INSULIN BINDING IS A SPECIFIC MARKER OF FETAL ERYTHROCYTES IN RUMINANTS\textsuperscript{1,2,3}

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Summary

The ability of erythrocytes (RBC) from sheep and cattle of various gestational and postnatal ages to bind insulin specifically was studied. Insulin binding to RBC decreased as gestational and postnatal age advanced and was absent in blood obtained from adult animals. Maximal percentage \(12^sI\)-insulin bound to RBC (3.6 \( \times \) \( 10^9 \)/ml) was highest in the fetuses of sheep and cattle (7.3 \( \pm \) .6 and 7.8 \( \pm \) .9, respectively) compared to postnatal animals (2.3 \( \pm \) .2 and 2.2 \( \pm \) .3, respectively), or adults (no binding) of the same species. The decrease in binding began antenatally, and binding was projected to be insignificant by the end of the second postnatal month. Most of the observed decrease was due to a progressive decrease in the number of receptors on the cell surface. The time course of this phenomenon, as well as the total absence of insulin receptors on the RBC of adult ruminants, provides independent evidence that two distinct populations of RBC in ruminants exist. The gradual appearance of the adult RBC with no insulin binding results in a decrease in observed binding to RBC in a given blood specimen as fetuses and postnatal animals age.

(Key Words: Ruminant Erythrocytes, Insulin Binding, Erythrocyte Markers.)

Introduction

Two distinct populations of erythrocytes (RBC) are present in fetal and postnatal ruminant animals, but only one type is present in older animals or adults. Studies by Perk et al. (1964) in sheep and Frei et al. (1963) in cattle showed that fetal-type RBC were distinguishable from adult-type RBC on the basis of differing osmotic fragilities. As the animals aged, the more osmotically stable fetal RBC gradually disappeared, leaving only the more fragile adult-type RBC. Evans and Blunt (1961) also suggested that two populations of RBC exist in fetal and postnatal sheep based on RBC Na and K concentrations, but only one type in adults. The fetal-type RBC was absent by approximately 8 wk of postnatal age.

Studies in this (Kappy et al., 1981) and another (Sinha et al., 1981) laboratory have shown that insulin binding to the RBC of sheep decreases throughout fetal and postnatal life, and that the RBC from adult sheep do not bind insulin, in contrast to those from adults in non-ruminant species (Ginsberg et al., 1977; Herzberg et al., 1978; Kappy and Plotnick, 1979). We have extended our earlier research with sheep (Kappy et al., 1981) and have now studied changes in osmotic fragility and insulin
binding in the RBC of another ruminant, cattle, to further document that insulin binding is specific to fetal RBC in ruminants. This would establish a distinct difference between ruminant and nonruminant species.

**Experimental Procedure**

**Animals.** Thirty-five time-dated fetal sheep (113 to 134 d gestation), 16 lambs (1 to 21 d) and 10 adult sheep were studied. In preparation for the fetal studies, anesthesia was induced in time-dated ewes (108 to 129 d gestation) with halothane and maintained with halothane, N\textsubscript{2}O and O\textsubscript{2}. Following a midline incision in the maternal abdomen, hysterotomy over the appropriate fetal part allowed placement of polyvinyl catheters in a fetal pedal or femoral artery. All catheters were tunneled sc to an incision in the maternal flank where they were stored. Ewes were allowed a minimum of 4 d for recovery from the operative stress before fetal studies. Complete insulin binding curves were obtained on 34 fetuses, 14 lambs and 10 adult sheep. Eight fetuses and one lamb were studied serially over a 2 wk period.

Six bovine fetuses (210 to 246 d gestation), 18 calves (1 to 19 d old) and eight adult cattle (2 to 5 yr) were studied in a similar manner. Complete insulin binding data were obtained on all animals except for one calf. Seven calves were studied serially over a 2 wk period.

