Immunological Abnormalities in Insulin Receptors on Cultured EBV-Transformed Lymphocytes From Insulin-Resistant Patient With Leprechaunism

ALESSANDRO CAMA, BERNICE MARCUS-SAMUELS, AND SIMEON I. TAYLOR

Defects in insulin-receptor structure can impair insulin-receptor function. We have previously identified qualitative abnormalities in insulin binding to insulin receptors from an insulin-resistant patient (Lep/Ark-1). The defects in insulin binding are probably caused by a defect in receptor structure. In this study, we used immunological probes to investigate the structural defect(s) responsible for the abnormal function. Several anti-receptor antibodies were impaired in their abilities to bind to the insulin receptor of Lep/Ark-1. For example, monoclonal antibody MoAb-51 was much less effective in inhibiting binding to insulin receptors from Lep/Ark-1 (ID_{50} 70 nM) than to those of normal subjects (ID_{50} 8 nM). In addition, there was a 10-fold reduction of the avidity with which human polyclonal antibody B-d immunoprecipitated the patient's insulin receptors. The avidity of antibody B-10 was also reduced, although to a lesser extent. In contrast, several site-specific antibodies against epitopes on the β-subunit of the receptor bound to receptors from Lep/Ark-1 with normal avidity. The data with monoclonal and polyclonal antibodies are consistent with the hypothesis that the structural defect resides in the extracellular domain of this patient's insulin receptor. The normal immunoreactivity of two putative phosphorylation sites on the β-subunit with site-specific antibodies gives further support to the conclusion that this patient's receptors have normal tyrosine kinase activity. *Diabetes* 37:982–88, 1988

Resistance to insulin action is an important feature of many disease states, for example non-insulin-dependent diabetes mellitus and obesity. In some insulin-resistant patients, there is an impairment in the function of the insulin receptor (1–6). In fact, in some patients with genetic forms of extreme insulin resistance, the receptor defect may be the primary cause of insulin resistance.

Leprechaun/Ark-1 (Lep/Ark-1) is a patient in whom extreme insulin resistance is associated with abnormal insulin binding (7). Although the number of insulin receptors present on Epstein-Barr virus (EBV)–transformed lymphocytes is normal, the affinity of the receptor for insulin is increased. Furthermore, several other qualitative abnormalities in insulin binding have been identified, including decreased sensitivity to changes in pH and temperature (8–10). Inasmuch as the receptor's affinity was increased, the primary cause of the patient's insulin resistance is not the failure to bind insulin but more likely a defect in transmembrane signaling. However, the insulin-stimulated tyrosine kinase activity of the patient's receptors appears to be normal (11,12). In this study, we have immunologically characterized the insulin receptor from the patient in an attempt to correlate the abnormalities in insulin binding with a structural defect in the receptor. Several antibodies that recognize the extracellular domain of the insulin receptor distinguished between the receptor from the normal subjects and the receptor from Lep/Ark-1. However, two antibodies directed against epitopes in the intracellular portion of the receptor did not distinguish between the two types of receptor. This result is consistent with the hypothesis that there is a structural alteration in the extracellular domain of the patient's insulin receptor.

MATERIALS AND METHODS

Patients and cell lines. Lep/Ark-1 is an insulin-resistant patient with leprechaunism who has been described previously (7–15). The normal subjects who participated in our study (P.L., Y.G., T.T., R.S., and A.R.) were 17–27 yr old and had no history of diabetes or obesity.

Lymphocytes from peripheral blood were transformed with EBV as described previously (7,16) and cultivated in RPMI-1640 medium supplemented with 10% fetal calf serum (Bio-Fluid, Rockville, MD).

From the Biochemistry and Molecular Pathophysiology Section, Diabetes Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland.

Address correspondence and reprint requests to Dr. Simeon I. Taylor, National Institutes of Health, Building 10, Room 8N-250, Bethesda, MD 20892.

Received for publication 18 September 1987 and accepted in revised form 3 February 1988.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/37/7/982/356350/37-7-982.pdf by guest on 24 June 2024

Antibodies. Monoclonal anti-insulin-receptor antibodies MoAb-51 (17) and MoAb-20 (18) were provided by R.A. Roth and I. Goldfine, respectively. These monoclonal antibodies interact with two different epitopes on the α-subunit of the insulin receptor, and both are able to inhibit insulin binding. Although the exact location of these epitopes is not known, previous studies suggest that MoAb-51 binds very close to or at the insulin-binding site, whereas MoAb-20 binds some distance from the insulin-binding site, perhaps inhibiting the binding in an allosteric fashion (18).

Human polyclonal anti-receptor antibodies B-d and B-10 were obtained from the sera of patients with type B extreme insulin resistance (19). The IgG fraction was prepared from these sera by chromatography over protein A–Sepharose (Pharmacia, Uppsala, Sweden). The IgG concentration was determined spectrophotometrically (20).

