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Application of Radioreceptor Assay to Circulating Insulin, Growth Hormone, and to Their Tissue Receptors in Animals and Man

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Introduction

MANY polypeptide hormones, such as adrenocorticotrophic hormone (ACTH), thyroid stimulating hormone (TSH), and the gonadotropins, act on target tissues which secrete substances that can be used to estimate the activity of the whole system. This is not true, however, for insulin or growth hormone. The latter hormones act on a variety of tissues and there is no unique response to indicate the activities of these agents. It is, therefore, no surprise that the advent of radioimmunoassay provides our first real glimpse into the physiology and pathophysiology of both insulin and growth hormone.

Radioimmunoassay is based on the observations of Berson and Yalow (5, 39) that it is possible to generate antibodies of sufficiently high affinity to reversibly bind polypeptide hormones present in minute concentrations in biological fluids. Their studies provided the ideas and techniques for: (a) measuring the concentration of a material by its ability to compete with a labeled hormone for antibody binding sites (39), and (b) quantitative techniques for studying the nature of the antibody combining site (5).

There is now a strong body of evidence to suggest that the initial action of insulin and probably all other polypeptide hormones is binding to a specific receptor in the cell membrane. Thus measurement of the potency of a polypeptide hormone to compete for binding to cellular receptors gives an estimate of the biological potency of the preparation (fig. 1).

Since it is now clear that both insulin and growth hormone exist in multiple forms in both their glands of origin and in plasma, it is important to assess the biological as well as the immunological properties of each component. The same techniques and analyses used to study the hormone may also be used to study the hormone receptor in experimental animals or in man in both normal and pathological states (fig. 1).

The essential ingredients of the radioreceptor system are a high specific activity, biologically-active labeled hormone, and a convenient cell or subcellular membrane fraction containing the specific receptor.

The remainder of this presentation will be devoted to how these conditions have been met and applied to insulin and growth hormone. For a more complete review of the history and application of these techniques to other hormones see Roth (32).

Radioreceptor Assay of Insulin

The mechanism whereby insulin exerts its potent and multiple effects in a variety of tissues has been the subject of much study, numerous publications and considerable controversy. For the purpose of this discussion we need only assume that at least one action of insulin is reversible binding to a biologically significant receptor. This as-

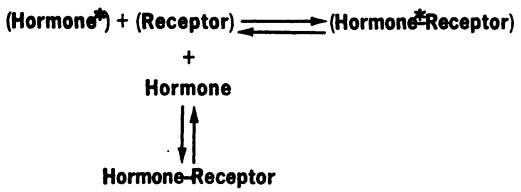

FIG. 1. Diagrammatic representation of the binding of ^{125}I-labeled hormone (*) to a biologically specific receptor and the competition for binding by unlabeled hormone. In this scheme the concentration of an unknown hormone can be measured by its ability to inhibit binding of the labeled hormone. Alternatively both the numbers and affinity constants of the receptors can be determined by appropriate manipulation of data acquired in this way (From S. A. Berson and R. S. Yalow: Quantitative aspects of the reaction between insulin and insulin-binding antibody. J. Clin. Invest. 38: 1996–2016, 1959, and C. R. Kahn, P. Freychet, D. M. Neville, Jr. and J. Roth: Quantitative aspects of the insulin-receptor interaction in liver plasma membranes. J. Biol. Chem., submitted for publication.)

TABLE 1

*Tissues shown to specifically bind insulin**

Liver
Plasma membrane fractions
Isolated hepatocytes
Adipose
Plasma membrane fractions
Isolated adipocytes
Lymphocytes
Established cultured cells
Circulating cells
Transformed cells
Fibroblasts
Chondrocytes
Placenta
Nerve
Muscle

* For complete reference list see table 1 in J. Roth: Peptide hormone binding to receptors: a review of direct studies *in vitro*. Metabolism, in press.

sumption is essential to the concept of a radioreceptor or radiobiological assay.

