Insulin Binding and Response in Erythroblastic Leukemic Cells

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SUMMARY

Mammalian erythrocytes have been shown to bind, but not to respond to, physiologic doses of insulin. Insulin binding was studied in normal rat erythrocytes and erythroblastic leukemic (EBL) cells by standard ^{125}I-insulin competitive binding assays. EBL cells exhibited marked insulin degradation, which was time, temperature, and concentration dependent and was mediated by both cell-bound and soluble enzymes. Bacitracin or bovine serum albumin was used to inhibit such degradation in a dose-dependent fashion to allow meaningful data analysis. Insulin binding studies showed a greater than 10-fold increase of specific binding to EBL cells compared with erythrocytes. Scatchard analysis was consistent with increases predominantly in the number of receptors on EBL cells. Concordant with increased insulin binding, EBL cells demonstrated increased transport of α-aminoisobutyric acid and increased incorporation of uridine into ribonucleic acid in response to physiologic doses of insulin (100 μU/ml). It can be concluded that EBL cells may serve as useful models of erythroblasts to explore the relationships between insulin binding, response, and cell maturation. DIABETES 29:571–578, July 1980.

Despite the demonstration of specific insulin receptors on human erythrocytes,[1–3] little is known concerning their function. In the differentiated cell, aging has been reported to decrease, increase, and have no effect on the number of insulin receptors.[4–6] However, in the Friend erythroleukemia cell, dimethyl sulphoxide-induced differentiation is associated with a decrease in insulin receptor number.[7] From these observations we inferred that immature erythroblasts, which are producing specialized proteins and are metabolically active, may require the action of insulin. Following maturation, the erythrocyte loses its nucleus and assumes an essentially passive transport function, which appears to be unaffected by physiologic levels of insulin. Thus, the coupling of insulin binding to response may be lost during maturation.

Ideally, we would have wished to test this hypothesis in the normal erythroblast. While it is possible to culture erythroblasts, their use was precluded in this study by the problems attendant on culture of sufficient yields for the relatively large numbers of cells required for binding studies. The erythroblastic leukemic (EBL) cell shows morphologic similarities to normal erythroblasts. In addition, it has been shown that EBL cells have three sodium-dependent amino acid transport systems, the reticulocyte has two, and the mature erythrocyte none.[8] However, the peculiar permeabilities of the erythrocyte to water and nonelectrolytes are mirrored in the EBL cell.[9] The EBL cell was therefore selected as the best available model of the normal erythroblast to test the above hypothesis with respect to insulin binding and response. The study described below was essentially performed in two stages: to demonstrate the existence of insulin receptors on the EBL cell and to demonstrate receptor-response coupling to biologic end points.

MATERIALS AND METHODS

Preparation of EBL cells. Tumor cells were induced by pulse doses of 7, 8, 12 trimethylbenz (a) anthracine in Long-Evans rats and maintained by intraperitoneal inoculation of 1–5-day-old pups with frozen or fresh cells.[10] Animals were killed at about the tenth day and EBL cells isolated from hepatic sinusoids by pressing through a wire mesh (60-μm diameter) followed by washing in sodium-potassium Ringer's solution. After centrifugation at 400 *g* for 5 min, the cells were resuspended in 0.166 M ammonium chloride for 6 min to lyse contaminating erythrocytes and again centrifuged.

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The resulting cell pellet was resuspended in Ringer's and cell indices were measured.

Preparation of erythrocytes. Whole blood was obtained by cardiac puncture from adult Long-Evans rats and drawn into vacutainer tubes containing 143 U of heparin. Erythrocytes were isolated by density gradient centrifugation after the method of Boyum[11] as modified by Gambhir et al.[2].