Site-specific anti-receptor antibodies were generated as follows. Synthetic peptides, corresponding to partial sequences of the human insulin receptor (Table 1), were obtained from Peninsula Laboratories (San Carlo, CA), where the peptides (15 μg) were also conjugated to keyhole-limpet hemocyanin (KLH, 75 μg). Rabbits were immunized with the KLH conjugates via standard techniques at Meloy Laboratories (Springfield, VA). Site-specific anti-receptor antibodies rAb-6 and rAb-50 were affinity purified for immunoblot studies. The peptides recognized by these two antibodies (6 and 50, 1 mg/ml) were cross-linked to 5 ml of Affigel 10 (Bio-Rad, Richmond, CA) in HEPES buffer (100 mM, pH 7.6) at 4°C according to the manufacturer's specifications. The reaction was quenched with ethanolamine (1 M, pH 10) at 1:10 dilution for 1 h at 4°C. The resin was washed extensively with 10 L of buffer [glycine hydrochloride, 100 mM, pH 3; Triton X-100, 0.1%; sodium dodecyl sulfate (SDS), 0.01%] followed by 2.5 L of phosphate-buffered saline (PBS, pH 7.4) containing Triton X-100 (0.1%).

The IgG fraction from 3 ml of sera (prepared as described above) was applied to the column (5 ml) and recycled four times. Columns were washed with 200 volumes of PBS (pH 7.4) containing Triton X-100 (0.05%). Specific IgGs were eluted with 10 ml of citrate buffer (100 mM; pH 2.5) and neutralized immediately. Eluates were concentrated to ~400 μl in the presence of bovine serum albumin (BSA, 1% final concn) with Centricon 10 (Amicon, Grace, Danvers, MA). The titer of the concentrated antibodies was comparable with that of the original sera as estimated by enzyme-linked immunosorbent assay.

Binding-inhibition studies. EBV lymphocytes from Lep/Ark-1 and normal subjects (10^7 cells/ml) were incubated for 2 h at 15°C with increasing amounts of antibody B-d or B-10 as previously described (22). Cells were washed and incubated for 2 h at 15°C with ^{125}I-labeled insulin (0.1 ng/ml; 2200 Ci/mmol), and the radioactivity specifically associated with the cells was determined in duplicate. Because of the relatively low affinity of the MoAbs, the wash step was omitted. Instead, cells were preincubated with MoAbs as indicated at 23°C for 1.5 h. ^{125}I-insulin (0.1 ng/ml) was then added, and cells were incubated for an additional 3 h at 15°C. At the end of this incubation, cell-associated radioactivity was determined as above (22).

Immunoprecipitation studies. ^{125}I-insulin was covalently cross-linked to insulin receptors on intact EBV lymphocytes (4×10^8 cells) with 0.1 mM disuccinimidyl suberate (Pierce, Rockford, IL) as described previously (23,24). Cells were solubilized in 1% Triton X-100 in a buffer containing NaCl (150 mM), HEPES (50 mM, pH 7.6), and phenylmethylsulfonyl fluoride (2 mM). Insoluble material was sedimented by centrifugation (200,000 × g for 15 min at 4°C). The supernatants were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE), either directly or after immunoprecipitation with anti-receptor antibodies (24). In preparing samples for electrophoresis, 10% β-mercaptoethanol was added to reduce disulfide bonds.

The radioactivity in the 135,000-M_r α-subunit of the insulin receptor was determined by γ-counting. The radioactivity in the 135,000-M_r band of the immunoprecipitated samples (duplicate determinations) is expressed as a percentage of the radioactivity in the 135,000-M_r band of the nonimmunoprecipitated samples (triplicate determinations).

We calculated the efficiency of cross-linking in five experiments with Lep/Ark-1 and three other subjects. With cells from Lep/Ark-1, 0.9 ± 0.2% (mean ± SE of 5 experiments) of the ^{125}I-insulin bound to the cell was covalently cross-linked to the α-subunit. In the three other subjects, the corresponding efficiencies of cross-linking were 1.1 ± 0.2, 1.2 ± 0.3, and 1.2 ± 0.3%. Because most of the ^{125}I was associated with tyrosine A-14 of insulin, it is a measure of the efficiency of covalent cross-linking of the A chain of insulin to the α-subunit of the insulin receptor. In experiments in which the reduction with β-mercaptoethanol was omitted, approximately fivefold more ^{125}I was associated with the oligomeric forms of the insulin receptor, suggesting that ~80% of the ^{125}I-insulin is cross-linked via the B chain and 20% via the A

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/37/7/982/356350/37-7-982.pdf by guest on 24 June 2024

TABLE 1
Epitopes recognized by site-specific anti-insulin-receptor antibodies

Antibody	Amino acid residue numbers	Amino acid sequence	Localization of epitope
rAb-6	242–253	Pro-Pro-Pro-Tyr-Tyr-His-Phe-Gln-Asp-Trp-Arg-Cys	α-Subunit cysteine-rich domain
rAb-13	906–917	Thr-Asp-Tyr-Leu-Asp-Val-Pro-Ser-Asn-Ile-Ala-Lys	β-Subunit extracellular domain
rAb-46	954–969	Ala-Ser-Ser-Asn-Pro-Glu-Tyr-Leu-Ser-Ala-Ser-Asp-Val-Phe-Pro-Cys	β-Subunit intracellular—near transmembrane domain
rAb-50	1309–1324	Ser-Leu-Gly-Phe-Lys-Arg-Ser-Tyr-Glu-Glu-His-Ile-Pro-Tyr-Thr-His	β-Subunit COOH-terminal

The numbering of amino acid residues follows the nomenclature of Ullrich et al. (21).

chain. In any case, the efficiency of cross-linking appeared normal with receptors from Lep/Ark-1.

