0014-4754/93/020126-07$1.50 + 0.20

Pharmacological modification of hemoglobin F expression in sickle cell anemia: an update on hydroxyurea studies

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Abstract. The most studied pharmacological intervention in sickle cell anemia aiming at elevating HbF expression is the use of hydroxyurea. At the present time the experience has been that after 1 year of treatment with maximum tolerated doses (MTD) all patients showed increases of percent HbF, with a mean of 15% HbF, without apparent side effects besides the reversible ones observed during the process of attaining the MTD. The question of efficacy is presently being investigated by a multicenter placebo controlled double blind clinical trial that involves more than 20 sites. The goal of the study is to determine if hydroxyurea can decrease the incidence of painful crises by 50%. Results of this study are not expected before the end of 1993.
Key words. Sickle cell anemia; HbF expression; hydroxyurea treatment; pharmacological intervention.

Background

Clinical observations on fetal hemoglobin and sickling

The first indication that fetal hemoglobin (HbF) interfered with sickling was Watson's observation that children with sickle cell anemia did not begin to develop symptoms until HbF levels had dropped to those seen in adults[55]. In 1948, Watson noted that others had reported few symptoms of sickle cell anemia (SS disease) in the first few months of life and that decreased sickling was seen when blood from African-American newborns ($n = 226$) was compared with blood from African-American adults[55]. Although the proportion of samples that showed sickling on sickle preparations was the same in both groups, all of the mothers' red cells sickled, but only 11% of the cells sickled, in average, in the newborns' blood. Watson knew that HbF disappeared over the first few months of life, so she hypothesized that 'fetal hemoglobin is unable to produce sickling, and [that] the sickling trait progressively becomes 100% with the gradual formation of the new red cells containing the adult type of hemoglobin which possesses the sickling property'[55]. She further suggested that inhibition of sickling by HbF played a clinical role by 'partially protecting the infant in the first four months of life, during which time it gradually disappears from the blood'[55]. Those seminal concepts have been extensively refined over the intervening 40 years, but the basic idea, based on correlation of clinical and laboratory findings, remains unchanged.

Much stronger clinical evidence was provided some years later by description of the compound heterozygous condition in which one chromosome carries the β^S gene and the other a deletion generating hereditary persistence of fetal hemoglobin[10]. Between 1955 and 1958, there were three reports of patients who appeared to have SS disease electrophoretically, but who did not manifest the clinical manifestations of sickling[18, 24, 56]. In 1960 and 1961, Conley and his coworkers described additional cases of 'hereditary persistence' of HbF (HPFH)[10, 44]. Carriers had 25% HbF, whereas compound heterozygotes had about 30%. The latter were neither anemic, nor symptomatic, despite the presence of 70% HbS and were thought to have the same condition reported by the earlier workers. All red cells contained HbF (a pancellular distribution)[5] in contrast to the heterogeneous (or heterocellular) distribution of HbF in red cells of more typical patients with high HbF levels. Based on the observations of Watson described above[55], and further in vitro studies (see below), it was concluded that the pancellular distribution of HbF protected the cells from sickling, and the patients from clinical illness. Black HPFH (to distinguish it from more recently described forms of HPFH[51]), is caused by deletion of the β-and δ-globin genes.

Another and more heterogeneous group of patients was reported in a paper that appeared at the same time, claiming that patients with HbF levels between 12% and 59% had less severe illness than those with trace or absent levels[23]. At least some of these patients probably had what is now colloquially called 'Saudi Arabian sickle cell anemia', but the features of that variant were not described until a decade later.

In 1972, Perrine et al. reported that SS patients in the eastern oases of Saudi Arabia had high levels of HbF (between 10 and 26%) and exhibited relatively mild disease[37]. While Saudi patients did have painful crises, and an increased frequency of meningitis and osteomyelitis when compared with normal controls, splenic function was preserved longer, serious complications were less frequent, childhood mortality was lower, and

anemia was less than in Black SS patients reported from the United States or Jamaica[6, 36, 38]. In contrast, SS patients from western Saudi Arabia, were more typical. Patients from other parts of the Middle East seemed to be a mixture of the 'African' and 'Saudi' forms of the disease in both their clinical features and their HbF levels. Of considerable interest to anthropologists, some SS patients in southern India are very similar to eastern Saudis[1, 25]. We now know that the Saudi Arabians (eastern oases), and the Indians have the β^S gene linked to the same haplotype of the β-cluster (the Arab-India haplotype)[25].

