Decrease in Insulin Receptors During Friend Erythroleukemia Cell Differentiation

BARRY H. GINSBERG, THOMAS BROWN, AND MOHAN RAIZADA

SUMMARY

The Friend erythroleukemia cell has an insulin receptor with all the properties of mammalian insulin receptors: rapid, reversible, and saturable binding of insulin; specific for insulin and insulin analogs; inversely proportional to temperatures; sharply pH dependent (optimum = 8.0); and demonstrated ligand-induced accelerated dissociation consistent with negative cooperativity. There were 17,200 sites per cell. After induction by dimethylsulfoxide, 80% of the cells became benzidine positive (i.e., contained hemoglobin). The receptor concentration dropped to 4300 sites per cell, while the remaining receptors retained all the initial binding characteristics. This loss of receptors could not be attributed directly to either dimethylsulfoxide or changes in cell size. Thus, during the process of differentiation, the concentration of insulin receptors in the Friend erythroleukemia cell decreases. DIABETES *28*:823-827, September 1979.

The Friend erythroleukemia cell (FLC), obtained from a murine leukemia, can be induced to differentiate in vitro from a cell resembling a primitive red cell precursor, the basophilic erythroblast, to one resembling a more differentiated cell, the orthochromic normoblast.[1] The process of in vitro differentiation appears to reflect the natural process of erythropoiesis. Thus, in an orderly manner, the enzymes of heme synthesis are sequentially induced, globin mRNA is synthesized, and hemoglobin is produced.[2-4] Although there are some subtle alterations in the process of in vitro red blood cell differentiation, the amount of α and β globin chains is equal and the entire process appears well integrated.[5]

The cytoplasmic and nuclear events accompanying differentiation have been well studied, but there have only been a few studies of plasma membrane-associated events. Flow cytofluorometric studies show that the fluorescent anisotropy increases, suggesting an increase in membrane rigidity as the cells differentiate.[6] There is an increase in erythrocyte-specific antigens and a decrease in H-2 antigens.[6,7] Rubidium transport (a model for K^+) and transferrin receptors increase while glucose transport decreases.[8-10] Hormone receptors have not been previously studied.

In the present study we demonstrate that insulin receptors are present on the FLC and investigate the properties of these insulin receptors during the process of differentiation. We find that the undifferentiated cells contain insulin receptors that have binding properties identical to those of all mammalian cells. During the process of differentiation there is a decrease in the total cellular insulin receptors, which does not appear to be due simply to a loss of surface area. The remaining receptors in the differentiated cells have normal binding properties.

MATERIALS AND METHODS

Cells. Friend erythroleukemia cells (FLC) clone 745 were obtained from the mammalian cell repository, Institute for Medical Genetics, Camden, N. J. (#GM86). They were grown for 7 passages, then frozen in liquid nitrogen. Cells were thawed and used for 30 passages. No differences in insulin binding to the seventh and last passage were noted.

Cells were grown in suspension culture in Temin's medium (Gibco) with 15% fetal calf serum from 1×10^5 to 2×10^6. Viability was routinely >95% as determined by Trypan blue dye exclusion. Differentiation of the cell was obtained by the addition of 280 mM dimethylsulfoxide (DMSO) media. The degree of differentiation was determined by benzidine staining.[11]

Hormones. Porcine insulin was obtained from Elanco (Indianapolis, In.), porcine proinsulin was the generous gift of Dr. Ronald Chance, and bovine desoctapeptide insulin the generous gift of Dr. Fred Carpenter. Human growth hormone was a gift of the National Pituitary Agency.