High specific activity, biologically active ^{125}I-insulin has been prepared by a modification of the chloramine-T method. A variety of tissues including hepatocytes, adipocytes or purified plasma membrane fractions of either of these cells (table 1) has been used for binding studies (32). Tissues of laboratory rodents have been used most extensively in receptor preparations. More recently it has been shown that both circulating (1, 12) and cultured human (11, 12) lymphocytes contain specific binding sites for insulin. While the lymphocyte is undoubtedly not a major site of insulin action, several effects of insulin on lymphocytes have been demonstrated. It is not clear whether the binding of insulin to the lymphocyte is related to a specific metabolic event, but the biological specificity of the lymphocyte receptor is exactly the same as receptors in the major target tissues of insulin. The cultured human lymphocyte provides a highly satisfactory tissue for use in the radioreceptor assay. It is convenient to handle, requires minimal preparation, and provides the required sensitivity and specificity.

When a tracer of ^{125}I-insulin is incubated with cultured human lymphocytes, binding occurs. The percentage of the tracer bound is a function of the cell concentration, and the binding reaction is time and temperature dependent. Binding of the labeled hormone is inhibited by as little as 0.1 ng/ml of unlabeled insulin. Thus, the sensitivity of this system is well within the physiological range of plasma insulin concentrations. Inhibition of ^{125}I-insulin binding to the lymphocyte receptor occurs only with the addition of insulin or insulin derivatives; other polypeptide hormones do not react. The stimulation of glucose oxidation in fat cells is a standard method to determine the biological potency of insulin. The ability of a given insulin preparation to inhibit the binding of ^{125}I-insulin to lymphocytes or to purified liver membrane receptors is directly proportional to the potency of that preparation to stimulate glucose oxidation in fat cells. Of particular interest for this discussion is that proinsulin has only about one-fifth the potency in both the receptor assay and the glucose oxidation assay as does insulin. By contrast, the radioimmunoassay may dis-

tinguish insulins of equal biological potency. For example, beef, pork, and human insulins, which react essentially the same in the radioreceptor or bioassay, are readily distinguished by the immunoassay. On the other hand, the radioimmunoassay may distinguish poorly between preparations of very different biological activity. For example, proinsulin, which is readily distinguished by radioreceptor and bioassay, is poorly distinguished by the standard insulin immunoassay (10, 11). [Proinsulin may be distinguished by a specific immunoassay designed to measure the connecting peptide (30)]. Proinsulin which exists both in the pancreas and in plasma provides an excellent example of how the receptor and immunoassay compliment each other.

Proinsulin is the single chain biosynthetic precursor of insulin (37); its molecular weight is approximately 9000 whereas insulin is about 6000. The molecule contains the complete insulin structure in which the A and B chains of insulin are joined by a connecting peptide which links the carboxy-terminus of the B chain with the amino-terminus of the A chain. Proinsulin is converted to insulin in the granules of the *beta* cell and usually constitutes less than 5% of the total immunoreactive insulin extracted from the pancreas (7, 36).

When plasma is filtered over Sephadex G-50 and the immunoreactivity of each effluent fraction is measured between markers of albumin and salt, two components are readily distinguished (13, 20, 31, 33). The major component elutes coincident with labeled insulin and is indistinguishable from pancreatic insulin. The less retarded component elutes coincident with labeled proinsulin and is proinsulin-like (18, 35). In the basal state the proinsulin-like component comprises from 10 to 30% of the total immunoreactive insulin in most subjects. Greater proportions are seen in some hypoinsulinemic subjects and the proinsulin-like component may constitute virtually all the immunoreactive insulin in some patients with islet cell tumors (19, 20). When insulin secretion is stimulated by glucose or a variety of other secretogogues, the insulin component is mainly released and results in a fall in the percentage of proinsulin-like component. By 2 hr after stimulation, the proinsulin-like component assumes the same proportions of the total immunoreactive insulin as seen in the basal state (18, 19).

