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Butyrate-induced reactivation of the fetal globin genes: A molecular treatment for the β-hemoglobinopathies

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Abstract. The inherited β-hemoglobinopathies (sickle cell disease and β thalassemia) are the result of a mutation in the adult (β) globin gene. The fetal globin chain, encoded by the γ globin genes, can substitute for the mutated or defective β globin chain, but expression of the γ globin gene is developmentally inactivated prior to birth. Reinducing expression of the normal fetal globin genes is a preferred method of ameliorating sickle cell disease and the β thalassemias. Stimulation of as little as 4–8% fetal globin synthesis in the bone marrow can produce >20% fetal hemoglobin in the peripheral circulation, due to enhanced survival of red blood cells containing both sickle and fetal hemoglobin, compared to those containing sickle hemoglobin alone. Butyric acid and butyrate derivatives are generally safe compounds which induce fetal hemoglobin production by stimulating the promoter of the fetal globin genes. An initial trial with the parent compound, delivered as Arginine Butyrate, has demonstrated rapid stimulation of fetal globin expression to levels that have been shown to ameliorate these conditions. Phase 1 trials of an oral butyrate derivative with a long plasma half-life have just begun. These agents now provide a specific new apporach for ameliorating these classic molecular disorders and merit further investigation in larger patient populations.

Key words. Fetal hemoglobin; sickle cell anemia; β thalassemia; butyrate; gene expression.

The molecular and cellular basis of the β globin disorders

The β-hemoglobinopathies, sickle cell anemia and the β thalassemia syndromes, are among the most common of the genetic diseases, afflicting millions of people worldwide. These disorders were widely selected for because the heterozygous state gave protection against falciparum malaria, particularly in early childhood. The tetrameric hemoglobin molecule consists of two α and two β chains in the adult, enfolding a heme group, to which molecular oxygen binds. The β-thalassemias and hemoglobinopathies are the result of mutations in the β globin gene or a region controlling its expression. In the case of sickle cell anemia, a single base substitution in the globin gene results in the polymerization of hemoglobin upon deoxygenation, with subsequent vaso-occlusion, vascular damage and infarction of internal organs. In the β-thalassemia syndromes, inadequate production of the β globin chain is the result of mutations in the gene or in the gene promotor, resulting in the accumulation of excess α globin chains, which are toxic to the red cell. The ensuing premature destruction of the red cell produces a severe anemia that usually requires transfusions to sustain life and subsequently the risks of transfusional iron overload[9, 26].

The effects of high fetal globin production on the beta globin disorders

While individuals with either sickle cell anemia or β thalassemia may be severely affected and often require frequent hospitalizations, it was noted that infants born with these diseases have no symptoms or signs of the disease until after four months of age. It was determined that fetuses and infants are spared the ravages of these diseases during fetal life and into the early perinatal period, the β^s globin gene is not expressed in large quantities and is substituted for by the γ (fetal) globin chain. Although this fetal γ globin chain functions normally in the hemoglobin tetramer, fetal maturation results in an inevitable suppression of γ globin synthesis with a reciprocal increase in β globin synthesis near the end of gestation. This developmental switch occurs even in the setting of defective β globin genes (sickle cell anemia) or the absence of functional β globin genes (β thalassemia).

Clinical studies carried out in Saudi Arabia in the 1970s by Perrine, Woods and Weatherall suggested that continued expression of fetal globin after birth could ameliorate many signs and symptoms of sickle cell disease[17, 27]. Populations of patients were identified there and in India who were homozygous for the sickle cell mutation. Whereas these patients should have been symptomatic from their disease, especially in their harsh environment, they were found to be in excellent health, to be largely free from sickle crises, and to have normal lifespans. Analysis of globin chain expression in these fortunate patients revealed a peristence of fetal globin expression into adulthood. This genetic abnormality provided sufficient γ globin chains to prevent sickling. Analysis of larger populations revealed that specific levels of fetal

globin production had to be achieved for partial or complete amelioration of sickle cell disease[17]. Circulating levels of 20 to 30% γ globin (expressed as γ globin/total $\gamma + \beta$ globin), or 8% γ globin chain synthesis, and with expression in 70% of the red blood cells were sufficient to protect an individual from serious complications of sickle cell disease[27]. More recently, Platt and Noguchi and colleagues have demonstrated that even small amounts of fetal hemoglobin can have ameliorating effects in sickle cell anemia[14, 24].

