Proc. Nat. Acad. Sci. USA
Vol. 69, No. 3, pp. 747–751, March 1972

Insulin Receptors in Human Circulating Cells and Fibroblasts

(lymphocytes/monoiodoinsulin/glucose oxidation)

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Communicated by Robert W. Berliner, January 14, 1972

ABSTRACT **Human lymphocytes obtained from fasted adult subjects and cultured human tumor lymphocytes were investigated for specific insulin receptors. By use of monoiodoinsulin, specific insulin binding sites were demonstrated in peripheral human lymphocytes, cultured human lymphocytes, and in other types of human circulating cells. Insulins and insulin derivatives that varied in their potency to stimulate glucose oxidation in the fat cell and to inhibit binding of [^{125}I]insulin to purified plasma membranes, varied in an analogous fashion in their ability to inhibit the binding of labeled insulin to human lymphocytes. Hormones that had no effect on the binding of insulin to fat cells or liver membranes also had no effect on the binding of insulin to lymphocytes. Binding was time and temperature dependent; dissociation of [^{125}I]insulin was rapid upon addition of 10 μM insulin. These findings afford a direct approach to the study of endocrine disorders in man.**

Using biologically-active iodinated hormones and various *in vitro* preparations of target tissue, we and others have studied directly the binding of polypeptide hormones to specific receptors on target cells (1–8). These techniques have not been applied to the study of clinical disorders in man largely because it has not been feasible to obtain sufficient quantities of these tissues under appropriate physiological conditions. In the present study, we have found that lymphocytes and granulocytes from human peripheral blood contain specific binding sites for insulin. The specificity, sensitivity, and kinetics of this binding are extraordinarily similar to those observed in two of the target tissues of insulin—highly purified plasma membranes of rat liver cells and isolated rat fat cells. Characterization of these readily-available specific binding sites for insulin in both normal adults and patients with endocrine disorders afford a direct approach to the study of such disorders.

MATERIALS AND METHODS

Insulin and Other Hormones. Unless otherwise indicated, insulin refers to porcine insulin (PJ 5589) obtained from Eli Lilly. Other hormones, insulin, and insulin derivatives used in this study have been described (1).

Iodination of Insulin. Insulin was iodinated by reaction of purified pork insulin with Na ^{125}I (carrier free, Union Carbide) and with chloramine T in equal molar ratios. 5 μg of insulin in 5 μl of 0.3 M phosphate buffer (pH 7.4) were added to 40 μl of 0.3 M phosphate buffer containing 3.5 mCi of ^{125}I. 5 μl of a solution of chloramine T (40 μg/ml) were added, and after 30 sec, 5 μl of a solution of sodium metabisulfite (200 μg/ml) was added to stop the reaction. These conditions of iodination were chosen in order to minimize the introduction of more than 1 atom of ^{125}I per insulin molecule and the possible deleterious effects of the oxidizing agent (9). The determination of the bioactivity of monoiodinated insulin has been extensively discussed (10). After the reaction was stopped, 100 μl of 0.3 M phosphate buffer, containing 2.5% bovine serum albumin (pH 7.4; Armour, Fraction V) was added to the reaction mixture, and the entire solution was transferred to a 5 × 50 mm cellulose column previously washed with 0.05 M veronal solution (pH 8.6). The column was then washed with 9.0 ml of the veronal solution to elute unreacted ^{125}I or damaged insulin. The iodinated hormone was eluted with 6.0 ml of 0.3 M phosphate buffer containing 12% bovine serum albumin and collected in 1.5-ml fractions. The extent of iodination was determined by chromatoelectrophoresis (11). 99% of the [^{125}I]insulin was precipitated by 5% trichloroacetic acid, and 95% was absorbed to 50 mg of talc (3).