Porcine insulin was iodinated to 155-200 μCi/μg by the chloramine-T method and separated from iodide and de-

From the Veterans Administration Hospital and The Diabetes-Endocrinology Research Center, the Departments of Internal Medicine and Physiology and Biophysics, University of Iowa, Iowa City, Iowa 52240.
Received for publication 4 May 1979 and in revised form 8 June 1979.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/28/9/823/350596/28-9-823.pdf by guest on 13 June 2024

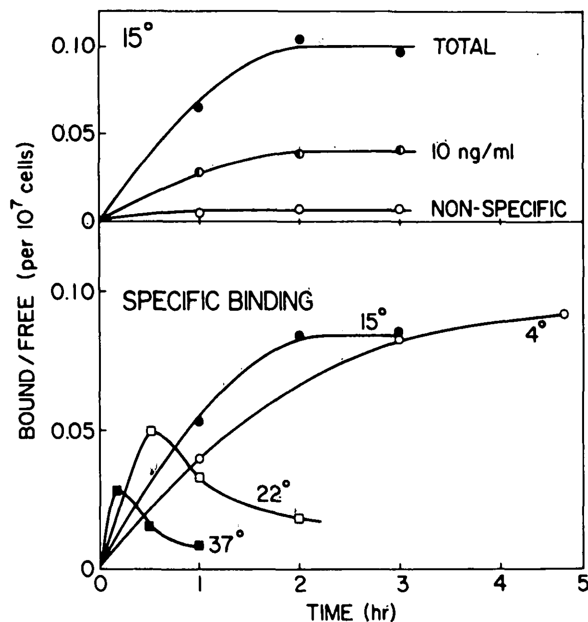


FIGURE 1. Insulin binding to FLC. ***Upper:*** **FLC were washed 3× in buffer HL and resuspended at 1 × 10⁷/ml in 5 ml containing ^{125}I-insulin, 20 pM, and either 0, 10 (1.7 nM), or 1000 ng/ml (174 nM) insulin. The mixtures were incubated at 15°C and at the times indicated two 200-μl aliquots were layered over 200 μl of cold HLB and sedimented at about 8000 × g for 1 min in a 6-mm microfuge tube.** ***Lower:*** **Effects of incubation temperature. Experimental details varied from above only in the incubation temperature and that only 0 and 1000 ng/ml of unlabeled insulin were added. Only saturable binding is shown.**

graded insulin by chromatography over Whatman cellulose (coarse[12]).

Binding. Cells were washed three times in HL buffer (100 mM Hepes; 120 mM NaCl; 25 mM Na acetate; 10 mM glucose; 2.4 mM KCl; 0.81 mM $MgSO_4$; 0.81 mM EDTA; 10 mg/ml BSA)[13] and resuspended at a final concentration of 1×10^7/ml in the assay (0.5 ml). ^{125}I-insulin was present at 20 pM. Nonsaturable binding was determined with 0.17 or 1.7 μM unlabeled insulin; there was no difference. Unless otherwise specified, binding was done at 15 °C in a refrigerated water bath for 3 h. Duplicate 200-μl aliquots were withdrawn, layered over 200 μl of cold HL buffer, and sedimented at 8000 × g for 1 min. The supernatant was aspirated and a portion of the tube containing the pellet counted for 10 min in a Beckman Gamma 300 at 75% efficiency.

RESULTS

Characterization of the insulin receptor. Friend erythroleukemia cells were grown in Temin's media[14] with 15% fetal calf serum and harvested in the stationary phase (2×10^6/ml). After washing, the cells were incubated at 10^7/ml with ^{125}I-insulin (20 pM) or with ^{125}I-insulin plus unlabeled insulin (10 ng/ml or 1 μg/ml, 1.67 or 167 nM). Binding of insulin to these cells was rapid, reaching a steady state of 10% bound hormone by about 2 h (Figure 1, upper panel). Unlabeled insulin, at 10 ng/ml, reduced binding to about 3%, while the nonspecific binding (defined as the nonsaturable component) was less than 1% of the added insulin. The binding was reversible and labeled insulin could be dissociated by either addition of excess insulin or by dilution of the receptor–insulin complex (data not shown).

The expected temperature sensitivity of insulin binding was found (Figure 1, lower panel).[15] The initial rate of binding was proportional to temperature, but the steady state binding was lower at higher temperatures.

The binding of insulin to its receptor demonstrated the characteristic specificity for insulin and insulin analogs (Figure 2). Porcine insulin was a good competitor for the insulin binding site. Other insulin analogs competed for binding in proportion to their biologic activity. Thus, porcine insulin reduced maximum tracer binding by 50% at 11 ng/ml, whereas porcine proinsulin required about 150 ng/ml and desoctapeptide insulin (DOP) about 700 ng/ml to reduce binding by 50%. The insulin-like growth factors, MSA and IGF 6.5,[16] caused 50% displacement at 1500 ng/ml (data not shown). These relative potencies in competition for receptor sites are the same as the relative biologic potencies (molar ratio of 1/0.11/0.013/0.008, respectively). Unrelated peptides such as human somatotropin (HGH) did not compete for binding.