Biophysical studies

Concentrated solutions of HbS gel when deoxygenated (that is, they form a tridimensional polymer structure), and HbF were shown to interfere with that process[46]. The structure of HbS polymers was described[57], and it became clear that only one valine (the site of mutation) per molecule, rather than both of them, was a contact point between polymers. This implied that in a mixture of HbA and S, the hybrid tetramer $\alpha_2\beta^A\beta^S$ could fit into polymers, as well as the homotetramer $\alpha_2\beta^S_2$. That was not true for the fetal/S hybrid ($\alpha_2\beta^S\gamma$), for the γ chain of fetal hemoglobin differs from the normal β-chain and lacks both the donor and the acceptor sites needed for polymerization. The effect of HbF then was twofold, for neither the intact molecule, nor the hybrid tetramer could polymerize.

The structural basis for non co-polymerization of HbS and HbF stems from the differences between the β-globin chains of HbS and the γ-globin chains of HbF, a total of 39 amino acids per chain. The particular residues involved were deduced from gelation experiments in which HbS was mixed with a variety of abnormal hemoglobins, each of which was produced by one of the amino acids that differ between β and γ chains[33]. The residues most likely to be responsible are γ80 and γ87, the latter of which forms part of the lateral (side-to-side) contact between molecules at the acceptor site for the β6 valine (see fig. 2). Residue γ22 may also inhibit formation of an axial (up-and-down) contact in the polymer.

Further knowledge of the inhibitory effect of HbF on sickling came from measurements of the delay time for gelation. Allison had shown that deoxy HbS did not gel at 10°C but did so when the solution was warmed[2]. Hofrichter and his co-workers described the kinetics of gelation induced by rapid warming of deoxy HbS solutions form 2 °C to 20 °C[22]. There was a delay time, during which no change was evident, followed by a rapid change as the solution polymerized and gelled. The delay time was interpreted as being caused by the difficulty of single molecules to form nuclei, followed by rapid addition of molecules of deoxy HbS to the nuclei to form fibers. Mixtures of HbS with HbA or HbF showed a prolongation of delay time relative to pure HbS, and much more marked effects were seen with HbF. It was suggested that a prolonged delay time might prevent sickling from occurring in vivo, because it might be sufficiently long that red cells could return to the lungs and be reoxygenated, or at least escape from the microvasculature, before polymerization had begun[31]. Sickling can occur very rapidly at very low oxygen pressures[30, 59] and the presence of some polymer within red cells at PO_2 values that could be found in arterial blood[34] complicates interpretation of the foregoing studies.

The delay time is remarkably sensitive to the concentration of deoxyhemoglobin in a solution under study[34] varying inversely with the ratio (hemoglobin concentration/hemoglobin solubility) raised to the 15–30th power. Small changes in mean corpuscular hemoglobin concentration (MCHC) might be expected to have a large effect on sickling tendency, and it was reported many years ago that extremely hypochromic sickle trait cells could not be induced to change shape on deoxygenation[21]. Irreversibly sickled cells (ISCs), which have very high MCHCs, might be expected to sickle very easily, and Serjeant et al. provided evidence to support that view[43].

'F Cells'

The distribution of HbF among red cells, as noted earlier, is an important determinant of its effect. Not all red cells contain HbF: those which do are called 'F cells'[5]. Among young red cells (reticulocytes), the F-containing erythrocytes are called 'F-reticulocytes' (F retics). Whether or not a given red cell sickles or not, under a particular set of circumstances, depends in part on the amount of HbF it contains ('F/F cell'). The first to show that HbF was heterogeneously distributed among SS red cells were Singer and Fisher[45], Shepard et al.[44] and others[4]. This concept was refined by Boyer et al.[5] and Dover and co-workers[23] who developed assays for F cells and, later, F reticulocytes. Using an immunoassay, Boyer et al. found that normal persons have their HbF confined to a subpopulation of F cells, which comprised 0.01%–4% of their RBCs[5]. The proportion of F reticulocytes was usually lower than the proportion of F cells in SS patients, reflecting preferential survival of the F-containing SS erythrocytes[12].

