Fetal Hemoglobin Synthesis Determined by γ-mRNA/γ-mRNA + β-mRNA Quantitation in Infants at Risk for Sudden Infant Death Syndrome Being Monitored at Home for Apnea

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ABSTRACT. *Objective.* **Fetal hemoglobin (HbF) levels in the hemolysates obtained from infants who died from sudden infant death syndrome (SIDS) are reported to be markedly increased compared with controls. This finding could have been explained by increased HbF synthesis caused by episodes of hypoxemia in the SIDS infants. A prospective study in a group of infants being monitored at home after an apparent life-threatening event (ALTE) and considered at increased risk for SIDS was conducted with an improved ribonuclease protection assay. The ribonuclease protection assay allowed for the quantitation of [γ/(γ+β)]-globin mRNAs, which has a highly significant correlation with the levels of HbF synthesis.**

Methods. **Thirty-five infants who were admitted for an ALTE were included in the study. All infants were at home under surveillance with a cardiorespiratory monitor and followed in an apnea clinic with monthly appointments. Seventy-three blood samples were obtained between 38 and 61 weeks of postconceptional age. For control purposes, a similar group of 37 normal infants (99 samples) whose HbF synthesis was previously determined were included.**

Results. **Mean [γ/(γ+β)]-globin mRNAs were increased in the ALTE group at 42 to 45 and 46 to 49 weeks of postconceptional age (mean: 55.2 ± 17.4% and 33.9 ± 14%) in comparison with HbF synthesis in controls (mean: 42.6 ± 13.7% and 23.6 ± 9.8%).**

Conclusions. **The data obtained in this report from infants who were considered at risk for SIDS show that HbF synthesis is increased between 42 and 49 weeks of postconceptional age. Determining HbF synthesis as described in this study may have value as a marker for episodes of hypoxemia for certain infants who are at risk for SIDS.** *Pediatrics* **2003;112:e285–e288. URL: http://www.pediatrics.org/cgi/content/full/112/4/e285;** ***sudden infant death syndrome, apparent life-threatening event, fetal hemoglobin.***

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The results of this study were presented at a platform session at the Pediatric Academic Societies Annual Meeting; May 3, 2003; Seattle, WA.

Received for publication Feb 21, 2003; accepted May 14, 2003.

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ABBREVIATIONS. SIDS, sudden infant death syndrome; ALTE, apparent life-threatening event; HbF, fetal hemoglobin; HbA, adult hemoglobin.

Sudden infant death syndrome (SIDS) is defined as the death of an infant at <1 year of age for which a complete investigation did not reveal a cause of death. In developed countries, including Canada, the incidence of death by SIDS is approximately 0.5/1000 live births. Death occurs principally between the age of 2 and 4 months. Infants who have had 1 or more apparent life-threatening events (ALTEs) for which there was a need for resuscitation have a 7% to 10% risk of SIDS.[1] The etiologic hypothesis that is frequently retained for SIDS is that there is an anomaly or a delay in maturation of the brainstem, where the cardiorespiratory centers are situated. This anomaly could cause repeated episodes of hypoxemia. Several markers of long-term hypoxemia have been described in SIDS victims, namely the retention of periadrenal fat, increases in smooth muscle about the small pulmonary arteries,[2] and extramedullary hematopoiesis.[3] Detailed neuropathology examination and specific studies that identified necrosis and apoptosis have also revealed evidence of previous episodes of severe hypoxemia/asphyxia in SIDS victims.[4] It can be postulated that victims of SIDS have experienced recurrent episodes of hypoxemia before death.

A study by Giulian et al[5] and confirmed by others[6–9] showed that fetal hemoglobin (HbF) levels in the hemolysates obtained from infants who died from SIDS were markedly increased compared with controls. This finding could have been explained by increased HbF levels caused by unsuspected episodes of hypoxemia in the SIDS infants. The differences in HbF levels were most marked in children who were older than 50 weeks postconceptional age (ie, the period of peak incidence for SIDS). A subsequent report by Zielke et al[10] failed to confirm the findings of Giulian et al.[5] They used 3 different assays for the determination of HbF and reported no significant differences in levels of HbF in postmortem blood samples of 67 infants who died of SIDS compared with 102 controls. However, a comprehensive study by Fagan and Walker[6] showed elevated levels of HbF in blood samples of 106 full-term SIDS cases compared with 425 full-term control cases of known gestational ages.