**Insulin Determinations.** Two milliliters of heparinized blood were collected at the same time as were samples for insulin binding studies. After centrifugation, the plasma was frozen for subsequent determination of insulin by the double antibody radioimmunoassay method using guinea pig antiporcine insulin and rabbit antiguinea pig serum as the first and second antibody, respectively (Morgan and Lazarow, 1963). Insulin concentrations were not determined for lambs or calves because of the difficulty in sampling them in a consistent relationship to their meals. Feeding had no effect on the binding of insulin to RBC.

**Erythrocyte Preparations.** Five to seven milliliters of blood were collected into sterile vacutainer tubes containing .7 ml citrate-phosphate-dextrose (Gibson et al., 1961) as an anticoagulant-preservative and stored for a maximum of 7 d at 4 C before assay. Previous studies showed that this treatment did not change insulin binding to RBC beyond the interassay variability of the method, and that 98% of the RBC were still viable (Moller, 1977).

Erythrocytes were separated from other blood cells by a modification of the method of Boyum (1968) as reported by Gambhir et al. (1977, 1978). The final RBC pellet was taken up in 50 mM Hepes buffer (Gambhir et al., 1978), pH 8.0 (on ice), to a volume of 3.5 ml. This suspension had a cell count of 6 to 12 \times 10\textsuperscript{9}/ml. Leukocytes and platelets were only rarely seen on Wright's stained smears of the RBC preparations. Reticulocytes were occasionally seen in RBC preparations from fetal animals only, but were never more than 1.1% of all RBC (mean = .4%). Binding of insulin by the RBC in fetal preparations with the two highest reticulocyte counts (1.0 and 1.1%) was no different than that in RBC preparations that had no reticulocytes.

**Osmotic Fragility Studies.** For osmotic fragility curve determinations .015 ml of each RBC suspension was added to duplicate test tubes containing 3.0 ml of decreasing concentrations (1.0 to 0%) of phosphate-buffered saline (PBS), pH 7.4. The suspensions were gently mixed several times by inversion, and then left at room temperature for 30 min. The tubes were remixed by inversion, and were centrifuged at 1,200 \(\times\) g for 10 min. The optical densities of the supernatants (hemolysates) were measured at 543 nm (hemoglobin), using the supernatant from the 1.0% PBS tube as a blank (no lysis). Percentage lysis for each blood sample was calculated as follows:

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\text{% lysis} = \frac{\text{OD}_{543} \times 100}{\text{OD}_{543} \text{ at } 0.0\% \text{ PBS} (100\% \text{ lysis})}
\]

The concentration of salt (PBS) that resulted in the lysis of 50% of a given RBC preparation (determined graphically) was designated the osmotic fragility 50 (OF 50) of the preparation.

**Radioiodination of Insulin.** Porcine insulin was kindly supplied by Dr. Robert Hosley. The \(^{125}\)I was purchased as labeled NaI. Insulin was iodinated by a modification of the method of Freychet et al. (1971) as described by Lesniak (1978). Monoiodinated insulin with a specific activity of 100 to 180 \(\mu\)Ci/\(\mu\)g was used for all binding studies. The \(^{125}\)I iodoinsulin was

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90 to 96% precipitable with trichloroacetic acid (TCA) and was greater than 80% precipitable in the standard insulin radioimmunoassay (Morgan and Lazarow, 1963).

**Incubation Procedure.** The incubation of RBC with insulin was performed in 12 × 75 mm sterile plastic test tubes. Each tube received 50 μl of either 50 mM Hepes buffer or unlabeled insulin stock solution, 50 μl tracer [125I] insulin (10,000 cpm) and 200 μl RBC suspension, for a total incubation volume of 300 μl. Twelve final insulin concentrations between .1 (tracer only) and 10 ng/ml were used. Tubes containing .1, 50 and 100 ng/ml were assayed in duplicate. Incubations were carried out routinely for 200 min at 15 C.