Immunoblot studies. EBV lymphocytes (~4 × 10^8 cells) were solubilized on ice in a buffer containing Triton X-100 (1%), NaCl (150 mM), HEPES (50 mM, pH 7.6), and the following protease inhibitors: phenylmethylsulfonyl fluoride (2 mM), aprotinin (0.1 mg/ml), leupeptin (0.1 mg/ml), pepstatin A (0.1 mg/ml), and α_2-macroglobulin (0.5 U/ml). Insoluble material was removed by centrifugation at 200,000 × *g* for 45 min, and insulin receptors were incubated with anti-receptor antibody rAb-50 (1:100) overnight at 4°C. Immune complexes were immunoprecipitated with Pansorbin (Calbiochem, La Jolla, CA). The immunoprecipitates were fractionated by SDS-PAGE under reducing conditions (10% β-mercaptoethanol). Electrophoretic transfer was carried out as described elsewhere (25). The nitrocellulose membranes were incubated with a buffer containing 10% nonfat milk (Carnation, Los Angeles, CA), Tris (20 mM, pH 7.5), and NaCl (500 mM) for 1 h at 24°C to block nonspecific sites for protein binding. Blots were incubated overnight with affinity-purified rAb-6 or rAb-50 (1:50 dilution) in the presence or absence of the specific corresponding peptide (0.3 mg/ml), in PBS (pH 7.5) containing BSA (1%). After extensive washes with TTBS buffer (Tween 20, 0.05%; NaCl, 500 mM; Tris, 20 mM, pH 7.5), blots were incubated for 1 h at 24°C with ~5 × 10^5 counts · min^{-1} · ml^{-1} of ^{125}I-protein A (130 μCi/μg) in TTBS containing 10% nonfat milk (26). After extensive washes in TTBS, blots were dried and autoradiographed. The regions corresponding to the α- or β-subunits were excised from the blot and counted in a γ-counter.

RESULTS

BINDING-INHIBITION STUDIES

MoAbs. MoAb-51 caused a concentration-dependent inhibition of ^{125}I-insulin binding to insulin receptors on EBV lymphocytes from normal subjects and Lep/Ark-1 (Fig. 1, *left*). However, the concentration required to inhibit ^{125}I-insulin binding by 50% was ~10-fold higher with receptors from Lep/Ark-1 than with receptors from normal subjects (ID_{50} 70 vs. 8 nM, respectively). Moreover, as the concentration of MoAb-51 was increased, ^{125}I-insulin binding to receptors from Lep/Ark-1 appeared to plateau at ~50% of maximal. In contrast, nearly complete inhibition of binding was achieved with normal insulin receptors. When similar studies were carried out with MoAb-20, the antibody had identical potencies to inhibit ^{125}I-insulin binding to normal insulin receptors and receptors from Lep/Ark-1 (ID_{50} 2.5 nM; Fig. 1, *right*), suggesting that the receptor from Lep/Ark-1 may be structurally abnormal in the region of the epitope recognized by MoAb-51 but not in the epitope recognized by MoAb-20.

Human polyclonal autoantibodies. To further investigate epitopes in the region of the insulin-binding site, we used anti-receptor antibodies from two patients with type B extreme insulin resistance (B-d and B-10). Both antibodies resembled MoAb-20 in having normal potencies to inhibit ^{125}I-insulin binding to insulin receptors from Lep/Ark-1 (Fig. 2).

IMMUNOPRECIPITATION AND IMMUNOBLOT STUDIES

Human polyclonal autoantibodies. To study binding of antibodies to epitopes not involved in insulin binding, we