Preparation of Cells for Binding. Cultured human lymphocytes (no. 4265) were generously supplied by Drs. Albert Einstein and Dean L. Mann (National Cancer Institute, NIH). These cells were originally obtained in 1965 from a 42 year-old American male with chronic myelogenous leukemia (12). The karyotype of these cells had a normal distribution of chromosomes, except for several small "marker" chromosomes that probably represented broken tetraploid metaphases (12). Normal circulating cells were obtained from adult men who were fasted for 8–12 hr. 500 ml of blood was drawn into acid–citrate–dextrose solution (29) and centrifuged at 1500 × *g* for 3 min at 20°. The plasma was removed and the white cell layer (buffy coat) was transferred to a Ficoll–Hypaque gradient for further fractionation into granulocytes and lymphocytes (13). Erythrocytes from the bottom of the container were resuspended in a 1:4 dilution of plasma with 0.9% NaCl and centrifuged. The packed cells were washed and centrifuged in this fashion three times to yield a final ratio of red to white cells of 5000:1.

Binding of [^{125}I]Insulin to Blood Cells. Human lymphocytes (30–50 × 10^6 cells), granulocytes (60–70 × 10^6 cells), erythrocytes (2.5 × 10^9 cells), leukemic granulocytes (150–200 × 10^6 cells), and cultured human lymphocytes (5–7 × 10^6 cells) were incubated in a water bath at 30° with labeled insulin in the presence or absence of unlabeled peptides in 0.5 ml of Tris buffer [25 mM Tris–10 mM dextrose–1 mM EDTA–1.4 mM sodium acetate–0.5 mM KCl–12 mM NaCl–0.24 mM $MgSO_4$–1% bovine serum albumin (pH 7.4)]. At the end of incubation, duplicate 200-μl aliquots of the suspension were layered onto 150 μl of cold Tris–Ringer buffer containing 2%

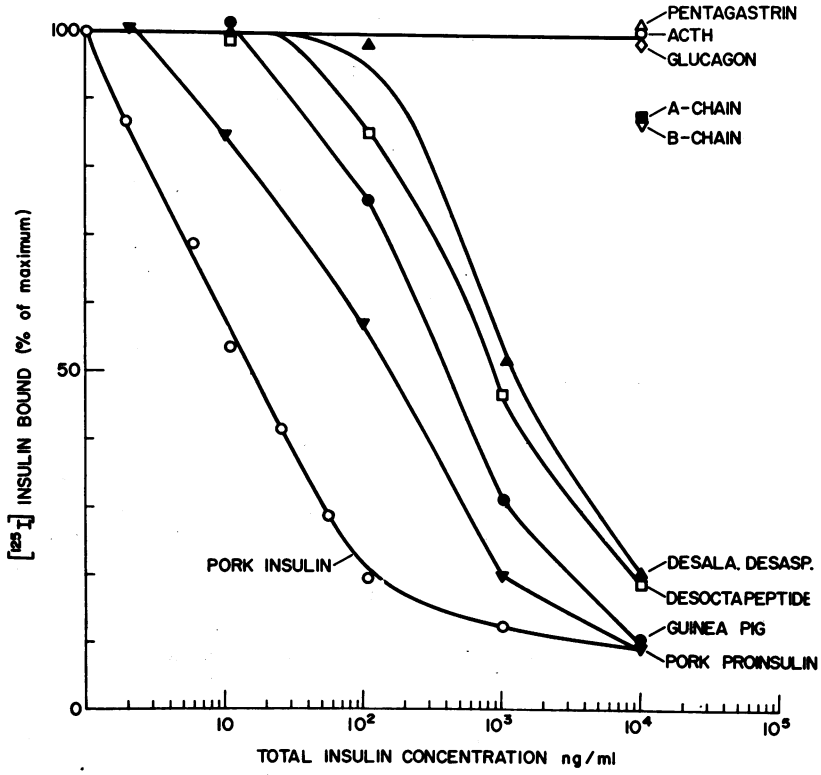


FIG. 1. Effect of insulins, insulin derivatives, and other peptides on [^{125}I]insulin binding to cultured human lymphocytes. Cultured cells (5–7 × 10^6 cells/ml) were incubated for 10 min under the conditions described in *Methods*. [^{125}I]insulin was added to give a final concentration of 0.12 nM. Desala. desasp., desalanine–desasparagine insulin.