Fetal hemoglobin levels in hemolysates, reflecting an average of all cells, depended on the proportion of F reticulocytes, the degree of their preferential survival, and the amount of HbF each of them contained. Each factor appeared to be separately regulated within a patient, as well as between patients, and under significant, but not complete, genetic control. Number of F cells produced, and the amount of HbF per F cell, also appeared to be independently affected by various forms of treatment which might affect either or both.

If there were enough HbF in a red cell to render it very unlikely to sickle (about 30%, based on observations of S/HPFH carriers), the proportion of F cells needed to achieve clinical benefit can be deduced, to some degree, from studies of transfused SS patients. About 50% AA cells have some apparent clinical benefit in uncontrolled studies[6,49], but 70% or more may be needed to prevent recurrent strokes[68]. If hemoglobin production were to be switched from S to F, those limits provide a considerable challenge.

5-Azacytidine

Shortly after De Simone and his coworkers administered 5-azacytidine to anemic baboons, and produced striking increases in their F cell production[11], Charache and others showed that 5-azacytidine administration increased HbF in SS and thalassemic patients[9,28], and observations of patients at Johns Hopkins suggested that there was clinical improvement.

The mechanism involved has been difficult to ascertain. For a time, the hypomethylation hypothesis was entertained. It became known in the early 1980s that one of the mechanisms involved in repressing genes was the degree to which they were methylated[40]. Methylation occurred on cytosine molecules adjacent to guanosines. In erythropoietic tissues, the γ-δ-β-globin gene cluster showed a low level of methylation, whereas the same sites were methylated in non-erythropoietic tissue[52]. DNA hypomethylation and active gene expression were linked in erythroid tissues[29,52]: for example, in postnatal tissues the γ chain gene was more heavily methylated than the β gene region.

The nucleoside analogue, 5-azacytidine, had been shown to interfere with methylation of DNA[19], and it was that observation which led to the use by De Simone. Unfortunately, observations of non-erythroid genes in patients who received the drug also showed hypomethylation, but they were not depressed, suggesting strongly that the drug had some other mode of action[9].

How much HbF is needed?

Considerable attention has been directed to the question of how much HbF is needed to ameliorate the clinical course of sickle cell anemia. 'Ameliorate' must be understood to mean 'lessen the frequency of painful vasoocclusive manifestations', for most patients can cope with their anemia. Platt et al.'s data from 3578 American patients enrolled in the Cooperative Study of the Clinical Course of Sickle Cell Disease, showed that HbF level is a significant predictor of pain rate, over the entire range of values encountered, without a threshold[38a], predicting that any increase in HbF would be beneficial. In another study, patients with both sickle cell anemia and α-thalassemia were less anemic than patients with sickle cell anemia alone, but had more vasoocclusive events[48]: a drug which converted sickle cell anemia to something resembling sickle cell anemia, plus α-thalassemia would not be a success.

Previously mentioned clinical observations are also relevant: 1) 25–35% HbF in every red cell would eliminate the disease (sickle/hereditary persistence of fetal hemoglobin); 2) 15–22% HbF spread over 60–70% of red cells would ameliorate the disease but not eliminate all manifestations (Saudi sickle cell anemia); 3) more than 50% F cells, containing enough HbF to completely inhibit sickling (25–35% HbF/F cell) would lessen crises, and more than 70% of such cells would probably eliminate them (based on results of transfusion studies with normal cells); and 4) patients with more than 20% HbF in their hemolysates show a lower frequency of such vasoocclusive events as chest syndrome[39].