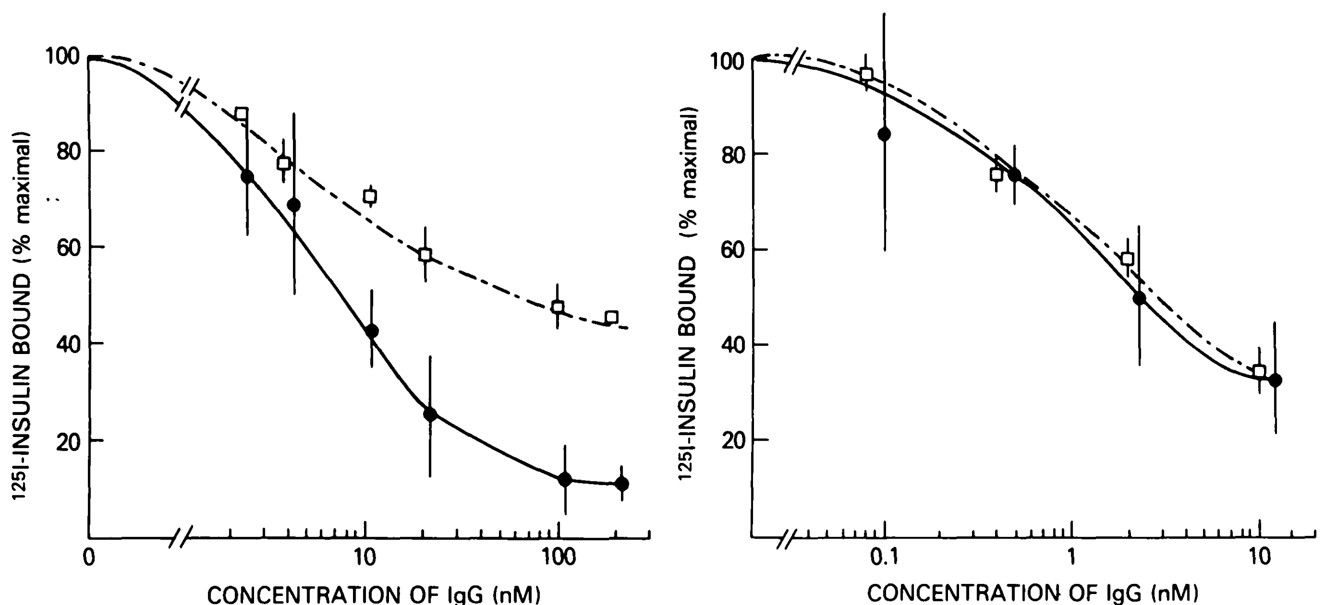


FIG. 1. Inhibition of insulin binding by monoclonal anti-receptor antibodies. Cells from Lep/Ark-1 (□) and normal subjects (●, *n* = 3) were incubated for 2 h at 15°C with ^{125}I-labeled insulin (0.1 ng/ml) in presence of increasing amounts of monoclonal anti-receptor antibody MoAb-51 (*left*, average of 4 experiments) or MoAb-20 (*right*, duplicate determinations in 1 experiment). ^{125}I-insulin bound is expressed as percentage of insulin specifically bound to cells in absence of antibodies. Data are presented as means ± 2SD for normal subjects and means ± SE for Lep/Ark-1. In these studies, mean ^{125}I-insulin binding observed in absence of antibody with cells from Lep/Ark-1 was 17 ± 2% per 10^7 cells/ml (range 14–21%). For comparison, we intentionally chose normal subjects with relatively low ^{125}I-insulin binding (4–16% per 10^7 cells/ml) to give data comparable with those obtained with Lep/Ark-1. In separate experiments, higher concentration (500 nM) of MoAb-51 was employed, which did not inhibit ^{125}I-insulin binding to cells of Lep/Ark-1 significantly more than did maximal concentration (200 nM) used in *left* panel. Because of limited availability of antibody, it was not possible to investigate higher concentrations.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/37/7/982/356350/37-7-982.pdf by guest on 24 June 2024

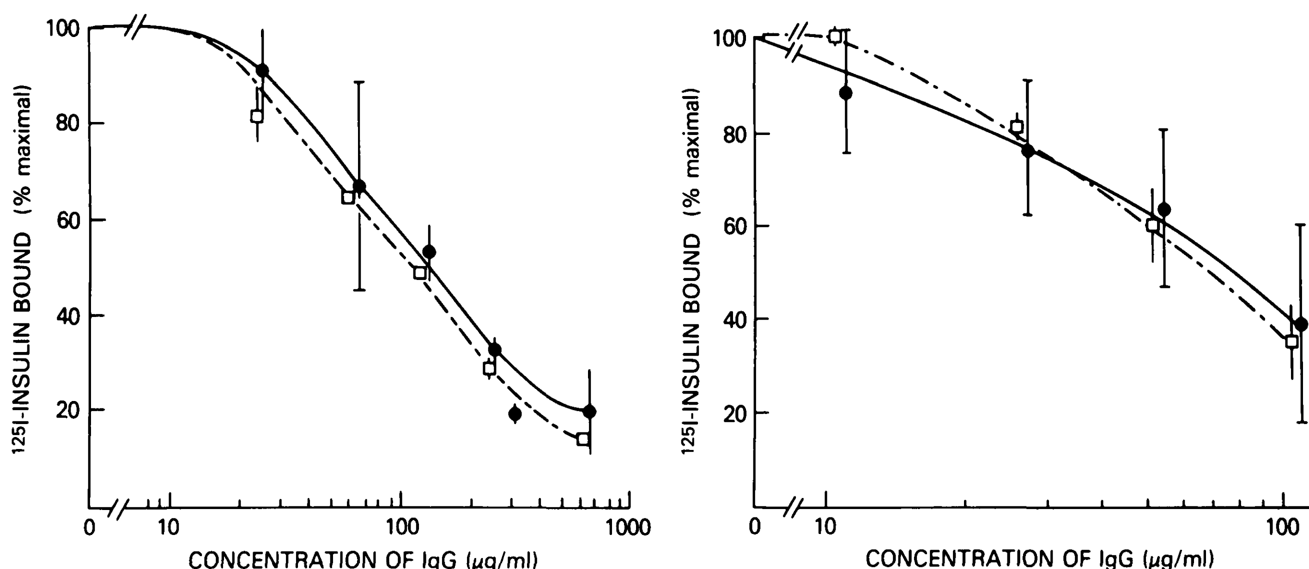


FIG. 2. Inhibition of ^{125}I-labeled insulin binding by human anti-insulin-receptor antibodies. Cells from Lep/Ark-1 (□) and normal subjects (●, n = 3) were preincubated for 2 h at 15°C with increasing concentrations of IgG derived from serum B-d (*left*, average of 3 experiments) or B-10 (*right*, average of 2 experiments). Cells were washed and incubated 2 h longer in presence of ^{125}I-insulin (0.1 ng/ml).

assayed the ability of anti-receptor antibodies to immunoprecipitate affinity-labeled insulin receptors. In contrast to the observations in the binding-inhibition assay (Fig. 2), antibody B-d was markedly impaired in its ability to immunoprecipitate insulin receptors from Lep/Ark-1 (ED_{50} 900 vs. 70 μg/ml; Fig. 3, *left*). Thus, antibody B-d contains populations of antibody molecules that can identify a structural abnormality in insulin receptors from Lep/Ark-1. This population of antibody molecules does not appear to be the same population that recognizes the epitopes involved in insulin binding (Fig. 2, *left*). Antibody B-10 also had a decreased ability to immunoprecipitate receptors from Lep/Ark-1 (ED_{50} 130 vs. 45 μg/ml; Fig. 3, *right*), although the decrease was less marked than with antibody B-d (Fig. 3, *left*).

