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Research Article

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Increased Erythropoiesis and Elevated Erythropoietin in Infants Born to Diabetic Mothers and in Hyperinsulinemic Rhesus Fetuses

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ABSTRACT The pathogenesis of the increased erythrocytosis and extramedullary erythropoiesis observed in infants of diabetic mothers (IDM) has been obscure. In the present studies, IDM were found to have elevated umbilical plasma erythropoietin (Ep) concentrations by radioimmunoassay. 22 of 61 IDM (36%) had levels above the range of 28 nonasphyxiated, appropriately grown normal infants. In 16 controls and 20 IDM, plasma Ep correlated directly with plasma insulin ($P < 0.001, r = 0.73$). To investigate this relationship further, a chronic rhesus model was studied with continuous fetal hyperinsulinemia for 21 d in utero in the last third of pregnancy. In five experimental fetuses, plasma insulin levels averaged 4,210 μU/ml at delivery, whereas plasma Ep was above the range of six controls. In addition, the experimental fetuses had elevated reticulocyte counts in umbilical cord blood. The mechanism for the increased plasma Ep associated with hyperinsulinemia in the fetus is unexplained but may be mediated by fetal hypoxia.

INTRODUCTION

Erythrocytosis, increased normoblastemia, and extramedullary erythropoiesis have been observed during the first days after birth in infants of diabetic mothers (IDM)[1] (1–3). Fetal hypoxia, which may be secondary to placental insufficiency, has been suggested as the cause for this increase in erythropoiesis in IDM (4).

Because the humoral control of erythropoiesis is dependent on erythropoietin (Ep) production in the fetus as well as the adult, we studied Ep levels in umbilical plasma from infants of normal and diabetic mothers. In addition, because fetal hyperinsulinemia is the major factor responsible for fetal macrosomia and cellular proliferation in IDM (5, 6), the relationship of fetal insulin and Ep was also investigated in a hyperinsulinemic fetal rhesus monkey (Macaca mulatta) model, in which all the mothers were nondiabetic (7).

METHODS

Human studies. Written informed consent was obtained from all study mothers. Two groups of subjects were studied, control infants and IDM. Controls were those singleton births whose birthweights were appropriate for gestational age (>10th percentile and <90th percentile) (8), whose APGAR scores were ≥7 at 5 min of life, and whose mothers were not known to have diabetes. The IDM group was likewise selected for being singleton and nonasphyxiated at birth, but not for appropriateness of birthweight. The mothers of these babies were subgrouped according to the earlier modified classification of White (9). This classification was based on both severity and duration of diabetes. The mildest group, class A, included those women with glucose intolerance but without insulin requirements, whereas those in classes D and F/R included

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[1] *Abbreviations used in this paper*: Ep, erythropoietin; Hb A_{1_c}, glycohemoglobin; IDM, infants of diabetic mothers; NRBC, nucleated erythrocyte count; RBC, erythrocyte count.

those insulin-dependent women who manifested vascular disease. Women from classes B and C required insulin but had no evidence of vascular disease.

Mixed umbilical cord plasma or serum, which had been refrigerated as whole blood for <24 h, was separated, frozen (−20°C) and saved for Ep determination. A doubly clamped umbilical cord blood sample was obtained within 15 s of delivery in a subgroup of 34 IDM and 5 controls. In these individuals, heparinized umbilical venous whole blood was analyzed for hemoglobin concentration, volume of packed erythrocytes, nucleated erythrocyte count (NRBC), reticulocyte count, and total erythrocyte count (RBC) by the usual laboratory procedures. In addition, for these IDM delivered by cesarean section, umbilical arterial and venous blood gases were analyzed using the Corning 164/2 Blood Gas Analyzer (Corning Glass Works, Corning, N. Y.).

