Glycosylated Fetal Hemoglobin

Correlation with Hyperglycemia and Birth Weight in Infants of Diabetic Mothers

FELIX FELDMAN, HARRY RUBIN, ISMAT NAWABI, ANN MARIE ABBONDANTE, NORMAN A. POSNER, AND NICHOLAS I. STEFANYSHYN

SUMMARY

Glycosylated fetal hemoglobin levels were measured in umbilical cord blood of normal infants and infants of diabetic mothers. The glycosylated fraction proved to be a stable compound; its level remained unchanged over a 19-day period. Exposure of fetal and adult hemoglobins to the same concentrations of glucose in vitro resulted in similar levels of glycosylated hemoglobins, suggesting that both types of hemoglobin are about equally reactive with glucose. Levels of glycosylated hemoglobin were significantly increased above normal in umbilical cord blood of infants of both Class A and Class B diabetic mothers. A significant relationship was found between macrosomia, reflected in birth weight ratios, and glycosylated hemoglobin from fetal erythrocytes in infants of diabetic mothers. While these data are consistent with the conclusion that glycosylated fetal hemoglobin levels are a function of fetal blood glucose concentrations in utero during the 2 mo before delivery, it is not known whether the glycosylated hemoglobin contributes to the abnormalities other than macrosomia found in infants of diabetic mothers. DIABETES *33*:81–85, January 1984.

It has been demonstrated that 5% of the hemoglobin content of normal adult erythrocytes consists of a minor fraction that is increased in red blood cells of diabetic persons and has been designated hemoglobin A_{1c}.[1] It accumulates in the erythrocyte as a result of posttranscriptional, nonenzymatic, stable glycosylation of the NH_2 terminal valine of the beta chain.[2] Glycosylation can also occur at other sites on the hemoglobin molecule.[2]

Studies indicated that the degree of glycosylation is a function of three factors: glucose concentration, erythrocyte life span, and permeability of red cell membrane to glucose.[3] Concentrations of hemoglobin A_1 are increased in the erythrocytes of diabetic persons and have been widely used as an indicator of diabetes control since they reflect blood glucose levels during the preceding 2–3 mo.[4–6]

Recently a glycosylated fetal hemoglobin counterpart, HbF1, has been separated chromatographically[7] and by isoelectric focusing.[8] This minor fetal hemoglobin fraction contains gamma chains, which are both glycosylated and acetylated at the NH_2 terminus. Fetal hemoglobin is one of a number of human proteins that can be acetylated in vivo. An acetyltransferase catalyzes the transfer from acetyl CoA to the NH_2 terminal glycine of the gamma chain. Normally 10–15% of fetal hemoglobin molecules are acetylated.[9] It is quite likely, therefore, that in previous reports[7,8] most of the HbF1 measured was acetylated fetal hemoglobin. With these methods, it was demonstrated that the F1 fraction is increased in umbilical cord blood of infants of diabetic mothers.[8,10]

More recently glycosylated hemoglobin in maternal and umbilical cord blood was measured using a thiobarbituric acid colorimetric method. The results were expressed as optical densities rather than as percentages of glycosylated hemoglobin. They found that optical densities were significantly elevated in infants of diabetic mothers. There was a high correlation between maternal and cord levels in the diabetic group. Cord blood glycosylated hemoglobin was not related to macrosomia in that study.[11]

Other investigators using a thiobarbituric method were unable to demonstrate significant increases in glycosylated hemoglobin in infants of diabetic mothers.[12] The thiobarbituric acid method may show inconsistencies because substances other than glucose can contribute to the development of color. In addition, the site of glucose attachment is an important determinant of the amount of color produced.[13] To our knowledge it is not known whether glycosylation of the amino terminal valine of the adult beta chain and glucose linked to the amino terminal glycine of the fetal gamma chain yield similar degrees of color with thiobarbituric acid. The