At the end of the incubation period, duplicate 100 μl aliquots were removed from each tube and placed in chilled 400 μl Microfuge tubes containing 200 μl dibutylphthalate. The tubes were centrifuged at 10,000 × g for 2 min. The RBC (containing the bound radioactivity) formed a pellet that was separated by the dibutylphthalate layer from the unbound radioactivity in the buffer layer from each aliquot. The buffer and dibutylphthalate layers were aspirated, and the RBC pellet was cut from the Microfuge tube and placed in a test tube for counting in a gamma counter. Intra- and interassay variability of the maximal percentage 125I-insulin bound were 3.2 and 9.5%, respectively.

Degradation of insulin by RBC occurring during the incubation was assessed by subtracting the percentage of TCA-precipitable counts in the supernatant buffer layer of two Microfuge tubes containing 200 μl dibutylphthalate. The tubes were centrifuged at 10,000 × g for 2 min. The RBC (containing the bound radioactivity) formed a pellet that was separated by the dibutylphthalate layer from the unbound radioactivity in the buffer layer from each aliquot. The buffer and dibutylphthalate layers were aspirated, and the RBC pellet was cut from the Microfuge tube7 and placed in a test tube for counting in a gamma counter. Intrarand interassay variability of the maximal percentage 125I-insulin bound were 3.2 and 9.5%, respectively.

Insulin Binding as a Function of Erythrocyte Concentration. In three separate experiments, RBC in final concentrations of 3.0 to 12.0 × 10⁸/ml were incubated with labeled insulin alone (total binding) and in the presence of 10⁵ ng/ml of unlabeled insulin (nonspecific binding) for 200 min at 15 C. In all binding studies, specific binding was taken as the difference between total and nonspecific binding. Specific binding increased linearly from 3.3 to 14.4% over the range of eight RBC concentrations from 3.0 to 12.0 × 10⁹/ml. In our studies, binding was measured at final RBC concentrations of 4.0 to 8.0 × 10⁹/ml. The results were all corrected to a final RBC concentration of 3.6 × 10⁹/ml for purposes of comparison with other studies.

**Calculations.** The mean maximal percentage 125I-insulin bound, receptor concentration and affinities for each group were calculated from values obtained for each individual in the group, as reported by DeMeyts and Roth (1975) and Gambhir et al. (1978), using the single receptor negative cooperativity theory of insulin binding. It is recognized that an alternative method of data interpretation based on two distinct receptors is also possible (Pollet et al., 1977; Olefsky and Chang, 1978), however, the conclusions reached using either method are identical. In the calculation of receptor concentration, the highest insulin concentration used was 100 ng/ml.

The significance of differences between means was calculated by Student's t-test.

**Results**

**Osmotic Fragility Studies.** The RBC osmotic fragility, expressed as the mean salt concentration that resulted in lysis of 50% of the RBC in a sample of blood (OFs0), increased with advancing developmental stage in both sheep and cattle (figure 1). The OFs0 of the RBC from adult sheep and cattle were greater than those from the fetuses (P's<.001).

The RBC osmotic fragility was measured serially in seven calves over a 2 wk period from a mean of 5 to 19 d old. The mean OFs0 of the RBC in these animals increased from .46 ± .02 gm/dl to .54 ± .02 gm/dl (P<.001).

**Insulin Binding Studies.** In both sheep and cattle, RBC binding of insulin decreased significantly during fetal and postnatal life. This was evident in both the cross-sectional (figures 2a and 3a) and longitudinal (figure 4) studies. The RBC from fetuses bound more insulin than did those from either lambs or calves (P<.001), while the RBC from adult animals of both species showed no specific binding of insulin (figures 2a and 3a). Postnatal animals of both species showed little binding by the end of the third week of life. A total loss of insulin binding to RBC by the end of the second postnatal month was predicted, based on both the cross-sectional and longitudinal studies.