When the plasma insulin and proinsulin-like components are separated completely from each other and each assayed in the isolated fat cell system, the ability of the insulin component to stimulate glucose oxidation is exactly the same as the insulin standard; the proinsulin-like component is much less active and is indistinguishable from the pancreatic proinsulin standard. Similarly, in the radioreceptor assay the insulin component inhibits binding of labeled insulin in the same fashion as the insulin standard, whereas the proinsulin-like component is less active and indistinguishable from the proinsulin standard (fig. 2).

In extracts of bovine and porcine pancreas, several partially cleaved proinsulin molecules have been described. These proinsulin intermediates may have the same biological activity as proinsulin or the biological activities may be greater than proinsulin (7, 36). When porcine proinsulin intermediates are assayed in either the liver membrane or lymphocyte radioreceptor assay, they inhibit the binding of labeled insulin in a fashion that would be expected from their biological activity (11a). Whether these intermediate components exist in human plasma is less certain. Gutman *et al.* (21) have demonstrated immunoreactive materials from plasma, by polyacrylamide electrophoresis, which they feel represent proinsulin intermediates. We have shown that the proinsulin-like material from plasma is heterogeneous based on gel filtration, immunological, and biological properties and that one form or the other may predominate in different disease states (15). The most distinct example of this heterogeneity is in islet cell carcinoma but our material appears to be different from the known proinsulin intermediates and its exact nature remains unclear (15). In addition, a still larger immunoreactive component has

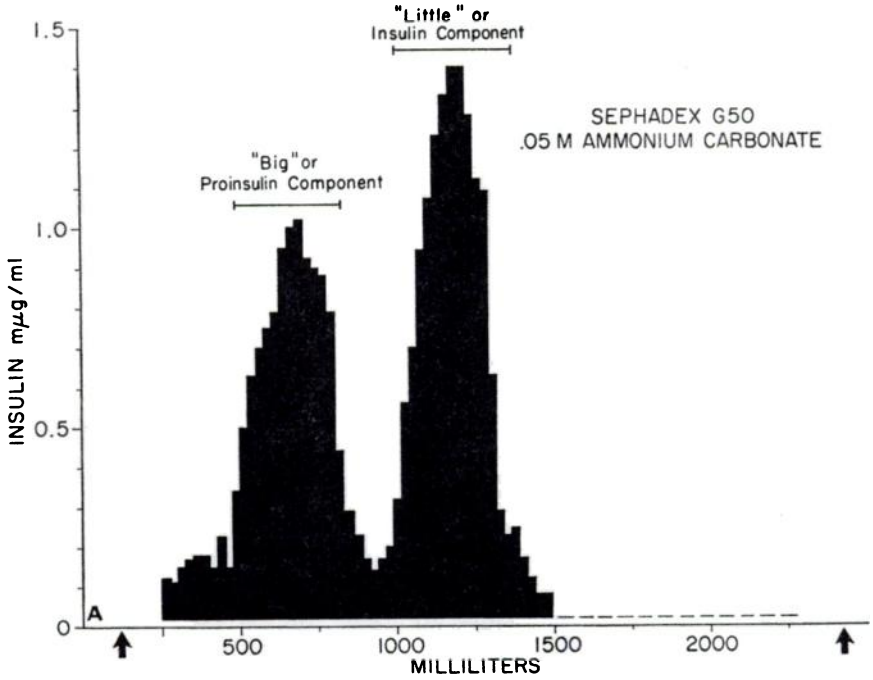

FIG. 2A. Sephadex gel filtration pattern. Plasma from an obese subject was filtered on a column of Sephadex G-50. The column was eluted with 0.05 M ammonium carbonate and the insulin concentration in each effluent fraction between markers of ^{125}I-albumin (↑) left and ^{125}I (↑) right is plotted as a function of the elution volume. The fractions shown under the bracket labeled "big" or proinsulin component and "little" or insulin component were separately pooled, lyophilized, and refiltered to give the patterns shown in figure 2B.

been demonstrated in a patient with islet cell carcinoma (6).