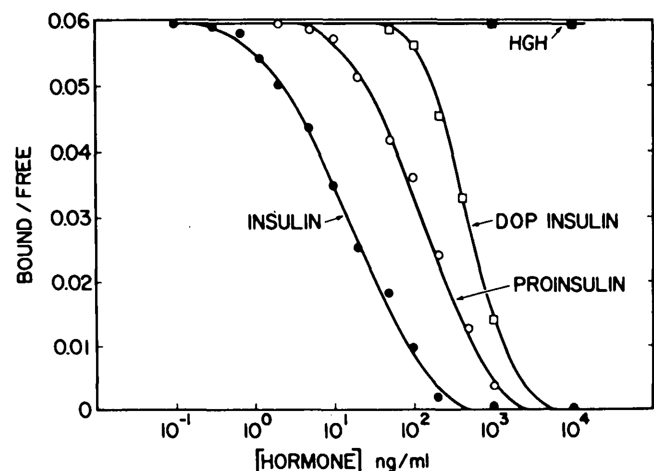
The competition data with porcine insulin were reanalyzed by the method of Scatchard[17] (Figure 3). The plot was curvilinear, consistent with negatively cooperative site–site interactions. Further evidence for negative cooperativity of FLC insulin receptors was demonstrated by the dilution method of DeMeyts et al.[18] As shown in Figure 4 the rate of dissociation of insulin from its receptor was considerably enhanced by the addition of unlabeled insulin. Since the dissociation rate of hormones from independent sites would not be altered, the data suggest negatively cooperative site–site interactions.[18] Although there is some controversy about the interpretation of these types of experiments,[19] they are characteristic of insulin receptors.[15]

The insulin receptor of the FLC also demonstrated a sharp pH optimum at pH 8.0. This, too, is a general property of mammalian insulin receptors.

Effects of differentiation on the insulin receptor. FLC were diluted from stationary phase to 1×10^5/ml in 280 mM DMSO. This routinely caused at least 80% of the cells to produce hemoglobin and turn benzidine positive by day 5–6. Cells were allowed to grow to early stationary phase ($1–2 \times 10^6$/ml) for all the following studies.

Differentiated cells were washed and tested for insulin binding as described above. Scatchard analysis[17] demon-

FIGURE 2. Specificity of binding of insulin to FLC. Cells were washed and resuspended at 1 × 10⁷/ml in 0.5 ml of HLB with ^{125}I-insulin and the indicated amount of hormone. Incubation was at 15°C for 3 h and bound and free hormone separated as described above.



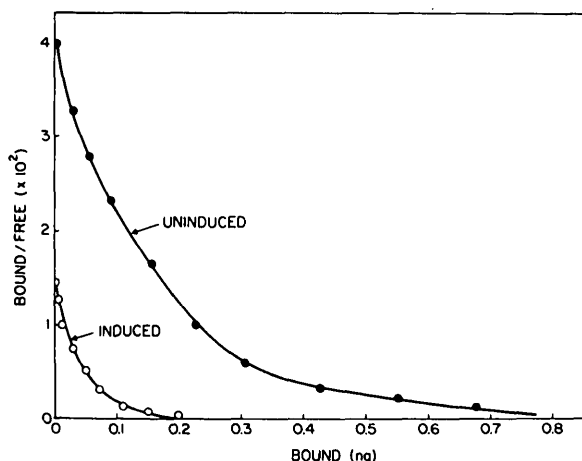
Downloaded from http://diabetesjournals.org/diabetes/article-pdf/28/9/823/350596/28-9-823.pdf by guest on 13 June 2024

strated that the differentiated cells bound less insulin at all insulin concentrations. The receptor concentration, estimated from the x intercept of the Scatchard plot, was 0.8 ng/ml (17,200 receptors/cell) for the undifferentiated FLC but 0.2 ng/ml (4,300 receptors/cell) for the differentiated cells. Since in this experiment 20% of the cells were not differentiated, these data probably overestimate the receptor concentration in the differentiated cells. Indeed, if the 20% benzidine-negative cells maintained their initial receptor concentration, the receptor concentration of the benzidine-positive cells would be only 1075 receptors/cell. Thus it appears that during the process of differentiation there was a marked decrease in the number of receptors/cell to between 6 and 25% of control.

