Proc. Natl. Acad. Sci. USA
Vol. 75, No. 7, pp. 3469–3473, July 1978
Medical Sciences

Insulin resistance due to a defect distal to the insulin receptor: Demonstration in a patient with leprechaunism

(receptor/glucose transport/fibroblasts)

MASASHI KOBAYASHI*, JERROLD M. OLEFSKY*, JOYCELYN ELDERS†, MARY E. MAKO‡, BRUCE D. GIVEN‡, HEINRICH K. SCHEDWIE†, ROBERT H. FISER†, RAYMOND L. HINTZ§, JAMES A. HORNER§, AND ARTHUR H. RUBENSTEIN‡¶

* Division of Endocrinology and Metabolism, Department of Medicine, Stanford University School of Medicine, Stanford, California 94305 and the Palo Alto Veterans Administration Hospital, Palo Alto, California 94305; †Department of Pediatrics, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72201;‡ Department of Medicine, University of Chicago, Chicago, Illinois 60637; and § Department of Pediatrics, Stanford University School of Medicine, Stanford, California 94305

Communicated by Donald F. Steiner, May 8, 1978

ABSTRACT **We have studied a 2-year-old girl with acanthosis nigricans, glucose intolerance, marked hyperinsulinemia, and somatic features characteristic of the leprechaunism syndrome. Circulating plasma insulin levels were increased up to 50-fold and the patient showed a blunted hypoglycemic response to an injection of exogenous insulin (0.2 units/kg), indicating the presence of severe insulin resistance. Insulin purified from the patient's plasma was normal on the basis of chromatographic, electrophoretic, and immunologic criteria. Furthermore, the purified insulin competed effectively with ^{125}I-labeled insulin for binding to insulin receptors on cultured IM-9 lymphocytes and rat fat cells and also exhibited normal biological potency when tested on rat fat cells. Anti-insulin receptor and anti-insulin antibodies were not detected in the patient's plasma, and plasma levels of glucagon, growth hormone, and cortisol were normal. Insulin binding to the patient's circulating mononuclear leukocytes was only slightly depressed into the low normal range and could not account for the severe insulin resistance. Studies on the patient's fibroblasts revealed normal levels of insulin receptors but a total absence of insulin's ability to accelerate glucose transport. Because rates of glucose transport and metabolism were normal in the basal state in the absence of insulin, we conclude that this patient's insulin resistance is due to an inherited cellular defect in the coupling mechanism between occupied insulin receptors and the plasma membrane glucose transport system.**

Leprechaunism is a rare, inherited disease characterized by an unusual facial appearance, hirsutism, cliteromegaly, and sparse subcutaneous fat stores (1–3). We have performed detailed metabolic studies on a 2-year-old girl with this syndrome who also presented with acanthosis nigricans and severe insulin resistance. Theoretically, insulin resistance can be due to abnormal beta cell secretory products, circulating insulin antagonists, or a cellular defect in insulin action (4). To elucidate the etiology of this patient's insulin resistance, each of these possibilities was examined in detail. Thus, we studied the integrity of her circulating insulin, assayed for potential insulin antagonists, measured cellular insulin receptors, and studied the insulin responsiveness of the patient's cultured fibroblasts *in vitro*. The results indicate that this patient's insulin resistance is due to a defect in insulin action distal to the insulin receptor.

CASE REPORT

The patient, an 18-month-old white girl, had been born after a full-term (41-week gestation) uncomplicated pregnancy and delivery to a gravida I para 1 28-year-old mother who had taken no medications during her pregnancy; birth weight was 2600 g. At birth she was noted to have bilateral inguinal and an umbilical hernia, increased body hair, prominent labia majora and clitoris, coarse facial features, and decreased subcutaneous fat. The infant did well and had no apparent difficulties during the immediate postnatal period and infancy. At age 11 months she was referred for evaluation of a possible mucopolysaccharidosis because of coarse facial features. The history at this time, given by the mother, suggested that she had intermittent polyuria and polydipsia, especially if she had consumed a large amount of free sugar. There was also a history of periods of profound deep sleep when the infant would be sweaty and difficult to arouse. There were other times when the infant would have sudden acute severe abdominal distention which would subside over a course of 4–6 hr. Growth and development were considered otherwise normal.