The possibility that plasma factors other than immunoreactive insulin may react with the insulin receptor was recently suggested by Hintz *et al.* (22). They found that somatomedin inhibited the binding of labeled insulin to receptors on adipocytes, liver membranes, and chondrocytes. Somatomedin is a plasma factor that is thought to mediate at least some of the effects of growth hormone (8). This material reacts very poorly with insulin antibodies but exhibits insulin-like effects on fat metabolism, protein synthesis and cell growth (8). This is an example of a material whose biological properties parallel its ability to compete with insulin for biological receptors but which is immunologically relatively inert. Furthermore, the activity of somatomedin on chondrocytes, its major target tissue, is greater than its effects on adipocytes and liver membranes (22). With an acid-ethanol soluble non-suppressible insulin-like activity from plasma (NSILA-S) (23), which is thought to be the same or closely related to somatomedin, we have shown that the potency of this material to compete for insulin receptors on liver membranes and cultured lymphocytes is much greater than can be accounted for by the immunological potency of the material (11a).

Radioreceptor Assay of Growth Hormone

The same features required for the insulin receptor system are also necessary for the growth hormone binding system. These include high specific activity labeled growth hormone and a suitable tissue containing the biologically significant receptor (28). Since human growth hormone is active only in man, a human tissue is required. As with the insulin system the cultured human lym-

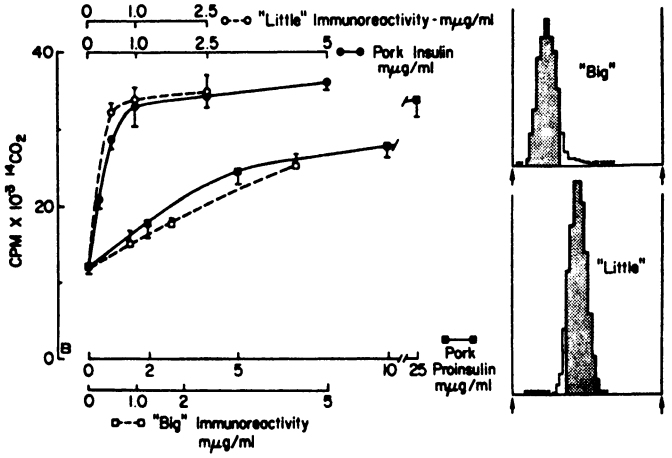

FIG. 2B. The fractions comprising the hatched areas of the gel filtration patterns (axes are the same as in fig. 2A) labeled "big" and "little" were separately pooled, lyophilized, and used for the fat cell bioassay shown on the left and the radioreceptor assay shown in figure 2C. In the bioassay shown on the left, labeled glucose is incubated with isolated rat adipocytes. $^{14}CO_2$ is plotted as a function of the insulin concentration. Since the plasma "big" and "little" components were measured by radioimmunoassay against an insulin standard, these components and the insulin standard are plotted on the same scale (see upper and lower scales). The pork proinsulin scale has been adjusted so that it corresponds to the insulin scale on a molar basis. (From B. M. Sherman, P. Gorden, J. Roth and P. Freychet: Circulating insulin: the proinsulin-like properties of "big" insulin in patients without islet cell tumors. J. Clin. Invest. **50**: 849–858, 1971.)