Recent pharmacological interventions

Hydroxyurea and the recruitment hypothesis

The hypomethylation hypothesis is inadequate to explain the effect of 5-azacytidine on HbF synthesis. An alternate hypothesis proposed by Papayannopoulou, Stamatoyannopoulos, and their colleagues in Seattle, proposed that the cytoxicity of 5-azacytidine on late erythroid progenitors leads to faster mobilization (recruitment) of early RBC progenitors (with their intrinsic high F production) into hemoglobin synthesis[35]. Data from patients treated with azacytidine conflicted with that hypothesis since the expected reticulocytopenia before appearance of F reticulocytes had not been observed[13a]. The hypothesis, nevertheless, remains viable, perhaps in modified form, because it predicts that any chemotherapeutic agent that is cytotoxic to the S phase would stimulate HbF production. Treatment of baboons with arabinosylcytosine[35], vinblastine[54,82], hydroxyurea[26,53] and combinations of those agents[27] showed the predicted effect.

Hydroxyurea pharmacokinetics

Because 5-azacytidine was potentially carcinogenic, and hydroxyurea probably was not[32], patients at Hopkins and NIH were switched to the latter drug[8,17]. Bone marrow depression was encountered initially, but reticulocytopenia was not found to be a good predictor of F-reticulocyte response. Experience with the drug resulted in HbF responses equal[13] to those obtained with azacytidine. As predicted by Alter and Gilbert[3], daily therapy was more effective than intermittent treatment[13,14]. Proliferation of theories as to the mechanism of the hydroxyurea effect[13,15,17] suggested to some observers that a definitive explanation was not yet available.

Carcinogenicity, mutagenicity and teratogenicity of hydroxyurea

Although probably not a carcinogen, patients with polycythemia vera treated with hydroxyurea for as long as nine years have not yet shown an excess incidence of

cancer[19]. Hydroxyurea is a mutagen[47,50] a clastogen[20,33], and a teratogen[42], and it induces sperm abnormalities in mice[58]. The relevance of these studies to clinical practice is in doubt: for example, teratogenicity of hydroxyurea was demonstrated in rats treated with doses 10 times greater than those used in patients[10a], and aspirin was equally teratogenic at such doses. Women with chronic myelocytic leukemia being treated with hydroxyurea have borne normal children, and no treated man has ever been reported to have fathered a child with genetic abnormalities. Nevertheless, the drug must be used with discretion, and should not be used in children until its clinical efficacy is proved.

Short-term clinical trials

Two patients have been treated with hydroxyurea at Johns Hopkins since November 1983[8]. Their most recent HbF levels are 22–25% with >80% F cells. In-patient crises have virtually been eliminated, but mild out-patient crises requiring treatment in the Emergency Room have continued. The practicality of chronic therapy was established. In these patients, blood counts are needed no more often than once in 6–8 weeks, for the patients' responses to the drug are well defined.

The hydroxyurea study group

In October 1987, a cooperative multicenter study of the effect of hydroxyurea on HbF synthesis was begun, with the organizational center at Johns Hopkins. The goal was to determine how much HbF could be produced at maximum non-toxic doses in a group of 25 patients to be followed until June 1990. 49 patients entered the study and 32 finished. The trial was 'open' for patients and physicians who knew that an active drug, rather than a placebo was being used. Clinicians were not told of HbF or F cell levels, hoping that a decision to admit, or not to admit a patient, would not be biased on that information. Six clinics participated in the study (figs 1 and 2, table 1)[7].

