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Insulin Resistance in a Boy with Congenital Generalized Lipodystrophy

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ABSTRACT. We have studied insulin resistance in a 12-year-old Japanese boy who presented with congenital generalized lipodystrophy. Oral glucose tolerance test exhibited a diabetic pattern with normal fasting plasma glucose. Results from euglycemic glucose clamp study showed decreases in both insulin sensitivity and responsiveness. Both the patient's erythrocytes and Epstein-Barr virus transformed lymphocytes showed low-normal insulin binding with a slight reduction in binding affinity in the latter. Insulin binding to the cultured fibroblasts was decreased due to a lowered affinity. In addition, they displayed a rightward shift of the insulin dose-response curve for D-^{14}C-glucose uptake with no decrease in the maximum uptake. Insulin-stimulated autophosphorylation and kinase activity of the wheat germ agglutinin purified receptors from the Epstein-Barr virus-transformed lymphocytes appeared normal. The reason for some discrepancies in insulin binding among the cells remains unknown, and we cannot formulate a conclusion as to whether or not a primary binding defect of insulin receptors exists and contributes to insulin resistance in the patient. The decrease in insulin responsiveness demonstrated in the glucose clamp study may result from a defect at the rate-limiting step in the postbinding process of insulin action, presumably a defect in the glucose transport system in muscle tissues. The defect may be secondary to changes in *in vivo* circumstances. (*Pediatr Res* 24: 668–672, 1988)

Abbreviations

CGL, congenital generalized lipodystrophy
EBV, Epstein-Barr virus
WGA, wheat germ agglutinin
src, the gene responsible for transformation by Rous sarcoma virus
BSA, bovine serum albumin
gMCR, steady-state glucose metabolic clearance rate
B/T, ratio of bound to total ^{125}I-insulin
Ro, total receptor number
Ke, binding affinity constant
IBC, insulin binding capacity
HGP, hepatic glucose production
ID_{50}, insulin concentration necessary to obtain the half-maximum inhibition of specific binding.

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CGL, a rare hereditary disease, was first documented by Berardinelli (1) and Seip (2). The clinical features are observed from birth or early infancy, and the hallmarks include a total lack of fatty tissue, peculiar face, accelerated growth, muscular hypertrophy, acanthosis nigricans, hepatomegaly, and hypertrophied genitals (1–10). In CGL, normal glucose tolerance is observed during the first few years of life. However, between the ages of 7 and 12 yr, glucose intolerance ensues and insulin-resistant and nonketotic diabetes mellitus develops in most cases (6–10). The reason for insulin resistance in CGL remains unknown. Previous studies on the insulin resistance have yielded conflicting results (6–10).

In this paper, we report the case of a 12-yr-old Japanese boy with CGL who showed insulin resistance. To gain further insight into the mechanisms of the insulin resistance, we performed euglycemic glucose clamp studies and investigated insulin binding to circulating erythrocytes, cultured skin fibroblasts and EBV-transformed lymphocytes. Similarly, we estimated insulin-stimulated autophosphorylation and kinase activity of WGA-purified receptors from the EBV-transformed lymphocytes and insulin-stimulated glucose incorporation into the cultured fibroblasts using D-^{14}C-glucose.

PATIENT

The patient had been born to healthy and unrelated parents after a 41-week uncomplicated term pregnancy and normal delivery. His weight at birth was 2040 g; his length 45 cm. At birth, he was noticed to have a generalized deficiency of subcutaneous fat and a short, peculiar face, which was characterized by large eyes, hollow cheeks, micrognathia and mandibular prognathism. Developmental milestones were normal (head control at 4 months, crawl at 8 months, walk alone at 15 months, and speech at 12 months). Between the ages of 8 and 12 yr, he was hospitalized at the Kyoto Municipal Toyo Hospital for the treatment of bronchial asthma. At age 8 yr, bone age was 5 yr, based on the criteria of Greulich and Pyle. At age 10 yr, pubic hair appeared. At age 12 yr, glucosuria was noticed, and hyperpigmentation of the skin in the bilateral axillae appeared.