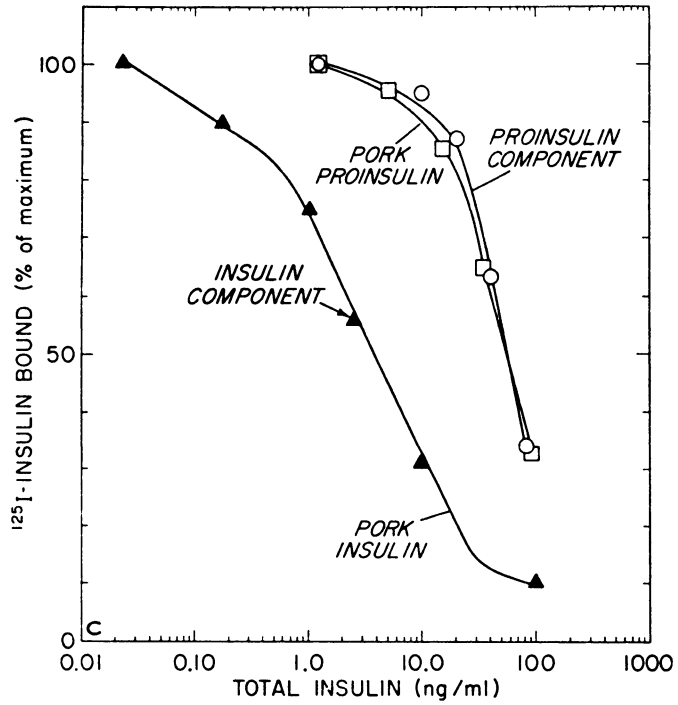

FIG. 2C. The same components as shown in figure 2B assayed in the cultured lymphocyte radioreceptor assay. Competition for binding of ^{125}I-insulin to cultured human lymphocytes is plotted as a function of the hormone concentration. (From J. R. Gavin III, P. Gorden, J. Roth, J. A. Archer and D. N. Buell: Characteristics of the human lymphocyte insulin receptor. J. Biol. Chem. **248**: 2202–2207, 1973.)

phocyte receptor provides the necessary sensitivity and specificity.

The mechanism by which growth hormone exerts its biological effects on tissues is complex and poorly understood. One set of effects appears to occur by way of a growth hormone-dependent serum factor while other effects appear to result from a direct action of growth hormone on a target tissue.

The stimulation of $^{35}SO_4$ uptake by cartilage appears to parallel the growth-promoting effects of growth hormone, yet growth hormone does not elicit this effect *in vitro*. Salmon and Daughaday (34) demonstrated that the administration of growth hormone *in vivo* produces a factor that stimulates sulfate incorporation into cartilage. In addition this plasma factor stimulates thymidine incorporation into deoxyribonucleic acid (DNA), uridine into ribonucleic acid (RNA), and converts proline to hydroxyproline in cartilage (8). This growth-promoting factor which previously went under a variety of names, depending on which activity was studied, is now referred to as somatomedin (8). As previously described, this material is insulin-like (8, 38), which is paradoxical since growth hormone is generally considered to be a counter-insulin hormone. Since the counter-insulin effects of growth hormone can be demonstrated both *in vivo* and *in vitro*, it seems reasonable to conclude that growth hormone exerts its metabolic effects both by generating somatomedin and by its direct effects on a variety of tissues.

Labeled human growth hormone binds to the cultured lymphocyte and is displaced only by human growth hormone or a closely related material such as human placental lactogen which has about 1% of the activity of growth hormone. Animal growth hormones as well as other polypeptide hormones are inactive. Furthermore, the ability of a given human growth hormone preparation to displace growth hormone from the lymphocyte receptor is a function of the biological activity of the preparation, determined by a rat bioassay. This activity may be the same or different from the immunological activity of the preparation. The lymphocyte assay is sensitive to less than 5 ng/ml of growth hormone which is well within the physiological range of plasma growth hormone (28).

When plasma is filtered over Sephadex G-100 and growth hormone immunoreactivity measured in each effluent fraction between markers of γ-globulin and iodide, at least two discrete components are evident (16). The major component corresponds to 22,000 molecular weight growth hormone ("little" growth hormone) and the second component is less retarded by the gel and approximately doubled in molecular weight ("big" growth hormone). In many samples there is a broad, less well defined peak that extends from "big" growth hormone to the void volume of the column. There is no conversion of one component to the other in plasma; the "little" component does not convert to the "big" component, but the "big" component when freed from plasma partially converts to the "little" component. Although the nature of "big" growth hormone remains unclear, the fact that it is present in both plasma and pituitary extracts suggests that growth hormone circulates in these heterogenous forms. Similar components have been described by Goodman *et al.* (14) with Sephadex G-75. In addition, Berson and Yalow (6) have described a higher molecular weight immunoreactive growth hormone component but this component appears to be larger than albumin and it is not completely clear which of our components is most comparable. Bala *et al.* (3) were the first to apply gel filtration to the characterization of plasma growth hormone, but they have not resolved discrete components and their findings are not directly comparable with other studies.