From the Departments of Pediatrics, Internal Medicine, and Obstetrics and Gynecology, Maimonides Medical Center, Brooklyn, New York.
Address reprint requests to Felix Feldman, M.D., Department of Pediatrics, Maimonides Medical Center, 4802 10th Avenue, Brooklyn, New York 11219.
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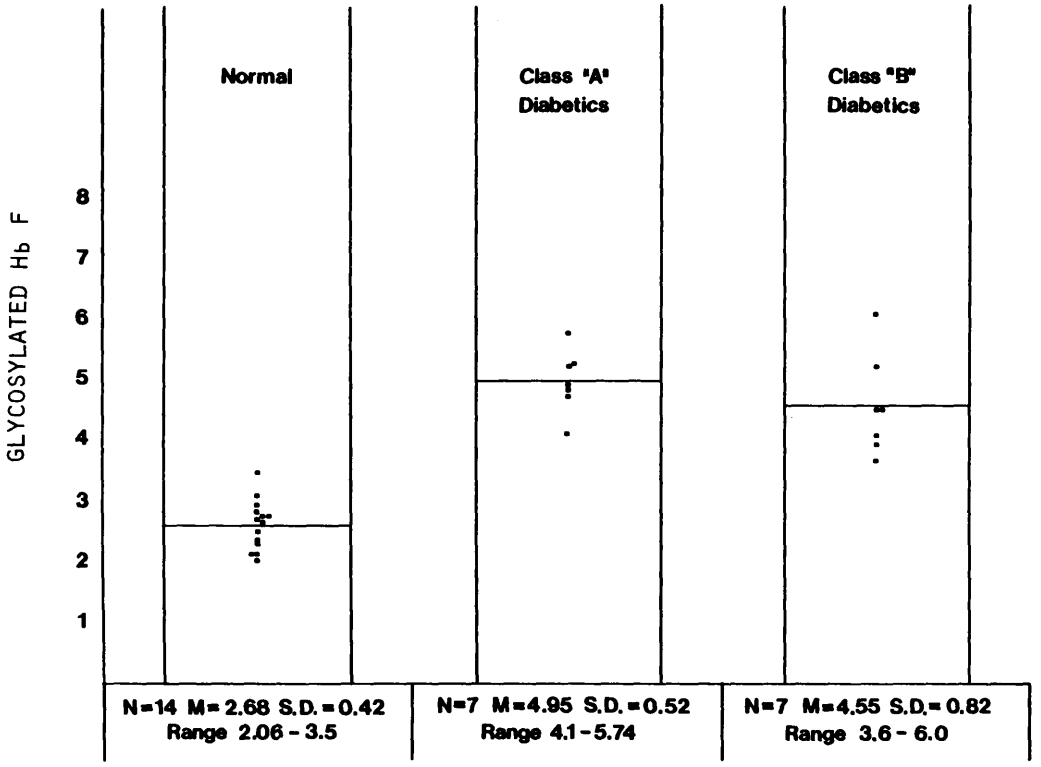

FIGURE 1. Percentages of glycosylated fetal hemoglobin in normal infants and infants of diabetic mothers. Percentages in infants of Class A and Class B diabetic women are significantly higher than normal ($P < 0.001$).

present study employs boronate affinity chromatography to measure levels of stable, glycosylated fetal hemoglobin in umbilical cord erythrocytes. The method is precise and quite specific;[14] in this study it measures only glycosylated fetal hemoglobin. The small amount of glycosylated adult hemoglobin present in umbilical cord blood is largely eliminated by lysis of cells that contain predominantly adult hemoglobin. Percentages of glycosylated fetal hemoglobin were found to be consistently elevated in infants of diabetic mothers. They were significantly related to birth weight in that group of infants.

MATERIALS AND METHODS

Aliquots of unclotted maternal blood were obtained when blood was drawn for other, clinically indicated reasons. Fetal blood was collected, using EDTA as anticoagulant, from the placental side of the umbilical cord of normal infants and infants of diabetic mothers. The fetal red blood cell content of umbilical cord blood was enriched by a method described by Boyer et al.[15] based on observations by Ørskov and Jacobs and Stewart. Erythrocytes from umbilical cord blood were washed 3 times and added to a solution containing 18 vol of 0.1844 M NH_4Cl and volumes of 0.1 mM acetazolamide in 0.15 M NaCl. After 2 min the solution was stirred gently and 2 vol of 3 mM NH_4HCO_3 was quickly added. The mixture was sedimented in cold Ficoll solution (10% in 0.15 M NaCl) and centrifuged in a refrigerated centrifuge for 30 min at 3000 rpm. The cells were then washed 3 times in 5% human albumin in physiologic saline. Following this method of treatment 99% of the cells recovered were fetal cells containing high concentrations of fetal hemoglobin, confirmed by Bethke-Kleihauer staining.