Regulation of the globin genes and the mechanisms underlying globin switching are far from understood. Yet, because definitive treatment is available only for the minority of patients with β hemoglobinopathies who have HLA-matched bone marrow donors, attempts have been made over the past decade to pharmacologically activate fetal globin expression in adult patients. Chemotherapeutic agents have been found to modestly increase hemoglobin production[5, 6, 10, 23, 25]. The mechanism by which these agents act is not understood, but it appears likely that some degree of cytotoxic suppression of bone marrow growth is required to produce increases in fetal globin production[6]. Studies using these drugs in adult patients have been underway for a number of years. The ability of these agents to prevent organ damage in sickle cell patients is being investigated in a multi-center trial. In addition, the fact that these drugs are mutagens makes them unattractive as life-long therapies for younger patients with β-hemoglobinopathies. Cytokines which affect erythroid cell progenitor growth have also been utilized to stimulate fetal globin expression, but these therapies have not resulted in consistent and predictable changes in fetal globin production.

A model of persistent fetal globin expression induced by α amino n-butyric acid

In 1985 we conducted a study to determine if the normal developmental fetal globin switch was inhibited in any infant populations. A goal was that study of such a population might lead to the discovery of a natural and safe regulator of fetal globin switching. We found that infants of diabetic mothers did not undergo fetal globin gene switching in utero and were born with a globin expression pattern appropriate for the early fetus of 20–28 weeks' gestation with 85 to 90% fetal globin synthesis, compared to the pattern of less than 50% fetal globin expression in the normal term newborn [18]. These infants of diabetic mothers underwent rapid switching to predominant β (adult) gene expression when they were delivered from the diabetic intrauterine environment, suggesting that an environmental factor present during their gestation was reversibly inhibiting the switch from γ to β globin chain synthesis. An aminated metabolite of ketones, α-amino-n-butyric acid, found in high levels in the plasma of infants of diabetic mothers, was found to be responsible for inhibiting the fetal globin switch in these infants. Despite the inhibition of fetal to adult globin switching, the infants of diabetic mothers in this study were healthy at birth with no signs of other developmental delays. One other developmental gene switch, the alpha fetoprotein to albumin switch, is accelerated in these infants.

Butyric acid, an analogue of α-amino-n-butyric acid, had been shown by others to regulate expression of certain genes, including globin, in experimental systems. Sodium butyrate induces expression of a specific embryonic gene globin gene (ρ) in adult chickens when given in combination with a chemotherapeutic agent[7]. In addition, butyrate had been shown by Partington[16] to regulate the expression of human globin genes after their microinjection into amphibian oocytes. We first tested α-amino-n-butyric acid and sodium butyrate for their ability to induce fetal globin gene expression in cultures of erythroid progenitor cells from patients with sickle cell anemia or thalassemia. In culture, exposure to butyrate resulted in significant increases in γ chain synthesis, compared to control untreated culture[20]. In addition, staining for the presence of fetal hemoglobin in young red blood cells, using a specific monoclonal antibody, revealed significant increases in the accumulation of this protein in nearly all the cells in the culture after butyrate exposure.