Site-specific rabbit antisera. The use of site-specific anti-receptor antibodies is advantageous because the epitope recognized by the antibody is known. We used three site-specific anti-receptor antisera, all of which immunoprecipitate the solubilized affinity-labeled insulin receptors: rAb-13, which binds to the extracellular domain of the β-subunit, and rAb-46 and rAb-50, which bind to tyrosine-containing peptides of the intracellular domain of the β-subunit. All three of these antisera have normal avidity for affinity-labeled insulin receptors from Lep/Ark-1 (Fig. 4). Note that rAb-13 has a much lower titer in the immunoprecipitation assay than does rAb-46 or rAb-50. Accordingly, the conclusions with rAb-13 must be moderated by the relatively low percentage (~5%) of receptors that were immunoprecipitated.

We have not obtained site-specific antisera raised against peptides derived from the α-subunit that are able to immunoprecipitate native insulin receptors. However, rAb-6 recognized the α-subunit of the insulin receptors on immunoblots. Therefore, we used rAb-6 and rAb-50, respectively, to identify the α- and β-subunits of the insulin receptor on immunoblots (Fig. 5). This method allows for detection of the α- and β-subunits on separate immunoblots. As judged by this immunoblot technique, the ratio of α-subunit to β-subunit appears normal for insulin receptors from Lep/Ark-1. Thus, we conclude that there is a normal interaction between rAb-6 and the α-subunit of receptors from Lep/Ark-1.

DISCUSSION

Genetic forms of extreme insulin resistance are often associated with abnormalities in the insulin receptor (6). In some patients, the number of insulin receptors on the cell surface is markedly reduced (16,27–29). In other patients, there is an alteration of the receptor structure that impairs receptor function (1–5). In previous studies, we have shown that receptors from Lep/Ark-1 exhibit multiple qualitative abnormalities in insulin binding suggestive of an altered structure (8). This study, with anti-receptor antibodies, provides direct immunological evidence of an abnormality in the structure of the patient's receptors.

Localization of receptor defect(s). The data with the various antibodies suggest somewhat conflicting conclusions with respect to localization of the structural defects in the patient's insulin receptors. Because MoAb-51 is thought to bind at or near the insulin-binding site (17), the data with MoAb-51 suggest there may be a structural defect in the region of the binding site (Fig. 1, *left*). In contrast, antibodies B-d and B-10 appear to detect an abnormal structure outside the binding site (Fig. 3). How can this apparent conflict be resolved? A mutation that alters the amino acid sequence in one region of the receptor might secondarily change the conformation of the molecule and alter epitopes in distant regions of the receptor. An alternate explanation might be based on the possibility that there is more than one type of insulin receptor on the surface of the patient's EBV lymphocytes. As we have concluded previously, Lep/Ark-1 may be a compound heterozygote, with a different mutant allele inherited from each parent at the insulin-receptor gene locus (10). [The actual situation may be even more complicated because the insulin receptor is an oligomeric protein. Thus,

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/37/7/982/356350/37-7-982.pdf by guest on 24 June 2024