Plasma glucose was measured using a glucose oxidase method (YSI 23A Yellow Springs Instrument Co., Yellow Springs, Ohio) and insulin by a double antibody radioimmunoassay technique of Hales and Randle (10). Only offspring of White's diabetic classes A and A/B[2] were evaluated for venous plasma insulin levels, since plasma from these infants do not have maternal antiinsulin antibodies that cross the placenta and interfere with the insulin assay.

Mixed umbilical serum as well as venous plasma was analyzed for Ep using a specific double antibody radioimmunoassay technique (11). The purity of the labeled Ep was 70,400 U/mg protein (kindly supplied by Dr. Eugene Goldwasser, Department of Biochemistry, University of Chicago). Confirmation of the three highest Ep values was obtained with the polycythemic mouse bioassay for Ep (12).

A retrospective chart review carried out on the study subjects and their mothers included: maternal age, parity, duration of pregnancy, route of delivery, smoking history, third trimester glycohemoglobin (Hb A_{I_c}) level nearest to delivery, and parameters of fetal distress during labor or delivery. Hb A_{I_c} was determined either by a macrocolumn analytical cation resin technique or by a high-performance liquid chromatography method as previously reported (13). For the infants, these details included: birthweight, placental weight, assessment of gestational age, and APGAR scores.

Rhesus primate studies. Five fetal rhesus monkeys were made hyperinsulinemic in utero by an osmotically driven minipump (Alzet, Alza Corp., Palo Alto, Calif.) delivering 19 U of porcine insulin per day as previously reported (7). To achieve this, a hysterotomy was performed during the final third of pregnancy in accurately dated rhesus monkeys and a minimpump implanted subcutaneously in the hind limb of the fetus. The minipump was left *in situ* and the pregnancy allowed to continue. 21 d later (between 134 and 148 d gestation with term gestation being 165 d), the fetus was delivered by cesarean section. Three of the six control pregnancies were treated identically, whereas the remainder were nonoperated.

Before delivery of the fetus, free-flowing umbilical venous and arterial samples were obtained and analyzed in 7 of the 11 cases for pH, pCO_2, and pO_2. Arterial and venous plasma samples were also analyzed for glucose, insulin, glucagon, and Ep. Glucagon was measured by a radioimmunoassay procedure using the Unger antibody (14). Anticoagulated venous whole blood samples were analyzed for hemoglobin, volume of packed erythrocytes, NRBC, reticulocyte count, and RBC.

Fetuses were exsanguinated moments before delivery and examined grossly. At autopsy, total fetal weight was determined by adding the blood volume removed to the weight of the fetal body. In addition, the placenta and fetal organs, viz. spleen, liver, and kidneys were also weighed. To assess the degree of hepatic extramedullary erythropoiesis, a histologic point counting technique was used in evaluating light microscopic sections of the fetal livers with the results expressed as a percentage of the total volume of the liver (15).

Statistical analysis included unpaired *t* test, chi-square, linear regression analysis, and the Wilcoxon rank-sum test, where applicable.

RESULTS

Human studies. In Table I are presented selected clinical features of the control and IDM subjects. Statistical comparisons are indicated in the table for comparisons of controls with all other groupings. The IDM were heavier than the controls ($P < 0.001$ unpaired *t* test), although there was no difference in gestational age. Of the 61 IDM, 18 were large for gestational age (>90th percentile) (8), three were small for gestational age (<10th percentile), and the remainder were appropriate for gestational age. Maternal third trimester Hb A_{I_c} levels nearest to the time of delivery were determined for 14 controls and for 56 of the diabetic mothers. When individually compared with controls, all diabetic classes had elevated Hb A_{I_c} values, as did the diabetic group as a whole.

