RAPID COMMUNICATION

Metabolic Persistence of Fetal Hemoglobin

By Jane A. Little, Nancy J. Dempsey, Mendel Tuchman, and Gordon D. Ginder

Hereditary persistence of fetal hemoglobin (Hb) has been described in patients with β-globin disorders either as a result of point mutations proximate to the γ-globin genes, or concomitant to more distant deletions in the β-globin gene locus. An elevated fetal Hb beyond infancy has been noted to be clinically ameliorating for patients with sickle cell anemia or β-thalassemia, which has led to attempts at pharmacologic upregulation of fetal Hb using hypomethylating and/or S-phase active agents (5-azacytidine and hydroxyurea), and acylating agents (phenylacetate and phenylbutyrate). Many of these compounds, including 5-azacytidine, hydroxyurea, butyrate, and acylating agents were first identified in cell culture or animal models.

The reported activity of both acylating agents and butyrate analogs in vivo and in vitro led us to speculate that an elevation of fetal Hb beyond infancy associated with an absence of amino acid and fatty acid intermediates. In this report we investigate embryonic globin gene upregulation in a murine adult erythroid cell culture was shown by RNase protection after induction with three short-chain fatty acids (C2-C5). Chart reviews and measurement of fetal hemoglobin in five patients with abnormalities in propionate (C3) metabolism were undertaken; SSCP/dideoxy fingerprint analysis of the γ-globin gene promoters was done in three of these five patients. Twelve patients with other metabolic derangements served as controls. Only the four patients with clinically severe abnormalities in propionate metabolism (ages 2 to 11), but without anemia, showed a sustained elevation in fetal hemoglobin (3% to 10%). The level of elevation of fetal hemoglobin in these patients, who lack erythropoietic stress, suggests that propionic acid and/or its metabolites are potent stimulators of fetal hemoglobin expression. Study of this group of patients should allow unique insights into the long-term effects of sustained exposure to elevations of short-chain fatty acid levels.

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MATERIALS AND METHODS

In vitro culture assay. Adult phenotype MEL cells were maintained in RPMI, 10% fetal calf serum, ampicillin, and streptomycin. Cells were induced for 5 days with 2.0% dimethylsulfoxide (DMSO), 1 mmol/L Na butyrate, or various concentrations of propionic acid. RNA was precipitated and quantitated after cell lysis and RNA extraction. RNase protection assays were performed as described previously, using 20 μg of MEL cell RNA hybridized to uniformly 32P-labeled RNA probes. These probes were complementary to embryonic murine β-globin (epsilon') and to a constitutively expressed murine glycolytic enzyme (triose phosphoisomerase, TPI) that served as an internal control.

Patient analyses. Subjects of interest were identified from a roster of patients being followed in the Metabolism Clinic at the University of Minnesota. Permission was obtained from the Institutional Review Board at the University of Minnesota to analyze residual anticoagulated blood samples that remained after clinically indicated lab tests.

Chart reviews were undertaken of all patients whose blood was analyzed as part of this study. Note was made of hematologic parameters at the time of the Hb F assay, and the patient’s clinical condition during these routine clinic visits. Clinical outlines are presented in Table 1.

Hb F assay. Red blood cells (RBC) lysates were prepared from 50 μL of anticoagulated whole blood that had been treated with potassium cyanide. Aliquots were electrophoresed through an am-
Table 1. Clinical Summary