Physical examination revealed a thin, hirsute infant with curly hair and coarse facial features; she was 73.5 cm in length, weighed 7.5 kg and had a head circumference of 44.5 cm. There was a generalized thickening of the skin over the entire body and especially at the knees, ears, elbows, and dorsum of the feet with hypertrichosis and velvety smooth hypermelanotic areas of skin in the axilla, around the neck, and in the postauricular areas. The lips were full and the mucosa of the lips, anus, and labia were all rugated and keratotic. The gums appeared to be somewhat hypertrophic, making the teeth appear short. The breasts were peculiarly shaped with very prominent nipples but no subcutaneous fat or breast tissue. There was enlargement of the clitoris (11 mm) and the labia majora. The nails were dysplastic, short, thin, and hyperconvex. The remainder of the physical findings were considered to be within normal limits.

Apart from the 24-hr urinary glucose excretion (5–14 g/24 hr), the results of routine laboratory tests were within normal limits. A skin biopsy was compatible with the diagnosis of acanthosis nigricans with no evidence of a mucopolysaccharidosis on histochemical staining.

MATERIALS AND METHODS

Materials. Porcine monocomponent insulin was generously supplied by Ronald Chance (Eli Lilly & Co., Indianapolis, IN). Na ^{125}I was purchased from New England Nuclear (Boston, MA), bovine serum albumin (fraction V) from Armour Pharmaceutical Co. (Phoenix, AZ), and [*1*-^{14}C]glucose and 2-

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviation: ^{125}I-insulin, ^{125}I-labeled insulin.

¶ To whom to address reprint requests at: Department of Medicine, University of Chicago, 950 E. 59th St., Chicago, IL 60637.

deoxy[1-^{14}C]glucose from New England Nuclear. Cyanogen bromide-activated Sepharose 4B was obtained from Pharmacia (Upsala).

Iodination of Insulin. ^{125}I-Labeled insulin (^{125}I-insulin) was prepared with a specific activity of 100–150 μCi/μg according to the modification by Freychet *et al.* (5) of the method of Hunter and Greenwood (6) as described (7).

Purification of Serum Insulin and Radioimmunoassay Procedure. Serum proinsulin and insulin were separated on a Bio-Gel P-30 column (8). The radioimmunoassay method for measuring insulin in serum and in the column fractions has been described (8). Screening of the infant's serum for anti-insulin antibodies was carried out according to Goldman *et al.* (9).

Coupling of anti-insulin globulin to cyanogen bromide-activated Sepharose-4B was accomplished by a technique similar to that of Akanuma *et al.* (10). One hundred twenty milliliters of a solution consisting of 1 vol of serum and 2 vol of buffer (0.113 M boric acid, pH 8.0) was passed over 2.0 ml of anti-insulin globulin-coupled agarose (binding capacity, 1.5 units/ml) in a 10 × 150 mm siliconized column at a rate of 15 ml/hr at room temperature. The beads were washed successively with 20 ml of borate buffer (containing 1% bovine serum albumin), 10 ml of borate buffer (without albumin), and 5.0 ml of distilled water. The material bound to the antibodies was eluted with 1 M acetic acid/0.01% albumin and collected in 1.0-ml fractions. Aliquots from each tube were dried under vacuum, dissolved in buffer, and assayed. The tubes containing the insulin peak were pooled and stored at −20°.

Binding Studies. Insulin binding to the patient's circulating mononuclear leukocytes was measured according to described methods (7, 11, 12). Insulin binding to cultured fibroblasts was measured by the method of Rechler *et al.* (13). Fibroblasts were cultured from a skin biopsy obtained from the deltoid region. Cells were propagated in culture at 37° in Eagle's minimal essential medium supplemented with 5 mM glutamine and 15% fetal calf serum. Cultures were trypsinized and divided each week and used between the sixth and eighth weekly passages. At the time insulin binding was determined, cells were brought into suspension by a brief exposure to trypsin under conditions shown not to destroy insulin receptors (0.5 mg of trypsin per ml for 30 sec) (13). Insulin binding studies were then performed by incubating cells (2×10^6) with various concentrations of labeled and unlabeled insulin at 15° for 90 min in 0.5 ml of buffer (pH 7.6) containing 35 mM Tris, 120 mM NaCl, 1.2 mM $MgCl_2$, 4 mM KCl, 10 mM glucose, 1 mM EDTA, and 1% bovine serum albumin.