Umbilical cord Ep values determined by radioimmunoassay for the two study groups are portrayed in Fig. 1. Because of the extreme range of values (5–30,000 mU/ml), the vertical axis for Ep is presented on a log scale for graphic purposes. Statistical comparison of the two groups using the Wilcoxon rank-sum test for nonparametric distributions is significant at $P < 0.01$. 22 of the 61 IDM (36%) fell above the range for the controls (5–69 mU/ml). The three highest Ep values in the IDM group, 30,000, 17,000, and 6,200 mU/ml, were corroborated using the polycythemic mouse bioassay in which the Ep values were 20,000, 13,100, and 5,000 mU/ml, respectively. These three infants were born of mothers from White's classes B (two) and D and, therefore, were not only from the groups with the most severe diabetes. Although significant differences in Ep values were noted when controls were compared with IDM of White's classes A, A/B, C, and D ($P < 0.02$) (classes B and F/R were too small in number to reach significance), no differences of Ep levels were evident between pairings of diabetic subgroups.

The two subsets of control and IDM subjects (class A and A/B only) who were studied with glucose, insulin, and Ep measurements at the time of delivery are portrayed in Table II. Although umbilical vein plasma glucose concentrations were no different between the two groups, plasma insulin values were significantly higher ($P < 0.01$, rank-sum test). Similarly, these 20 IDM individuals manifested higher Ep values compared with the 16 control infants ($P < 0.01$). Compari-

[2] Class A/B refers to chemically diabetic women who received insulin only during pregnancy.

TABLE I
Clinical Features of Human Study Groups at Delivery

Subject group	No.	Gestational age	Birth weight	Maternal Hb A_{I_c}
		wk	*g*	*% total Hb*
Controls	28	37.0±3.0*	2,750±670	4.6±0.7 (14)
IDM	61	37.1±2.1	3,360±720‡	6.7±1.4 (56)
White's class				
A	14	38.4±1.4	3,430±520§	5.8±1.1§ (13)
A/B‖	9	38.4±1.9	3,850±590‡	6.1±0.9‡ (7)
B	5	37.0±0.0	3,300±580	6.6±1.1‡
C	16	36.0±1.5	3,110±720	7.0±1.5‡ (15)
D	12	36.5±2.2	3,400±860	7.3±1.2‡ (11)
F/R	5	36.4±3.8	3,050±930	7.5±1.5§

* Mean±SD.
‡ $P < 0.001$ relative to control group.
§ $P < 0.01$ relative to control group.
‖ Refers to chemically diabetic women who received insulin therapy only during pregnancy.
( ), number of subjects included, if different from that already indicated.

son of plasma Ep and insulin by linear regression analysis in the combined control and IDM groups resulted in a positive correlation ($P < 0.001$, $r = 0.73$).

No correlations were found between plasma Ep level and maternal Hb A_{I_c} when analyzed by linear regression. Similarly, the 28 control infants did not manifest any relationship of Ep level with gestational age. This was also true for the IDM alone as well as for the combined groups. In the subset of 31 IDM who had umbilical blood gases measured at delivery, positive correlations were observed for Ep concentration and ΔP_{O_2} ($P_{uv}O_2 - P_{ua}O_2$) ($P < 0.012$, $r = 0.42$) as well as ΔPCO_2 ($P_{ua}CO_2 - P_{uv}CO_2$) ($P < 0.003$, $r = 0.49$). However, no relationship between umbilical arterial P_{O_2} and Ep was observed. There was no correlation between plasma Ep and either RBC, NRBC, or reticulocyte counts.

In review of the data in mothers' and infants' charts, few differences could be ascertained in comparisons of the IDM with the controls. In addition, those 22 IDM whose Ep values were above the control range (>69 mU/ml) were compared with those 39 IDM within the normal range. There were no differences for the maternal features of age, parity, or smoking history. Cesarean section was, however, more common in the IDM group ($P < 0.02$ by chi-square). For the infants, no differences could be found for the placental weight or 5-min APGAR scores. However, the IDM with elevated Ep values also had significantly higher birthweight to placental weight ratios compared with IDM with normal Ep levels ($P < 0.02$). Furthermore, the

