Fetal Hemoglobin Induction With Butyric Acid: Efficacy and Toxicity

By C. Anthony Blau, Pantelis Constantoulakis, C.M. Shaw, and G. Stamatoyannopoulos

Butyric acid induces fetal hemoglobin (HbF), a property of potential therapeutic advantage in patients with disorders of globin chain synthesis. We performed dose escalation studies of this compound in baboons to assess whether clinically significant increases in HbF are achievable, and to define the associated toxicities. Additionally, the effect of butyrate in combination with erythropoietin on HbF induction was assessed. HbF induction in response to butyrate was dependent on the dose and duration of treatment. Doses of butyrate less than 4 g/kg/d were associated with minimal toxicity (hypokalemia) and significant HbF induction in these nonanemic animals, with 1 g/kg/d producing an increase in HbF-containing reticulocytes (F reticulocytes) from 0.9% to 8.7% and an increase in HbF from 0.8% to 1.4%. A dose of 2 g/kg/d resulted in an increase in F reticulocytes from 2.1% to 27.8% and an increase in HbF from 0.7% to 2.2%. Doses of 4 g/kg/d in another animal produced an increase in F reticulocytes from 1% to 21.6% and in HbF from 1.9% to 5.3%. Infusions in excess of 4 g/kg/d were complicated (after a variable amount of time) by a decreased level of alertness (caused by hyperosmolality or butyrate itself) and hematologic toxicity with declines in reticulocyte, white blood cell, and platelet counts. Prolonged infusions of high doses of butyrate (8 to 10 g/kg/d) were associated with peak F reticulocyte percentages reaching 38% to 64.5% and HbF reaching levels in excess of 20%. These high doses (8 to 10 g/kg/d) were complicated in two animals with a striking and unique neuropathologic picture and, in one animal, multiorgan system failure. Erythropoietin in combination with butyrate, induced F reticulocytosis in an additive manner. We conclude that butyric acid is a strong inducer of HbF, particularly when administered in combination with erythropoietin. As chronic toxicities remain undefined, patients in future clinical trials of this and similar compounds should be monitored closely for evidence of neurologic toxicity.

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RESULTS

Table 1 outlines the doses and durations of seven courses of butyrate administration in five different animals. Doses of

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butyrate ranged between 1 and 10 g/kg/d. Sodium butyrate (NaB) was used in treatment courses 1 through 6, and arginine butyrate was used in course 7.

**HbF Induction**

NaB at a dose of 1 g/kg/d produced an increase in F reticulocytes from 0.9% to 8.7% (Fig 1). An increase in dose to 2 g/kg/d resulted in a further increase to 25% F reticulocytes. F cells increased from 4% to 6.7% with the 1 g/kg/d dose, and reached 15.2% with 2 g/kg/d. HbF increased from a baseline of 0.8% to 1.4% with 1 g/kg/d of butyrate, and increased further to a maximum of 2.2% with 2 g/kg/d. The response observed in animal B (Table 1) to 2 g/kg/d was less pronounced, with an increase in F reticulocytes from 0.8% to 6.2% and no discernible increase in HbF. The responses to higher doses of butyrate were studied in treatment courses 1 to 6, and except in experiment 4, all dose increases were separated by at least 2 days.

Abbreviation: NA, not applicable because of the short duration of treatment.

* On day 8 of treatment course 2, butyrate was interrupted for 10 days due to catheter malfunction.

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**Summary of Experiments**

<table>
<thead>
<tr>
<th>Animal</th>
<th>Treatment Course</th>
<th>Butyrate Dose (g/kg/d)</th>
<th>Duration (d)</th>
<th>Maximal % F Reticulocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>1</td>
<td>35</td>
<td>8.7</td>
</tr>
<tr>
<td>B</td>
<td>2</td>
<td>2</td>
<td>25</td>
<td>27.8</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>8, 18*</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>4</td>
<td>4</td>
<td>27</td>
<td>21.6</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>16</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>16</td>
<td>6.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>22</td>
<td>9.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>29</td>
<td>64.5</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>6</td>
<td>8</td>
<td>3</td>
<td>NA</td>
</tr>
<tr>
<td>E</td>
<td>7</td>
<td>10</td>
<td>1.5</td>
<td>NA</td>
</tr>
</tbody>
</table>

Summary of doses and durations of butyrate infusions, and peak F reticulocyte responses. Sodium butyrate was used in treatment courses 1 to 6, and arginine butyrate was used in course 7.