The differentiated cells varied from the undifferentiated ones in a number of ways. Aside from the obvious biochemical alterations, the differentiated cells were smaller and had been exposed to DMSO. Either of these factors might be responsible for the decrease in receptor concentration.

Table 1 presents the data from an experiment that investigated the effects of DMSO and cell size. Friend cells were grown in 280 mM DMSO for 0, 2, or 5 days, harvested by centrifugation, washed three times with buffer, and studied for insulin binding by Scatchard analysis. Before the addition of DMSO, FLC were found to have 15,000–17,000 insulin binding sites per cell or 42–48 sites/μ^2 (assuming cells to be uniform spheres). Two days after the addition of DMSO, a time at which the cells were irreversibly committed to differentiation,[20] the receptors per cell and per unit surface area were unchanged. Thus, DMSO did not affect the amount of insulin bound to these cells. Five days after the addition of DMSO, about 80% of the cells were benzidine positive and the receptor number had decreased to about 6000/cell. The cells, however, were smaller, and the receptor concentration was 19 sites/μ^2. Thus, correction of receptors for gross surface area did not explain the decrease in receptor concentration. Scanning electron microscopy suggested that microvilli accounted for an additional 25–30% of the surface of the undifferentiated cells and 5–10% in the differentiated ones. Correcting for this additional plasma membrane, the receptor concentration was 38 sites/μ^2 in the undifferentiated and 17 sites/μ^2 in the differentiated cells. Again correcting the benzidine-negative cells (as above) in the differentiated population would bring the receptor concentration down to 3 sites/μ^2 in the benzidine-positive cells. There were no differences in the affinity of the receptor for insulin at any of these times (Table 1). Thus, it appears that in the process of differentiation there is a marked decrease in the insulin receptor concentration.

FIGURE 3. Scatchard plot of insulin binding to FLC. Cells were diluted to 1 × 10⁵/ml in media or media + 280 mM DMSO and grown for 6 days. At this time both cultures were 2 × 10⁶ cells/ml and the cells grown in DMSO were 80% benzidine positive. Both cultures were washed and tested as in Figure 2.



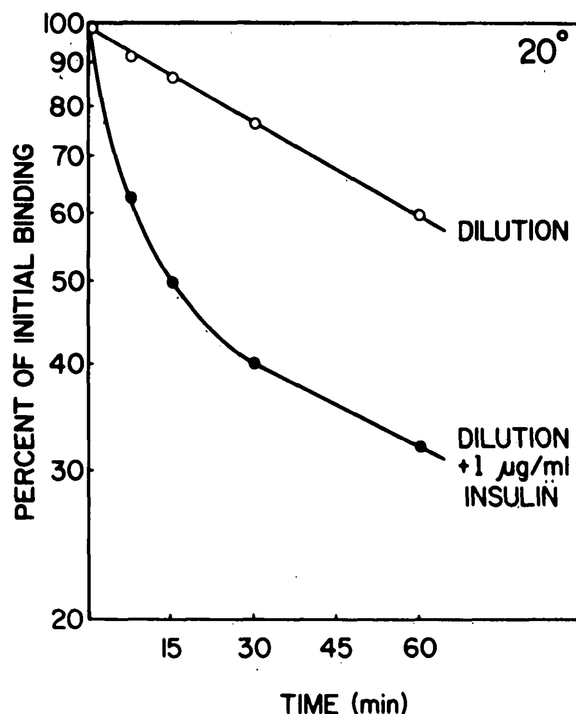


FIGURE 4. Negative cooperativity of the insulin receptor of the FLC. Cells 1 × 10⁷/ml were incubated with ¹²⁵I-insulin (20 pM) for 3 h at 15°C. One hundred microliter aliquots were diluted in 10 ml of buffer (dilution) or 10 ml of buffer with unlabeled insulin (dilution + 1 µg/ml insulin). The dilutions were incubated at 20°C and at the indicated periods triplicate tubes were sedimented at 15,000 × g for 2 min. The supernatants were aspirated and the tubes counted. Identical results were obtained if the cells were washed once before dilution.