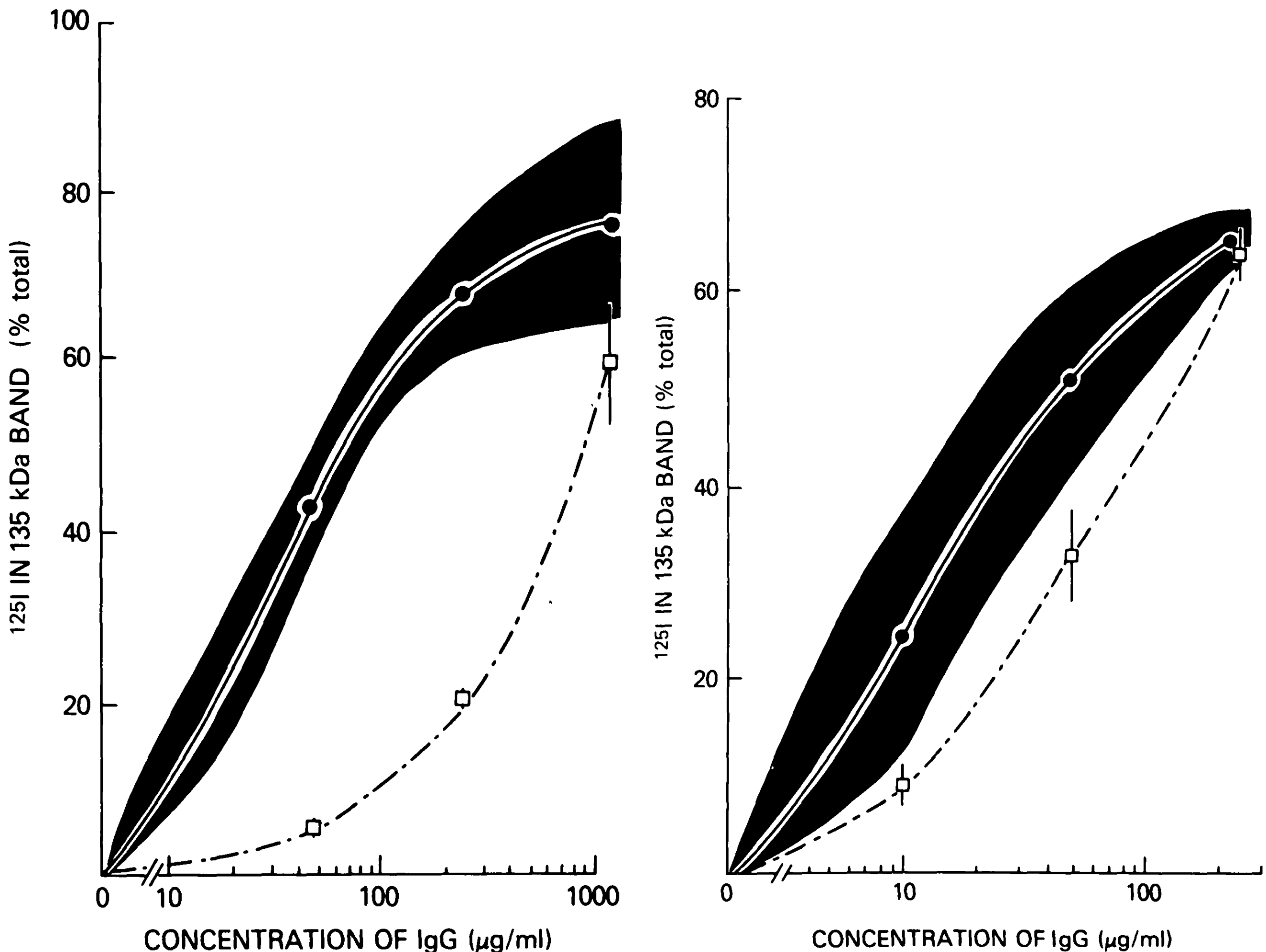


FIG. 3. Immunoprecipitation of insulin receptor by human anti-receptor antibodies. Affinity-labeled insulin receptors from Lep/Ark-1 (□) and normal subjects (●, n = 3) were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis either directly or after immunoprecipitation with increasing amounts of anti-insulin-receptor antibodies B-d (*left*, duplicate determinations in 1 experiment) and B-10 (*right*, average of 2 experiments). Data are expressed as percentage of radioactivity associated with α-subunit of insulin receptor in nonimmunoprecipitated samples. Normal range in *black area* is mean ± 2SD of values obtained in normal subjects. Data from Lep/Ark-1 are expressed as means ± SE.

the molecules encoded by the two different alleles can aggregate either symmetrically or asymmetrically. If p and m represent the receptors encoded by the paternally and maternally derived genes, respectively, then at least 3 types of oligomeric receptors can be formed: pp, mm, and mp (or pm). In principle, anti-receptor antibodies could have different affinities for each type of molecule.] One allele may encode a receptor that is impaired in its ability to interact with MoAb-51. This hypothesis is supported by the observation that maximal concentrations of MoAb-51 inhibit only 50% of the ^{125}I-insulin binding to EBV lymphocytes of Lep/Ark-1, and 50% of the binding was not inhibited by MoAb-51 (Fig. 1, *left*). However, we have not obtained proof of the existence of two pools of insulin receptors on the surface of the patient's cells.

Efforts are under way to isolate and sequence cDNA clones encoding the patient's insulin receptors. This approach has the potential to detect alterations in the primary amino acid sequence of the receptor molecules. Considering the immunological and binding data, we predict that the putative abnormality in the amino acid sequence will be mapped to the extracellular domain of the receptor—probably the α-subunit. Of course, the structural defect might result from abnormalities in the posttranslational processing of the insulin receptor. This possibility seems less likely to be specific for the insulin receptor. However, it would deserve consideration if the cDNA-cloning studies fail to detect mutations in the insulin-receptor gene. Furthermore, cDNA-cloning studies should answer whether the patient's two alleles encode insulin receptors with different amino acid sequences. If so, it would be possible to study the properties of the products of each gene by transfection of the cloned cDNA molecules separately into cultured cells.

Other laboratories have reported insulin binding to cultured fibroblasts of Lep/Ark-1 to be either normal (13) or decreased (15). We have reported that insulin binding to circulating monocytes is decreased but conclude that this decrease may be due at least partly to downregulation in response to the patient's extreme hyperinsulinemia (10). In this study, we used EBV lymphocytes for two reasons: *1*) this is the cell type in which the qualitative abnormalities in insulin binding have been identified, and *2*) this type of study

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/37/7/982/356350/37-7-982.pdf by guest on 24 June 2024