When the fractions corresponding to the "little" growth hormone component are pooled and lyophilized, the reconstituted material gives about the same activity in the lymphocyte receptor assay as in the radioimmunoassay. In contrast, when the fractions corresponding to the "big" growth hormone component are handled in an iden-

tical fashion, the potency of the material in the lymphocyte receptor assay is less than 20% of that in the radioimmunoassay. The decreased radioreceptor activity of the "big" growth hormone component in pituitary powder is exactly the same as seen in plasma (17). The radioreceptor activity of the growth hormone components eluted from Sephadex are, therefore, strikingly similar to the Sephadex components of insulin.

If it is true that the major growth promoting action of growth hormone is by way of the generation of somatomedin and that other actions of this hormone as a counter insulin factor are mediated in another way, the cultured lymphocyte uniquely qualifies for the measurement of both activities. When labeled insulin is used, somatomedin activity is measured, and when labeled growth hormone is used, the intrinsic activity of growth hormone is measured.

Concept of a Receptor Disease (Animal Model)

At the outset we stated that methods used in radioimmunoassay or in radioreceptor assay could be used either to study the hormone, the antibody, or the receptor. Berson and Yalow (5) demonstrated that antibodies contain a heterogeneous population of binding sites of varying affinities and that both the number of sites and affinity constants can be determined. The same techniques can be applied to hormone receptors and in many instances it can be shown that a heterogeneity of binding sites is present (11).[1] Thus, hormone receptors as well as antibody combining sites may be described in terms of their affinity constants and their binding capacity (fig. 1).

Insulin resistance is seen in a variety of pathological states. For the purpose of this discussion it may be defined as a higher than normal requirement of insulin to produce a quantitatively normal response. The abnormality involved in insulin resistance resides at the level of the insulin receptor, at some site distal to the receptor or in multiple sites. The receptor studies now allow us to determine whether or not a defect in the insulin receptor may be involved in at least some states of insulin resistance.

Obesity is an excellent example of an insulin resistant state and is characterized by endogenous hyperinsulinemia, resistance to exogenously administered insulin and frequently by glucose intolerance and hyperglycemia. The genetic obese hyperglycemic mouse (ob/ob mouse) is one of the the most extreme examples of insulin resistance. Kahn *et al.* (24, 25) have demonstrated that under identical conditions of preparation and incubation, the liver membranes of the ob/ob mouse bind only 20 to 25% as much insulin per mg of protein as those of their thin litter mates. This decrease in binding appears mainly to result from a decrease in the receptors of higher affinity (fig. 3). The insulin binding is decreased per mg of protein, per cell, or when compared to the binding of isoproterenol, or other hormones such as growth hormone or glucagon. A similar abnormality has been demonstrated by Freychet *et al.* (9) in adipose cell membranes from these animals. In this animal model the decrease in insulin receptors correlates well with their insulin resistance. The same abnormality appears to extend to other non-genetic states of obesity and insulin resistance (26). The extent to which receptor abnormalities may exist in insulin-resistant states is as yet unclear. Bennett and Cuatrecasas (4) did not demonstrate a defect in insulin binding in adipocytes of steroid and streptozotocin-treated rats who showed insulin resistance *in vitro*, nor was a defect in insulin binding shown in large adipocytes when compared to small adipocytes from rats (29).

Whether the differences in the studies that have shown receptor deficiencies in insulin resistance and those that have not are due to the models or tissues chosen for study is as yet unclear.

[1] Kahn, C. R., Freychet, P., Neville, D. M., Jr. and Roth, J.: Quantitative aspects of the insulin-receptor interaction in liver plasma membranes. J. Biol. Chem., submitted for publication.