**Table 1. Summary of Experiments**

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**Toxicities**

In five of the seven courses, infusions were discontinued because of the development of obtundation (Table 2). Three animals died (C, D, and E), one during parenteral administration of potassium for hypokalemia with a presumed arrhythmia (animal C, treatment course 5), a second (animal E) following the development of multiorgan system failure within 36 hours after starting the highest administered dose of butyrate (10 g/kg/d), and the third (animal D) in a control experiment in which hypertonic solutions (without butyrate) were used to produce hyperosmolality.

**Electrolytes.** Hyperosmolality (calculated or measured serum osmolality >300 mOsm/L) was noted during six courses of butyrate administration (Table 2, courses 2 through 7). In courses 2 through 6 (in which NaB was used), hypernatremia was the predominant contributor to hyperosmolality. In course 7, in which arginine butyrate was used instead of NaB, the serum sodium concentration was markedly low (114 mEq/L), and the major contributors to hyperosmolality were unmeasured compounds (presumably arginine, butyrate, or metabolites of these compounds), with a difference between measured and calculated osmolality (osmolar gap) of 68 mOsm/L (normal for humans is <10 mOsm/L).

Hypokalemia occurred during five courses of butyrate administration (Table 2, courses 2 through 6). Its severity appeared to correlate with dose and was, at times, accompanied by an elevated serum bicarbonate concentration, presumably a metabolic product of butyrate. Moderate hypocalcemia and hypomagnesemia occurred only in the animal (Table 2, animal E) receiving the highest dose of butyrate.

**Hematologic toxicity.** Reticulocytes and platelets decreased in a dose-dependent fashion as shown in Fig 3. Leukocyte counts closely mirrored these changes (data not shown). Whereas no decrease in reticulocytes occurred at
Fig 1. HbF induction in response to escalating doses of NaB. Data from animal A are shown on the left, and animal C on the right. Doses of NaB are shown in shaded rectangles above.
Fig 2. HbF induction in animal A in response to butyrate alone (left, transposed from Fig 1), Epo alone, and Epo in combination with butyrate (right). Epo (1,000 U/kg) was administered intravenously thrice weekly. Individual doses of Epo are designated by vertical arrows. Doses of NaB are shown in shaded rectangles. These data suggest that Epo and NaB augment HbF additively.
doses of butyrate up to 2 g/kg/d, declines were observed at doses of 3 to 4 g/kg/d. Reticulocytopenia persisted despite significant decreases in the hematocrit in courses 5 and 7 (Fig 2 and data not shown). The mean corpuscular volumes (MCV) did not significantly change. Platelet counts decreased slightly at 2 g/kg/d, and decreased more significantly at higher doses. A profound decrease in hematocrit occurred in concert with multiorgan system failure in animal E (Table 1), in which, over 2 days, the hematocrit decreased from 40% to 23%. Evidence of hemolysis included an abrupt elevation in serum lactate dehydrogenase (to 7,780 U/L), undetectable haptoglobin, and a serum-free hemoglobin of 6 mg/dL. More gradually progressive anemia developed during prolonged butyrate infusions at doses of 8 g/kg/d (Fig 1 and data not shown), and occurred in the absence of apparent blood loss or abrupt hemolysis.

Also in animal E, which received arginine butyrate at 10 g/kg/d, a fulminant coagulopathy developed within 33 hours of starting the infusion, with a PT of 3.13 seconds (2.6 times mean), activated PTT of 71 seconds (2.54 times mean), fibrinogen levels, and platelet levels were normal in this animal.