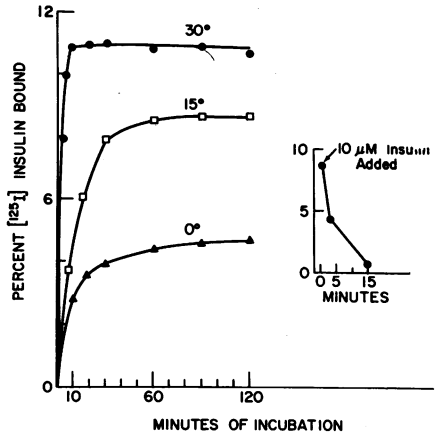


FIG. 2. Binding of [^{125}I]insulin to cultured human lymphocytes (No. 4265) as a function of time. 6–9 × 10^6 cells/ml were incubated in 0.5 ml buffer (see *Methods*) in the presence of 0.1–0.2 nM [^{125}I]insulin at each temperature. *Inset*, Dissociation of labeled insulin from cultured cells. Cells (4–6 × 10^6 cells/ml) were incubated at 30° for 10 min in the presence of 0.2 nM of [^{125}I]insulin. After an aliquot was removed for determination of uptake of labeled hormone at 10 min, 60 μg of unlabeled insulin was added in a small volume (15 μl) to the incubation mixture (1.0 ml) to give a final concentration of 10^{-5} M.

bovine serum albumin in plastic microtubes (1). After centrifugation in a Beckman microfuge, supernatants were discarded and the radioactivity in each pellet was determined. Cell viability was determined at the beginning and conclusion of each experiment by the Trypan Blue dye exclusion method (12). In general, 90% or more of the cells were viable at each determination.

Binding of [^{125}I]Insulin to Human Fibroblasts. Human skin fibroblasts were obtained by punch biopsy from nondiabetic volunteers of both sexes. Fibroblasts were cultured in monolayer at 37° for 10–22 days in 10 ml of Eagle's nonessential medium reinforced with 10% fetal calf serum and 50 μg of neomycin per ml. Media were changed every 3 days. On the day of experiment, the medium was removed and cells were washed five times with 5-ml aliquots of cold Dulbecco's solution (14).

Fibroblasts (2–4 × 10^6 cells) were incubated in 2 ml of Dulbecco's medium containing 15 mg of bovine serum albumin (Armour Fraction V) and 0.05 mg of bovine growth hormone per ml. Serum albumin did not prevent "nonspecific" or random binding of labeled and unlabeled insulin to the plastic incubation flasks. Bovine growth hormone, for reasons not yet clear, was effective in preventing this random adsorption and was therefore routinely included in the incubation mixtures with the fibroblasts. [^{125}I]insulin (0.20 nM) plus various amounts of unlabeled insulins and insulin derivatives were added and the assay mixtures were incubated for 45 min at 37° in a humidified incubator. At the end of incubation, the medium was aspirated and the cells were washed six times with 5-ml aliquots of Dulbecco's media containing 15 mg of bovine serum albumin per ml. After the final wash, the cells were removed from the surface of the flask by incubation with 1 ml of 2 N NaOH for 2 hr at room temperature, followed by a 5- to 15-min heating in a water bath at 80°. The cells were then assayed for radioactivity in an automatic well-type counter. In order to determine cell populations, the protein content of aliquots of cell suspensions was measured by the method of Lowry *et al.* (15).