For the HbF observations to tell us more about what causes SIDS, how to prevent it, or how identify infants who are at risk, the high levels of HbF documented in SIDS must be either supported or rejected and, if confirmed, understood. A prospective study therefore was conducted in a group of infants who were being monitored at home after an ALTE, with precise laboratory methods that could determine HbF synthesis in a small sample of blood. This was done using an improved ribonuclease protection assay that is a highly sensitive method for mapping and quantitation of specific RNA molecules. The ribonuclease protection assay allows for the quantitation of [$\gamma/(\gamma+\beta)$]-globin mRNAs. This assay has a highly significant correlation with the levels of HbF synthesis. However, HbF synthesis as determined in the past is a time-consuming method requiring a minimum of 2 mL of blood for red cell incubation with radioactive leucine, column chromatography separation, and eventual scintillation counting of the separated globin chains.[11] The recent mRNA methodology requires only 50 μL of blood, which could easily be obtained in a capillary tube by heel stick, and provides an accurate measure of HbF synthesis.[12]

METHODS

Study Patients

Infants who were admitted at 3 centers for an ALTE (defined according to the National Institutes of Health Consensus Statement on Apnea of Infancy)[13] were included in the study. These were infants who were admitted for an event that necessitated vigorous and prolonged stimulation and sometimes mouth-to-mouth respiration and cardiac massage. All infants under surveillance by the monitors are followed in an apnea clinic with monthly appointments. When infants presented with subsequent events (clinical events and/or alarms on the monitor), they were reevaluated immediately to confirm the nature of the events.

Type of Monitor and Data Acquisition

The 3 clinics are part of a large network of infants undergoing home cardiorespiratory monitoring in the Province of Quebec. They all use the Healthdyne 970 Smart Monitor (Healthdyne Technologies, Marietta, GA), a thoracic impedance-type apnea monitor with a memory capability to store events that violated the preset alarms. At each visit, the monitor's memory content was downloaded and analyzed, and the events were classified by visual inspection. A significant event was defined as a central apnea longer than 20 seconds or a bradycardia exceeding 10 seconds (limits at 80 bpm if <1 month old and at 60 bpm thereafter). The choice of 20 seconds for a significant apneic event is based on the recommendation of the National Institutes of Health Consensus Statement on Apnea of Infancy.[13]

Thirty-five infants who were admitted for ALTE at Hôpital Ste-Justine, at the Montreal Children Hospital, or at the Centre Hospitalier de l'Université de Sherbrooke between February 1998 and October 2000 were included in the study. Some infants had up to 3 blood samples taken at different ages during the study. Seventy-three blood samples were obtained between 38 and 61 weeks of postconceptional age. For control purposes, 37 infants (99 samples) who had a postconceptional age of 38 to 60 weeks and whose HbF synthesis was determined postnatally in our laboratory and previously reported[14] were included in this report. The gestational age of the infants was based on an ultrasound examination at 18 to 20 weeks of pregnancy and confirmed by the physical examination at birth.