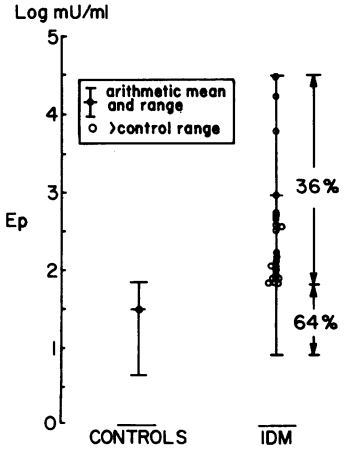

FIGURE 1 Human umbilical plasma or serum levels of erythropoietin in controls ($n = 28$) and IDM ($n = 61$). Arithmetic mean and range for both groups are indicated along with 22 individual IDM values that exceeded the control range. (Note log scale.) $P < 0.01$.

TABLE II
Mean Umbilical Venous Plasma Glucose, Insulin, and Ep Levels for Human Study Groups

Subject group	No.	Glucose	Insulin	Ep
		mg/dl	*μU/ml*	mU/ml
Controls	16	108 (48–258)	24 (10–77)	32 (7–64)
IDM*	20	119 (39–224)	73‡ (5–330)	96‡ (14–520)

( ), range.
* Class A and A/B only.
‡ $P < 0.01$ by Wilcoxon rank-sum test.

three infants with the extraordinarily elevated Ep levels could not be segregated from any of the other subjects on the basis of severity of maternal diabetes, diabetic control during pregnancy, or possible acute or chronic hypoxia. However, because of possible interference of maternal insulin antibody in the umbilical plasma, insulin determination was not possible for these three individuals.

Fetal rhesus studies. Fig. 2 shows the results of the fetal rhesus monkey experiments with respect to the glucose, insulin, and Ep values measured in umbilical arterial plasma at the time of delivery. Both groups of fetuses were born of nondiabetic normal mothers. The fetal arterial plasma insulin levels achieved in the five hyperinsulinemic fetuses were two logs higher (2,900–5,300 μU/ml) than the controls (29–64 μU/ml) with no overlapping of values. Arterial plasma glucose levels were lower ($P < 0.03$) in the hyperinsulinemic group, but whether this finding per se had any adverse physiologic effect is questionable, since simultaneously obtained umbilical venous glucose values were not different between the groups (7). Similarly, the hyperinsulinemic fetuses manifest plasma Ep levels that were one order of magnitude higher than the controls and without overlap. The level for the six control fetuses (8–45 mU/ml) fell within the range of those obtained from the control human subjects (8–69 mU/ml). The plasma glucagon levels in the hyperinsulinemic group were lower than the controls, also with no overlap of values.

Parameters of fetal erythropoiesis for both groups of monkeys are shown in Fig. 3. There was a tendency towards increased hepatic extramedullary erythropoiesis and absolute number of NRBC in the hyperinsulinemic group, but this did not reach the 5% level

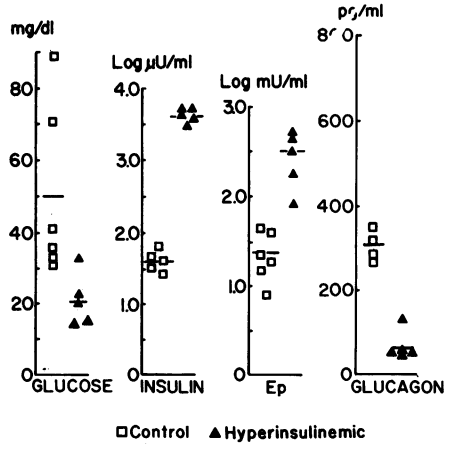

FIGURE 2 Rhesus umbilical arterial glucose ($P < 0.03$), insulin ($P < 0.001$), Ep ($P < 0.001$), and glucagon ($P < 0.001$) levels in control and hyperinsulinemic fetuses. (Note log scale for insulin and Ep values.) Mean values for each group are indicated by the horizontal line.