Glycosylated fetal hemoglobin was separated and quantitated in fetal erythrocytes using boronate agarose affinity chromatography.[14] Red blood cells were separated by centrifugation and hemolyzed by mixing with 20 parts of distilled

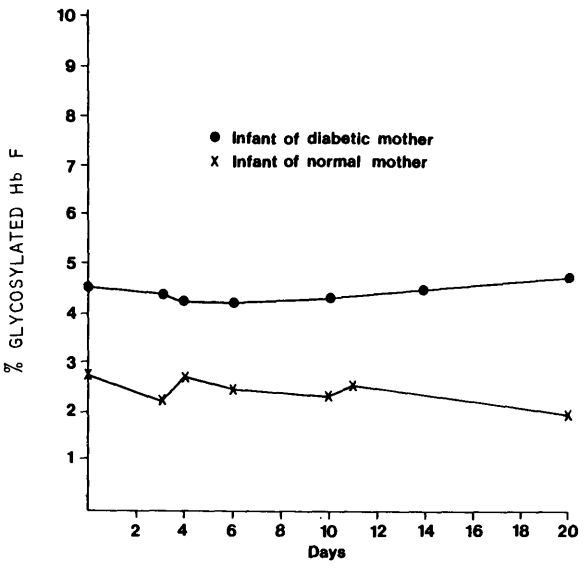

FIGURE 2. Serial determinations of the glycosylated fetal hemoglobin in a normal infant and an infant of a diabetic mother showing no change over a 20-day period, indicating stability on storage.

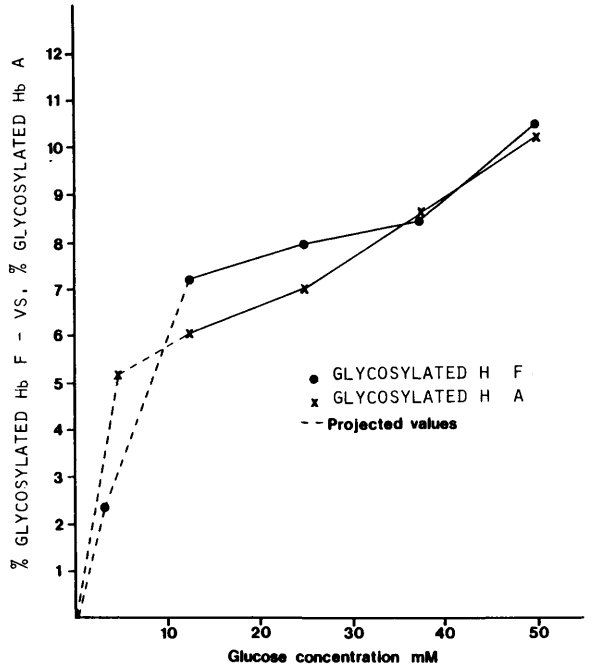

FIGURE 3. Glycosylated fetal versus adult hemoglobin after incubation with various glucose concentrations in vitro. HbA and HbF combine with glucose to a similar extent.

water. After cellular debris was removed by centrifugation, hemolysate (100 μl) was added to a 1-ml column of immobilized *m*-amino-phenyl boronic acid on a support of 6% beaded agarose in an aqueous medium containing 0.02% sodium azide (Pierce glyco-gel test kit), which had been equilibrated with a buffer containing ammonium acetate, magnesium chloride, and 0.02% sodium azide. The column was washed with three 5-ml aliquots of the same buffer to wash off the nonglycosylated hemoglobin, which was collected for quantitation; the total volume of the effluent was adjusted to 20.1 ml. The glycosylated hemoglobin was eluted with a buffer containing sorbitol, tris, and 0.02% sodium azide; the total volume of eluate was 5.0 ml. The absorbance of the effluent fractions was read in a Beckman DU[2] spectrophotometer (Beckman Instruments, Fullerton, California) at 414 nM. Percentage of glycosylated hemoglobin was calculated using the following formula:

$$\% \text{ glycosylated Hb} = \frac{5.0\ (A414B)}{20.1\ (A414NB) + 5.0\ (A414B)}$$

A414NB = absorbance at 414 nM of the unbound (nonglycosylated) hemoglobin fraction