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Age (yrs) (1994)</th>
<th>Diagnoses</th>
<th>Hb F (%)</th>
<th>Date</th>
<th>Complete Blood</th>
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<tbody>
<tr>
<td>A.L.*</td>
<td>3</td>
<td>Propionic acidemia</td>
<td>8</td>
<td>3/93</td>
<td>WBC 7,300/μL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Seizures, controlled</td>
<td></td>
<td></td>
<td>Hb 13.1 g/dL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Developmental delay, mild, stable to improved</td>
<td>4</td>
<td>5/94</td>
<td>WBC 4,900/μL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hb 12.9 g/dL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Plt 265,000/μL</td>
</tr>
<tr>
<td>K.F.*</td>
<td>11</td>
<td>Propionic acidemia</td>
<td>5</td>
<td>2/93</td>
<td>WBC 4,100/μL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Precocious puberty (status post pulsatile GnRH therapy)</td>
<td></td>
<td></td>
<td>Hb 12.6 g/dL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Developmental delay, mild, stable</td>
<td>3</td>
<td>7/93</td>
<td>WBC 5,200/μL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hb 12.2 g/dL</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Plt 166,000/μL</td>
</tr>
<tr>
<td>M.S.*</td>
<td>5</td>
<td>Propionic acidemia</td>
<td>12</td>
<td>1/93</td>
<td>WBC 4,700 (1,100 PMNs)</td>
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<tr>
<td></td>
<td></td>
<td>Seizures, controlled</td>
<td></td>
<td></td>
<td>Hb 9.8 g/dL</td>
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<tr>
<td></td>
<td></td>
<td>Developmental delay, mild, stable</td>
<td>9</td>
<td>3/93</td>
<td>WBC 6,300/μL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Intermittent neutropenia</td>
<td></td>
<td></td>
<td>Hb 11.1 g/dL</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Relapsing metabolic decompensation</td>
<td>8</td>
<td>4/94</td>
<td>WBC 7,000/μL</td>
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<tr>
<td>I.M.</td>
<td>2</td>
<td>Methylmalonic acidemia</td>
<td>6</td>
<td>3/93</td>
<td>Hb 12.3 g/dL</td>
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<tr>
<td>M.D.</td>
<td>4</td>
<td>Methylmalonic acidemia (mild)</td>
<td>&lt;1</td>
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<td>WBC 4,800/μL</td>
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<td></td>
<td></td>
<td>Homocystinuria</td>
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<td>M.G.§</td>
<td>23</td>
<td>Citrullinemia</td>
<td>&lt;1</td>
<td>2/93</td>
<td>Hospitalized at analysis with hyperammonemia</td>
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<td></td>
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<td>Intermittent neutropenia</td>
<td></td>
<td></td>
<td>Hb 11.7 g/dL</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Plt 226,000/μL</td>
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<td>T.C.§</td>
<td>10</td>
<td>Ornithine transcarbamylase (OTC) deficiency</td>
<td>&lt;1</td>
<td>1/93</td>
<td>Hb 14.4 g/dL</td>
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<td>J.C.§</td>
<td>12</td>
<td>OTC deficiency</td>
<td>&lt;1</td>
<td>1/93</td>
<td>WBC 4,200/μL</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Hb 12.6 g/dL</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Plt 192,000/μL</td>
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<tr>
<td>B.B.§</td>
<td>45</td>
<td>Hyperammononemia, hyperornithinemia, homocitrullinuria (&quot;HHH&quot;) syndrome</td>
<td>4/84†</td>
<td></td>
<td>WBC 4,900/μL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hb 13.6 g/dL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Plt 264,000/μL</td>
</tr>
<tr>
<td>S.C.</td>
<td>4.5</td>
<td>Medium chain acyl-CoA dehydrogenase (MCAD) deficiency</td>
<td>&lt;1</td>
<td>1/93</td>
<td>WBC 6,700/μL</td>
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<td>D.R.</td>
<td>7</td>
<td>MCAD</td>
<td>&lt;1</td>
<td>1/93</td>
<td>WBC 5,200/μL</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hb 13.2 g/dL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Plt 533,000/μL</td>
</tr>
<tr>
<td>A.R.</td>
<td>5.5</td>
<td>MCAD</td>
<td>&lt;1</td>
<td>1/93</td>
<td>WBC 7,100/μL</td>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Hb 11.8 g/dL</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Plt 422,000/μL</td>
</tr>
</tbody>
</table>

All samples were obtained, except as noted, during clinic visits while patients were metabolically stable. No patients had signs/symptoms or family history suggestive for a concomitant hemoglobinopathy. All patients were supplemented with multivitamins, minerals, and trace elements.