Studies of Fibroblast Glucose Metabolism and Transport. [1-^{14}C]Glucose uptake was determined by incubating a cell suspension in Krebs–Ringer bicarbonate buffer containing 1% bovine serum albumin and 5 mM glucose for 1 hr at 37° in a shaking water bath. The reaction was terminated by rapidly centrifuging the incubation tubes in an Eppendorf centrifuge (model 3200). After being washed three times in iced buffer, the pellet was resuspended and cell-associated radioactivity determined.

Glucose transport was assessed by measuring the initial rates of uptake of 2-deoxyglucose (14, 15). 2-Deoxyglucose uptake was determined in the same way as that of glucose, except that the assay was carried out for 3 min at 37° at a 2-deoxyglucose concentration of 0.1 mM. This assay measures the total uptake of the radiolabeled 2-deoxyglucose and is based on the principle that, although 2-deoxyglucose is transported and phosphorylated by the same processes as D-glucose, it cannot be further metabolized (16).

Adipocyte Studies. Insulin binding and glucose oxidation by isolated rat adipocytes were determined according to methods previously described in detail (14).

Table 1. Oral glucose tolerance test

Time after glucose ingestion, min	Glucose conc., mg/100 ml	Insulin conc., μunits/ml
0	45	135
30	146	360
60	220	2970
90	241	4400
120	223	4610
180	147	3609

RESULTS

***In Vivo* Studies of Insulin Sensitivity.** An oral glucose tolerance test demonstrated a low fasting plasma glucose concentration, modestly increased plasma glucose levels at 30–180 min, and markedly increased plasma insulin levels (Table 1). The plasma glucose levels exceed the normal range for this patient's age according to Rosenbloom *et al.* (17), and the plasma insulin levels are 50-fold greater than the expected values. These findings strongly suggest an insulin-resistant state, and this was confirmed by demonstrating that intravenous injections of porcine insulin (0.15 unit/kg on one occasion and 0.2 unit/kg on a second occasion) elicited less than a 45% decrease in the plasma glucose level, whereas the expected decrease in normal children with 0.1 unit/kg is greater than 50%.

Integrity of Circulating Insulin. Radioimmunoassay of the patient's plasma gave the same insulin values with three different antisera. The immunoreactive material in the patient's plasma behaved identically to a porcine insulin standard upon serial dilution, and the contribution of proinsulin (10%) to the total immunoreactive insulin material was normal (8).

Insulin isolated from the patient's plasma by affinity chromatography was indistinguishable from standards by chromatographic and electrophoretic criteria. Receptor binding and biological potency of the insulin purified from the patient's plasma were also normal. The patient's insulin inhibited ^{125}I-insulin binding to isolated rat adipocytes (Fig. 1 *left*) to the same extent as porcine and human standards. Comparable results were found with IM-9 cultured lymphocytes. The biologic potency of this insulin was also intact as demonstrated by its ability to promote [1-^{14}C]glucose oxidation by isolated rat adipocytes (Fig. 1 *right*).

Insulin Antagonists. Because anti-insulin receptor antibodies have been reported in patients with insulin resistance and acanthosis nigricans (18, 19), it seemed possible that this mechanism was the cause of the insulin resistance in our patient. Therefore, the patient's plasma was studied for the presence of anti-insulin receptor antibodies according to the procedures described by Flier *et al.* (18). Fig. 2 demonstrates that preincubation of IM-9 lymphocytes with the patient's serum, control serum, or buffer did not affect the subsequent measurements of insulin binding. Comparable findings were observed when human placental membranes were used as a source of insulin receptors (data not shown).

Other known circulating insulin antagonists were assessed with negative results. Thus, the patient's plasma did not contain anti-insulin antibodies (9), and plasma levels of glucagon, growth hormone, and cortisol were normal.

Insulin Binding to Circulating Monocytes. On the basis of the above results it seemed likely that this patient's insulin resistance was due to a cellular defect in insulin action. Most insulin-resistant states in man have been associated with a de-