Based on the above-mentioned clinical findings, he was diagnosed as CGL and referred to Kyoto University School of Medicine Hospital at 12 yr, 7 months of age for further evaluations. Informed consent for all studies was obtained from the patient and his parents. There was no family history of diabetes mellitus, lipodystrophy, or peculiar face. He had a weight of 26.5 kg (−1.9 SD), a height of 141 cm (−1.3 SD), and a head circumference of 48 cm (−3.4 SD). His peculiar face gave him an elderly look. Subcutaneous fatty tissue was diminished everywhere, especially in the face and extremities, and superficial veins were prominent in the extremities. Hyperpigmented skin lesions were present in the bilateral axillae, and the findings of histolog-

ical examination were compatible with those of acanthosis nigricans. His bilateral fifth fingers were short and were similarly observed in his mother. He did not have cardiomegaly but presented mild hepatomegaly. His pubertal development was at stage IV (Tanner's criteria), and bone age was 14 yr. Phallic enlargement was not observed. Karyotype was 46 XY. He had mild perceptive hearing disturbance.

Hematologic study and liver and renal function tests yielded normal results. He had increased serum concentration of immunoglobulin E (1297 IU/ml), and cold activation of complement system was observed (CH_{50}: < 4.3 U/ml in serum and 45.1 U/ml in plasma after freezing). Hyperlipidemia was not observed (serum total cholesterol, triglyceride, and free fatty acid concentrations: 138 mg/dl, 92 mg/dl, and 370 μEq/liter after overnight fasting, respectively). Oral glucose tolerance test (1.75 g/kg of glucose) revealed that plasma glucose concentration increased from 108 mg/dl to 248 mg/dl (at 120 min) and that serum insulin concentration increased from 34.9 to 251 μU/ml (at 120 min). A bolus injection of exogenous insulin (1.0 U/kg, intravenously) showed no remarkable reduction in plasma glucose concentrations. Neither anti-insulin nor anti-insulin receptor antibodies were found in the patient's plasma, and the concentrations of circulating insulin antagonists, *i.e.* glucagon, growth hormone, and cortisol were not elevated. These findings suggested that insulin resistance in the patient was due to a target cell defect in insulin action.

MATERIALS AND METHODS

Reagents. Na-^{125}I and [γ-^{32}P]ATP were purchased from New England Nuclear and src-related peptide, Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly, was from Peninsula Laboratories, Inc. WGA sepharose was obtained from Pharmacia (Piscataway, NJ). Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis were purchased from Bio-Rad. α minimum essential medium, RPMI-1640 medium, trypsin and fetal calf serum were from Flow Laboratories, Inc. HEPES, Tricine, tris, phenylmethyl sulfonyl fluoride, N-acetyl-D-glucosamine, BSA and bovine γ-globulin were from Sigma. Porcine insulin was from Novo Inc., and Triton X-100 was from Wako Chemical Co. All other chemicals used were reagent grade.

Porcine insulin was iodinated by the chloramine T method with a specific activity of 110 to 220 μCi/μg and purified on a Sephadex G-75 column (1 × 60 cm).

Euglycemic Glucose Clamp Technique. In vivo peripheral insulin action was measured using the euglycemic glucose clamp technique as previously described by Greenfield *et al.* (11). The study was performed with two different rates of insulin infusion. Insulin was initially infused at 40 mU/m^2/min and thereafter raised to 200 mU/m^2/min. Blood glucose concentration was held constant at the basal level by a variable glucose infusion using the negative feedback principle. Because basal blood glucose concentrations varied among the subjects, we used the gMCR, which was calculated by dividing the glucose infusion rate by the steady-state plasma glucose concentration (11, 12). Controls were five healthy 20-yr-olds.

Cell Culture. Skin fibroblast lines were established from forearm incision biopsy. Cells were cultured as monolayers in a CO_2 incubator with α minimum essential medium supplemented with 10% fetal calf serum.

Lymphoblastoid cell lines were established by infecting peripheral blood lymphocytes with EBV (conditioned medium of marmoset B95-8 cells) (13). Cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. All studies were performed when cells were in late log phase growth.

