threshold of toxicity above which a decrease in the HbF-response occurs. The mean

decrease in hemoglobin was greater (HU+ISB, -42%; HU, -31%) and the mean percentage of reticulocytes significantly increased (HU+ISB, 6.3%; HU, 3.8%) in the HU+ISB combination treatments versus HU alone, indicating increased hematological toxicity due to combination treatment. It may be significant that the lack of HbF induction observed in mice treated with didox alone corresponded to low hematotoxicity especially in comparison to treatment with HU. It is possible that there was not sufficient erythropoietic stimulation to induce the production of HbF. In contrast, the high degree of toxicity in the mice treated with HU and ISB actually prevented increases in or had a negative effect on HbF. This is demonstrated by the decrease in the amount of HbF per cell and the decline in the percentage of F cells to almost baseline after initiating ISB treatment in the HU+ISB mice. Several studies have indicated that butyrate or other fatty acids cause decreased HbF expression and increased hematologic suppression at high doses. An anemic baboon infused with arginine butyrate at 1 g/kg/day had a 5-fold increase in the percentage of F reticulocytes but when the dose was increased to 2 g/kg/day, a rapid decline in F reticulocytes to almost baseline levels occurred

with a concurrent decline in reticulocytes (42). Acetate treatment of a baboon elicited a decrease in F reticulocytes from peaks of more than 3-fold to baseline levels upon increasing the dose from 4 to 6 g/kg/day (43). In a comprehensive toxicity study of butyrate, non-anemic baboons experienced hematologic toxicity as defined by declines in reticulocytes and platelets in a dose-dependent fashion at doses ranging from 3-10 g/kg/day (44). In the present study, the dose of ISB was the maximum (1.25 g/kg) tolerated by the mice as determined in a pre-trial in which mice were treated with various dose combinations of HU and ISB. The 1.25 g/kg dose used in this study is equivalent to 300 mg/kg in primates, and is thereby much lower than the suppressive doses discussed above. However, this dose of ISB, in combination with HU, appears to exceed the threshold between HbF induction and suppression.

#### *Lethargy Caused by Combination Treatment*

Administration of ISB with HU or didox in concurrent injections led to significant lethargy in the mice which began 3-5 minutes after the injections and lasted up to 30 minutes. The effect was dependent on the doses of each drug given. In a pre-trial prior to this study, the intensity of the lethargy increased as the dose of HU increased (ranging from 200 to 400 mg/kg) when ISB was injected immediately afterwards at a dose of 2 g/kg (data not shown). HU, didox, and ISB given individually at any of the above doses had no observable side effects. Blau et al. (44) did a comprehensive study using sodium butyrate in baboons to test the toxic effects at varying doses. Obtundation and lethargy were observed in different animals given infusions of 4-8 g/kg/day. The mice in the pre-trial and the present study were administered 1-2 g/kg/day of ISB which would convert to doses of about 0.3 g/kg/day for the baboons (37), which did not cause any observable toxicity in the study by Blau et al. (44). A possible explanation for the negative effects of the combination of drugs in the study mice is the method of drug delivery. ISB (and HU or didox)

was administered intraperitoneally, not by infusion as in the Blau et al. study. It is possible that there was a physical interaction between the two drugs caused by the concentrated method of administration that led to adverse effects in the mice. For example, the presence of HU or didox could cause a more rapid uptake of ISB and cause lethargy at a lower dose of ISB. If this is the case, then the same negative effects would be unlikely to occur in a clinical situation. However, the results of this study indicate that combination therapy involving HU or didox with butyrate derivatives may lead to adverse effects, and that the doses and timing of administration are important factors in the response to drug therapy.

#### *Phlebotomy Stimulates Increases in the Percentage of F Cells*

The results of the F cell assay in control mice injected with PBS indicate that phlebotomy five days a week is sufficient to stimulate increases in the percentage of F cells. Hematopoietic stress has been reported to result in increases in the percentage of F cells in patients with sickle cell anemia (45). Reports in the literature support the theory that phlebotomy stimulates increases in the percentage of F cells. In a study by DeSimone et al. (46), an increase in F cells from 0 to almost 80% was observed in a baboon after two weeks of phlebotomy alone. One baboon treated with acetate had an increase in HbF which could not be distinguished from the effects of phlebotomy alone before treatment (43). Therefore, the increases in F cells observed in the control mice confirm reports in the literature that phlebotomy can stimulate F cells. Our results indicate that a phlebotomy control is essential when monitoring F cells in transgenic mouse experiments.

#### *An Increase in the Percentage of F Cells Does Not Necessarily Reflect an Increase in the Percentage of HbF*

Measurement of the percentage of F cells is not appropriate as the sole basis for evaluating the

pharmacologic induction of HbF in transgenic mice. The results of this study demonstrate that substantial increases in the percentage of F cells do not necessarily correlate with similar increases in the amount of HbF. Similarly, in the study by DeSimone et al. (46), F cells increased from 0 to 80% with repeated phlebotomy, but there was only a small increase in HbF from 0 to 4 pg/cell (which would be undetectable with the ELISA used here). These data are in contrast to a study by Marcus et al. (47) in which there was a positive correlation between the changes in the percentage of F cells and the percentage of HbF in 242 children with sickle cell disease. In our study, the percentage of F cells increased by 2 to 4-fold in almost all mice without similar increases in the amount of HbF as determined by ELISA assay. These results indicate that the number of red blood cells producing HbF increased more than the overall amount of HbF and that individual red blood cells exhibit only minute increases in their quantities of HbF. It is doubtful that the induction of such low concentrations of HbF would be clinically relevant and, therefore, the evaluation of HbF-inducing agents in transgenic mice should not be based on the percentage of F cells alone.

Recently, two mouse models of sickle cell disease were developed (48,49). These mice have the human  $\alpha$ - and  $\beta^s$ -globin genes rather than the corresponding mouse genes. They exhibit many of the same findings as patients with sickle cell anemia, including sickled erythrocytes, anemia and multi-organ pathology. Although these mice have not yet been tested, they may be useful in future investigations of the efficacy of HbF-inducing agents.

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