RESULTS

Human erythrocytes, lymphocytes, granulocytes, granulocytic leukemic white cells, cultured human lymphocytes, and cultured human fibroblasts, all have specific insulin binding sites. These cells bound [^{125}I]insulin, and the bound hormone was displaced by nanogram quantities of unlabeled peptides. The ability of insulins and insulin derivatives to displace labeled insulin from these binding sites was proportional to their ability to stimulate glucose oxidation in isolated fat cells (1). The binding of [^{125}I]insulin to erythrocytes and fibroblasts was different in several respects from that observed in liver, fat, and white cells.

Binding of [^{125}I]insulin to cultured human lymphocytes

At a concentration of 0.05–0.10 nM [^{125}I]insulin per ml cultured lymphocytes (5–7 × 10^6 cells) bound about 10% of the total radioactivity. Unlabeled insulin at a concentration of

1–2 ng/ml, which is well within the range of circulating insulin concentration *in vivo* (1, 16), displaces 12% of the bound radioactivity; 10 and 100 ng of insulin per ml displace 50 and 100% of the bound radioactivity, respectively (Fig. 1).

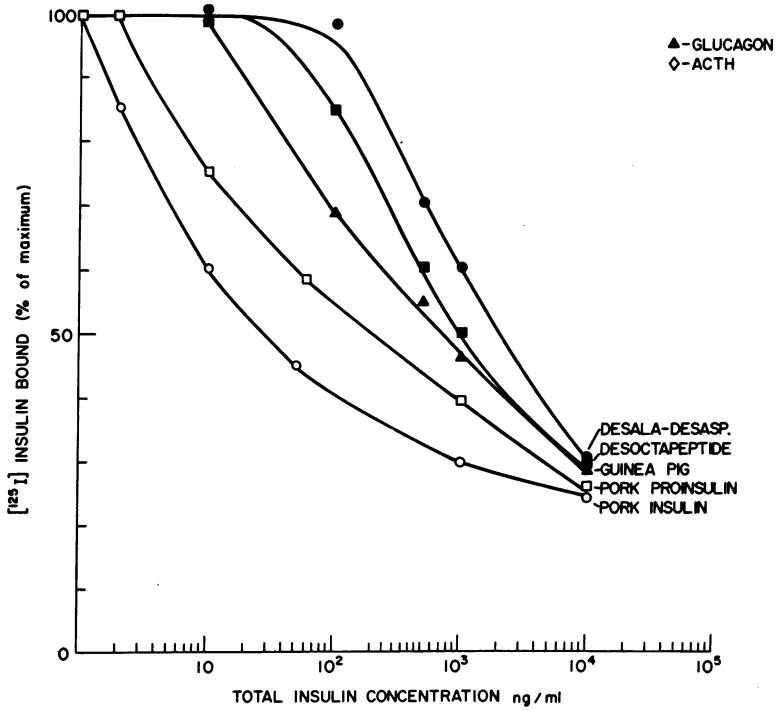
Pork proinsulin, which has 10% of the activity of insulin (on a weight basis) in stimulating glucose oxidation in fat cells and in displacing [^{125}I]insulin from liver cell receptors (1), had about 10% of the activity of insulin in displacing labeled insulin from lymphocytes (Fig. 1). Similarly, guinea pig insulin, desoctapeptide insulin, and desalanine–desasparagine insulin, which have 1–2% of the activity of insulin in the isolated fat cells and liver membranes, had about 3, 1.2, and 1%, respectively, of the activity of insulin in displacing the labeled hormone from lymphocytes. "A" chain and "B" chain of the insulin molecule had small effects that could be completely accounted for by contaminations of these preparations with less than 0.005% of insulin. Other hormones that do not affect insulin binding in fat cells or liver membranes also had no effect on binding of hormone to the lymphocytes (Fig. 1).