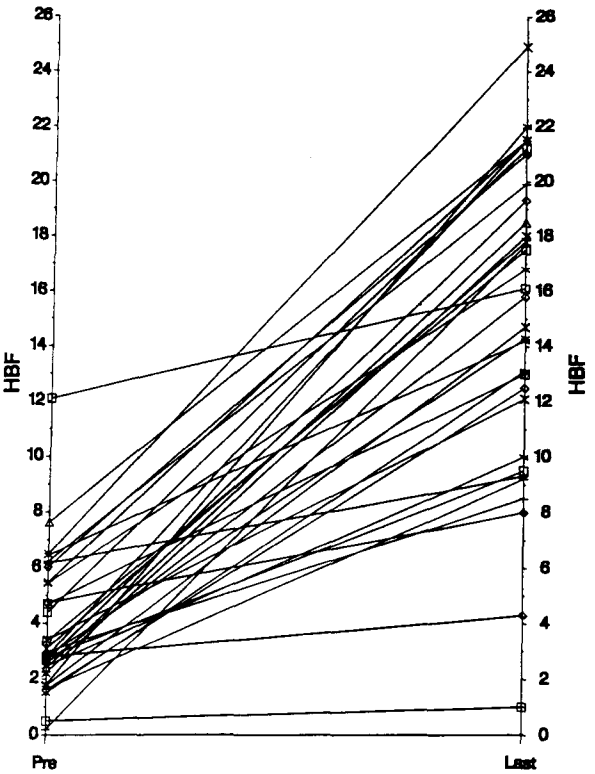

Figure 1. Change in HbF (pretreatment level to last measurement during therapy) for individual patients. Men did not differ from women in Lat F oder ΔF (p = 0.7, 0.3). From Blood 79 (1992) 2555, with permission.

Preliminary studies suggested that maximal non-toxic doses of hydroxyurea could be predicted from the renal clearance of that drug[16]. That concept proved correct for the dose at which toxicity was first encountered (r = 0.41, p = 0.03), but did not hold true when therapy

Table 1. Comparison of pretreatment measurements with measurements at MTD

	Pretreatment value	Value at MTD	p*
HbF (%)	4 ± 2	15 ± 6	0.0001
F reticulocytes (%)	8 ± 5	23 ± 10	0.0001
F cells (%)	28 ± 14	73 ± 17	0.0001
F/F cell (pg)	5 ± 2	8 ± 2	0.0001
Enrichment ratio	4.1 ± 2.6	2.6 ± 0.8	0.0001
Hb (g/dl)	8.5 ± 1.4	9.7 ± 1.8	0.0001
Reticulocytes (× 10^9/l)	401 ± 157	243 ± 73	0.0001
MCV (fl)	94 ± 8	117 ± 15	0.0001
Median CHC (g/dl)	34 ± 3	34 ± 2	0.39
Epo (U/l)	202 ± 189	471 ± 673	0.03
WBC (cells × 10^9/l)	13.4 ± 3.2	8.4 ± 1.4	0.0001
Neutrophil count (cells × 10^9/l)	7.4 ± 2.7	4.6 ± 1.1	0.0001
Platelets (× 10^9/l)	447 ± 136	364 ± 73	0.0003
Total bilirubin (mg/dl)	3.9 ± 3.4	1.9 ± 1.2	0.0001
ALT (IU/l)	36 ± 33	37 ± 29	0.78

Values are mean ± standard deviation. *p by paired t-test.
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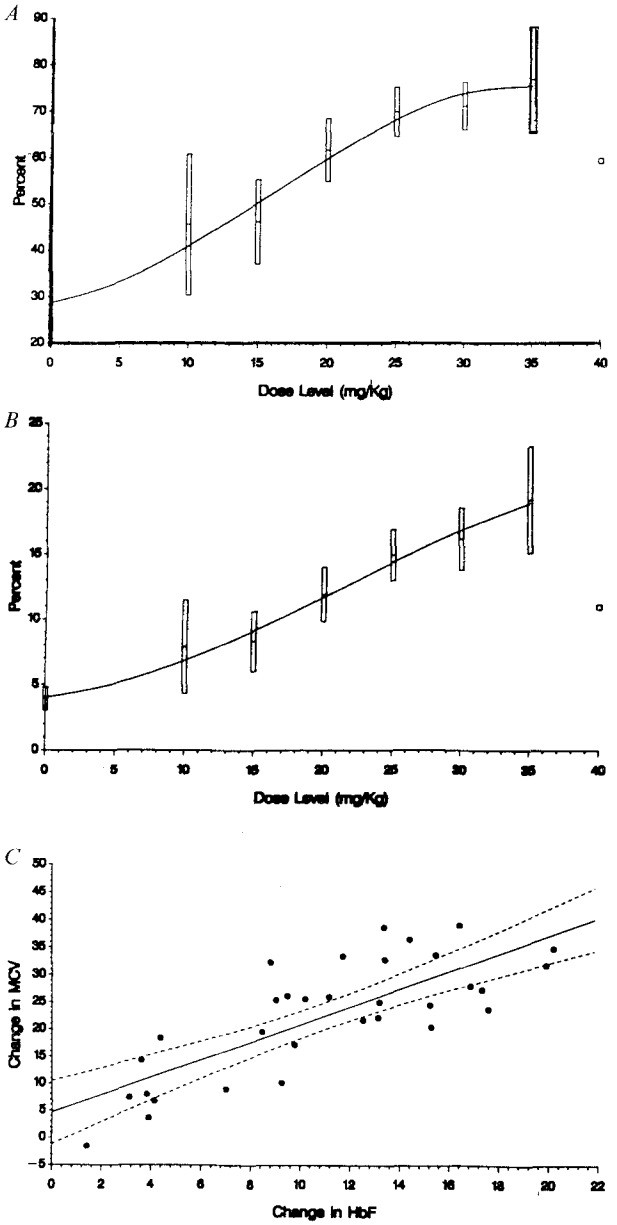