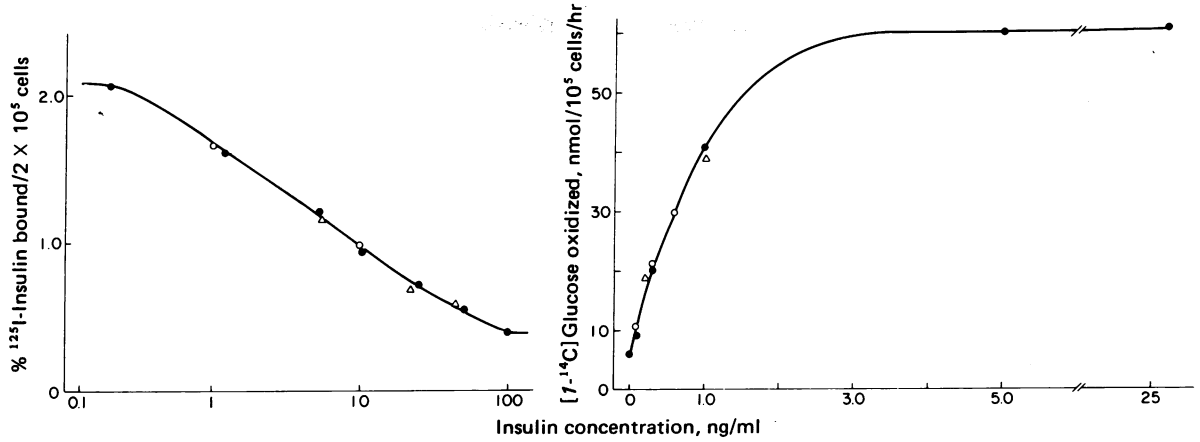

FIG. 1. (*Left*) Ability of insulin purified from the patient's plasma to inhibit ^{125}I-insulin binding to isolated rat adipocytes. ^{125}I-Insulin (0.2 ng/ml) was incubated with isolated rat adipocytes (2×10^5 cells per ml) with the indicated unlabeled insulin concentrations for 90 min at 24°. The concentration of the patient's insulin was determined by radioimmunoassay. ●, Porcine standard; ○, control subject's purified insulin; △, patient's purified insulin. (*Right*) Ability of insulin purified from the patient's plasma to stimulate [*1*-^{14}C]glucose oxidation by isolated rat adipocytes. Adipocytes (2×10^5 cells per ml) were incubated with [*1*-^{14}C]glucose at a total glucose concentration of 2 mM, at 37°, for 60 min. ●, Porcine standard; ○, control subject's purified insulin; △, patient's insulin.

crease in insulin receptors (20, 21), and therefore insulin binding to freshly isolated circulating mononuclear leukocytes was examined. Fig. 3 shows that the insulin binding to this patient's circulating mononuclear cells was in the low normal range. Thus, although insulin binding was somewhat depressed, sufficient numbers of insulin receptors were present to result in adequate insulin action, especially at the supraphysiologic levels found in the patient's plasma (20, 23). Consequently, it seemed probable that the insulin resistance was due to a defect distal to the binding of insulin to its receptor.

Insulin Binding and Insulin Action in Cultured Fibroblasts. Insulin binding was studied by using fibroblasts that had been cultured for 8 weeks. The results (Fig. 4) indicate that the ability of the patient's fibroblasts to bind insulin was normal. On the other hand, insulin's ability to augment glucose uptake was distinctly abnormal in the patient's cells. The ability of fibroblasts from the patient and a control subject to take up [*1*-^{14}C]glucose in the absence (basal state) and presence of a maximally effective insulin concentration (100 ng/ml) is shown in Fig. 5 *left*. Basal uptake rates were similar in both cases, but insulin was unable to stimulate glucose uptake in the patient's fibroblasts compared to a nearly 2-fold increase in control cells. Glucose uptake reflects glucose transport, glycolysis, and incorporation of glucose carbons into cell-associated macromolecules. However, insulin's effects on cellular glucose metabolism are primarily mediated through acceleration of glucose transport (25). In order to study this aspect of insulin action specifically, 2-deoxyglucose uptake was measured (14, 15, 23). Basal rates of transport were normal in the patient's fibroblasts, but insulin was unable to accelerate uptake in the patient's cells compared to a 2-fold increase in control fibroblasts (Fig. 5 *right*). Comparable data on glucose uptake and 2-deoxyglucose transport were obtained when fibroblasts were studied without trypsinization, while still adherent to the culture flasks (data not shown).