The amount of [^{125}I]insulin that bound to cultured lymphocytes was directly proportional to the cell concentration over a 48-fold range (0.5–24 $\times$ 10^6 cells). Binding was rapid; at 30° maximal binding was observed within 10 min (Fig. 2). The degree of binding was temperature-dependent in the same fashion observed for binding of insulin to liver (1), ACTH to adrenal (17), and glucagon to liver (5) cells. Upon addition of 60 μg (10 μM) of unlabeled insulin per ml at 30°, the bound insulin was rapidly dissociated from the lymphocytes; 50% of the bound insulin was released within 3 min and more than 80% was dissociated in 15 min (Fig. 2).

Proteolytic degradation of the insulin exposed to lymphocytes at 30 and 15° was investigated by measurement of the trichloroacetic acid precipitability, adsorption to 50 mg of talc, and antigenic integrity of the labeled hormone recovered from the supernatant as discussed by Freychet *et al.* (manuscript in preparation). By these criteria, insulin exposed to the cells up to 90 min at either temperature (15 or 30°) was not degraded (data not shown). We have calculated an apparent binding constant of 1.5 $\times$ 10^9 M^{-1} and about 12 $\times$ 10^8 binding sites per cultured human lymphocyte (18).

Binding of [^{125}I]insulin to normal human lymphocytes

When [^{125}I]insulin (0.05–0.10 nM) was incubated with normal human lymphocytes (50 $\times$ 10^6 cells) at 30° for 10 min, about 5% of the total radioactivity was bound. The kinetics of [^{125}I]insulin binding with normal human lymphocytes were identical to those obtained with the cultured lymphocytes. However, at 30° the normal human lymphocytes showed "clumping" and decreased viability with prolonged incubations of highly-concentrated cell suspensions. This phenomenon has been observed and discussed earlier (19, 28). No clumping was observed in lymphocyte preparations incubated at 15°. Quantitatively, the displacement of labeled hormone by unlabeled insulin, pork proinsulin, guinea pig, desoctapeptide, and desalanine–desaparagine insulins and other hormones was very close to that observed with cultured



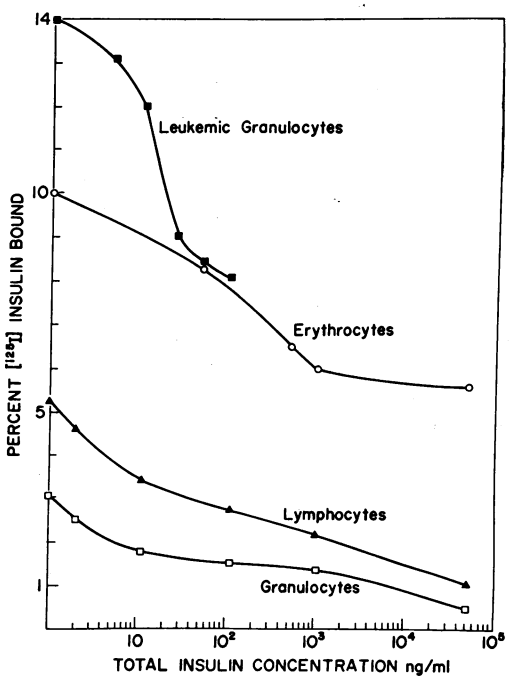


FIG. 3 (*left*). Effect of insulins, insulin derivatives, and other peptides on [^{125}I]insulin binding to normal human lymphocytes, which (60 $\times$ 10^6 cells/ml) were incubated under identical conditions as cultured lymphocytes. In most experiments, 20–30% of the bound radioactivity was not displaced by insulin at a concentration of 1 μM. This represented material trapped in the pellet or nonspecifically adsorbed to the cell surfaces. Results of three experiments were combined.

FIG. 4 (*right*). Effect of unlabeled insulin on binding of [^{125}I]insulin to human circulating cells. Granulocytes and lymphocytes were incubated in the presence of 0.1–0.2 nM [^{125}I]insulin for 10 min, and erythrocytes were incubated in the presence of 1.2 nM [^{125}I]insulin for 40 min at 30° under conditions described in *Methods*. Results of two experiments were combined.