* Neither SSCP nor sequence analysis indicated known HPFH point mutations.
† Nierhaus-Betke stain: 8% to 10% cells with Hb F.
‡ Nierhaus-Betke stain negative.
§ On acylator agents.

Phosphate-embedded polyacrylamide gel; densitometric Hb quantitations were made after Coomassie Brilliant Blue staining. The percentage of cells containing Hb F was estimated using a modified Nierhaus-Betke stain, as follows: air-dried blood smears from heparinized blood were fixed in 80% alcohol eluted in hematoxylin, ferric chloride, and alcohol at pH 1.5 and then counterstained with eosin. The percentage of bright pink to RBCs (containing fetal Hb) in 1,000 RBCs was obtained by manual count.

DNA analysis. The structural integrity of the immediate 5′ region of the human γ-globin genes was assessed in all three patients.
with propionic acidemia using the single-stranded conformational polymorphism (SSCP) technique on a DNA ladder generated through dideoxy nucleotide sequencing (dideoxy fingerprinting)\(^3\); the wild-type \(\gamma\)-globin gene promoter and two known \(\gamma\)-globin HPFH promoter point mutations served as controls. Using standard techniques, DNA was isolated from peripheral blood mononuclear cells\(^7\) and then amplified via polymerase chain reaction (PCR). The PCR products were cloned en masse into vectors; DNA for analysis was obtained from pools of clones. Control plasmids, analyzed identically, were \(^5\)\(\gamma\) wild-type promoter, \(^4\)\(\gamma\) \(\rightarrow\) A \(-117\) promoter, and \(^5\)\(\gamma\) \(\rightarrow\) T \(-196\) promoter. Human \(\gamma\)-globin primers were designed which spanned \(-268\) to \(-62\) upstream of the start site; thus, amplified sequences contained previously described sites of HPFH point mutations between \(-117\) and \(-202\). The \(5\)' primer at \(-268\) was GAATCGGAACAAGGCAAAGC; the \(3\)' primer at \(-62\) was CTCACTGGAATCTCTAAGAC. The \(5\)' \(\gamma\)-globin primer was used to generate a dideoxy nucleotide-terminated DNA sequence ladder that was run on an acrylamide gel under nondenaturing conditions.

**RESULTS**

*In vitro culture assay.* Expression of the embryonic murine \(\beta\)-globin gene \((\epsilon^g)\) in an adult phenotype murine erythroleukemia cell line was used to screen for agents that could upregulate embryonic globin gene expression in an adult phenotype erythroid cell. Both 1 mmol/L sodium butyrate and 5 mmol/L sodium propionate upregulated embryonic globin gene expression in vitro (Fig 1). Pentanoate was active in this assay system, whereas methylmalonate and acetate, at concentrations in the range of 1 to 20 mmol/L, were not (data not shown).

*Patient analysis.* Fetal Hb was quantitated in 17 blood samples obtained from 12 patients with metabolic abnormalities; only one patient, as noted, was hospitalized at the time of examination, and none were experiencing acute decompensation.

Fetal Hb was elevated in four of five patients with inherited abnormalities in the metabolism of branched-chain and other amino acids (ie, valine, isoleucine, methionine, and threonine); the clinical history of these patients is discussed below and outlined in Table 1. All five children were being managed with protein restriction and L-carnitine, and none had been treated with phenylactate or phenylbutyrate during this investigation. All evaluations were made at routine clinic follow-up, when the patients were metabolically stable. Serum propionate levels were not available in these children; the diagnoses were typically made shortly after birth through clinical and enzymatic analyses. Glycine levels were obtained intermittently, and serve as an indirect measure of disease severity. When drawn, glycine levels throughout these patients' lives were typically two to three times normal and did not change markedly over the course of this study.