Insulin-Binding Assay. Insulin binding was performed on circulating erythrocytes, monolayer cultures of skin fibroblasts, and EBV-transformed lymphocytes according to the methods of Gambhir *et al.* (14), Baldwin *et al.* (15) and Taylor *et al.* (13), respectively.

In the figures, B/T was plotted as a function of total insulin concentration. All the insulin-binding data were analyzed by the method of Scatchard (16). Ro and Ke were calculated from Scatchard plots by the method of De Meyts *et al.* (17). The insulin binding data were also analyzed by the two binding-site model (18).

Phosphorylation Assays of the WGA-Purified Insulin Receptors from EBV-Transformed Lymphocytes. Solubilization and partial purification of insulin receptors from EBV-transformed lymphocytes was performed as described by Grunberger *et al.* (19). Insulin binding to the WGA-purified receptors was carried out according to the method of Hedo *et al.* (20). Data on autophosphorylation and kinase activity were expressed as the amount of ^{32}P incorporated into the β-subunit and an exogenous substrate per IBC, respectively.

In vitro autophosphorylation of the β-subunit. Ninety microliters of the WGA-purified receptor preparation was preincubated with or without insulin (10^{-10} to 10^{-6} M) at 22° C for 45 min. Phosphorylation was then commenced by incubating the mixture with 40 to 50 μM [γ-^{32}P]ATP, 3 mM $MnCl_2$ and 10 mM NaF at 22° C for 15 min. The reaction was terminated by adding 5-fold concentrated Laemmli's sample buffer with 500 mM dithiothreitol. The mixture was then boiled for 5 min and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5% resolving gel). After electrophoresis, the gels were stained with Coomasie blue, destained, and autoradiographed. Molecular weight was estimated using protein standards composed of myosin, β-galactosidase, phosphorylase b, BSA and ovalbumin. The molecular mass = 95,000 protein bands were excised and counted by a liquid scintillation counter. Control cells were obtained from eight healthy subjects with a mean (±SD) age of 36.8 ± 18.4 yr.

Phosphorylation assay of src-related peptide. src-Related peptide was used as an exogenous substrate, and the assay was performed as previously described (21). In brief, 90 μl of WGA-purified receptor preparation was preincubated at 22° C for 45 min with or without insulin (10^{-7} M) in a total volume of 100 μl. After the mixture was incubated with 5 mM ATP for an additional 10 min at 22° C, phosphorylation was commenced by incubating the mixture with 1.5 mM src-related synthetic peptide and 30 to 40 μM [γ-^{32}P]ATP at 22° C for 15 min, in a final volume of 170 μl of 50 mM tris-HCl buffer, pH 7.6, containing 3 mM $MnCl_2$ and 10 mM NaF. The reaction was terminated by the addition of 40 μl of 1% BSA and 50 μl of 10% (wt/vol) trichloroacetic acid. The assay tube was ice-chilled for 30 min, and then centrifuged in a Beckman Microfuge B for 4 min. Forty microliters of the supernatant fluid were spotted onto phosphocellulose paper, washed in acetic acid, dried, and counted in a liquid scintillation counter. Control cells were obtained from four healthy subjects with a mean (±SD) age of 35.0 ± 16.4 yr.

Glucose Incorporation Study. D-[U-^{14}C] glucose (0.5 mM, 1 μCi/ml) uptake to monolayer cultures of fibroblasts was measured in triplicate at 37°C for 20 min, as described by Howard *et al.* (22). Control cells were obtained from four healthy subjects with a mean (±SD) age of 29.0 ± 2.6 years.

Protein Assay. Protein content was determined with a Bio-Rad protein assay Kit (Bio-Rad Laboratories) using BSA as a standard.