Total volume = 20.1 ml

A414B = absorbance of 414 nM of bound (glycosylated) hemoglobin fraction

Total volume = 5.0 ml

RESULTS

Glycosylated fetal hemoglobin concentrations were measured after concentrating the fetal erythrocytes in the umbilical cord blood of three groups of patients (Figure 1). There were no differences noted in red blood cell behavior in response to Ørskov lysis among the three groups. In 14 normal full-term infants of normal mothers glycosylated hemoglobin from fetal RBC ranged from 2.1% to 3.50%, with a mean of 2.7% (SD = 0.42). Gestational ages throughout were estimated by the criteria of Dubowitz carefully applied by an experienced neonatologist. In the seven full-term infants of Class A diabetic mothers, values were significantly higher than the infants of normal mothers ($P = 0.001$); percentages ranged from 4.1% to 5.7% with a mean of 5.0% (SD = 0.52). In the third group, which included 7 infants of well-controlled Class B diabetic mothers and 1 infant of a Class C diabetic mother,[16] percentages of glycosylated hemoglobin from fetal red blood cells were higher than in the infants of normal mothers ($P = 0.001$) but were not significantly different from the infants of Class A diabetic mothers.

To demonstrate the stability of glycosylated fetal hemoglobin during storage, seven measurements were done over a 19-day period with umbilical cord blood from a normal infant and from an infant of a Class A diabetic mother (Figure 2). In both instances the values were fairly constant. In the normal infant they varied from 2.0% to 2.8%. In the infant of the diabetic mother the values ranged from 4.2% to 4.7%. These data indicated that the glycosylated fetal hemoglobin fraction being measured is a stable compound on storage.

Concentrations of glycosylated fetal hemoglobin in umbilical cord blood are low compared with values in adult blood. Accordingly, we tested whether fetal hemoglobin and adult hemoglobin A formed a stable conjugate with glucose to a comparable extent. Fetal hemoglobin containing umbilical cord red cells was enriched by removal of adult erythrocytes by the Ørskov-Jacobs-Stewart reaction. Increasing concentrations of glucose were added to 0.5-ml aliquots of fetal and adult erythrocytes to reach final concentrations of glucose of 12.5, 25, 37.5, and 50 mM. The specimens were incubated for 24 h at 37°C, then washed 3 times with saline and dialyzed against H_2O for 5 days. Glycosylated hemoglobin was then measured in the fetal and adult specimens. The results are shown graphically in Figure 3. Initially glycosylated hemoglobin in the fetal erythrocytes was only about half the percentage of that in the adult red blood cells. Following incubation with known concentrations of glucose the percentages of glycosylation were comparable in both fetal and adult hemoglobin.

Birth weight ratios were calculated in normal infants and in infants of diabetic mothers by dividing the actual birth weight by the 50th percentile weight for the corresponding gestational age on the Colorado intrauterine growth chart. Birth weight ratios were markedly increased in infants of diabetic mothers (range 0.92–1.57, mean = 1.25) as compared with normal mothers (range 0.87–1.17, mean = 1.03). Birth weight ratios were significantly related to glycosylated fetal hemoglobin levels in infants of diabetic mothers ($r = 0.66$, $P < 0.004$) (Figure 4). Eleven of fifteen infants of diabetic mothers but none of the normal infants in this series were above the 90th percentile for weight.

DISCUSSION

The present study shows that there is a small percentage of glycosylated fetal hemoglobin in umbilical cord blood of infants of normal mothers, which amounts to 2–3% of the hemoglobin content of fetal erythrocytes. This is a much