TABLE 1
Effect of differentiation on the insulin receptor concentration of FLC

	Time			
	Expt. 1		Expt. 2	
Days with DMSO	0	2	0	5
% Benzidine positive	0	0	0	80
Receptors/cell	15,000	15,000	17,000	6000
Receptor/μ^2 membrane	42	42	48	19
Receptor/μ^2 membrane including microvilli	34	34	39	16
Affinity constants (nM^{-1})				
$\bar{K}_e$	0.28	0.29	0.29	0.32
$\bar{K}_f$	0.04	0.03	0.04	0.03

Experiment 1: Cells were diluted to 5 × 10⁵/ml and grown for 2 days with or without 280 mM DMSO. The cells were harvested at 2 × 10⁶/ml. Experiment 2: Cells were diluted to 1 × 10⁵/ml and grown for 5 days with or without 280 mM DMSO. Cells were harvested in early stationary phase and treated as described in Figure 3.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/28/9/823/350596/28-9-823.pdf by guest on 13 June 2024

The other properties of the insulin receptor of the differentiated cells were unchanged. Thus, at higher temperature binding was more rapid but reached a lower steady state (B/F = 0.035 at 4 °C, 0.03 at 15 °C, 0.01 at 24 °C, and 0.002 at 37 °C); insulin analogs competed for binding in proportion to their biologic potency (molar ratios of 1/0.09/0.008 for porcine insulin, proinsulin, and DOP insulin); the pH optimum was 8.0; and a ligand-induced increase in dissociation consistent with negative cooperativity was kinetically demonstrable.

DISCUSSION

Our data demonstrate that the Friend erythroleukemia cell possesses an insulin receptor with properties identical to those of other insulin receptors and that, during the process of differentiation of these cells, the number of receptors decreases. This decrease does not appear to be a direct effect of DMSO, the inducer, or of the reduction in size that accompanies differentiation.

A loss of receptors might be the reason that the normal mature red blood cells of mammals are not sensitive to insulin.[21] In these cells glucose transport is rapid and intracellular glucose is only slightly less than extracellular. Red blood cells do appear to have insulin receptors, but the number of receptors/cell is very limited.[22] Little data are available about the effect of insulin on erythroid precursor cells. There is evidence, however, to suggest that insulin can affect the undifferentiated FLC, at least to cause an increase in the cloning efficiency.[23]

The regulation of insulin receptor concentration is a very complex process. Prior studies demonstrated that regulation of the insulin receptor in other systems may be altered by changes in ambient insulin concentrations[13,24,25] during changes in the growth phase and cell cycle and during differentiation. Thomopoulos et al.[26,27] and Bar et al.[28] previously showed that rapidly growing cells have fewer insulin receptors than cells at stationary phase. In addition, Thomopoulos et al.[27] showed that, in fibroblasts, this is due to an increase in receptors at the beginning of the G_1 phase, so that at stationary phase, when all the cells were at G_0, the insulin receptor concentration is maximal. A similar phenomenon occurs in the FLC (unpublished observations). In order to make comparable observations in the differentiated cells, both were studied during early stationary phase. The system is further complicated by a recent study of Friedman and Schildkraut,[29] which suggests that the differentiated cells exist as two types—those that are terminally differentiated and arrested in G_1, and those that contain hemoglobin but continue to divide. Our studies could not separate these populations, but since our differentiated cells were in stationary phase, presumably all cells were arrested in early G_1 phase. Further studies of these populations will await correlative studies on individual cells.

Alterations in insulin receptor concentrations have also been shown during the in vitro differentiation of the 3T3-L1 fibroblast.[30] When this cell differentiates to an adipocyte, the number of insulin receptors is increased and the cell becomes more sensitive to insulin. In the FLC the opposite effect appears to occur. During the process of differentiation there is a loss of insulin receptors and fully differentiated red blood cells are completely resistant to insulin actions.