RESULTS

Euglycemic Glucose Clamp Technique. With the low-dose insulin infusion (40 mU/m^2/min), the steady-state plasma insulin concentration reached to 93.0 μU/ml in the patient, which was comparable to those of the controls (mean ± SD = 105.5 ± 23.9 μU/ml). Under this condition, the gMCR ($gMCR_{40}$: 4.6 ml/kg/min) was low compared with the controls (mean ± SD = 9.0

± 2.4, range = 6.1 ~ 11.4 ml/kg/min). With the high-dose insulin infusion (200 mU/m²/min), the steady-state plasma insulin concentration reached to a value (1233 μU/ml) higher than the controls (mean ± SD = 862.9 ± 243.5 μU/ml), but the gMCR ($gMCR_{200}$: 8.7 ml/kg/min) was decreased compared with the controls (mean ± SD = 14.8 ± 3.0, range = 11.3 ~ 19.1 ml/kg/min). Previous studies (23) showed that insulin infusion at 200 mU/m²/min yielded almost near-maximum glucose disposal. To see if this is the case with our patient, an additional glucose clamp study was performed on the patient with a higher insulin infusion rate (800 mU/m²/min). This study demonstrated a slight increase in gMCR to 10.5 ml/kg/min. The ratio of gMCR obtained at low-dose (40 mU/m²/min) to the maximum gMCR (max gMCR: gMCR at 200 mU/m²/min for the controls and that at 800 mU/m²/min for the patient) was decreased in the patient (0.44) compared with the controls (mean ± SD = 0.61 ± 0.08, range = 0.54 ~ 0.74).

Insulin-Binding Studies. The specific binding (B/T) to circulating erythrocytes at a tracer amount of ^{125}I-insulin (3×10^{-11} M) was in the lower part of normal range (Fig. 1*A*). Scatchard analysis of the binding data shows that insulin binding to the circulating erythrocytes was within normal values, with respect to both total receptor number and binding affinity (Table 1).

The specific binding (B/T) to cultured fibroblasts at a tracer amount of ^{125}I-insulin (7.5×10^{-11} M) was decreased in the patient (Fig. 1*B* and Table 1). The decreased insulin binding was attributed mainly to a lowered receptor affinity.

The specific binding (B/T) to EBV-transformed lymphocytes at a tracer amount of ^{125}I-insulin (4.5×10^{-11} M) was in the lower part of normal range (Fig. 1*C*). As shown in Table 1, receptor affinity was slightly decreased.

Two binding-site analyses on cultured fibroblasts and lymphocytes showed that the number of low-affinity sites was increased in the patient compared with the controls (data not shown). The results were in agreement with those obtained from the analysis of binding by De Meyts *et al.*

Phosphorylation Assays of the WGA-Purified Insulin Receptors from EBV-Transformed Lymphocytes. In vitro *autophosphorylation of the β-subunit.* Autoradiograms of *in vitro* autophosphorylation using the WGA-purified receptor preparation demonstrated that the protein with molecular weight of 95,000 (β-subunit) was dominantly phosphorylated by the preincubation with insulin. Autophosphorylation of the β-subunit reached to the maximum at 10^{-7} M of insulin in each subject. The basal ^{32}P incorporation value was undetectable in the patient (controls, mean ± SD = 0.12 ± 0.06, range = 0.05 ~ 0.19 ^{32}P mol/mol·IBC). Insulin (10^{-7} M) stimulated the autophosphorylation maximally to 0.33 ^{32}P mol/mol·IBC, which was within normal range (controls, mean ± SD = 0.57 ± 0.29, range = 0.29 ~ 0.99 ^{32}P mol/mol·IBC). The insulin dose-response curve for autophosphorylation in the patient was almost superimposable to those of the controls.

Phosphorylation assay of src-related peptide. We used the insulin concentration of 10^{-7} M, which gave maximum autophosphorylation as described above. Both the basal (8.66 ^{32}P mol/mol·IBC) and maximally insulin-stimulated phosphorylation (43.55 ^{32}P mol/mol·IBC) of src-related peptide were within normal ranges (controls, basal: mean ± SD = 14.15 ± 3.49, range = 2.44 ~ 18.95 ^{32}P mol/mol·IBC; maximum: mean ± SD = 64.02 ± 58.84, range = 23.65 ~ 150.78 ^{32}P mol/mol·IBC, respectively).