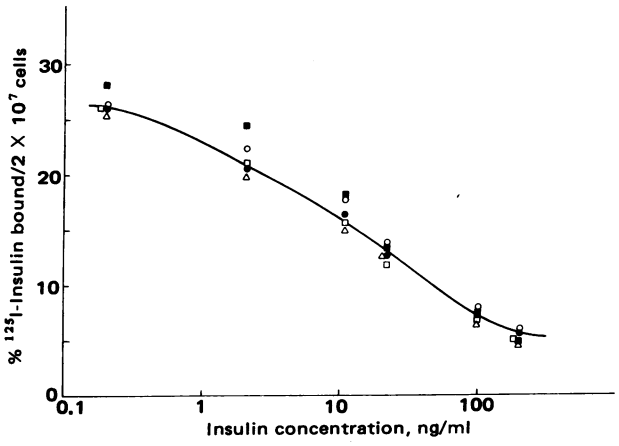

FIG. 2. Effect of preincubating IM-9 cultured lymphocytes with the patient's serum on subsequent measurements of insulin binding to receptors. IM-9 lymphocytes were preincubated with patient's serum (■), normal serum (○), buffer (△), or patient's serum diluted 1:10 (□) for 90 min at 15° as described by Flier *et al.* (18). The curve drawn through the solid circles (●) represents insulin binding to IM-9 lymphocytes without preincubation. Although exposure to the patient's undiluted serum and control serum resulted in slightly higher insulin binding at same insulin concentrations, none of these differences was statistically different from control incubations.

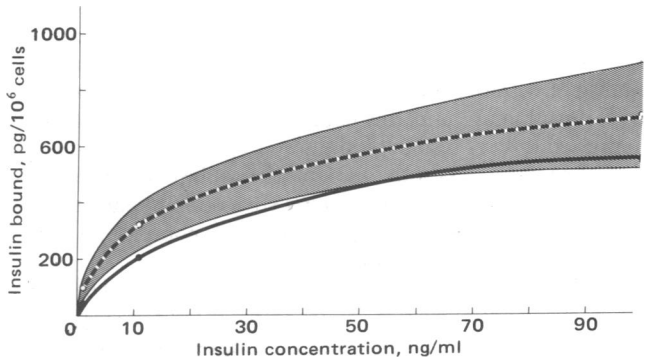

FIG. 3. Ability of patient's isolated circulating mononuclear leukocytes to bind insulin. Mononuclear leukocytes were isolated from heparinized blood according to the method of Boyum (22) as described (7, 11, 23). Cells were 97% viable as determined by trypan blue exclusion, and monocytes were determined according to the method of Yam *et al.* (24). Cells were incubated with ^{125}I-insulin (0.2 ng/ml) at the indicated total insulin concentrations for 100 min at 15° in Tris buffer as described (7, 11, 23). Hatched area represents mean ±2 SEM for a series of 20 normal individuals, and the unbroken line (●) indicates the data from the patient's cells.

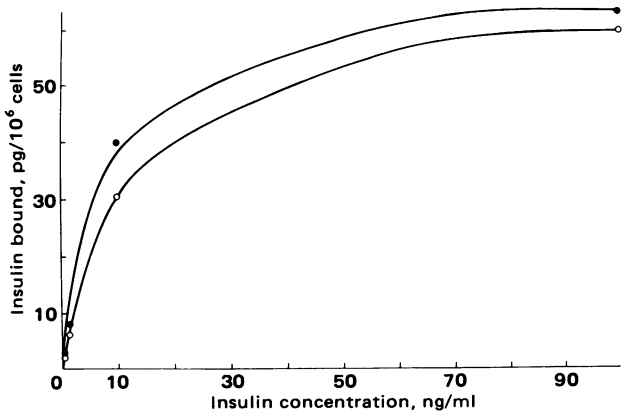

FIG. 4. Insulin binding to control (○) and the patient's (●) cultured fibroblasts. Insulin binding studies were performed after six weekly passages by incubating 10^6 cells per ml with ^{125}I-insulin (0.2 ng/ml) and unlabeled insulin for 90 min at 15° in 35 mM Tris/1% bovine serum albumin, pH 7.6, in 2-mm plastic microtubes. Control cells were from a 2-year-old child.