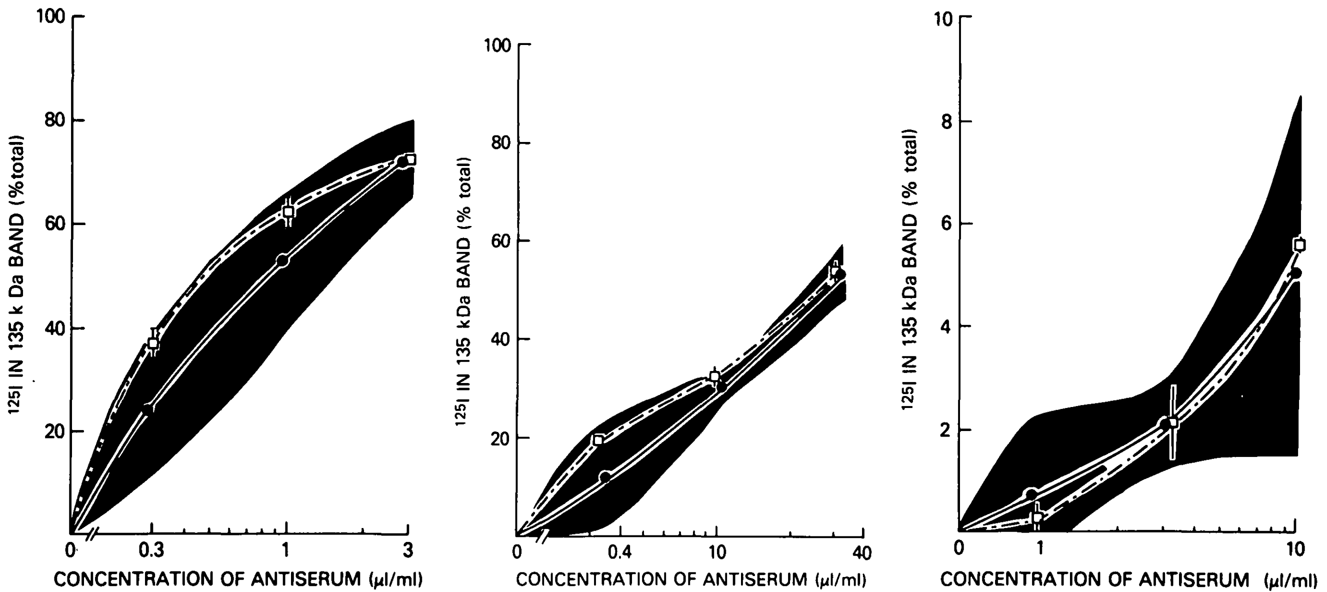
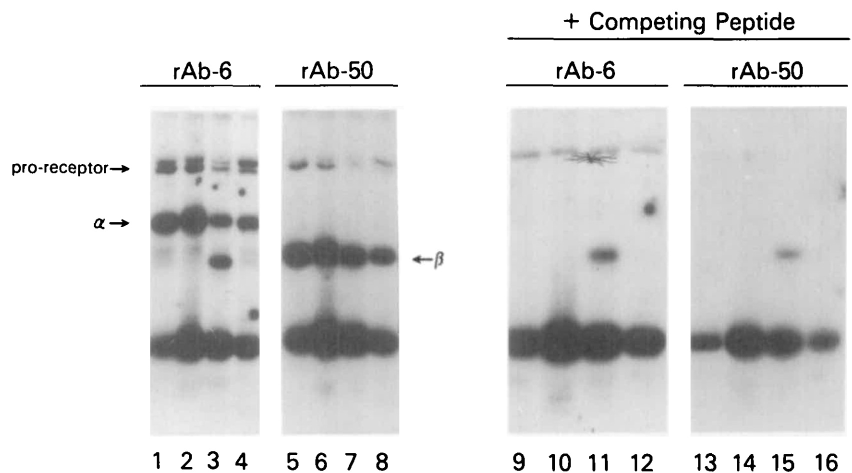


FIG. 4. Immunoprecipitation of insulin receptor by site-specific anti-receptor antibodies. Immunoprecipitation of affinity-labeled insulin receptor from Lep/Ark-1 (□) and normal subjects (●, *n* = 3) by site-specific antibody rAb-50 (*left*, average of 3 experiments), rAb-46 (*center*, duplicate determinations in 1 experiment), and rAb-13 (*right*, duplicate determinations in 1 experiment) was performed as described in Fig. 3 legend. Normal range (*black area*) is mean ± 2SD of values obtained in normal subjects. Data from Lep/Ark-1 are expressed as means ± SE.

is greatly facilitated because of the easy availability of many insulin receptors from EBV lymphocytes. We have not completely eliminated the possibility that viral transformation may alter the expression of the insulin-receptor gene. Nevertheless, the qualitative abnormalities appear specific to the cell lines derived from both the patient and her mother (7–10). Accordingly, we believe that the abnormalities are a reflection of mutations that would be present in all of the cell types derived from the patient. When the cloning studies are completed, it will be possible to study genomic DNA as well as insulin-receptor mRNA sequences in all of the cell types; studies with all cell types will probably yield the same conclusions.