Glucose Incorporation Study. Both the basal (15.2 nmol/mg protein/20 min) and maximally insulin-stimulated D-^{14}C-glucose uptake (24.6 nmol/mg protein/20 min) to the monolayer cultures of fibroblasts were within normal ranges (controls, basal: mean ± SD = 16.1 ± 5.2 nmol/mg protein/20 min; maximum: mean ± SD = 24.9 ± 7.6 nmol/mg protein/20 min, respectively). But when the uptake was expressed as percentage of the maximum uptake, the resulting insulin dose-response curve was shifted to the right (Fig. 2). Insulin concentration for a half-maximum stimulation was between 100 and 1000 ng/ml in the patient, higher than those of the controls (mean = approximately 50 ng/ml).

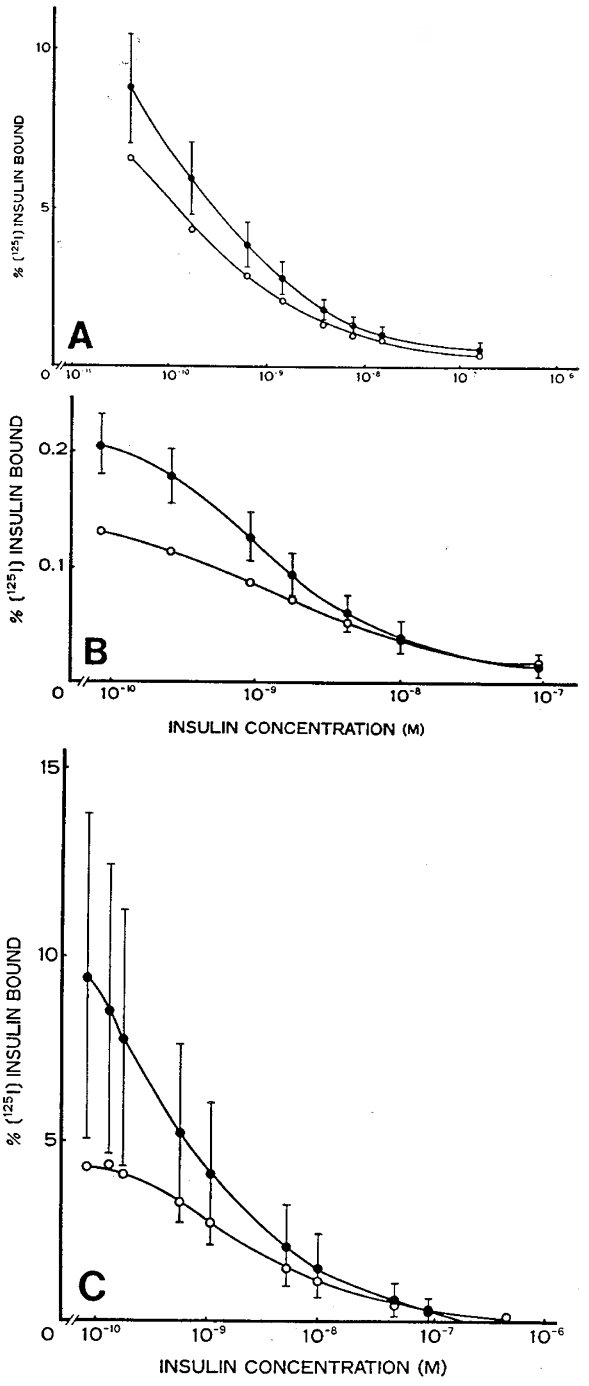

Fig. 1. ^{125}I-insulin binding to circulating erythrocytes (*A*), monolayer cultures of fibroblasts (*B*), and EBV-transformed lymphocytes (*C*) from the patient with CGL (O) compared with the controls. The percentage of specific ^{125}I-insulin bound to erythrocytes (per 3×10^9 cells), cultured fibroblasts (per 10^6 cells), and EBV-transformed lymphocytes (per 10^7 cells) is expressed as a function of total insulin concentration (M). ●, mean ± SD for the respective control subjects. The controls are 11, 7, and 10 in number, respectively, and the mean (±SD) age is 26.9 ± 1.4, 33.8 ± 5.7, and 37.9 ± 17.6 years, respectively.