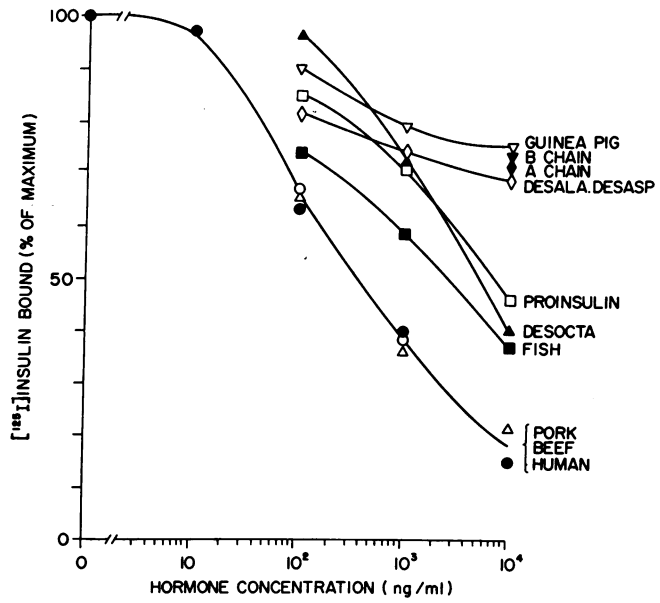


FIG. 5. Effect of insulins and insulin derivatives on binding of [^{125}I]insulin to human skin fibroblasts. Details of assay procedure are described in *Methods*. The inhibition of [^{125}I]insulin binding, expressed as percent of maximum, is plotted as a function of concentration of unlabeled peptide. All points were corrected for the "nonspecific" binding and trapping of labeled hormone as discussed (1). Results of six experiments were combined.

human lymphocytes (Fig. 3). This finding is consistent with the similarity between the apparent binding constants of the cultured and normal human lymphocytes; we have calculated an apparent affinity constant of 0.97×10^9 M^{-1} for the normal cells. In addition, we have calculated about 10^3 binding sites per isolated human lymphocyte. It should be noted that no degradation of insulin was observed upon exposure to normal lymphocytes at 30° for 45 min or at 15° for 90 min.

Binding of [^{125}I]insulin to human granulocytes and erythrocytes

When labeled insulin (0.05–0.1 nM) was incubated with normal human granulocytes (about 60×10^6 cells), about 3.0% of the total radioactivity was bound (Fig. 4). Physiologic amounts (1–2 ng/ml) of unlabeled hormone produced displacement of the [^{125}I]insulin. To be certain that this binding was to granulocytes and not to other cellular elements that contaminated the preparation, we prepared granulocytes from a patient with granulocytic leukemia. With $150–200 \times 10^6$ cells/ml, 14% of the radioactivity was bound, and the [^{125}I]insulin was displaced by unlabeled insulin (Fig. 4).

When 2.5×10^9 human erythrocytes were incubated with unlabeled insulin in the presence of [^{125}I]insulin (0.10–20 nM) at 30° for 40 min, 10% of the added radioactivity was bound; 45% of the total amount bound was displaced by 50 μg of unlabeled insulin (Fig. 4) per ml, and 20% of the insulin-specific binding was inhibited by 100 ng of hormone per ml. The relatively small amount of "specific" binding of insulin to erythrocytes was consistent with earlier observations (3, 20).

Binding of [^{125}I]insulin to fibroblasts

In an attempt to locate insulin binding sites in readily-available fixed tissue cells with characteristics similar to those of the blood-cell receptors, we investigated human skin fibroblasts. In a series of experiments, [^{125}I]insulin (0.20 nM) was incubated with human skin fibroblasts; 1.9–4.4% of the total radioactivity was bound to the cells at 37° for 45 min. In the presence of 50 μg of insulin per ml, the binding ranged from 0.9 to 1.3% of the total radioactivity. 50% of the binding of labeled insulin was inhibited by 400 ng of insulin per ml (Fig. 5). Pork, beef, and human insulins were equipotent. Fish insulin, desoctapeptide insulin, and pork proinsulin were significantly less effective. Desalanine–desasparagine insulin, A and B chains, and guinea pig insulin had modest effects (Fig. 5). These results are similar to the specificity observed in inhibition of [^{125}I]insulin binding to rat liver membranes (1).

