125 I-Insulin Receptor Binding to Cord Blood Erythrocytes of Varying Gestational Age and Comparison with Adult Values

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Summary

The measurement of 125 I-insulin binding to erythrocytes from 34 samples of cord blood (% B/T = 11.2 ± 3.8) showed statistically significant differences when compared to adult erythrocytes (% B/T = 6.6 ± 1.7). Preterm erythrocytes (% B/T = 14.7 ± 3.4) bound significantly more insulin when compared to term erythrocytes (% B/T = 10.1 ± 3.2). The negative correlation ($r = -0.613$; $P = 0.001$) between insulin binding and gestational age indicates that the trend during the final trimester of gestation is toward the lower binding observed for adult cells.

The major differences in binding between adult and term cord blood erythrocytes may be primarily attributed to differences in the number of binding sites per cell. Additionally, there are small differences in both affinity and number of sites per cell in preterm as compared to term and adult erythrocytes.

Speculation

The mechanisms governing insulin release from the pancreas are not mature *in utero*. Insulin released directly into the circulation from the fetal pancreas may be bound to tissue receptors in relation to the affinity and concentration of specific surface receptors, thereby, providing direct control at the cellular level for rapidly growing fetal tissues. Erythrocyte receptors may serve to transport insulin for this purpose.

Fetal growth and development is influenced by the quantitative presence of insulin as evidenced by fetal overgrowth in hyperinsulinemia and growth retardation in the absence of insulin (8). Insulin has been detected in fetal pancreatic islets 12 wk after conception (9), and the extractable pancreatic insulin exhibits a progressive rise during gestation (12, 17). Serum insulin levels in newborns are increased compared to normal adult values (12); however, in contrast to pancreatic insulin, serum insulin concentration does not correlate with gestational age (9). The insulin receptor on mononuclear leucocytes from term cord blood exhibits a higher affinity for insulin and has more sites per cell than receptors from adult mononuclear leucocytes (18). Little information is available concerning the ontogeny of the receptor for insulin through which the direct action of the hormone is mediated. The following study provides an assessment of the ontogeny of insulin receptors utilizing cord blood erythrocytes. The binding of insulin to erythrocytes was first reported in 1954 (7), but has been investigated infrequently until recent improvements in methodology reduced the high nonspecific binding (6). The circulating erythrocyte provides an easily obtainable homogeneous cell system which may reflect insulin binding activity in other tissues not ordinarily available for survey.

MATERIALS AND METHODS

SUBJECTS

Thirty-four cord bloods were collected during routine deliveries on the University of Arkansas for Medical Sciences Obstetrics Service. The gestational ages ranged from 33-42 wk as ascertained by Dubowitz scoring (3). The type of delivery (vaginal vs. Caesarean section), head circumference, weight, and length were recorded for each subject. Fifteen healthy, fasting adults whose ages ranged from 25-45 yr donated blood for comparative studies.

HORMONE

Porcine insulin (Eli Lilly Company) was iodinated to a specific activity of 125-150 μ Ci/ μ g by the stepwise addition of Chloramine-T (4, 15). Purified 125 I-insulin was separated from the iodination mixture by chromatography on Sephadex G-50 and frozen at -20°C . An aliquot of 125 I-insulin was repurified chromatographically before each assay.

PREPARATION OF CELLS

Whole blood samples (5-10 ml) were collected in EDTA tubes and refrigerated for no longer than 3 days before assay. The hematocrit was determined for each sample. After centrifugation, each serum was frozen and assayed for insulin at a later date. The red cells were diluted 1:1 with saline, layered on isolymp (8 ml RBC:3 ml isolymp: Gallard-Schlesinger Chemical Mfg. Corp), and centrifuged for 20 min, $400 \times g$ at 20°C . The gradient step was repeated with red cells which passed through the first isolymp gradient. The red cells were washed 1:2 with assay buffer (see *Binding Studies*) and diluted in buffer to a concentration of 3.75×10^9 cells/ml as determined by hemocytometer count.