K.F., 11 years old, was diagnosed at 3 days of age with propionic acidemia when she presented with severe acidosis and hyperammonemia, hyperglycinemia, and characteristic excess urinary excretion of methylcitrate, propionylglycine, and 3-OH propionate. Fetal Hb has ranged from 3% to 10% (Table 1). Of note, K.F. had been hospitalized with metabolic decompensation 2 weeks before a clinic visit on February 21, 1993 (Hb F 5%) and again at 2 months before a February 9, 1994 visit (Hb F 10%). SSCP analysis of the \(\gamma\)-globin genes shows no evidence for a \(5\)' HPFH point mutation (Fig 2).

M.S. is a 5-year-old child who presented at 2 days of age with neonatal hyperammonemia, hypotonia, and acidosis. Analysis of urinary organic acids suggested propionic acidemia, and enzymatic analysis confirmed propionyl-CoA carboxylase deficiency. Fetal Hb has ranged from 8% to 12%. She has had a clinically difficult course characterized by frequent metabolic decompensations, but was stable at these analyses, except for a pneumonia before the March 1993 visit. She has had intermittent neutropenia, typically associated with metabolic decompensation, and was neutropenic
(1,100 neutrophils/mm³) during the first analysis of fetal Hb, but not subsequently. A Nierhaus-Betke stain from April 20, 1994, when Hb F was 8%, showed that approximately 8% to 10% of peripheral blood cells contained acid-insoluble Hb F (Fig 3B). SSCP analysis of genomic DNA showed a wild-type pattern only (Fig 2).

A.L. is 3 years old. She was diagnosed with propioninemia and hyperammonemia when she presented with neonatal hyperammonemia and acidosis. At 11 months of age she was diagnosed with infantile spasmst for which she receives phenobarbital. She has been characterized as partially biotin-responsive, and is treated with biotin. Fetal Hb levels have been 3% and 8%. γ-Globin gene analysis showed a wild-type pattern on SSCP (Fig 2).

I.M. is a 2-year-old with methylmalonic acidemia that was diagnosed at 3 days of age when he presented with acidosis and hyperammonemia. Molecular analyses showed a cobala min B mutation and he receives vitamin B-12 supplementation; however, his clinical course and biochemical parameters have not been characterized by significant B-12 responsiveness. A single analysis of fetal Hb, at 11 months old, showed a level of 6%. Throughout his life, I.M.'s methylmalonate levels have been elevated 1,000-fold, even when clinically stable.

M.D. is a 5-year-old with a cobalamin C mutation and resultant methylmalonic acidemia and homocystinuria. He was diagnosed at 6 months of age when he presented with developmental delay, failure to thrive, and blindness; nonetheless, his metabolic abnormality has been characterized as being less severe clinically, because of therapeutic vitamin B-12 responsiveness. M.D. has had thrombocytosis throughout life, intermittent neutropenia, and epilepsy. His treatment has included betaine, vitamin B-12, and phenobarbital. A single fetal Hb analysis on February 12, 1993 showed less than 1% Hb F. Methylmalonate levels drawn during his life have shown a sustained 10-fold elevation above normal.

Three patients were being observed for medium-chain acyl-CoA dehydrogenase (MCAD) deficiency, a defect in the β-oxidation of fatty acids manifest as fasting hypoglycemia, hepatomegaly, a Reye-like syndrome, and, during exacerbations, by an elevation in the blood and urinary metabolites of medium-chain fatty acids and acyl-carnitine derivatives. Once diagnosed in infancy or early childhood, patients are managed on a fat-restricted diet, in which fasting is avoided, and by supplementation with L-carnitine.25 Four additional patients were being observed for various defects in the urea cycle, including citrullinemia and ornithine transcarbamylase (OTC) deficiency. These conditions are characterized by recurrent hyperammonemia and protein intolerance. These patients received oral phenylbutyrate, an ammonia scavenger, and L-citrulline or arginine as an adjunct to dietary restrictions.26-38 Phenylbutyrate had been prescribed for more than 1 year, at 250 mg/kg/d or 5.5 g/m²/d in all urea cycle disorder patients. Clinical data are outlined in Table 1. None of these patients has had detectable fetal Hb above 1% as determined by isoelectric focusing. Patient B.B., 45 years old with hyperammonemia/hyperornithinemia/hypercitrullinemia (“HHHH” syndrome) for which she received phenylbutyrate, showed no elevation in Hb F on Nierhaus-Betke stain (Table 1 and Fig 3A).