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Neurologic toxicity. A diminished level of consciousness complicated butyrate administration in five of the seven treatment courses (Table 1). The dose of butyrate at which this occurred varied between animals, with animal B developing obtundation within 3 days after beginning a butyrate infusion at 4 g/kg/d, and animal C not showing apparent changes in alertness until 2.5 and 4 weeks of treatment at 8 g/kg/d (courses 4 and 5, respectively, Table 2). We are unable to distinguish whether this complication is attributable to butyrate itself, its metabolites, or other factors such as hyperosmolality. With most treatments, these changes were rapidly reversible with discontinuation of the infusion, the only exceptions being animals C and E.

Animal C received two courses of NaB (Table 2 and Fig 2). In the first course, NaB was administered at a dose of 4 g/kg/d for nearly 4 weeks followed uninterruptedly by an increase in dose to 8 g/kg/d. Three days before the discontinuation of treatment, ammonium butyrate was substituted for NaB. The infusion was discontinued when the animal was noted to rapidly become lethargic. The calculated serum osmolality was 329 mOsm/L (increased from a baseline value of 319 mOsm/L), and serum ammonia level and BUN were normal. The animal’s level of consciousness improved noticeably within 12 hours after discontinuation of the drug, despite an increase in the calculated serum osmolality to 332 mOsm/L. However, the animal was found to have a striking cerebellar ataxia that appeared to gradually and completely resolve over a period of 8 weeks. This animal was subsequently retreated with escalating doses of sodium butyrate (Table 2), before again developing obtundation after nearly 10 weeks of treatment, with 4 weeks of treatment at a dose of 8 g/kg/d. There was no evidence of ataxia, and the animal died abruptly 12 hours after discontinuation of the infusion of a probable arrhythmia.

Animal E received 10 g/kg/d of arginine butyrate for 8 hours before the infusion was interrupted because of technical error. The following day butyrate was resumed, but was again discontinued after 10 hours (33 hours after it was first started) because of the development of obtundation. The animal developed multiorgan system failure (see below) and was killed 30 hours later.

Other toxicities. Animal E developed elevated transaminases, acute renal failure, hyperglycemia, hypoalbuminemia, and an elevated amylase before death. In other animals, only modest (<twofold) elevations in transaminases were observed. Diarrhea was noted in animals C and E just before interruption of the infusion.

Table 2. Treatment Toxicity

<table>
<thead>
<tr>
<th>Animal</th>
<th>Treatment Course</th>
<th>Butyrate Dose (g/kg/d)</th>
<th>Toxicities</th>
<th>Peak Osmolality (mOsm/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>None detected</td>
<td></td>
<td>Not done</td>
</tr>
<tr>
<td>B</td>
<td>2</td>
<td>None detected</td>
<td></td>
<td>Not done</td>
</tr>
<tr>
<td>C</td>
<td>4</td>
<td>Hypokalemia*</td>
<td>319†</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>Obtundation</td>
<td>315†</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>6</td>
<td>Hypokalemia, obtundation</td>
<td>380</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>7</td>
<td>Hypokalemia, obtundation</td>
<td>351</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Multiple</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Summary of toxicities and peak osmolalities with each dose or butyrate.

* Calculated serum osmolality. Other values are measured.
† Hypokalemia at lower doses persisted at higher doses in the same animal. Hematologic toxicity includes coagulopathy, anemia, leukopenia, or thrombocytopenia.
Fig 3. Changes in percent reticulocytes, hematocrit, and platelets in response to increasing doses of NaB. Results from animal A are shown on the left, and animal C on the right. Doses of NaB are shown in shaded rectangles above.
Pathology. Neuropathologic examination in animal C, which had developed cerebellar ataxia 5 months before death, showed cystic necrosis within the dentate nuclei bilaterally (Fig 4A). On microscopic examination, approximately one-third of the lesion was cystic, and the remaining two-thirds contained macrophages encircling blood vessels. The lesion was surrounded by reactive astrocytes. In addition, sections of medulla showed a poorly defined area of demyelination in the median floor of the fourth ventricle, with preservation of the axons.