^{125}I-insulin. Porcine monocomponent insulin was generously donated by Dr. Ronald Chance of Eli Lilly Research Laboratories (Indianapolis, Indiana) and carrier-free ^{125}I-sodium iodide purchased from New England Nuclear (Boston, Massachusetts). Iodination was modified after the method of Marchalonis[12] using lactoperoxidase coupled to insoluble beads (Bio-Rad, Lebanon, Pennsylvania), which yielded ^{125}I-insulin of specific activity 100–200 mCi/mg. Labeled insulin was desalted using Sephadex G-25 and stored at −70°C until use, when it was further purified by adsorption to, and elution from, a Whatman CF11 cellulose column[13] (Whatman, England). ^{125}I-insulin prepared as above was 98–100% precipitable in trichloroacetic acid (TCA) and greater than 95% precipitable by excess anti-insulin antibody.

Chemicals and reagents. Bovine serum albumin, fraction V, was purchased from Armour Pharmaceutical Co. (Phoenix, Arizona); dibutyl phthalate from A. R. Thomas Co. (Philadelphia, Pennsylvania), and [^{14}C]-α-aminoisobutyric acid and ^{3}H-uridine from New England Nuclear. Bacitracin (50,000 U/ml) was purchased from Upjohn Co. (Kalamazoo, Michigan), Penicillin G from Squibb Pharmaceuticals (Princeton, New Jersey), and gentamicin from Schering Pharmaceuticals (Kenilworth, New Jersey). All other chemicals were of reagent grade.

^{125}I-insulin binding assay. These were performed essentially after the method of Gambhir et al.[2] Briefly, cells were incubated at temperatures and times shown in the figures with 40 pg of ^{125}I-insulin in buffer "G" (Hepes/tris, pH 8.0, 300 mosmol/L) and varying concentrations of unlabeled insulin. The incubations were terminated by layering 200-μl aliquots of the cell suspension onto dibutyl phthalate at 4°C and centrifuging in an Eppendorf microfuge at 12,800 *g* for 2 min. Following aspiration of the supernatants, the cell pellets were cut into counting tubes containing 1 ml, 1 N sodium hydroxide and counted in a Beckman γ-counter. Nonspecific binding was defined as the percentage of ^{125}I-insulin bound in the presence of 10^5ng/ml of unlabeled insulin and was subtracted from total binding at each point to yield specific binding. Where indicated, insulin degradation was assessed by the percentage of counts soluble in 15% TCA. Cell viability as assessed by trypan blue dye exclusion[14] was always greater than 90% at the end of the incubations.

[^{14}C]-α-aminoisobutyric acid (AIB) transport. Transport was measured in sodium-potassium Ringer's solution[15], pH 7.4, 300 mosmol/L. One millimolar [^{14}C]-α-AIB (0.5 μCi) was incubated with 1.5×10^7 cells in a final volume of 1 ml at 37°C for 1 h, with a 10-min preincubation without the radiolabeled tracer. Insulin (50–200 μU/ml), when present, was added during preincubation and incubation. Incubations were terminated by washing cells in 2 ml choline Ringer's[15] at 4°C and centrifuging at 25,000 *g* for 2 min, following which supernatants were aspirated and cell pellets washed twice more. Cells were then solubilized in 0.5 ml of 0.5 N KOH for 1 h and counted in scintillation fluid in a β-scintillation spectrometer. Flux was measured at 5 min and steady state distribution ratios (SSDR) at 60 min.