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7 Beckman Industries, Palo Alto, CA.
Specific insulin binding of insulin by RBC was measured serially (on two occasions over a 2 wk period) in each of eight fetal sheep (from a mean of 121 to 135 d), one lamb (from 4 to 18 d) and seven calves (from a mean of 5 to 19 d). The data are shown in figure 4. All but two of the 16 animals showed decreasing binding of insulin to their RBC over the 2 wk interval. In 11 animals, the decrease was greater than the interassay variability of the method. The mean maximal percentage of $^{125}$I-insulin binding decreased from 7.7 ± 1.0 to 4.8 ± .7% in the sheep and from 3.3 ± .4% to 2.0 ± .3% in the calves during this interval. Both decreases were significant.

Further processing of the binding data by Scatchard analysis (DeMeyts and Roth, 1975) showed that the decrease in insulin binding during development was secondary to significant decreases in receptor sites on the RBC (figures 2b and 3b) from lambs and calves compared with fetal animals, with an absence of receptors on the RBC from adults. Decreases in empty-site binding affinity ($K_e$) for insulin during development (figures 2c and 3c) were also seen,
Figure 3. Binding characteristics in the RBC of cattle at three developmental stages. Height of the bar graph in each instance is mean ± SE. Numbers of animals in each group (fetal, postnatal and adult) are given in parentheses. Data are calculated from single measurements on each animal. a) Maximal percentage ¹²⁵¹-insulin bound at a final RBC concentration of 3.6 × 10⁹/ml. *P<.001, fetuses vs postnatal animals. b) Receptor concentration (sites/RBC). These data were determined by graphically establishing the maximal binding capacity (R₀) for each RBC preparation with a Scatchard plot (see Methods). *P<.001, fetuses vs postnatal animals. c) Empty-site (Kₑ) and full-site (Kᶠ) affinities of the RBC insulin receptor. The data were calculated according to the method of DeMeyts and Roth (1975).

Discussion

A significant increase in RBC osmotic fragility occurred as the animals aged. Although we did not observe a bimodal distribution of osmotic fragilities in our RBC preparations, this was probably due to the fact that our technique was not as sensitive as the continuous infusion chamber used by previous authors (Frei et al., 1963; Perk et al., 1964). In any event, the gradual increase in RBC osmotic fragility suggests that old cells are being continually replaced by more fragile younger cells as the animals age.

Consistent with the studies of Evans and Blunt (1961), Frei et al. (1963) and Perk et al. (1964), the results of osmotic fragility studies suggest that an adult-type RBC with increased osmotic fragility is released from the marrow in late fetal life and gradually becomes the sole RBC type at approximately 8 wk of postnatal age.

A gradual decrease in insulin binding by RBC also occurred as the animals aged, and no specific insulin binding by RBC was present in the adult animals. This supports the hematologic evidence that an adult-type RBC gradually replaces a fetal-type RBC in the whole blood of ruminants with advancing age.

It has been suggested that binding differences between human neonates and adults could be due to the relatively larger size of the RBC in cord blood when compared with the RBC in the adult, or due to the greater contribution of "younger" RBC, including reticulocytes, to the total RBC population in the neonate (Thompson et al., 1978; Kosmakos et al., 1979; Polychronakos et al., 1982). Although it has been shown that reticulocytes and younger (less dense) RBC bind more insulin than do more mature RBC (Thompson et al., 1978;
Figure 4. Maximal percentage $^{125}$I-insulin bound in the blood of the same animal on two occasions, 2 wk apart. Nine sheep (●) (eight fetuses and one lamb), and seven calves (○) were studied. The eight fetal sheep were 113 to 126 d gestation (mean = 121 d) at the start of the experiment, and the lamb was 4 d old. The seven calves were 1 to 7 d old (mean = 5 d) at the start of the experiment.