One last point deserves mention. During the process of differentiation there appear to be major shifts in membrane lipid mobility. Flow microfluorometry has shown a major increase in fluorescence anisotropy during differentiation, suggesting that the membrane becomes more rigid.[6] In very recent studies (B. Ginsberg and A. Spector, in preparation) we have demonstrated that an increase in membrane fluidity is associated with an increase in insulin receptors. Thus, the decreases in membrane fluidity that accompany differentiation may themselves be the signal for a fall in insulin receptor concentration.

The process of differentiation of the FLC involves an orderly induction of the enzymes of heme synthesis, synthesis of α and β globin, and the induction of transferrin receptors and erythrocyte membrane antigens. There is also a decrease in glucose transport. It now appears that there is also a decrease in the concentration of insulin receptors.

ACKNOWLEDGMENTS

The authors wish to thank Dr. James Gavin, III, for making available some preliminary data, Shelly Plattner and Dr. C. Shih for their assistance with electron microscopy, and Drs. R. S. Bar, J. H. MacIndoe, and D. K. Granner for suggestions. We would also like to thank Vicki Kramer for her secretarial assistance.

This research was supported by the Medical Research Office of the Veterans Administration and by research grants from the National Institutes of Health and the American Diabetes Association and its Iowa Affiliate.

REFERENCES

[1] Friend, C., Scher, W., Holland, J. G., and Sato, T.: Hemoglobin synthesis in murine virus-induced leukemic cells in vitro: stimulation of erythroid differentiation by dimethyl sulfoxide. Proc. Natl. Acad. Sci. USA *68*:378–82, 1971.

[2] Sassa, S.: Sequential induction of heme pathway enzymes during erythroid differentiation of mouse Friend leukemia virus-infected cells. J. Exp. Med. *143*:305–15, 1976.

[3] Ross, J., Gielen, J., Packman, S., Ikawa, Y., and Leder, P.: Globin gene expression in cultured erythroleukemic cells. J. Mol. Biol. *87*:697–714, 1974.

[4] Scher, W., Holland, J. G., and Friend, C.: Hemoglobin synthesis in murine virus-induced leukemic cells *in vitro*. I. Partial purification and identification of hemoglobins. Blood *37*:428–37, 1971.

[5] Kabat, D., Sherton, C. C., and Evans, L. H.: Synthesis of erythrocyte-specific proteins in cultured Friend leukemia cells. Cell *5*:331–38, 1975.

[6] Arndt-Jovin, D. J., Ostertag, W., Eisen, H., Klimek, F., and Jovin, T.: Studies of cellular differentiation by automated cell separation. Two model systems: Friend virus-transformed cells and hydra attemuata. J. Histochem. Cytochem. *24*:332–47, 1976.

[7] Furusawa, M., Ikawa, Y., and Sugano, H.: Development of erythrocyte membrane-specific antigen(s) in clonal cultured cells of Friend virus-induced tumor. Proc. Japan Acad. *47*:220–31, 1971.

[8] Mager, D., and Bernstein, A.: The program of Friend erythroid differentiation: early changes in Na^+/K^+ ATPase function. J. Supramolec. Struct. *8*:431–38, 1978.

[9] Hu, H. Y., Gardner, J., and Aisen, P.: Inducibility of transferrin receptors on Friend erythroleukemia cells. Science *197*:559–61, 1977.

[10] Germinario, R. H., Kleiman, L., Peters, S., and Olivera, M.: Decreased deoxy-D-glucose transport in Friend cells during exposure to inducers of erythroid differentiation. Exp. Cell Res. *110*:375–85, 1977.

[11] Gusella, J. F., and Housman, D.: Induction of erythroid differentiation *in vitro* by purines and purine analogs. Cell *8*:263–69, 1976.

[12] Roth, J.: Peptide hormone binding to receptors: a review of direct studies *in vitro*. Metab. Clin. Res. *22*:1059–73, 1973.

[13] Gavin, J. R., III, Roth, J., Neville, D. M., Jr., DeMeyts, P., and Buell, D. N.: Insulin dependent regulation of insulin receptor concentrations: a direct demonstration in cell culture. Proc. Natl. Acad. Sci. USA *71*:84–88, 1974.