DISCUSSION

It is striking that all leukocytes as well as normal erythrocytes and cultured fibroblasts contain specific insulin binding sites. Although insulin is reported to have effects in all of these cells (19, 21–25), it is not known whether insulin plays a physiologic role in their metabolic control, especially since the concentrations of insulin that have been effective *in vitro* far surpass those that occur *in vivo*.

Most remarkable is the similarity of the binding sites in the white cells to those that are present in the liver and fat cells—the apparent affinity, specificity, kinetics, and temperature sensitivity. This strongly suggests that the molecular structures that recognize insulin in the three cells are very similar and possibly identical.

The findings reported here require some mention of insulin effects in cultured cells and in isolated peripheral human leukocytes. Insulin has growth stimulating effects in several classes of cultured mammalian cells. It can replace the serum requirement for growth of mouse fibroblasts (26) and cultured Hela cells (27). From these and numerous other studies, it is clear that insulin has stimulatory chronic effects in many cultured mammalian cells. Similarly, Moore *et al.* reported that chronic growth-promoting effects of insulin could be observed with the cell line used in these studies (no. 4265) when the cells were grown in defined media or in media unsupplemented with serum (12). It is as yet unclear whether insulin produces a specific acute response in cultured or circulating lymphocytes (19, 22). Esmann found that a slight increase in glucose uptake and lactic acid production in white cells could be observed only upon prolonged incubations of the cells with insulin (28). Further studies are necessary to resolve the question of acute insulin effects in white cells.

Although it is entirely possible that the white cells are target tissues for insulin, it is also possible that most or all cells possess specific recognition sites for many or all of the polypeptide hormones, and that a target cell is one in which the recognition apparatus is present in high concentrations and is coupled to an effector system, such as the adenyl cyclase system that is found in many of the hormone-sensitive tissues (5).

The cultured tumor lymphocytes are probably the most convenient source of insulin-specific binding molecules for radioreceptor assays of insulin, as well as a source of large quantities of tissue for isolation of the insulin-specific binding molecules. It is interesting that the cultured tumor-lymphocytes and the isolated normal lymphocytes have very similar apparent binding constants, specificities, and kinetics of

binding, but about a 10-fold difference in the calculated number of sites per cell. The cultured lymphocytes have about 10 times more surface area than the normal human lymphocytes‡, so the apparent difference observed in sites per cell may be simply a function of available cell surface area in the two cells.

In the white cells, the insulin binding can be studied under conditions that are most nearly physiological. An important application of these findings is to studies of insulin binding in clinical disorders that are characterized by altered sensitivity to the effects of insulin such as obesity, diabetes, hyperlipidemia, as well as states of hormone excess or deficiency such as acromegaly. Data from preliminary experiments in this laboratory suggest that insulin binding is decreased in the lymphocytes of patients with acromegaly. This finding is consonant with the insulin resistance in acromegaly and suggests that the easiest way for determination of the role of altered receptors in human disorders may be a study of the circulating white cells.

NOTE ADDED IN PROOF

A recent study has shown an effect of insulin on plasma membrane ATPase in human lymphocytes [Hadden, J. W., Hadden, E. M., Wilson, E. E. & Good R. A. (1972) *Nature New Biol.* **235**, 174–176].

We thank P. Gorden, C. Pallayicini, D. Neville, R. Kahn, and I. Goldfine for helpful advice in many aspects of this study, and M. Lesniak for advice and expert technical assistance.

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‡ When cell diameters were micrometrically determined, normal lymphocytes measured 5–12 μm and cultured lymphocytes were 25–35 μm.