^{3}H-uridine incorporation into RNA. Incorporation was measured essentially after the method of Jagus.[16] Cells were prepared as described above except that NH_4Cl erythrocyte lysis was omitted, which gave more reproducible results. Contaminating erythrocytes were <2% by differential count. Cells were incubated in 12 × 35-mm culture dishes (Falcon) in Eagles medium with Earles Salts (Gibco) supplemented with twice the concentrations of essential amino acids, five times the concentration of glutamine, 10% fetal calf serum (Sterile Systems), penicillin G (50 U/ml), and gentamicin (50 μg/ml), pH 7.4, osmolarity 310 mosmol/L. This media is that described by Ostertag et al.[17] and maintained the cells at a >95% viability for up to 7 h at 37°C in a 95% O_2/5% CO_2 atmosphere (Forma scientific incubator). Cells were incubated at 1×10^6/ml density, with a final volume of 2 ml, for 1–3 h, with and without insulin (100 μU/ml), which was supplemented with an additional equal dose at 2 h. This supplementation was adopted following a parallel incubation of cells with ^{125}I-insulin, which showed 50% degradation of insulin after 2 h incubation (assessed by TCA precipitability). One hour before harvesting, the cells were pulsed with 3–6 μCi/ml ^{3}H-uridine in different experiments. Following incubation, the dishes were put on ice, and following gentle blowing, the cells were harvested immediately by aspiration and placed into polypropylene tubes to minimize adsorption of RNA. The dishes were washed with 2 ml of ice-cold phosphate-buffered saline (pH 7.4, 0.15 M). Cells were centrifuged at 400 *g* at 4°C for 5 min.

The supernatants were aspirated and the pellet washed twice more in 2 ml of ice-cold phosphate-buffered saline, following which 2 ml of 0.5 N perchloric acid (PCA) was added to the cell pellet, and the tubes were left at 4°C for approximately 18 h. Following centrifugation, the supernatants were removed; the pellets were incubated with 3 ml of 0.3 N potassium hydroxide at 37°C for 1 h, cooled, and reprecipitated with 1 ml of 2 N PCA at 4°C. Aliquots of supernatants were taken for scintillation counting and for estimation of total RNA by spectrophotometry against standards of yeast RNA.

Calculations and statistics. For insulin binding studies $4.5-5 \times 10^9$/ml erythrocytes were used as previously reported.[2] EBL cell binding assays were run at 4×10^7 cells/ml to enable sufficient binding for counting accuracy commensurate with greatest economy of the limited numbers of EBL cells available. AIB transport assays were run at the EBL cell density previously reported[8] and uridine incorporation assays at 1×10^6/ml, which provided sufficient counts for accuracy while ensuring reproducible cell viability during primary culture.

Cell concentrations and hematocrits were measured on a Coulter counter. Mean corpuscular volumes and radii were calculated from the above indices, and are in close agreement with those of Hempling and Wise.[9] These indices were used to calculate areas of 357 μm² for EBL cells and 95 μm² for erythrocytes.

Insulin binding data are expressed as means ± SEM. Specific binding is total binding less nonspecific binding, which is defined as binding in the presence of 10^5 ng/ml un-

labeled insulin. For Scatchard analysis,[18] bound is calculated as the percent specific binding of the total concentration of insulin at each point. Free is total less bound. Receptor affinities were calculated by the negative cooperative model of DeMeyts and Roth.[19] K_e is defined as the affinity of the receptors at minimal insulin occupancy; K_f is the affinity at maximal occupancy. Receptor number was calculated by multiplying the abscissa intercept from the Scatchard plot by Avogadro's number and dividing by the cell concentration per liter.

Flux and SSDR of AIB transport assays were calculated as described by Wise.[8] Uridine incorporation was measured as dpm/μg RNA. Since the incorporation of uridine into RNA by controls varied between experiments, in each experiment uridine incorporation by control and insulin-treated cells was expressed as a percentage of the mean incorporation observed in controls (range 8500–18,900 dpm/dish; mean 12,550 dpm). Four dishes were assayed for each group per time point per experiment. Data are presented as means ± SEM. The significance of differences between means was tested by Student's *t* test.