[14] Temin, H. M.: The control of cellular morphology in embryonic cells infected with Rous sarcoma virus *in vitro*. Virology *10*:182–87, 1960.

[15] Ginsberg, B. H.: The insulin receptor: properties and regulation. *In* Biochemical Actions of the Hormones, Vol. IV. Litwak, G., Ed. New York, Academic Press, 1977, Chap. 7.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/28/9/823/350596/28-9-823.pdf by guest on 13 June 2024

[16] Ginsberg, B. H., Kahn, C. R., Roth, J., Megyesi, K., and Baumann, G.: Identification and high-yield purification of insulin-like growth factors (NSILA and somatomedius) from human plasma by use of endogenous binding proteins. J. Clin. Endocrinol. Metab. *48*:43-49, 1979.

[17] Scatchard, G.: The attractions of proteins for small molecules and ions. Ann. N.Y. Acad. Sci. *51*:660-72, 1949.

[18] DeMeyts, P., Bianco, A. R., and Roth, J.: Site-site interactions among insulin receptors. Characterization of the negative cooperativity. J. Biol. Chem. *251*:1877-88, 1978.

[19] Pollet, R. H., Standaert, M. L., and Haase, B. A.: Insulin binding to the human lymphocyte receptor: evaluation of the negative cooperativity model. J. Biol. Chem. *252*:5828-34, 1977.

[20] Levy, J., Masaaki, T., Rifkind, R. A., and Marks, P. A.: Induction of erythroid differentiation by dimethylsulfoxide in cells infected with Friend virus: relationship of the cell cycle. Proc. Natl. Acad. Sci. USA *72*:28-32, 1975.

[21] Harvey, J. W., and Kaneko, J. J.: Glucose metabolism of mammalian erythrocytes. J. Cell Physiol. *89*:218-24, 1976.

[22] Gambhir, K. K., Archer, J. A., and Bradley, C. J.: Characteristics of human erythrocyte insulin receptors. Diabetes *27*:701-08, 1978.

[23] Golde, D. W., Bersch, N., and Li, C. H.: Growth hormone modulation of murine erythroleukemia cell grown *in vitro*. Proc. Natl. Acad. Sci. USA *75*:3437-39, 1978.

[24] Kahn, C. R., Neville, D. M., Jr., Gorden, P., Freychet, P., and Roth, J.: Insulin receptor defect in insulin resistance: studies in the obese-hyperglycemic mouse. Biochem. Biophys. Res. Commun. *48*:135-42, 1972.

[25] Bar, R. S., Gorden, P., Kahn, C. R., Roth, J., and DeMeyts, P.: Fluctuations in the affinity and concentration of insulin receptors on circulating monocytes of obese patients. Effects of starvation, refeeding and dieting. J. Clin. Invest. *58*:1123-35, 1976.

[26] Thomopoulos, P., Roth, J., Lovelace, E., and Pastan, I.: Insulin receptors in normal and transformed fibroblasts: relationship to growth and transformation. Cell *8*:417-23, 1976.

[27] Thomopoulos, P., Kosmakos, F. C., Pastan, I., and Lovelace, E.: Cyclic AMP increases the concentration of insulin receptors in cultured fibroblasts and lymphocytes. Biochem. Biophys. Res. Commun. *75*: 246-52, 1977.

[28] Bar, R. S., Koren, H., and Roth, J.: Physiological insulin concentrations affect macrophage function. Diabetes *25* (Suppl. 1):348, 1976.

[29] Friedman, E. A., and Schildkraut, C. L.: Terminal differentiation in cultured Friend erythroleukemia cells. Cell *12*:901-13, 1977.

[30] Rubin, C. S., Hirsch, A., Fung, C., and Rosen, O. M.: Development of hormone receptors and hormonal responsiveness *in vitro*. Insulin receptors and insulin sensitivity in the preadipocyte and adipocyte forms of 3T3-L1 cells. J. Biol. Chem. *253*:7570-78, 1978.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/28/9/823/350596/28-9-823.pdf by guest on 13 June 2024