In animal E (which died within 3 days after beginning arginine butyrate), horizontal sections of the brainstem and cerebellum showed gray brown discoloration in the floor of the fourth ventricle. Microscopic examination showed symmetrical demyelinating lesions of this region. The lesions were well defined and localized to the dorsolateral aspect of the vagus nucleus, immediately ventral to the area postrema. Additionally, a minute unilateral area of spongy degeneration in the dentate nucleus was seen, as well as a relatively well-defined area of demyelination in the globus pallidus. No inflammatory reaction or phagocytic activity was observed in these lesions.

There were no neuropathologic changes noted elsewhere in either animal. Specifically, there was no evidence of intracranial hemorrhage, and the region of the central pons was normal. Pathologic evaluation was otherwise notable only for marked cachexia in animal C.

Other studies. To discern whether the neuropathologic changes observed with butyrate administration were more likely due to hyperosmolality alone rather than butyrate toxicity, we infused hypertonic solutions (without butyrate) into animal D. This animal developed obtundation after receiving 8 g/kg/d of sodium butyrate for a total of 3 days, and returned to his baseline neurologic status within 48 hours following discontinuation of the infusion (Table 2). In this experiment, the serum sodium concentration had reached 178 mEq/L, with a serum osmolality of 360 mOsm/L. Nine months later, an equivalent osmotic load (adjusted for weight) was administered to the same animal via continuous intravenous infusion using sodium chloride alone. This solution had a concentration of 3,044 mOsm/L, approximately 10-fold higher than isotonic saline. The animal tolerated this infusion, lasting 13 days, and a slightly more concentrated infusion (3,352 mOsm/L), lasting an additional 12 days, without difficulty. There was no significant change in serum osmolality, due to a marked increase in water consumption and urine output. A combination of glucose and sodium chloride was then administered at a concentration of 6,160 mOsm/L (20-fold more concentrated than isotonic saline). Within 18 hours, the animal developed obtundation, intractable seizures, and death. Serum osmolality before death was 511 mOsm/L, with a serum sodium concentration of 209 mEq/L, and glucose of 1,324 mg/dL. At autopsy, no gross or microscopic abnormalities in the brain could be identified (Fig 4B).

DISCUSSION

A large body of evidence indicates that the presence of sufficient amounts of HbF can temper the clinical course of patients with sickle cell disease and β-thalassemia. Thus, much attention is currently focused on the development of drugs that stimulate HbF production. Most known inducers of HbF, such as hydroxyurea and Epo, are thought to exert their effects indirectly by abruptly accelerating the rate at
which erythroid cells divide and mature. In contrast, butyric acid induces the synthesis of HbF via direct effects on globin gene expression, largely independent of changes in cell cycling. The mechanism for this effect has yet to be determined, although cis-acting sequences localized between −569 and −725 bp upstream of the chicken embryonic γ-globin gene have been identified as necessary for butyrate inducibility in stably transfected mouse erythroleukemia (MEL) cells. To estimate the potential therapeutic value of this class of drugs, we performed in vivo dose escalation studies, measuring HbF induction at each dose while continuously monitoring for, and carefully characterizing, toxicities. The results show that at doses of up to 3 g/kg/d, butyrate appears to be well tolerated and produces a 1.5- to 2.5-fold increase in HbF. Butyrate is a potent inducer of HbF when administered at high doses for prolonged periods of time. The HbF induction observed in these experiments should be magnified in circumstances of continuous erythroid stimulation and preferential F-cell survival, as occurs in hemoglobinopathies or β-thalassemia. Thus, doses required for the effective treatment of these disorders will likely be less than those used in our studies. A significant increase in fetal globin biosynthetic ratios has been noted in recent studies of arginine butyrate using doses between 0.5 and 1.5 g/kg/d in patients with sickle cell disease and β-thalassemia. The additive effect observed when butyrate was administered in combination with Epo is similar to that described with other reported combinations of HbF inducers such as Epo (plus iron) and hydroxyurea. HbF levels of 9% were achieved with this combination using a relatively nontoxic dose of butyrate (2 g/kg/d).