Figure 2. *A* Plasma HU concentration versus drug dose. *B* HbF levels for all patients at all doses. Each patient is represented several times. Average HbF level was correlated with dose ($r = 0.32$, $p = 0.0001$) and plasma HU level ($r = 0.39$, $p = 0.0001$). *C* Change in RBC volume as a function of change in HbF level. Each patient is represented only once.

was continued for months rather than a few weeks, probably because of cumulative effects of the drug on bone marrow. The only toxicity noted was bone marrow depression. The most common dose at which toxicity ($< 80{,}000$ reticulocytes/mm^3, $< 80{,}000$ platelets/mm^3, < 2000 neutrophils/mm^3) developed was 20 mg/kg.

After only one year of treatment, all patients showed increases in % HbF (figs 1 and 2), with a mean of 15% HbF, but the increase in HbF during therapy could not be predicted by any pretreatment measurement. Beta-globin DNA haplotypes (Benin, Senegal or Central African Republic), and α-globin gene numbers were not related to a final HbF level, while white cell count before treatment was a predictor of response (table 2). No consistent effort was made to define or record crises in this preliminary trial, leaving the question of therapeutic efficacy unanswered.

Table 2. Prediction of HbF response

	Partial r^2	Model r^2	Probability
Last F			
Variables used:			
WBC_0	0.29	0.29	0.001
HbF_0	0.20	0.49	0.002
Last plasma HU	0.10	0.59	0.02
ΔF			
Variables used:			
WBC_0	0.39	0.39	0.0001
Last plasma HU	0.11	0.50	0.02

Variables: zero random plasma percent; last plasma HU, HbF_0, F $cell_0$, F $retic_0$, Hb_0, weight, age, sex, $AUC6$, Cr, MCV_0, T $Bili_0$, Abs $Retic_0$, WBC_0, ALT_0, Plt_0, Epo_0, last Epo, ΔEpo, last dose. Abbreviations: HbF_0, initial HbF; ALT_0, initial ALT; zero random plasma, fraction of random samples containing no HU; last plasma HU, last plasma HU level.
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The multicenter study of hydroxyurea in sickle cell anemia

At present, more than 20 clinics are participating in a multicenter placebo-controlled double blind clinical trial of hydroxyurea. The primary goal is to determine whether hydroxyurea therapy can decrease the crisis attack rate by 50%. That goal was chosen to try to balance the inconvenience of frequent clinic visits and blood tests against an improvement in clinical status. The first preliminary examination of results will be in the Spring of 1993, but the study is expected to continue for some months longer, before a significant difference between the treatment arms might be obtained.

Acknowledgment. Many of the studies at Johns Hopkins Hospital described here were supported by the In- and Out-Patient Clinical Research Centers, under grants 5M01RR00035 and 5M01RR00722.

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