Baumann et al., 1979; Kosmakos et al., 1979; Eng et al., 1980; Polychronakos et al., 1982), the differences in binding only become significant when reticulocytes are greater than 5% of the RBC in a given preparation (Thomopoulos et al., 1978; Kosmakos, et al., 1979; Eng et al., 1980). Reticulocyte counts of that magnitude were not found in our present study (maximum 1.1%), in other sheep studies (Ullrey et al., 1965; Upcott et al., 1971) or in our previous study in humans (Kappy and Plotnick, 1979). The RBC preparations showing the two highest reticulocyte counts (1.0 and 1.1%) were found in twin sheep fetuses of a gestational age of 127 d. Neither the maximal percentage $^{125}$I-lodoin- insulin bound nor the receptor concentration in these preparations was greater than those in corresponding age-matched fetuses whose RBC preparations had no demonstrable reticulocytes. In addition, if the youngest RBC bound insulin avidly, even the adult animals’ blood should have shown some insulin binding, but such was not the case. Thus it is unlikely that the occasional presence of reticulocytes in some RBC preparations had any measurable effect on the binding data.

Size differences between the RBC of the fetus vs that of the postnatal animal could account for some of the observed differences in binding, but the maximal ratio in surface areas between their RBC calculated from the data of Karvonen (1954), Ulrey et al. (1965) and Upcott et al. (1971) would only be 1.3 to 1.4, whereas the fetal cells bound two to three times as much insulin and had twice as many receptors. Thus, neither changes in the size (surface area) nor age of the RBC in the fetuses as compared with that of the postnatal animals and adults is likely to account for the observed differences in insulin binding.

From data in the present and previous studies (Kappy et al., 1981; Sinha et al., 1981), a total loss of receptors on RBC could be predicted to occur at approximately 8 wk of postnatal life. This is an excellent agreement with the previously cited hematologic studies that suggested that the adult-type RBC was the sole type present after 8 wk of age. Thus, specific insulin binding is a definitive marker of the fetal RBC in sheep and cattle blood. Estimations from our data suggest that fetal RBC could be detected if they represented as little as 5% of the total RBC in a blood specimen, because the specimen would then show approximately .4% maximal specific binding of insulin, the lowest meaningful value in our assay.

The lack of insulin binding to the circulating RBC of adult ruminants is in distinct contrast to the situation in several nonruminant species. Insulin binding to RBC does decrease with age of the cell and the age of the animal in non-ruminant species, but the binding in the adult RBC is still 50 to 60% that of the RBC at birth. This has been demonstrated for humans (Kappy and Plotnick, 1979; Herzberg et al., 1980; Hendricks et al., 1981), guinea pigs (Herzberg et al., 1978), turkeys (Ginsberg et al., 1977), and monkeys, rats and dogs (M. S. Kappy, unpublished data).

The significance of these findings (other than hematologic) is not clear. The loss of insulin binding in RBC begins antenatally, i.e., before the introduction of the ruminant diet to the young animals. In addition, the loss of
binding is complete by the time the final transition from monogastric to ruminant type metabolism occurs (Ralston, 1971). In other words, a transition occurs from a period when glucose and amino acids constitute the major metabolic fuels to one in which volatile fatty acid utilization predominates. This is not the case in nonruminant species.

Thus, the decline in insulin binding to ruminant RBC might reflect a genetically preprogrammed transition in other tissues to a relatively low insulin-requiring state in the animals, because insulin action would favor the utilization of carbohydrates and amino acids as opposed to volatile fatty acids. The research of Bartos and Skarda (1970) using the adipose tissue of goats is in keeping with this interpretation of the binding data. They showed a gradually decreasing ability of adipose tissue in the goat to synthesize fatty acids from glucose and an increasing ability to synthesize them from acetate, as the goats aged from 10 to 120 d of age. Furthermore, the ability of insulin to stimulate fatty acid synthesis by adipose tissue decreased with increasing age of the animals and was negligible by 120 d of age.

We are currently examining the changes in insulin binding during fetal life using an insulin target tissue (liver) to determine whether or not the observed changes in the RBC are significant from other than a hematologic standpoint.

**Literature Cited**