RESULTS

Insulin binding. Our initial binding studies were performed at 15°C and 24°C. However, insulin degradation as assessed by solubility in 15% TCA reached unacceptably high levels during EBL cell incubations. No significant degradation was detected during erythrocyte incubations, in the course of which insulin remained always more than 95% precipitable in TCA. Scatchard analyses are invalidated in the presence of degradation of ligand so this phenomenon and methods for its inhibition were studied. Figure 1(a) illustrates the dependence of the ^{125}I-insulin degradation rate on temperature during 3-h incubations of EBL cells. Figure 1(b) shows that the ^{125}I-insulin degradation rate also depends on the total insulin concentration present during incubation of EBL cells at 24°C. Incubation of labeled insulin with cell-free media obtained from a 1-h preincubation of EBL cells at 4°C also resulted in insulin degradation, which was time and temperature dependent [Figure 1(c)]. Trypan blue dye exclusion tests showed greater than 95% viability at the end of the preincubation period at 4°C; therefore, subsequent degradation was not thought to be mediated by leakage of proteolytic enzymes from effete or damaged cells.

Addition of bacitracin, an antibiotic with nonspecific protease inhibitory action, produced a dose-dependent inhibition of insulin degradation in the media, which was partial at 37°C and complete at 4°C, as shown in Figure 2. Similar, although quantitatively less, inhibition of insulin degradation was achieved by the addition of bovine serum albumin (50 mg/ml) to the incubation media.

Figure 3(a) shows the competitive insulin binding curve obtained at 37°C in the presence of 50 mg/ml albumin. Under these conditions insulin binding to EBL cells reached apparent equilibrium by 5 min of incubation (time course data not shown), at which time nonspecific binding accounted for an average of 45% of maximum total binding, and degradation at trace concentration of ^{125}I-insulin only was an average of 22.5%. No further significant decrease in degradation was achieved by adding either bacitracin alone, or in conjunction with albumin, during incubation.