DISCUSSION

Impaired insulin action in its target tissues has been recently recognized as an important pathogenic mechanism in several

Table 1. *Maximum specific binding of ^{125}I-insulin and stoichiometric binding parameters obtained from the insulin binding studies on circulating erythrocytes, cultured fibroblasts, and EBV-transformed lymphocytes**

	Patient	Controls
Erythrocytes		(n = 11)
Specific binding	6.6	(8.7 ± 1.7;
(%/3 × 10^9 cells)		6.3 ~ 11.6)
Ro(/cell)	82	(93 ± 29)
Ke(×10^8/M)	1.9	(2.5 ± 1.1)
Monolayer cultures of fibroblasts		(n = 7)
Specific binding	0.13	(0.21 ± 0.03;
(%/10^6 cells)		0.15 ~ 0.30)
Ro(/cell)	10,500	(8,100 ± 2,500)
Ke(×10^8/M)	1.5	(2.1 ± 0.6)
ID_{50}(×10^{-9} M)	2.1	(1.3 ± 0.3)
EBV-transformed lymphocytes		(n = 10)
Specific binding	4.3	(9.5 ± 4.4;
(%/10^7 cells)		2.5 ~ 20.0)
Ro(/cell)	38,000	(28,000 ± 11,000)
Ke(×10^8/M)	0.73	(2.4 ± 0.9)
ID_{50} (×10^{-9} M)	2.0	(0.94 ± 0.78)

* The Ro and Ke were calculated from Scatchard plots by the method of De Meyts *et al.* (16, 17). Ro, Ke, and ID_{50} are expressed in per cell, in M^{-1}, and in M, respectively. Normal data are expressed as mean ± SD and range for specific binding, and mean ± SD for Ro, Ke, and ID_{50}.

genetic diseases with insulin resistance, including type A syndrome of insulin resistance and acanthosis nigricans (13, 19, 24–26), leprechaunism (13, 27), Rabson-Mendenhall syndrome (28), and CGL (6–10).

In recent years, much information has been obtained about the interaction of insulin with its receptors (29–34). The insulin receptor is a heterotetrameric glycoprotein, consisting of two α- and two β-subunits (29, 30). The α-subunit (Mr = 135,000) is an extracellularly oriented protein which contains an insulin binding site. The β-subunit (Mr = 95,000) is a transmembrane protein which possesses a tyrosine-specific protein kinase (31, 32). Upon binding of insulin to the receptor, the kinase is activated, resulting in the autophosphorylation of the β-subunit on tyrosine residues (33) and also in the increase of kinase activity toward some exogenous substrates such as a src-related peptide (34). It has been proposed that receptor kinase activity may play a significant role in signal transduction from insulin binding to the effector units. Therefore, defects in any of these steps could give rise to impaired insulin action and insulin resistance.

Most previous studies on insulin resistance associated with CGL have concentrated on assessing the function of the α-subunit of insulin receptor. Oseid *et al.* (6) reported that insulin binding to monocytes from four patients with CGL was decreased due to a decrease in the affinity. Kodama *et al.* (8), studying insulin binding to monocytes from one patient with CGL, also showed decreased insulin binding. Conversely, Howard *et al.* (9) found normal receptor binding to cultured fibroblasts from one patient with CGL. Furthermore, Wachslicht-Rodbard *et al.* (10) observed three different patterns of insulin binding to monocytes from four patients with CGL: two had decreased binding due to a decreased receptor capacity, one had normal tracer binding with a decreased receptor affinity, and one had normal insulin binding. These findings suggest that the pathogenesis of insulin resistance in CGL seems to be heterogeneous.