Studies of butyrate in animal models

In vivo studies with butyrate and butyrate analogs were then begun, using the ovine fetal globin switching model. In these studies, ovine fetuses were infused with butyrate (or saline, as a control) during the later stages of their gestation. In contrast to the human fetal globin switch, which is still underway at term, the ovine switch is complete by the time of birth (day 140). Infusions of butyrate begun prior to the start of the ovine fetal switch were capable of preventing the switch[19, 21]. Butyrate-treated fetuses were found to express up to 100% fetal globin synthesis at the time of birth. Although butyrate treatment was quite effective at preventing globin switching if the infusion was begun before globin switching began in this model, the rapid metabolism of this short-chain fatty acid made it difficult to achieve plasma levels sufficient to reverse globin switching once it had begun. A number of analogs of butyrate acid were then designed to provide a longer half-life and prevent rapid metabolism. Intrauterine infusion of ovine fetuses with these analogues demonstrated that some of them were not only able to inhibit the fetal globin gene switch prior to its initiation but were also able to reverse the switch once it has occurred. Furthermore, infusion of these analogues could be interrupted for several days, yet high levels of fetal globin expression were still maintained[21].

We and other investigators have subsequently tested butyrate and butyrate analogs in a number of other animal models[3, 19]. Infusion of an adult non-human primate with butyrate resulted in profound and rapid increases in fetal globin gene expression and in production of mature erythrocytes containing fetal globin chains. A five-day infusion of a butyrate analog into mice transgenic for the human non-alpha globin cluster resulted in preferential expression of human γ chain over human β chain. The mechanisms by which butyrate and butyrate analogs influence fetal globin gene expression have been under investigation. These drugs do not appear to be cytotoxic and thus likely work by a different mechanism than do the chemotherapeutic agents in producing fetal globin production. Butyric acid has pleiotropic effects on cells in culture. Butyrate has been shown to stimulate adenyl cyclase, inhibit histone deacetylase, decrease DNA methylation, and force differentiation of certain tumor cell lines in culture. By using butyric acid and the butyric acid analogs, we have demonstrated that none of these previously described activities of the parent compound, butyrate, correlates with the ability of butyrate analogs to affect γ globin gene expression. Results from our laboratory, which have been recently confirmed by others, have demonstrated that butyrate appears to act specifically on the promotor of the fetal globin gene[12, 21]. Chimeric gene constructions, containing relatively short segments of the γ globin gene promotor driving reporter genes, are strongly up-regulated by 30 to 100-fold during transient and stable expression by treatment of the transfected erythroid cells with butyric acid or butyric acid analogs. Similar constructions utilizing other gene promotors, including the beta globin gene promotor, are only modestly affected or unaffected by butyric acid treatment, thus demonstrating the specificity of this drug for the fetal globin gene promotor.

A trial of butyrate in patients with beta globin diseases

Butyrate has previously been administered to children and adults to assess its activity as a cancer cell differentiating agent[4, 13, 15]. No significant adverse side effects were observed in these studies. In light of this information, and after formal toxicity testing with arginine butyrate further demonstrated its safety, a Phase 1/11 trial of intravenous arginine butyrate was begun in patients with β globin disorders. The arginine rather that the sodium salt was utilized to preclude potential problems with hypernatremia and hyperosmolarity during drug delivery, as arginine has superior neutralizing activity. In addition to assessing the safety of infusions of this agent into patients with β hemoglobinopathies, the study design permitted determination of the efficacy of short-term infusions in increasing γ globin gene expression, and assessment of what doses and plasma concentrations were necessary to achieve this effect. The results of this study, in which infusions of arginine butyrate were given intravenously for 14 to 21 days, demonstrated the potential efficacy of this class of compounds in stimulating fetal globin synthesis. Significant increases in fetal globin gene expression occurred in all six patients who were treated with intravenous arginine butyrate[22]. The results of treatment in a patient with sickle cell anemia is shown in figure 1. There was a rapid increase in fetal globin chain synthesis at even the lowest level of drug infused. A dose-dependent increase in fetal globin gene expression was also observed in patients with β thalassemia, as shown in the results of treatment of a 6-year-old child (fig. 2). The increase in fetal globin chains produced correction of the globin chain imbalence enough to create thalassemia trait ratios, which is not a disease state. There was also a striking decrease in the levels of plasma free hemoglobin in these patients, suggesting a significant decrease in