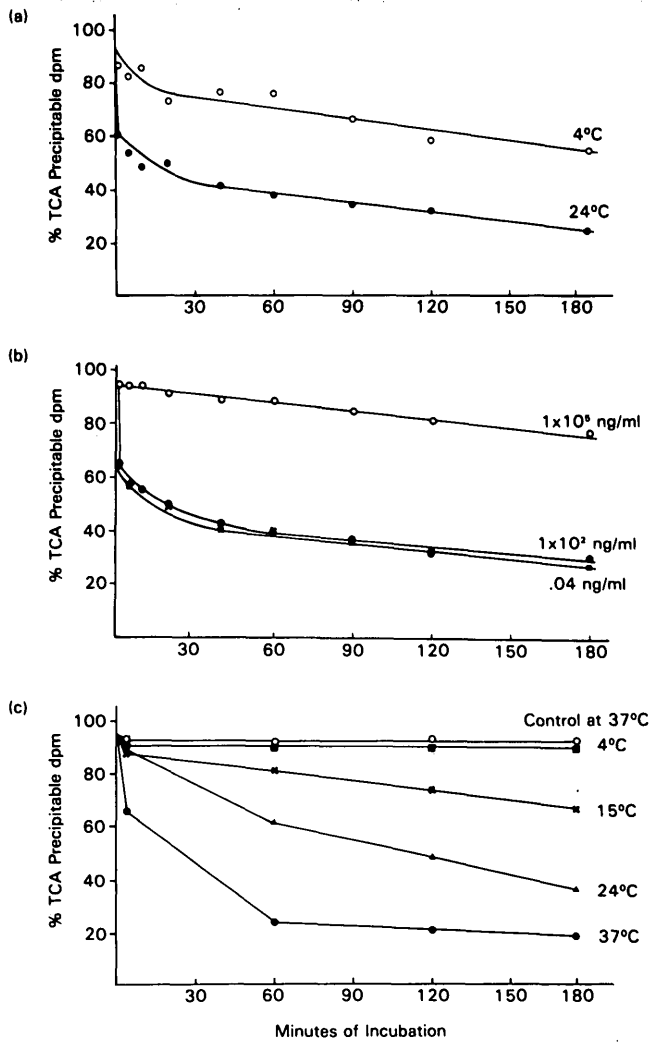

FIGURE 1. ^{125}I-insulin degradation was assessed by solubility in 15% cold TCA. EBL cell concentration was 4 × 10^7/0.5 ml of incubation medium. (a) Effect of temperature on degradation of trace concentrations of ^{125}I-insulin during incubation with EBL cells. (b) Effect of varying concentrations of unlabeled insulin on degradation of trace concentrations of ^{125}I-insulin (80 pg/ml) during incubation of EBL cells at 24°C. (c) Effect of cell-free supernatant on ^{125}I-insulin degradation. Cells were preincubated at 4°C for 1 h and centrifuged at 400 *g* for 20 min at 4°C. Cell-free media was aspirated and incubated at the temperatures indicated with trace concentrations of ^{125}I-insulin and degradation estimated during the time course. Cell viability as assessed by trypan blue dye exclusion was greater than 95% at the end of the preincubation.

Under these conditions erythrocyte insulin binding was entirely nonspecific.

Figure 3(b) shows competitive binding curves of insulin binding to EBL cells and erythrocytes obtained at 4°C for 4 h in the presence of 1 mg/ml bacitracin. Under these conditions insulin degradation was undetectable by TCA solubility and nonspecific binding accounted for 45% of maximum total binding by erythrocytes and 13% for EBL cells.

Maximum specific binding of EBL cells was at least 10 times greater than that of erythrocytes. Note that the data in Figure 3(b) are expressed per 4 × 10^7 EBL cells and per 2 × 10^9 erythrocytes. The difference in cell concentration tends to falsely minimize the difference in maximum binding but is shown uncorrected, as there were insufficient quantities of EBL cells to establish linearity of binding into the erythrocyte concentration range. Additionally, the EBL cell

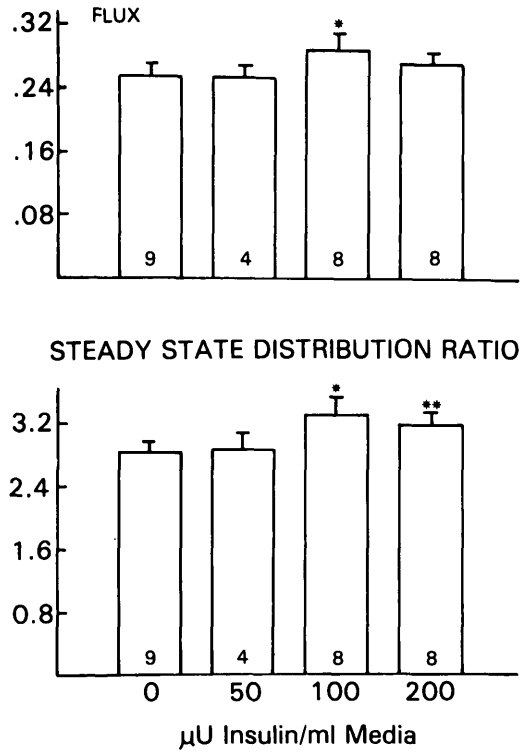

FIGURE 5. Effect of insulin on AIB transport in EBL cells. 1.5 × 10^7 cells were incubated at 37°C with 1 mM [^{14}C]-α-AIB, pH 7.4, for 1 h, with 10-min preincubations in the presence or absence of insulin (0–200 μU/ml media). Flux, defined as mM AIB/ml cell H_2O/min, was measured at 5 min. SSDR, defined as the ratio of the concentration of AIB in the cell water to the concentration in the media, was measured at 1 h. Means ± SEM are indicated; the number of experiments is shown at the base of the bars. *P < 0.05; **P < 0.02.