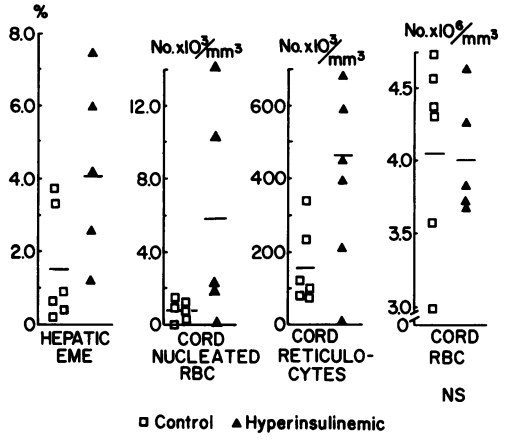

FIGURE 3 Rhesus fetus parameters of erythropoiesis: hepatic extramedullary erythropoiesis (EME) ($P < 0.06$), umbilical vein NRBC ($P < 0.08$), reticulocytes ($P < 0.007$) and RBC. (See legend for Fig. 2.)

of significance in these small groups. Reticulocytes were, however, increased in the experimental group, while RBC was no different between the two groups.

As shown in Fig. 4, the body, placenta, and organ weights of the hyperinsulinemic fetuses were heavier than their control counterparts with the exception of the kidneys. The ratio of body weight to placenta weight was no different between the two groups. There were no differences in the free-flowing umbilical arterial or venous blood gases between the groups with respect to P_{O_2}, P_{CO_2}, or pH.

DISCUSSION

Ep is the major hormonal regulator of erythropoiesis in the mammalian fetus and infant (16). In the present study, Ep was found to be elevated in umbilical plasma at delivery of infants of diabetic mothers. In one-third

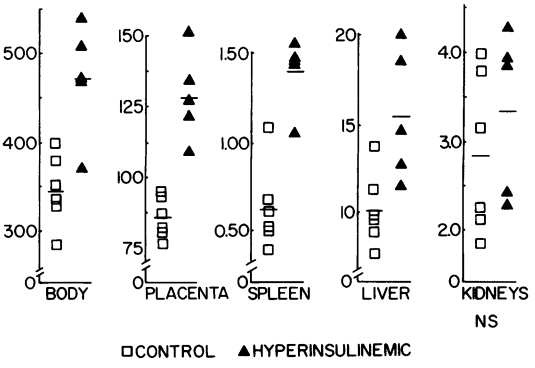

FIGURE 4 Rhesus fetus somatic ($P < 0.003$), placenta ($P < 0.001$), and organ (spleen [$P < 0.001$], liver [$P < 0.015$], and kidneys) weights (in grams) at delivery. (See legend for Fig. 2.)

of the infants studied, plasma Ep at delivery exceeded the range of values observed in normal infants. Three values were among the highest previously reported by either bioassay or the more sensitive radioimmunoassay used here. There was no relationship observed between plasma Ep and maternal insulin therapy or maternal vascular disease. At least two important questions arise from this observation: (*a*) what factors determined this abnormality in utero and (*b*) what is the biological significance of this elevation of Ep?

The fetus and newborn of the diabetic mother are subject to the altered maternal substrate milieu that can affect a variety of developing fetal systems (17). Most data have been interpreted to support the concept that maternal hyperglycemia results in fetal hyperglycemia, which stimulates fetal insulin production and release (5). Fetal hyperinsulinemia in the presence of adequate substrate produces macrosomia and selective organomegaly (7). Although several of the diverse complications of the infant of the diabetic mother have been related to fetal hyperinsulinemia, the etiology of some problems including polycythemia and extramedullary erythropoiesis has remained obscure (17). The finding of elevated plasma Ep in IDM and the correlation of umbilical plasma Ep with umbilical insulin in control and class A and A/B IDM may be interpreted as evidence supporting another role for fetal hyperinsulinemia.