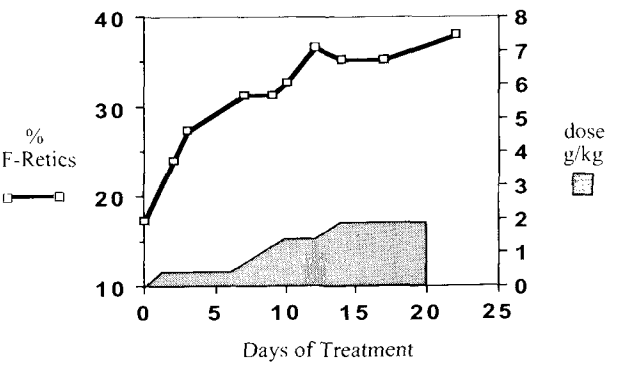

Figure 1. An increase in F-reticulocytes, the percentage of reticulocytes expressing some fetal globin, occurred rapidly in a sickle cell anemia patient treated with arginine butyrate. This effect persisted for more than one month after the drug was stopped.

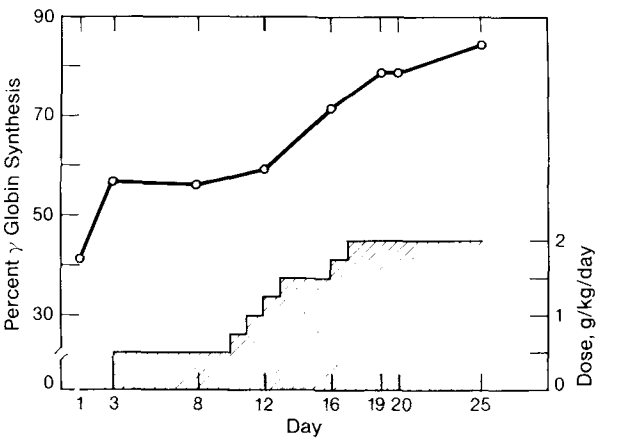

Figure 2. Fetal globin chain synthesis stimulated by arginine butyrate therapy in a 6-year-old boy with Hb E- β thalassemia. A dose-dependent increase in γ globin synthesis occurred with treatment. The increased γ globin synthesis corrected globin chain ratios into the thalassemia trait range. Reprinted with permission from the New England Journal of Medicine *328* (1993) 81–85.

their rate of red blood cell destruction. Infusions of arginine butyrate therefore produced rapid and impressive increases in fetal globin gene expression assayed as F-cells which were followed by dramatic increases in fetal globin chain protein synthesis, even over these short treatment periods.

We subsequently screened butyrate analogs to identify compounds with a longer plasma half-life and oral bioavailability. We have identified a number of butyrate analogs with activity in stimulating fetal globin gene expression in cultured human erythroid cells. These analogs were then tested in the fetal sheep globin switch model and demonstrated activity in maintaining expression of fetal globin. The fetal sheep model also served as an informal but very sensitive test of potential toxicity. One particular analog of butyrate, isobutyramide, has been chosen for further development. The drug has a long plasma half-life (7.5–10 h, fig. 3) and no demonstrable toxicity, even in the fetal subject in whom drug levels of 18 mM were achieved. Treatment of fetal sheep with isobutyramide results in profound inhibition of globin gene switching, with 80 to 90% fetal globin actively synthesized at the time of birth. Oral administration of isobutyramide to adult non-human primate has resulted in prompt elevations of fetal globin chain synthesis. Isobutyramide has been approved by the U.S. Food and Drug Administration for testing in human subjects in a pilot study and a trial of this oral drug is now in process.