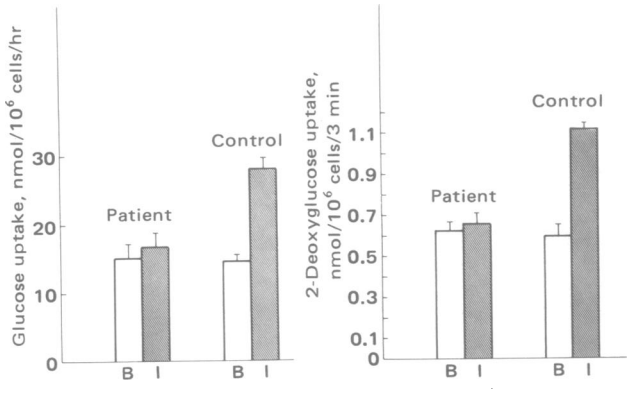

FIG. 5. Ability of the patient's fibroblasts and control fibroblasts to take up and metabolize [1-^{14}C]glucose (*Left*) or transport 2-deoxy[1-^{14}C]glucose (*Right*). Studies were performed in the basal state (B) in the absence of insulin (open bars) and in the presence of a maximally effective insulin (I) concentration (100 ng/ml) (hatched bars). Each bar represents the mean of two separate experiments performed on separate passages of cultured cells on different days.

DISCUSSION

We have studied a patient with severe insulin resistance, acanthosis nigricans, and the somatic features characteristic of the leprechaunism syndrome. This patient was markedly hyperinsulinemic and demonstrated a blunted hypoglycemic response to exogenous insulin. Her circulating insulin was intact by chromatographic, electrophoretic, and immunologic criteria and was normal in its ability to bind to insulin receptors and to promote adipocyte glucose oxidation *in vitro*. The circulating proinsulin concentration was normal. Therefore, this patient's insulin resistance cannot be due to an abnormal beta cell secretory product. Circulating insulin antagonists are also an unlikely cause of the insulin resistance because anti-insulin receptor and anti-insulin antibodies were absent and plasma levels of glucagon, growth hormone, and cortisol were normal.

The above findings point to a cellular abnormality in insulin action. Insulin binding to receptors was in the low normal range when the patient's freshly isolated circulating mononuclear leukocytes were used but was normal when cultured fibroblasts were studied. Gavin *et al.* (26) have previously demonstrated that high levels of insulin can down-regulate its own receptor. *In vitro* and *in vivo* evidence for this regulatory mechanism has also been presented (20, 21). The findings in our patient are consistent with this formulation. Thus, this patient was severely hyperinsulinemic and this could account for the minimally depressed level of insulin binding to circulating monocytes. On the other hand, when the patient's fibroblasts were removed from the hyperinsulinemic *in vivo* environment and cultured for 8 weeks, insulin binding was normal, compatible with a reversal of the receptor loss.

It is highly unlikely that the depressed *in vivo* level of insulin binding accounted for this patient's insulin resistance. First, the slight decrease in insulin binding is out of proportion to the severe hyperinsulinemia and absence of response to exogenous insulin (12, 27). Second, a sufficient number of insulin receptors were present to mediate normal insulin responsiveness, especially at the very high plasma insulin levels observed (20, 23). Therefore, it is most likely that the major cause of this patient's insulin resistance involves a cellular defect in insulin action distal to the insulin receptor. The studies utilizing the patient's fibroblasts support this suggestion. Thus, insulin binding to receptors on cultured fibroblasts was normal, whereas insulin's ability to stimulate cellular glucose metabolism was not. Although basal rates of glucose transport and metabolism were normal, insulin was unable to accelerate these rates in the patient's cells. Because basal transport rates were normal it seems probable that the glucose transport system *per se* was intact. This finding indicates that the specific defect in this patient's cells involves an abnormality in the coupling mechanism between occupied insulin receptors and the plasma membrane glucose transport system. Nonetheless, further studies will be necessary to be certain that the glucose transport system in the patient's fibroblasts is entirely normal. Additionally, it will be of great interest to utilize these cells as a genetic mutant model to explore the specific biochemical alterations that account for this defect in the coupling process.

Kahn *et al.* (19) and Flier *et al.* (18) have reported extensive studies on a series of patients with acanthosis nigricans and insulin resistance. None of their patients had features suggestive of leprechaunism, although some had a mild degree of virilism and hirsutism. All of their patients had markedly decreased insulin binding to circulating monocytes, and in half of the patients this was due to circulating anti-insulin receptor antibodies (18, 19). Although the mechanism of insulin resistance is clearly different in our patient, it is interesting to note the association with acanthosis nigricans in both syndromes. This association suggests that either acanthosis nigricans is related to a closely linked genetic abnormality in both disorders or, more likely, is in some way due to the metabolic abnormalities of insulin resistance.