BINDING STUDIES

1.5×10^9 cells were incubated in 16×100 mm polystyrene test tubes in a shaking water bath for 150 min with 0.2 ng/ml 125 I-insulin and increasing concentrations of unlabeled insulin at 15°C in a total final volume of 0.5 ml of assay buffer [tris-(hydroxymethyl) methylamine, 50 mM; 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, 50 mM; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 10 mM; CaCl_2 , 10 mM; ethylenediaminetetracetic acid, 2 mM; dextrose, 10 mM; NaCl, 50 mM; and KCl, 5 mM; 0.1% BSA], pH 8.0. The osmolarity of the buffer was determined to be 284 mOsm/kg. After incubation, 0.4 ml of the incubation mixture was removed from each assay tube and layered on an ice cold, biphasic medium containing 0.4 ml assay buffer and 0.4 ml dibutyl phthalate. After centrifugation for 3 min at 12,000 rpm, 4°C , the supernatant was aspirated

and the radioactivity in each cell pellet was determined on a Packard auto- γ scintillation spectrometer at ¹²⁵I-settings.

ADDITIONAL STUDIES

Insulin levels were determined by radioimmunoassay (20). Glucagon was obtained from Eli Lilly Company and growth hormone was obtained from the National Pituitary Agency. The data were analyzed on a DEC system-10 computer.

RESULTS

Investigation of the intrassay variability showed an average coefficient of variation of 3.6%. Two or three different samples drawn from four of the adult controls on different days gave an interassay coefficient of variation of 7.5%. Nonspecific binding was defined as the radioactivity that remained bound in the presence of 10,000 ng/ml of unlabeled insulin and was subtracted from all data points. For all samples included in the study, the average nonspecific binding was $1.7 \pm 0.8\%$ of the total ¹²⁵I-insulin available.

The percent of ¹²⁵I-insulin bound to cord blood erythrocytes in the absence of cold insulin (B_0) is illustrated as a function of gestational age in Figure 1. The negative correlation ($r = -0.613$; $P < 0.001$) reflects an apparent decrease in insulin binding with an increase in gestational age. Increasing concentrations of cold insulin resulted in decreased binding of ¹²⁵I-insulin as demonstrated in Figure 2, whereas the presence of increasing concentrations (1–1000 ng/ml) of glucagon or growth hormone did not inhibit binding of ¹²⁵I-insulin.

The Scatchard plot (13, 18) of the binding data, Figure 3, was analyzed for relative numbers of binding sites per cell by extrapolating the last 2 points to the abscissa intercept (R_0), converting the moles of insulin bound into number of insulin molecules bound [dividing by the cell concentration, and multiplying by the number of molecules per mole (1).] The results are given in Table 1.

An average affinity constant ($\bar{K}$) was calculated (2) and plotted against the fractional occupancy ($\bar{Y}$) resulting in an average affinity profile. The average affinity of insulin binding sites in the

empty configuration ($\bar{K}_e$), and the average affinity for filled sites ($\bar{K}_f$) determined from the average affinity profiles are shown in Table 1.

The sex of the newborn and the type of delivery did not significantly influence the binding of insulin to cord blood eryth-

Fig. 2. Comparison of specific ¹²⁵I-insulin binding to erythrocytes from preterm ($N = 8$) and term ($N = 26$) cord blood, and adult blood ($N = 15$). Erythrocytes (1.5×10^9 cells) were incubated for $2\frac{1}{2}$ hr at 15°C in the presence of 100 pg of radiolabeled insulin and increasing concentrations of cold insulin in a total volume of 0.5 ml.

Fig. 1. Specific ¹²⁵I-insulin binding to cord blood erythrocytes in relation to gestational age. Twenty-five B_0 determinations are included in addition to the 34 samples described in the text. The calculated regression ($r = -0.61$; $P < 0.0001$) is included for the 59 samples.