**DISCUSSION**

We report here a persistence of fetal Hb found in four of five patients with an abnormality in amino acid metabolism that is characterized by increased metabolites of propionate, but without an associated molecular alteration in the β-globin gene cluster itself. To our knowledge, this is the first report of a persistence of fetal Hb beyond infancy that is of a genetic metabolic origin. A modified Nierhaus-Betke stain with a value of approximately 8% to 10% in a 5-year-old patient suggests an heterocellular persistence of fetal Hb. Hb F levels in these children were consistently elevated and varied over time but were consistently higher after metabolic decompensation (ie, K.F. February 1994) or frequent exacerbations (ie, M.S.). Propionate levels were not available retrospectively because these children were managed clinically, so the exact relationship between levels of propionate and fetal Hb is not yet available. Of note, all propionate patients had sustained twofold to threefold elevations of glycine throughout this study, implying ongoing metabolic derangements even when these patients were clinically well. The two children with methylmalonic acidemia are additionally enlightening, as M.D., with a relatively mild, chronic 10-fold elevation in methylmalonate, showed no increased fetal Hb at 5 years of age, whereas I.M., with a sustained 1,000-fold elevation in methylmalonate, had 6% fetal Hb at 2 years of age.

We also evaluated patients with two other types of metabolic abnormalities but with normal fetal Hb levels. Children with MCAD deficiency would be expected to have excessive accumulation of C-6- and C-8-CoA derivatives during exacerbations but not when metabolically stable. At the time of Hb F analysis, these children were in excellent metabolic control and it is possible that results would differ during exacerbations, or at diagnosis. Patients with enzymatic abnormalities in the urea cycle who were treated with nitrogen scavenging agents, such as phenylacetate or phenylbutyrate, that had been associated with an increase in F cells, showed no detectable elevations in fetal Hb in our study. However, those earlier reports that showed an elevation in F cells used a highly sensitive antibody technique, whereas this study used less sensitive but potentially more physiologically relevant methods, ie, isoelectric focusing and Nierhaus-Betke staining. These results highlight the potency of propionate metabolites in stimulating fetal Hb synthesis.

Fetal Hb is 50% to 90% of total Hb at birth and decreases, by mechanisms not yet fully characterized, to less than 1% at 6 to 12 months postpartum, as Hb A predominates.

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**Fig 3.** Nierhaus-Betke stain for acid-insoluble fetal Hb in peripheral blood, at 105 x magnification. Sample (A), from patient B.B., shows less than 1% F cells; sample (B), from patient M.S., shows approximately 8% to 10% F cells in an heterocellular pattern.
Patients with HPFH mutations have likewise been clinically illuminating, as the observation that elevated Hb F in patients with concomitant $\beta$-thalassemia or sickle cell disease ameliorates the typical clinical sequelae of these diseases has led to ongoing pharmacologic trials in an attempt to upregulate fetal Hb.$^{9,17}$ Perrine et al$^{28}$ described a persistence of fetal Hb in infants of diabetic mothers which was felt to be metabolic in origin and was subsequently ascribed to elevated levels of butyric acid derivatives.$^{32}$ More recently, Dover et al$^{18}$ reported that phenylacetate and phenylbutyrate upregulated F cells in patients with urea cycle disorders, for whom these agents serve as ammonia scavengers. Neither phenylacetate nor phenylbutyrate is metabolized to butyrate or its analogs,$^{43,45}$ however, both butyrate and phenylacetate are metabolized in the mitochondria via thiol-esters with acetyl-CoA, to acetate/acyetyl-CoA and phenylacetylglutamine, respectively. This common metabolic pathway for phenylacetate and butyrate led us to investigate the possibility that propionate and other short-chain fatty acids could upregulate embryonic/fetal globin genes in vitro. Propionate, although derived predominantly from branched-chain and other amino acids rather than odd-chain fatty acids, is also metabolized through an acetyl-CoA ester, propionyl-CoA, to succinyl-CoA, and the tricarboxylic acid cycle. Our studies showed that 5 mmol/L propionate was capable of upregulating murine embryonic $\beta$-globin gene expression in cell culture (Fig 1), as were butyrate and pentanoate, (data not shown) at, respectively, 1 mmol/L and 5 mmol/L concentrations. As had previously been shown for the butyrate-induced upregulation of a transfected avian embryonic $\beta$-type globin in MEL cells,$^{46}$ this effect was not caused by terminal erythroid differentiation induced by such compounds,$^{47}$ because DMSO, which also induces differentiation of the MEL cells to an adult erythroid phenotype, did not upregulate $\epsilon$ expression.