Euglycemic glucose clamp technique is used to assess *in vivo* insulin action in terms of peripheral glucose disposal (11, 35). Previous studies (36, 37) have shown that under conditions of euglycemic hyperinsulinemia, muscle tissue is primarily responsible for disposal of an intravenous glucose load. During glucose clamp studies, total glucose disposal is equal to glucose infusion rate plus HGP (23, 35). The estimation of HGP requires an infusion of ^{3}H-glucose. Inasmuch as the use of radioactive isotopes in humans for experimental aims is restricted in our hospital, we were unable to measure HGP. However, previous studies (23) have indicated that HGP is almost completely inhibited during hyperinsulinemia, comparable to the levels obtained in this study. We assumed, therefore, that glucose infusion rate represents total glucose disposal. We assessed the extent of a rightward shift of the insulin dose-response curve for gMCR using the ratio of $gMCR_{40}$ to max gMCR. In the patient, both the $gMCR_{40}$/max gMCR and the maximum response were decreased compared with the controls. The results suggested that insulin resistance in the patient evaluated by the glucose clamp study was characterized by decreases in both insulin sensitivity and responsiveness (38–40).

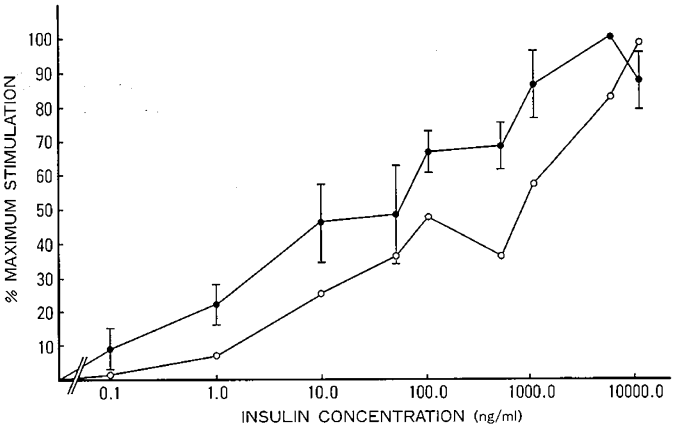

Fig. 2. Insulin stimulation of D-^{14}C-glucose uptake to cultured fibroblasts from the patient with CGL (○). The percentage of maximum stimulation is expressed as a function of insulin concentration (ng/ml). ●, mean ± SD for triplicate experiments on cultures from four control subjects.

Next, in order to understand the molecular basis of the defect causing the decreases in insulin sensitivity and responsiveness shown in the glucose clamp and to see if the defect is primary or not, we performed *in vitro* study using circulating erythrocytes and cultured lymphocytes and fibroblasts. The study exhibited that insulin binding to the patient's erythrocytes and EBV-transformed lymphocytes was low-normal with a slight reduction in binding affinity in the latter. By contrast, insulin binding to cultured fibroblasts was decreased due to a lowered affinity. In addition, they showed a rightward shift of the insulin dose-response curve for D-^{14}C-glucose uptake (a decrease in insulin sensitivity) with no decrease in the maximum uptake. The results obtained with the cultured fibroblasts suggested a possibility that a primary defect of insulin receptor binding exists, thereby causing the decrease in insulin sensitivity. However, there were some discrepancies in insulin binding among the cells for which the reason remains unknown. Thus, we cannot at the present time formulate a conclusion as to whether or not a primary binding defect of insulin receptor exists and contributes to insulin resistance in the patient. Recently, Grigorescu *et al.* (41) have described structural and kinase abnormalities of erythrocyte insulin receptors from a lipodystrophic patient. However, in our patient, we could not detect any abnormality of insulin-stimulated autophosphorylation and kinase activity toward an exogenous substrate using WGA-purified receptors from EBV-transformed lymphocytes.

In addition to the decrease in insulin sensitivity, the glucose clamp study demonstrated a decrease in maximum glucose disposal. This may result from a defect at the rate-limiting step in the postbinding process of insulin-mediated glucose disposal,

presumably a defect in the glucose transport system in muscle tissues (39, 40). Such a defect could not be exhibited in the cultured fibroblasts, favoring the idea that the defect may be secondary to changes in *in vivo* circumstances. Further studies with muscle tissue could elucidate a better understanding of the mechanisms underlying insulin resistance in the patient.

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