Fig. 3. Scatchard analysis of the data in Figure 2. The ratio of bound to free insulin is plotted as a function of the molar quantity of insulin bound.

Table 1. Comparison of adult and cord blood: affinity and number of insulin receptors

	Average affinity constant $\times 10^8 \text{ M}^{-1}$		Sites per cell
	K_e (empty sites)	K_f (filled sites)	
Adult	3.75	1.20	32
Cord blood			
Preterm	5.27	2.00	54
Term	4.02	1.20	46

rocytes. Although height, weight, head circumference, hematocrit, and serum insulin were analyzed for correlation with insulin binding, the only measurements approaching statistical significance of the correlation were height ($r = -0.4$; $P < 0.01$) and weight ($r = -0.4$; $P = 0.01$) in all 34 newborns and the hematocrit in the 26 term cord bloods ($r = 0.5$; $P = 0.015$).

Statistically significant differences ($P < 0.001$) are noted when ^{125}I -insulin binding to adult erythrocytes at the B_0 point (% B/T = 6.6 ± 1.7) is compared to binding of either preterm (% B/T = 14.7 ± 3.4) or term (% B/T = 10.1 ± 3.2) erythrocytes. Preterm erythrocytes bind significantly more insulin when compared to term erythrocytes ($P = 0.001$).

DISCUSSION

Measurement of insulin binding to erythrocytes provides an easily accessible cell type for assessment of insulin receptors. The decreased blood sample volume required for this assay provides an opportunity for conducting population surveys to define normal insulin binding and to delineate clinical manifestations concomitant with abnormal binding parameters. The survey of cord blood erythrocytes described here corroborates the report of increased insulin binding to cord blood mononuclear leucocytes (18) and, in addition, provides evidence that binding to preterm cord blood erythrocytes is higher than binding to term cord blood erythrocytes.

The average nonspecific binding for all samples in this study ($1.7\% \pm 0.8$) represent a substantial improvement over previously reported values for erythrocyte insulin binding studies (5, 6), and may be attributed to the repurification of the ^{125}I -insulin label immediately before use in this assay system. The coefficients of variation for both inter- and intrassay variability indicate that this assay system is highly reproducible. The mean percent binding at B_0 for the 15 normal adults determined in this study ($\bar{X} = 6.6 \pm 1.7$) compares favorably with the mean percent binding for the 11 adults initially reported by Gambhir *et al.* (6) ($\bar{X} = 7.2 \pm 1.4$) under identical assay conditions.

The significant negative correlation between gestational age and insulin binding indicates that the trend during the final trimester

of gestation is toward the lower binding observed for adult cells. Comparison of adult and cord blood values verify a significantly increased binding to cord blood erythrocytes. The difference is accentuated when cord blood samples are grouped according to term (≥ 38 wk) and preterm (< 38 wk) gestational ages.

The binding of insulin (B_0) to erythrocytes from term cord bloods is approximately 55% higher than binding to adult erythrocytes. This finding parallels to a lesser extent the 5-fold increase of insulin binding to cord blood mononuclear leucocytes compared to adult mononuclear leucocytes (18). Although the adult controls were fasting when sampled in both studies, it is doubtful that the increased binding observed with cord blood cells could be explained by increased blood glucose concentrations, because reported modification of insulin receptor binding after oral glucose ingestion (10) is minor compared to the observed differences between fasting adult and cord blood receptors.

The increased binding observed on cord blood erythrocytes may be related to an increase in surface area of the individual cells. The normal mean diameter of adult erythrocytes ranges from 7.2–7.9 μ , whereas the relatively macrocytic cord blood erythrocytes range from 8.2–8.6 (19). The surface area of a cord blood erythrocyte is approximately 25% greater than an adult erythrocyte and may account for part of the observed 43% increase in the number of sites per cell. The diameter of a fetal erythrocyte gradually decreases from 10.5 μ at 12 wk to 8.5 μ at 34 wk (11) and then remains constant until birth. Cord samples in this study range from 33 wk through term and, therefore, the negative correlation of insulin binding and gestational age should not be a reflection of decreasing cell surface area. The *in vivo* cell age is decreased in cord blood cells and the greater mobility of the membrane (14), or another related parameter may also account for part of the increased binding.