The mechanism by which propionate, butyrate, or phenylacetate and phenylbutyrate cause elevated expression of fetal and embryonic globin genes is not known. We and others have shown cis-acting sites for butyrate responsiveness in globin genes, but the specific mechanisms involved have not yet been elucidated.$^{48}$ It is possible that butyrate and propionate affect the level or modification of one or more sequence-specific trans-acting factors, perhaps by altering acetyl-CoA flux in the mitochondria.

It is expected that ongoing detailed studies of these children with metabolically mediated upregulation of fetal Hb will offer insight into both underlying pathophysiologic mechanisms and potential long-term clinical benefits and sequelae of exposure to short-chain fatty acids, such as butyrate, which are currently under study. In addition, these findings strongly support the notion that ongoing therapeutic trials of SCFAs hold considerable promise, as the children in this study had sustained Hb F elevations of 3- to 10-fold over controls without obvious hematologic stress; it seems likely that similar agents, applied therapeutically, would be much more active in anemic patients with ongoing stress erythropoiesis. This possibility is supported by the observation that none of the urea cycle disorder patients who received high doses of phenylbutyrate had detectable elevations of fetal Hb in our studies, whereas treatment with similar doses has been associated with elevated Hb F in patients with stress erythropoiesis caused by $\beta$-globin gene disorders.$^{48}$

None of the children with propionic acidemia or methylmalonic acidemia have had obvious progressive neurologic deterioration beyond those changes that likely arise from a difficult perinatal course and subsequent intermittent metabolic decompensation and protein restriction. Nonetheless, despite reports in the literature of adolescent children with propionic acidemia who, at diagnosis or with careful management, were of normal intelligence and without neurologic sequelae, this is not typical.$^{49-53}$ The presence of seizures and developmental delay in our patients, coupled with reports by Blau et al$^{54}$ regarding neurologic abnormalities in baboons infused with massive doses of sodium butyrate, do suggest the possibility that very high serum levels of short-chain fatty acid compounds and their metabolites could result in neurologic toxicity.

A number of other clinical sequelae have been associated with propionic acidemia, including cardiomyopathy, developmental delay, transient neutropenia, etc.$^{55,56}$ but similar syndromes have not developed in patients with $\beta$-globin gene disorders who are participating in short-term trials to upregulate fetal Hb by treatment with short-chain fatty acids. Nonetheless, future trials need to be structured so as to carefully define the therapeutic index of these compounds.

In summary, this report describes a metabolic persistence of fetal Hb in patients with inherited derangements in amino acid metabolism, as predicted in a tissue culture model. The uniformity of this effect at the level of total fetal Hb in nonanemic children suggests that propionate may be a potent agent for pharmacologic stimulation of fetal Hb in patients with $\beta$-globin gene disorders. Further study of individuals with elevated levels of short-chain fatty acid metabolites should afford insights into new pharmacologic therapies for $\beta$-globin disorders that may be ameliorated by upregulation of fetal Hb, and offer a unique opportunity to examine potential long-term sequelae from therapy with such compounds.

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