The preparation of DNA probes, the ^{32}P-labeled cRNA probes and ribonuclease protection assays were conducted as previously published.[12] In brief ^{32}P-labeled cRNA probes for human α_2-, β-, and $^{G}\gamma$-globin mRNAs were prepared using an in vitro transcription kit (Promega, Madison, WI). Total RNAs from samples were prepared by using the RNeasy blood mini kit (Qiagen, Chatsworth, CA). Total RNAs were subjected to RNase protection assays according to a published protocol[15] with the following modifications. Total RNA was mixed with 5×10^4 cpm each of cRNA probes for α_2-, β-, and $^{G}\gamma$-globins in 20 μL of hybridization buffer, denatured at 85°C for 5 minutes, and incubated for 30 minutes at 50°C. The RNA hybrids were digested with RNase A and RNase T_1 in 200 μL of digestion buffer for 30 minutes at 25°C. Proteinase K was added, and the incubation was continued at 37°C for 30 minutes. Denaturing buffer, containing 30 μg of yeast tRNA followed by 450 μL of isopropanol, was added to the samples. The RNA hybrids were precipitated by keeping the samples at −80°C for 15 minutes followed by centrifugation at 14 000 × g and 4°C for 20 minutes. Then the samples were washed with 70% ethanol, briefly dried, and solubilized in formamide sample buffer. The protected RNA fragments were resolved on urea-6% polyacrylamide gels and autoradiographed using Kodak X-Omat film. In addition, the bands were visualized and quantified by phosphorimaging (Molecular Dynamics, Sunnyvale, CA). The mRNAs encoding for HbF were expressed $[\gamma/(\gamma+\beta)] \times 100$ and for adult hemoglobin (HbA), as $[\beta/(\gamma+\beta)] \times 100$. The errors of measurement of replicates in the RNA assay and globin synthesis were 1.6% and 1.2%, respectively.

Statistical Analysis

Data obtained from the study group and controls from a previous published study[14] were compared. The main objective of the study was not to show that means between the 2 groups were different across the entire study period but at specific weeks of observation. A t test was considered appropriate because each of the subjects used had only 1 measure per period. For comparing the results between the 2 groups at each of the 4-week intervals, an unpaired t test was used (Prism 3.0, GraphPad Software Inc, San Diego, CA). $P \leq .05$ was considered as statistically significant. The present study was approved by the Institutional Review Boards of the participating department and conducted with informed consent.

RESULTS

The mean levels of [$\gamma/(\gamma+\beta)$]-globin mRNA from infants with ALTE were compared with the globin synthesis among controls at 4-week interval using an unpaired t test as shown in Fig 1. There was a significant increase in the ALTE group compared with controls at 42 to 45 weeks (mean: 55.2 ± 17.4% vs 42.6 ± 13.7%; $t = 2.25$, $P = .03$) and at 46 to 49 weeks (mean: 33.9 ± 14% vs 23.6 ± 9.8%; $t = 2.40$, $P = .02$), respectively. The other periods of observation were not different. There was no correlation between HbF synthesis and significant recorded events of apnea and/or bradycardia (16 significant events were recorded on monitors). No difference was noted between preterm and term infants included in this study. None of the infants included in this study succumbed to SIDS. Although these infants were not followed prospectively until 1 year of age, we know from other sources of data that none died suddenly and unexpectedly. This information is based on the results from the National Databank of SIDS victims and ongoing studies that are being conducted on the epidemiology of sudden death (Aurore Côté, personal communication).

DISCUSSION

Normally, the switchover from HbF to HbA synthesis is dependent on postconceptional age.[16] We used a control group from a previous study because of the difficulty of obtaining blood samples from normal infants. Because of the very close correlation