The level of HbF required for therapeutic benefit remains controversial. In a large prospective study of the natural history of sickle cell disease, Platt et al. found the frequency of painful crises to be inversely correlated with the square of the HbF level. Although there was no direct correlation between mortality and the percentage of HbF, the results suggest that even modest (threefold to fivefold) elevations in HbF may be of detectable therapeutic benefit. In contrast, a smaller prospective study by Powars et al. identified “threshold” levels of HbF, below which no benefit was seen. In this study, an HbF level of greater than 10% was associated with a lower incidence of stroke or aseptic necrosis, and a level of greater than 20% was associated with fewer crises or pulmonary complications. Thus, whereas the definition of “clinically significant” increases in HbF production remains to be defined imprecisely, there is evidence to support efforts to augment HbF to levels approaching 20%, while limiting toxicity. In the setting of preferential F-cell survival, or in combination with other inducers of HbF, it appears that with butyrate one may achieve this goal.

Butyrate has previously been evaluated in 10 to 14 day trials involving small numbers of patients with leukemia (at doses of 500 mg/kg/d), and hemoglobinopathies (in doses up to 1.5 g/kg/d) with minimal side effects. Thus, although it appears that these doses of butyrate are associated with little acute toxicity, there is as yet no experience regarding chronic toxicity. Predicting chronic toxicities is of obvious importance because patients with hemoglobinopathies will require long-term treatment, probably lasting many years. In an effort to characterize potential toxicities, we used doses in baboons 0.67- to 6.7-fold higher than used previously in humans. Our results may provide useful guidelines by which to develop protocols for future clinical trials involving butyrate. With one exception, short-term infusions of doses up to 4 g/kg/d were well tolerated. Laboratory evidence of hyperosmolality occurred with doses greater than 1 g/kg/d. Consistent with previous reports, hypokalemia occurred with butyrate doses as low as 2 g/kg/d. Although the cause of hypokalemia in these animals is unclear, at times it occurred in concert with elevated serum bicarbonate levels, consistent with a metabolic alkalosis. Doses in excess of 4 g/kg/d were accompanied by decreases in reticulocyte, WBC, and platelet counts, and neurologic abnormalities including obtundation and cerebellar ataxia. An even higher dose (10 g/kg/d) produced fulminant multiorgan system failure and death. This is consistent with previous studies in rats, in which the maximal tolerated oral dose of sodium butyrate was 8.79 g/kg. It is unclear whether the obtundation observed in four of the five animals resulted from hyperosmolality, butyrate, or both. As previously mentioned, there was one instance in which an animal’s level of alertness improved with discontinuation of butyrate while hyperosmolality transiently worsened. Additionally, reports of poisoning associated with the illicit use of the butyrate analogue γ-hydroxybutyric acid describe manifestations similar to those we observed, including drowsiness and loss of consciousness.

Similarities in the neuropathologic changes observed in the two animals that died are striking. Both animals had focal pathologic changes within the dentate nuclei and floor of the fourth ventricle. Animal E also had changes within, but not strictly localized to, the globus pallidus. Although this complication may have been caused by factors other than butyrate (ie, hyperosmolality), similar lesions have not previously been described, despite a large body of literature concerning the neurologic complications of hyperosmolality. In fact, whereas intracranial hemorrhage is the most common manifestation associated with hyperosmolality, and central pontine myelinolysis may occur during rapid correction of severe hyponatremia, no such lesions were observed in our study. Intriguingly, receptors for the neurotransmitter γ-ABA, have been found to be particularly abundant within the globus pallidus of baboons and the dentate nuclei in rabbits. An animal that had previously developed reversible neurologic abnormalities while on butyrate tolerated butyrate-free solutions of equal or greater osmolality without difficulty for 25 days. This animal was then treated with a twofold greater solute load than had been administered with butyrate, resulting in a serum osmolality approximately 1.5-fold higher than had been achieved in any of the butyrate-treated animals. Death occurred but no neuropathologic changes were seen.

The current method required for butyrate administration (long-term continuous intravenous infusion) is impractical for broad application in clinical trials. However, the potent effect of butyrate on HbF induction holds promise for future investigations using more readily administered members of this class of compounds.

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REFERENCES


Fetal hemoglobin induction with butyric acid: efficacy and toxicity

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