The number of sites per erythrocyte determined in this study is several orders of magnitude less than the number reported for other cell types, and is 1.6×10^2 lower than the 2000 sites per erythrocyte reported by Gambhir *et al.* (5). Determination of the number of sites per cell is related to the highest concentrations of insulin in the assay system. Thus the difference can be accounted for by the fact that the determination of Gambhir *et al.* is based on binding in the presence of cold insulin concentrations up to, and including 10,000 ng/ml, whereas specific binding in this study is measured in the range of insulin concentrations up to, and including 100 ng/ml. Considering that insulin concentrations of greater than 100 $\mu\text{U/ml}$ (4.2 ng/ml) exceed normal physiologic levels, it is doubtful that interactions of the receptor with insulin concentrations exceeding 100 ng/ml ($1.67 \times 10^{-8} \text{ M}$) play an important physiologic role. Reinterpretation of the data of Gambhir *et al.* excluding insulin concentrations exceeding 100 ng/ml results in less than 100 sites per cell. These figures more closely correspond to the 25 sites per rabbit erythrocyte reported under equilibrium conditions of prolonged incubation at 0°C (16).

The binding data were analyzed to determine the average affinity constants so as to allow comparison between groups (Table 1). Both the K_e and K_f of the preterm erythrocytes are increased compared to adult and term cells. The major differences in binding between adult and term cord blood erythrocytes may be primarily attributed to differences in the number of binding sites per cell. Measurements of binding parameters of preterm cells, however, do show small differences in both affinity and number of sites per cell. The K_f determined for adult and term cord blood erythrocytes ($1.2 \times 10^8 \text{ M}^{-1}$) corresponds well with the K_f reported by Thorsson and Hintz (18) for adult and cord blood mononuclear leucocytes (approximately $1.3 \times 10^8 \text{ M}^{-1}$). Investigation and comparison of the kinetics of the binding process can provide further information on the nature of the observed differences between preterm, term, and adult erythrocytes.

The negative correlation of insulin binding with height and weight may be attributed to the high correlation of these measurements to increasing gestational age. The relationship between these measurements and insulin binding may become more meaningful when larger numbers of samples are reported. The influence

of the hematocrit on the insulin binding reaches the highest correlation with term erythrocytes ($r = -0.48$; $P = 0.015$). The correlation of hematocrit and insulin binding values for adult and preterm samples are not significant, however, and no conclusions can be drawn on the basis of the data available from this study.

The lack of correlation between plasma insulin and insulin binding to erythrocytes confirms the reported observations on cord blood monocytes (18). The lack of inhibition by growth hormone and glucagon in this assay system verified previous reports of the lack of crossreactivity of the erythrocyte insulin receptor (6, 16). Both somatotropin and human chorionic gonadotropin have also been ruled out as possible inhibitors (6), although this does not preclude the possibility that the same receptor may recognize other, as yet unidentified, molecules.

The number of days or months after birth required for the binding to drop to adult levels has not been determined. Age-related studies of insulin binding for infants are possible using erythrocytes because the data can be obtained from a small sample of blood. B_0 and nonspecific binding points can be measured on approximately a ml of blood. Data for newborns as well as more samples of preterm cord bloods are necessary to substantiate the negative association between gestational age and insulin binding.