In a small study of eight IDM, Finne could not detect any abnormality in umbilical plasma Ep using a mouse bioassay system (18). In contrast, the present study included several infants whose Ep levels, when measured by both polycythemic mouse bioassay and radioimmunoassay, were extraordinarily high. It is unclear why certain infants had such high levels. As yet there is no evidence to support the possibility of an increased Ep production in contrast to a relative decrease in Ep receptors, which would permit the accumulation of exceptionally high circulating levels. The differences in magnitude of Ep levels observed in the IDM subjects compared with the hyperinsulinemic rhesus fetuses may be related to species differences, the limited number of rhesus fetuses studied, or unknown adverse uteroplacental factors present in the human diabetic subjects.

Unlike the human IDM, the experimental fetal monkey has been made hyperinsulinemic in utero within a metabolically normal mother. Under these conditions, it is unlikely that fetal hypoxia occurs secondary to maternal/placental insufficiency. However, the increased erythropoiesis and elevated Ep levels observed in the hyperinsulinemic fetal monkeys might result from fetal hypoxia secondary to hyperinsulinemia. Carson et al. (19) have recently observed that hyperinsulinemic fetal sheep have a progressive decrease in arterial oxygen content over several days possibly due to increased fetal and/or placental oxygen consumption or to a decrease in umbilical blood flow (19).

Since the hyperinsulinemic monkey fetus is macrosomic and has hepatosplenomegaly (7), a hypoxic stimulus for Ep production may occur secondarily to the increased cellular proliferation. Alternatively, Ep production in the fetus might be directly stimulated by insulin. Golde et al. (20) have demonstrated that a variety of nonhematologic hormones, e.g. growth hormone, thyroxin, and prolactin may stimulate murine and human bone marrow erythroid progenitors in vitro (20). In addition, insulin alone was shown to have some stimulatory effect in vitro on Friend erythro-leukemia cells. Zanjani et al. (21) have recently demonstrated in vivo that the increased erythropoiesis produced by testosterone and thyroxine in the fetal sheep is Ep mediated (21).

In the present studies, the hyperinsulinemic rhesus fetus with increased erythropoiesis and Ep levels had low plasma glucagon concentrations. Naets and Gans (22) have suggested from recent studies of rats and mice that increased glucagon may have an inhibitory role in the control of erythropoiesis (22). Whether decreased glucagon would result in enhanced erythropoiesis in the presence of Ep has not yet been established.

Studies involving extirpation of the kidneys and/or liver in the sheep fetus have localized the site of Ep production to the liver (21, 23, 24). However, there is little data from direct organ extraction of Ep in the fetus. In contrast, the adult can produce Ep from extrarenal sites under unusual conditions (25).

In the present study, the elevated Ep levels did not correlate with the RBC in either the IDM or the hyperinsulinemic fetal monkeys. This could be due to a mild, compensated hemolytic state and/or to ineffective erythropoiesis. The increased pulmonary excretion of carbon monoxide, an index of bilirubin production, observed by Stevenson et al. (26) in IDM may also reflect ineffective erythropoiesis or hemolysis. Although the hyperinsulinemic fetal monkeys had suggestive increases in erythropoiesis correlating well with increased Ep levels, measurements of erythrocyte production with ferrokinetics, and of erythrocyte survival may further elucidate this problem.

In summary, the rhesus and human umbilical plasma data on Ep levels presented here are most consistent with a secondary effect of fetal hyperinsulinemia on both Ep levels and erythropoiesis. This effect may be related to an increase in cellular proliferation and metabolism resulting in increased oxygen consumption. The fetus, being in a relatively hypoxic state compared to the adult may be more sensitive to changes in its internal environment. There is little direct evidence for fetal hypoxia in diabetic pregnancies in the absence of advanced vascular disease. The present data

are compatible with a major role for fetal insulin in the increased erythropoiesis of IDM.

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