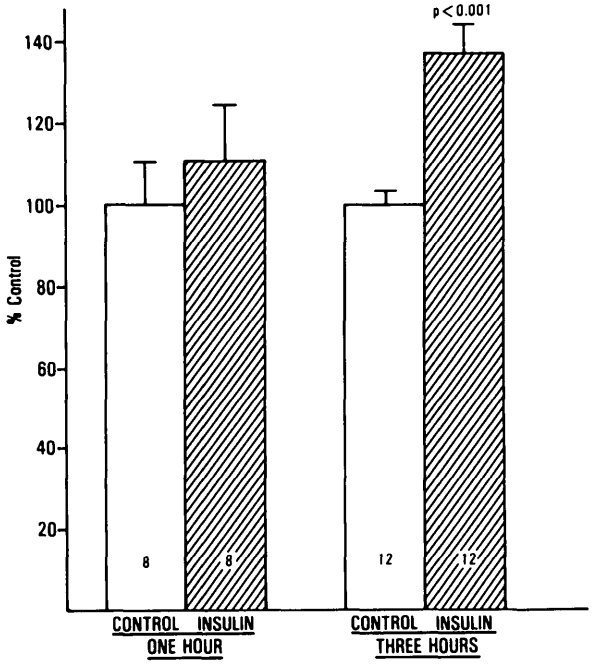

FIGURE 6. Effect of insulin on incorporation of ^{3}H-uridine into RNA of EBL cells. 1 × 10^6 cells were incubated at 37°C as described in METHODS, with or without insulin (100 μU/ml) added at 0 and 2 h. One hour before harvest, cells were pulsed with ^{3}H-uridine (3–6 μCi/ml). Data are normalized as percent of control (see Calculations and Statistics) and bars represent means ± SEM. The number of observations is shown at the base of each bar.

ditional increments of insulin dosage (400–800 μU/ml) resulted in no significant stimulation above basal incorporation. Maximal stimulation ranged from 26% to 37% in different experiments.

DISCUSSION

We have demonstrated the presence of specific insulin receptors on normal rat erythrocytes and EBL cells. There is a marked quantitative difference in insulin binding to these cells, which was initially minimized by the presence of insulin degradation in the EBL system. We have shown the insulin degradation to be, at least in part, independent of binding to, or the presence of, the EBL cell. This finding is in contrast to that of Terris and Steiner,[20] who showed that insulin degradation in rat hepatocytes correlated with insulin binding. Since the initial velocity and total amount of insulin degradation was greater with the EBL cells present than with the cell-free media only, it is likely that degradation in our system was due to both enzymes released by the cells and to cell-bound enzymes, as was found by Fussganger et al.[21] in human granulocytes. In addition, the partial inhibition of degradation by 50 mg/ml bovine serum albumin in our system suggests that the released protease is not specific for insulin, as was found by Gliemann and Sonne in adipocytes.[22] The degree of insulin degradation by EBL cells is, to our knowledge, quantitatively without precedent. This is especially so in view of the fact that TCA solubility tends to underestimate the degree of degradation when compared with its estimates by anti-insulin antibody and rebinding experiments. This finding is surprising if the EBL cell is a valid precursor model for erythrocytes, which are not noted for their proteolytic activity. It is possible, however, that this is merely a function of their malignant nature.

Ideally we would wish to measure insulin binding parameters under the conditions used to measure insulin response. However, at 37°C, Scatchard analyses of our data are fraught with uncertainty. First, valid Scatchard analysis demands several requirements,[23,24] one of which is equilibrium conditions. The presence of degradation alters the concentration of insulin both at and during attainment of equilibrium and necessitates correction factors before Scatchard analysis, the validity of which are dubious. Furthermore, even when corrected for degradation in the medium, at 37°C many cells have been shown to internalize the insulin-receptor complex.[25,26] It is at present unclear whether the receptor is recycled or degraded and replaced by de novo synthesis. We have preliminary data from electron microscope radioautography (unpublished) that EBL cells exhibit rapid internalization of the ligand. It is clear that, until these processes are more clearly defined, it is not possible to be sure that equilibrium has been attained or whether indeed it can even exist under these conditions. It is also possible that falsely high specific binding may be measured due to accumulation of ligand within the cell and further binding to newly synthesized or recycled receptors.