REFERENCES AND NOTES

- DeMeyts, P.: Insulin and growth hormone receptors in human cultured lymphocytes and peripheral blood monocytes. In: Blecher M.: *Methods in Receptor Research*. Part 1, pp 301-383 (Marcel Dekker, Inc., New York, 1976).
- DeMeyts, P., and Roth, J.: Cooperativity in ligand binding: a new graphic analysis. *Biochem. Biophys. Res. Commun.*, **66**: 1118 (1975).
- Dubowitz, L., Dubowitz, V., and Goldberg, C.: Clinical assessment of gestational age in the newborn infant. *J. Pediatr.*, **77**: 1 (1970).
- Freychet, P., Roth, J., and Neville, D. M.: Monoiodoinsulin: demonstration of its biological activity and binding to fat cells and liver membranes. *Biochem. Biophys. Res. Commun.*, **43**: 400 (1971).
- Gambhir, K., Archer, J., and Bradley, C.: Characteristics of human erythrocyte insulin receptors. *Diabetes*, **27**: 701 (1978).
- Gambhir, K., Archer, J., and Carter, L.: Insulin radioreceptor assay for human erythrocytes. *Clin. Chem.*, **23**: 1590 (1977).
- Haugaard, N., Haugaard, E., and Stadie, W.: Combination of insulin with cells. *J. Biol. Chem.*, **211**: 289 (1954).
- Hill, D.: Insulin and fetal growth. In: New M. I. and Fisher R. H.: *Diabetes and Other Endocrine Disorders During Pregnancy and in the Newborn*. pp 127-139 (Alan R. Liss, Inc., New York, 1976).
- Kaplan, S., Grumbach, M., and Shepard, T.: The ontogeny of human fetal hormones. I. Growth hormone and insulin. *J. Clin. Invest.*, **51**: 3080 (1972).
- Muggeo, M., Bar, R., and Roth, J.: Change in affinity of insulin receptors following oral glucose in normal adults. *J. Clin. Endocrinol. Metab.*, **44**: 1206 (1977).
- Oski, F.: Hematological problems. In: Avery G. B.: *Neonatology: Pathophysiology and Management of the Newborn*. pp 379-415 (J. B. Lippincott, Co., Philadelphia, 1975).
- Rastogi, G., Letarte, J., and Fraser, T.: Immunoreactive insulin content of 203 pancreases from fetuses of healthy mothers. *Diabetologia*, **6**: 445 (1970).
- Scatchard, J., Coleman, J., and Shen, A.: Physical chemistry of protein solutions: VII. The binding of some small anions to serum albumin. *J. Am. Chem. Soc.*, **79**: 12 (1957).
- Schekman, R., and Singer, S.: Clustering and endocytosis of membrane receptors can be induced in mature erythrocytes of neonatal but not adult humans. *Proc. Natl. Acad. Sci. USA*, **73**: 4075 (1976).
- Schneider, B., Straus, E., and Yalow, R.: Some considerations in the preparation of radioiodoinsulin for radioimmunoassay and receptor assay. *Diabetes*, **25**: 260 (1976).
- Shinomiya, Y., Hata, M., Ino, T., Nomura, S., Morimoto, H., Takashima, S., Kobayashi, K., Kagawa, S., and Matusoka, A.: Erythrocyte-insulin interaction. *Jpn. J. Clin. Pathol.*, **25**: 3 (1977).
- Steinke, J., and Driscoll, S.: The extractable insulin content of pancreas from fetuses and infants of diabetic and control mothers. *Diabetes*, **14**: 573 (1965).
- Thorsson, A., and Hintz, R.: Insulin receptors in the newborn. *N. Engl. J. Med.*, **297**: 908 (1977).
- Wintrobe, M.: *Clinical Hematology*. 6th edition, p. 93, (Lea and Febiger, Philadelphia, 1967).
- Yalow, R., and Berson, S.: Immunoassay of endogenous plasma insulin in man. *J. Clin. Invest.*, **39**: 1157 (1960).
- All numbers preceded by $\pm$ refer to 1 SD from the mean unless otherwise specified.
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