In summary, in a short term trial of butyrate, fetal globin synthesis increased from 6 to 43% over base line γ globin synthetic levels in six patients with sickle cell anemia and β thalassemia syndromes. These increases in fetal globin met the previously established criteria required for amelioration of sickle cell disease. In patients with thalassemia, these increases in fetal globin synthesis resulted in α:non-α globin chain ratios which could be expected to decrease hemolysis and render the patients free of the need for chronic blood transfusions. One patient with thalassemia has been treated for over three months with intravenous arginine butyrate given several times per week. This patient demonstrated normalization of α:non-α globin ratios and a rise in hemoglobin from 4.7 to 10 grams/dl (fig. 4). This level of hemoglobin has rendered her independent of the need for packed red blood cell transfusions and the response has been maintained with only 2 to 3 short infusions of butyrate per week. Cessation of therapy in this patient resulted in progressive globin chain imbalance, but still did not return to pre-treatment levels for more than sixty hours after discontinuing therapy.

Therapy with butyrate-based compounds thus represents an exciting new approach to the treatment of the β hemoglobinopathies, sickle cell disease and the β thalassemia syndromes. The development of a drug which reinduces expression of the developmentally-inactivated fetal globin gene by stimulating transcription marks a major advance in the treatment of these serious genetic disorders. Trials in larger patient populations are required to determine long-term tolerance.

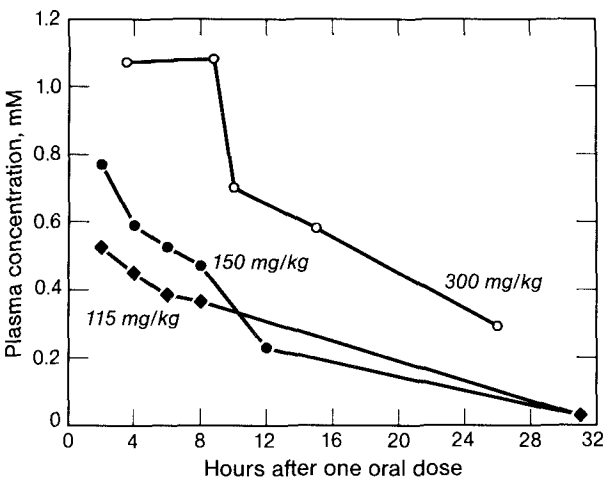

Figure 3. Plasma levels achieved after single oral doses of the butyrate derivative isobutyramide in two normal adults. The required plasma level for stimulating fetal globin production is predicted to be 0.3 mM. This can be maintained with 2 or 3 oral doses per day.

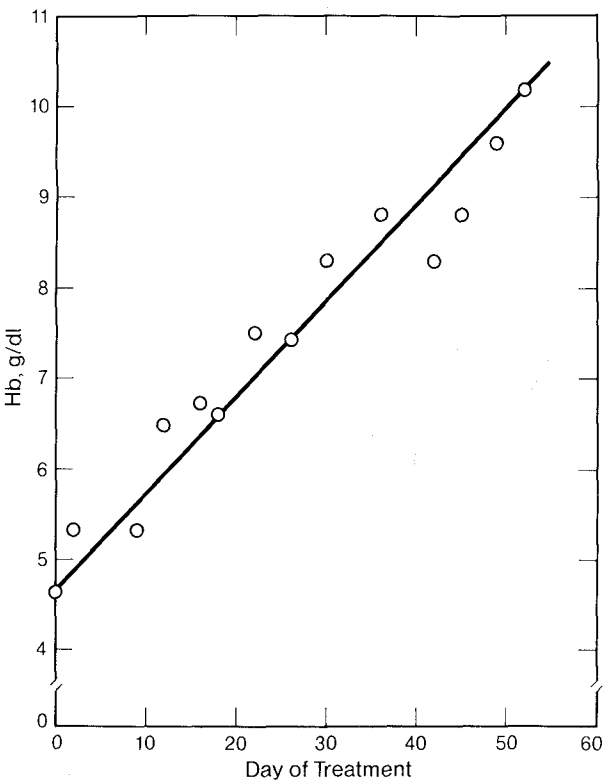

Figure 4. An increase in hemoglobin resulted from improvement in globin chain ratios in a thalassemia patient treated with arginine butyrate as an outpatient. Reprinted with permission from the New England Journal of Medicine *328* (1993) 81–85.

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