In contrast, at 4°C in the presence of bacitracin, conditions obtain wherein degradation is absent and internalization, if occurring, is believed (by virtue of its presumed energy dependence) to be minimal. Scatchard analysis under these conditions is mathematically valid, but the parameters derived may not be biologically valid. Temperature-induced changes in binding of receptors have been reported,[27] and

the force driving the association of insulin and its receptor has been shown to be highly temperature dependent.[28] Such thermodynamic considerations may contribute to the discrepancies between the K_e of EBL cells calculated at 4°C (0.09 nM^{-1}) and the dosage of insulin required to produce a response at 37°C (25–100 μU/ml). Additionally, it is reasonable to expect that larger doses of insulin are required at 37°C in the presence of degradation than would be predicted from a K_e obtained at 4°C without degradation. Alternatively, biologic response in the EBL cell may only be triggered by a high percentage receptor occupancy.

We were unable to demonstrate a dose response to insulin of AIB transport or uridine incorporation into RNA. This may be partly related to the presence of degradation or to the presence of an on/off rather than a graded response. The latter would be consistent with the observed relatively narrow range of maximal responses of uridine incorporation mediated by different doses of insulin. Another consideration pertinent to AIB transport is that basal rates are high in the EBL cell, possibly as a reflection of the hypermetabolism common in tumor cells. It may be that 100 μU/ml insulin produces an increase in transport above basal that is maximal; hence, further increments of insulin produce no additional response. Alternatively, this may be an example of the phenomenon of insulin insensitivity or inhibition at high doses, which has been reported in other systems.[29] Last, the different insulin doses which produced a maximal response on uridine incorporation in different experiments suggest variability in the EBL cell's responsiveness to insulin. This is not surprising, since there is variation both in the growth and maturity of the tumor and in the metabolic state of the host from which the cells are isolated and grown in primary culture.

Despite the paradox of relating insulin response to insulin binding either at 4°C, which is mathematically valid, or at 37°C, which is biologically valid, we would emphasize two points. First, at 37°C there was no demonstrable specific binding to erythrocytes compared with that demonstrated for EBL cells. Second, at all temperatures (including data at 15°C and 24°C not shown here) and under all conditions, EBL cells bound at least 10 times the amount of ^{125}I-insulin as did erythrocytes. Scatchard plots show a consistently larger number of receptor sites per cell. If analyzed with a negative cooperative model, EBL cells also exhibit greater affinities; if analyzed by a two-site model, initial slopes are more compatible with a greater erythrocyte affinity.

Notwithstanding our inability to demonstrate the precise correlation of pharmacokinetics with biologic function, we believe that our findings support the hypothesis that immature erythrocytes may be insulin dependent during differentiation and growth. It is also possible that the observed differences may be accounted for by the fact that EBL cells are tumor cells. Activation of T-lymphocytes is associated with emergence of insulin receptors,[27] and some mammary tumor cells showed increased insulin binding.[28] The EBL cells used in our study have been passaged at least 180 times and remained constant in character. If the increased insulin binding is a reflection of the initial transformation, then its expression must presumably be genetic. Studies of insulin binding and response in reticulocytes and normal erythroblasts should resolve this question.

Since it has been shown that there is a gradation of maturity and sodium-dependent amino acid transport in the erythroid series,[8] we sought coupling of insulin binding and AIB transport. We have demonstrated a small but significant acceleration of AIB transport in response to a physiologic concentration of insulin. We have also demonstrated an increase in uridine incorporation into RNA in response to the same physiologic dose of insulin. The coupling of insulin binding to responses in EBL cells and the absence of such coupling in erythrocytes may reflect their difference in degree of maturation. In this case the erythrocyte precursors may become useful models for the study of receptor function during cell differentiation, especially if receptor-function coupling persists in permanent culture.

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