This work was supported in part by funds from the National Foundation–March of Dimes, Medical Research Service of the Veterans Administration, and CA 13907 and by Grants AM 19905, AM 15901, AM 13941, AM 20595, and AM 20993 from the National Institutes of Arthritis, Metabolism, and Digestive Diseases of the National Institutes of Health. J.O. is a Clinical Investigator with the Veterans Administration. A.H.R. is an Established Investigator of the American Diabetes Association.

1. Donohue, W. L. & Uchida, I. (1954) *J. Pediat.* **45**, 505–519.
2. Dekaban, A. (1965) *Arch. Dis. Child.* **40**, 632–636.
3. Adam, J. M., Gordon, L. P., Dutton, R. V., Rosenberg, M. S. & Rudolph, A. J. (1977) *South. Med. J.* **70**, 998–1001.
4. Reaven, G. M. & Olefsky, J. M. (1978) *Adv. Mod. Nutri.* Vol. II (Part 1), 229–266.

5. Freychet, P., Roth, J. & Neville, D. M., Jr. (1971) *Biochem. Biophys. Res. Commun.* **43,** 400–408.
6. Hunter, W. M. & Greenwood, F. C. (1962) *Nature* **194,** 495–496.
7. Olefsky, J. M. & Reaven, G. M. (1974) *J. Clin. Endocrinol. Metab.* **38,** 554–560.
8. Starr, J. I. & Rubenstein, A. H. (1974) in *Methods of Hormone Radioimmunoassay,* eds. Jaffe, B. M. & Behrman, H. R. (Academic, New York), p. 289.
9. Goldman, J., Baldwin, D., Pugh, W. & Rubenstein, A. H. (1978) *Diabetes* **27,** 653–660.
10. Akanuma, Y., Kuzuya, T., Hayashi, M., Ide, T. & Kuzuya, N. (1970) *Biochem. Biophys. Res. Commun.* **38,** 947–953.
11. Olefsky, J. M. & Reaven, G. M. (1974) *J. Clin. Invest.* **54,** 1323–1328.
12. Olefsky, J. M. & Reaven, G. M. (1977) *Diabetes* **26,** 680–688.
13. Rechler, M. M. & Podskalny, J. M. (1976) *Diabetes* **25,** 250–255.
14. Olefsky, J. M. (1976) *J. Clin. Invest.* **58,** 1450–1460.
15. Olefsky, J. M. (1978) *Biochem. J.,* in press.
16. Wick, A. N., Drury, D. R., Hakada, H. I. & Wolfe, J. B. (1957) *J. Biol. Chem.* **244,** 963–969.
17. Rosenbloom, A. L., Starr, J. I., Juhn, D. & Rubenstein, A. H. (1975) *Diabetes* **24,** 753–757.
18. Flier, J. S., Kahn, C. R., Roth, J. & Bar, R. S. (1975) *Science* **190,** 63–65.
19. Kahn, C. R., Flier, J. S., Bar, R. S., Archer, J. A., Gorden, P., Martin, M. M. & Roth, J. (1976) *N. Engl. J. Med.* **294,** 739–745.
20. Olefsky, J. M. (1976) *Diabetes* **25,** 1154–1165.
21. Roth, J., Kahn, C. R., Lesniak, M. A., Gorden, P., DeMeyts, P., Megyesi, K., Neville, D. M., Jr., Gavin, J. R., III, Soll, A. M., Freychet, P., Goldfine, I. D., Bar, R. S. & Archer, J. A. (1975) *Rec. Prog. Horm. Res.* **31,** 95–140.
22. Boyum, A. (1968) *Scand. J. Clin. Lab. Invest.* **21** (Suppl. 97), 51–76.
23. Olefsky, J. M. (1976) *J. Clin. Invest.* **57,** 842–851.
24. Yam, L. T., Li, C. Y. & Crosby, W. H. (1971) *Am. J. Clin. Pathol.* **55,** 283–290.
25. Morgan, M. E. & Neeley, J. R. (1972) in *Handbook of Physiology,* eds. Greys, G. R. & Astwood, E. B. (Williams and Wilkins, Baltimore, MD), Vol. I, p. 323.
26. Gavin, J. R., III, Roth, J., Neville, D. M., Jr., DeMeyts, P. & Buell, D. N. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 84–88.
27. Bar, R. S., Gorden, P., Roth, J., Kahn, C. R. & DeMeyts, P. (1976) *J. Clin. Invest.* **58,** 1123–1135.