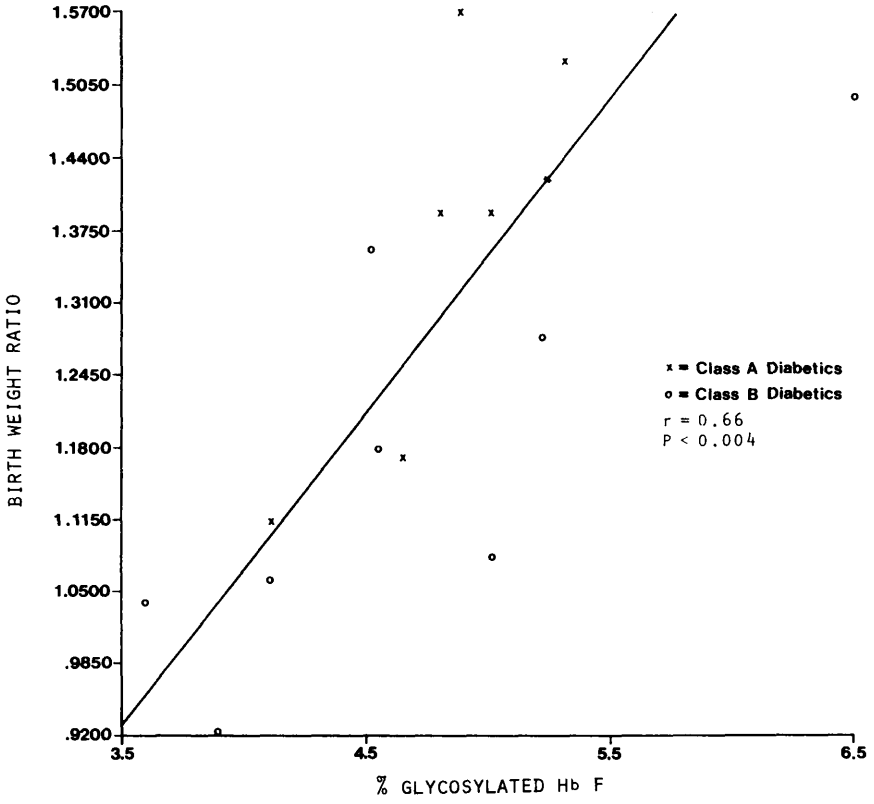

FIGURE 4. Relationship between birth weight ratio and umbilical cord glycosylated hemoglobin in infants of diabetic mothers. The relationship is highly significant ($r = 0.66$, $P < 0.004$).

smaller concentration than was reported previously by authors using methods that did not differentiate between glycosylated and acetylated fetal hemoglobin.[8,9] The present investigation was done using an affinity chromatography method specific for glycosylated hemoglobin. The glycosylated fetal hemoglobin measured was shown to be a stable compound since its concentrations remained constant over a 19-day storage period.

Although the percentages of glycosylated fetal hemoglobin in umbilical cord blood of normal infants were consistently lower than normal adult values following exposure to known concentrations of glucose in vitro, hemoglobin F and hemoglobin A formed comparable percentages of stable glycosylated hemoglobin. This indicates that the gamma chain of fetal hemoglobin interacts with glucose in a manner comparable to the beta chain of adult hemoglobin in vitro.[7]

There are very few reports of fetal blood glucose levels before delivery. Levels as low as 20 mg/dl have been reported in a 22-wk fetus, and concentrations of 50–60 mg/dl have been recorded in second trimester fetuses.[17] It has also been demonstrated that the life span of the fetal red cell is significantly shorter than that of the normal adult erythrocyte.[18] Both factors might contribute to the low levels of glycosylated hemoglobin in umbilical cord blood of normal newborns compared with normal adult values.

Glycosylated hemoglobin concentrations in this study were significantly increased in the umbilical cord blood of infants of class A and class B diabetic mothers due presumably to an elevation of blood glucose in these infants in utero. Glycosylated fetal hemoglobin levels in the infants of overt diabetic mothers were perhaps somewhat lower and more variable than might have been expected. There are a number of possible reasons why this might be true. Infants of all normal and class A diabetic women in this series were born at or close to term. By contrast, among the infants of overt diabetic women four were born at 36 wk, one was born at 37 wk, and only three were born at 40 wk. A shortened gestational period might result in a lower level of glycosylated hemoglobin in fetal erythrocytes since red-blood-cell life span might be shorter and blood glucose levels lower earlier in gestation. Also, close control of maternal glucose levels with insulin might have resulted in lower than expected fetal blood glucose levels in this group of infants. Determinations of maternal glycosylated hemoglobin, if they had been made, might have given important information regarding the effectiveness of control of blood glucose in this group of mothers. Because of the lack of definitive information the reasons why glycosylated fetal hemoglobin concentrations in infants of overt diabetic women might be lower and more variable than expected must remain speculative.

A significant correlation was found between the birth weight ratio and glycosylated fetal hemoglobin in the cord blood of infants of diabetic mothers. This strongly suggests that some of the same factors (such as fetal hyperglycemia) that produce the increase in glycosylated hemoglobin in the fetus contribute significantly to the macrosomia that so frequently occurs in these infants. This is at variance with findings in a previous report.[11] The disparate results might be explained by differences in methodology and by differences in the populations investigated. Unlike the previous study, ours included infants of Class A diabetic women, the group in which macrosomia is most prominent.

The increase in glycosylated hemoglobin in infants of diabetic mothers, although significant, is relatively modest, a

twofold increase to about 5%. With the limited knowledge available about glucose homeostasis in the fetus, more studies are needed to determine whether increased levels of glycosylated hemoglobin of this magnitude are likely to contribute to the polycythemia and other abnormalities seen in infants of diabetic mothers.

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