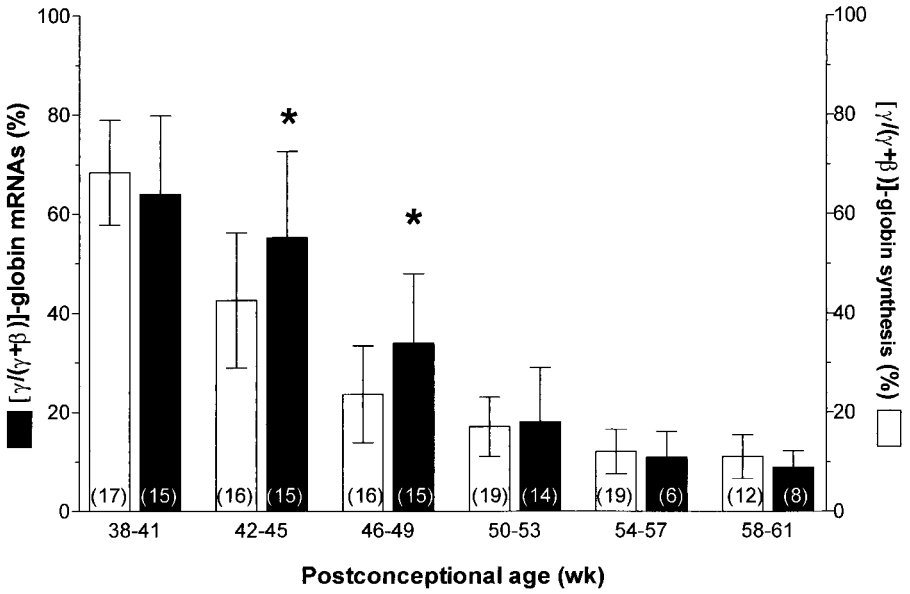

Fig 1. The comparison of $\gamma/(\gamma+\beta)$ levels at 4-week intervals for globin synthesis of controls (□) and the globin mRNA of the study group (■). Values shown are the mean and standard deviation; *n* in parentheses; $^*P < .05$.

between HbF synthesis and $[\gamma/(\gamma+\beta)]$-globin mRNA demonstrated in an earlier study,[14] the method of $[\gamma/(\gamma+\beta)]$-globin mRNA determination can be considered as an accurate measure of HbF synthesis.

Hypoxia has been shown to increase HbF synthesis in juvenile baboons[17] and in hyperglycemic fetal lambs.[18] In an animal experimental study, using the fetal lamb, late in gestation, hypoxemia, without any additional complicating factors, resulted in higher amounts of HbF being synthesized than could be expected for the time in gestation.[19] In human studies, increased levels of HbF synthesis have been documented in human newborns as a result of prenatal complications associated with maternal hypoxemia,[20] placental insufficiency,[21] and diabetes.[22] This increase in HbF synthesis was considered to be the result of an erythropoietic response to hypoxemia. In other human studies, infants with oxygen-dependent bronchopulmonary dysplasia[23] and anemia and children with congenital cyanotic heart disease were synthesizing greater amounts of HbF when compared with controls.[24]

There is evidence in this study that HbF synthesis is increased in infants being followed because of an ALTE, the increase taking place between 42 and 49 weeks' postconceptional age. There is no clear explanation for increased HbF synthesis found in this study. It could be attributable to unknown factors that temporarily decrease the rate of switchover from HbF to HbA synthesis. Because of stress erythropoiesis, immature red cells are released into the circulation from erythropoietic tissues that have not completed their full program of maturation; hence, these cells are synthesizing higher-than-normal levels of HbF.[25] Because the half-life of the red cells in newborn is approximately 60 days,[26] the observed increases in HbF synthesis could result in higher-than-normal levels of HbF that persist in these infants for several months beyond the period of increased HbF synthesis.

Infants who have experienced an ALTE could have had silent episodes of hypoxemia before the event that brought them to medical attention. They also could have experienced repeated episodes of hypoxemia afterward, episodes that would not be diagnosed at home with the type of monitor used in this study. This could explain our results. It is only very recently that monitors (pulse oximeters) that continuously record and store oxygenation data and have a reasonably low rate of false alarms as a result of movement have become available. Unfortunately, these monitors have not been accepted as standard monitoring devices for infants who have sustained an ALTE.

The data from the Collaborative Home Infant Monitoring Evaluation study[27] report that a certain proportion of infants on home monitors for an ALTE experienced what the authors described as "extreme events," which are often accompanied by bradycardia and desaturation. The "extreme" events were not frequent in the ALTE group, and their frequency of occurrence was not different from that of the healthy control group. What is not known, however, is whether some infants in the ALTE group experienced isolated episodes of hypoxemia not associated with an apnea or a bradycardia. The monitors used in the Collaborative Home Infant Monitoring Evaluation study recorded events only when the violation of alarm limits was for apnea or bradycardia, not oxygenated hemoglobin saturation. It is interesting that in studies using transcutaneous partial pressure of oxygen recordings, isolated episodes of hypoxemia could occur without an apnea or a bradycardia.[28,29]

Much is to be learned from new data concerning the oxygenation status that exists long before death occurs in SIDS victims. The correlation of HbF synthesis and hypoxemia would certainly be important in the evaluation of infants at risk for SIDS.

ACKNOWLEDGMENT

This work was supported by the Canadian Institutes of Health Research grant MT11552.

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Pediatrics 2003;112;e285
DOI: 10.1542/peds.112.4.e285

PEDIATRICS
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