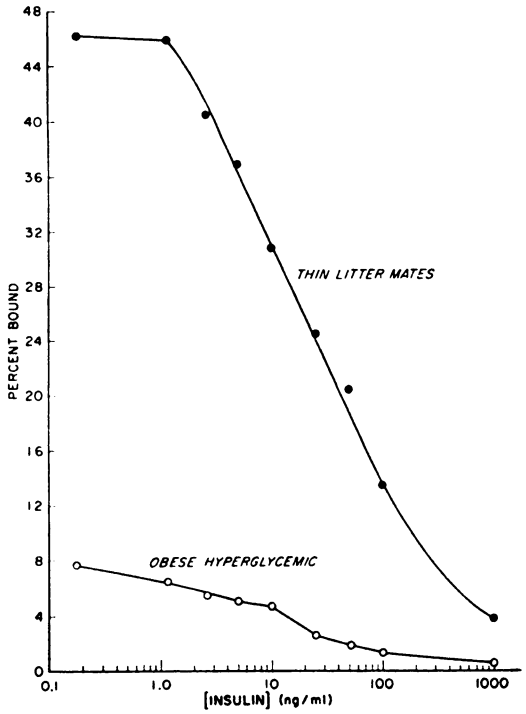

FIG. 3. Inhibition of binding of ^{125}I-insulin by unlabeled insulin from identical liver membrane preparations obtained from the obese hyperglycemic mouse and their thin litter mates. (From C. R. Kahn, D. M. Neville, Jr., P. Gorden, P. Freychet and J. Roth: Insulin receptor defect in insulin resistance: studies in the obese-hyperglycemic mouse. Biochem. Biophys. Res. Commun. **48**: 135–142, 1972.)

Concept of a Receptor Disease (Studies in Human Insulin Resistance)

To study the question of whether a defect in the insulin receptor exists in insulin resistant states in man, a suitable tissue that can easily be obtained under physiological conditions is required. The circulating lymphocyte is ideally suited for these studies. ^{125}I-insulin binds to circulating lymphocytes and is displaced by unlabeled insulin. The binding of labeled insulin to the circulating cell has the same specificity as binding to the cultured lymphocyte, liver, and fat cells (11, 12). The only difference thus far demonstrated between the cultured and circulating lymphocyte is the lower number of binding sites per cell in the circulating lymphocyte (11).

With this tissue, Archer *et al.* (1) have demonstrated that the insulin-lymphocyte receptor interaction in patients who are obese, hyperglycemic and insulin resistant is different from controls of normal-weight. At very low tracer concentrations the binding of ^{125}I-insulin to the lymphocyte is higher in the normal controls than in the obese patients and the shape of the displacement curve is different (1, 2). At each point in the displacement curve more insulin is required to displace labeled insulin from the lymphocyte receptor in obese insulin-resistant patients than in normal-weight controls. In addition, maneuvers that decrease insulin resistance in obesity, such as caloric restriction and weight loss, cause both the lymphocyte insulin receptor in man (2) and the liver receptors in animals (26) to return toward normal. The heterogeneity of binding sites in the lymphocyte complicates interpretation of the data but we suspect that the observed changes in insulin-resistant obese man are due to loss of receptors of the highest affinities which is analogous to what has been observed in the obese hyperglycemic mouse. Finally, we have studied one patient with extreme insulin resistance in whom virtually no binding of labeled insulin to the lymphocyte receptor was demonstrable when compared to either normal-weight or obese controls.

The abnormalities in this patient and the obese patients are in sharp contrast to insulin resistant, hyperinsulinemic acromegalic patients (1) who show no abnormalities in the insulin-receptor interaction.

The binding of labeled insulin to circulating lymphocytes has been a highly reproducible phenomena in our laboratory; the failure of Krug *et al.* (27) to demonstrate this has been discussed (11).

In summary, preliminary data have been presented from both animals and man to suggest that the insulin-receptor interaction is involved in pathological insulin resistance. Undoubtedly this is not the sole defect in all forms of insulin resistance but it is clear that we now have tools to isolate the component

parts and study this important pathological condition directly in man.

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