Anti-receptor antibodies as probes of receptor structure. Anti-receptor antibodies have been invaluable as reagents in studies of receptor structure, function, biosynthesis, and degradation (1–4,6–8,11,12,16–19,28,29). Generally, when anti-receptor antibodies have been used to



	Patient	Lanes	Ratio ($\frac{\alpha\text{-subunit}}{\beta\text{-subunit}}$)
Normal Subjects	P.L.	1,5,9,13	0.53
	Y.G.	2,6,10,14	0.68
	T.T.	4,8,12,16	0.70
	Lep/Ark-1	3,7,11,15	0.62

FIG. 5. Immunoblot detection of α- and β-subunits of insulin receptors by site-specific anti-receptor antibodies. Receptors from Lep/Ark-1 and 3 normal subjects were analyzed by SDS–PAGE after immunoprecipitation with rAb-50 (1:100). Proteins were transferred to nitrocellulose paper as described in MATERIALS AND METHODS. Specific radioactivity was radioactivity detected in absence of competing peptide (*lanes 1–8*) minus that detected in corresponding region of blots in presence of competing peptide (*lanes 9–16*). At least 2 types of nonspecific bands were noted with both antibodies. Lowest band (M_r ~52,000) may correspond to Ig heavy chain and was detected in samples from all 4 subjects and in blots probed with antibodies rAb-6 and rAb-50. Immunodetection was not prevented by competing peptides (*lanes 9–16*). In samples from Lep/Ark-1 (*lanes 3, 7, 11,* and *15*), nonspecific 95,000-M_r band was detected in blots probed with both antibodies in presence or absence of competing peptides. Although this band was not detected in samples from 3 normal subjects, nonspecific 95,000-M_r band was detected in subject P.L. in another experiment (data not shown). Identity of this band is not known, but data suggest it is not specifically detected by anti-receptor antibodies and is probably not structurally related to insulin receptor. We immunoprecipitated insulin receptors with rAb-50, which did not distinguish between normal receptors and those of Lep/Ark-1. Concentration of rAb-50 should have immunoprecipitated virtually all receptors (Fig. 4, *left*).

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/37/7/982/356350/37-7-982.pdf by guest on 24 June 2024

study insulin receptors from patients with insulin resistance or diabetes, it has been implicitly assumed that the antibodies interact normally with the patient's insulin receptor (e.g., refs. 2–4,11). However, as we have demonstrated herein, it is possible for a structural abnormality in the patient's insulin receptors to interfere with the binding of anti-receptor antibodies to a particular patient's insulin receptors. To avoid this pitfall, it is important to choose anti-receptor antibodies that recognize the patients' insulin receptors.

Most of the antisera used in previous clinical investigations have been used to immunoprecipitate the native solubilized insulin receptor. However, the site-specific antibodies are ideally suited to recognize denatured insulin receptors. Furthermore, site-specific antibodies provide the ability to obtain independent measures of the number of α- and β-subunit molecules (Fig. 5). In the case of the two site-specific antibodies directed against tyrosine-containing peptides on the intracellular portion of the β-subunit (rAb-50 and rAb-46), the normal immunoprecipitation of the patient's receptor is consistent with previous reports of normal tyrosine kinase activity (11,12).

In conclusion, immunological probes may be useful tools in detecting structural abnormalities of the insulin receptor. Furthermore, they may complement the molecular-biological approach by focusing attention on specific regions of the defective insulin-receptor gene.

NOTE ADDED IN PROOF

After we submitted the manuscript, we identified point mutations in the cDNA molecules derived from both alleles of the patient's insulin receptors (Kadowaki T., Bevins CL, Cama A, Ojamaa K, Marcus-Samuels B, Kadowaki H, Beitz L, McKeon C, Taylor SI: Two mutant alleles of the insulin receptor gene in a patient with extreme insulin resistance. *Science* 240:787–90, 1988). In the allele inherited from the father, there is a nonsense mutation that terminates translation after amino acid 671 in the α-subunit. In the allele inherited from the mother, glutamic acid replaces lysine at position 460 in the α-subunit.

ACKNOWLEDGMENTS

We are grateful to Drs. Phillip Gorden, Jesse Roth, and Takashi Kadowaki for helpful discussions. We also appreciate the cooperation of Dr. M. Joycelyn Elders for allowing us to study this patient.

REFERENCES

1. Grunberger G, Zick Y, Gorden P: Defect in phosphorylation of cells from an insulin-resistant patient with normal insulin binding. *Science* 223:932–34, 1984
2. Grigorescu F, Flier JS, Kahn CR: Defect in insulin receptor phosphorylation in erythrocytes and fibroblasts associated with severe insulin resistance. *J Biol Chem* 259:15003–5006, 1984
3. Grigorescu F, Flier JS, Kahn CR: Characterization of binding and phosphorylation defects of erythrocyte insulin receptors in the type A syndrome of insulin resistance. *Diabetes* 35:127–38, 1986
4. Caro JF, Ittoop O, Pories WJ, Meelheim D, Flickinger EG, Thomas F, Jenquin M, Silverman JF, Khazanie PG, Sinha MK: Studies on the mechanism of insulin resistance in the liver from humans with noninsulin-dependent diabetes. *J Clin Invest* 78:249–58, 1986
5. Comi RJ, Grunberger G, Gorden P: Relationship of insulin binding and insulin-stimulated tyrosine kinase activity is altered in type II diabetes. *J Clin Invest* 79:453–62, 1987
6. Taylor SI: Receptor defects in patients with extreme insulin resistance. *Diabetes Metab Rev* 1:171–202, 1985
7. Taylor SI, Roth J, Blizzard RM, Elders MJ: Qualitative abnormalities in insulin binding in a patient with extreme insulin resistance: decreased sensitivity to alterations in temperature and pH. *Proc Natl Acad Sci USA* 78:7157–61, 1981
8. Taylor SI, Hedo JA, Underhill LH, Kasuga M, Elders MJ, Roth J: Extreme insulin resistance in association with abnormally high binding affinity of insulin receptors in a patient with leprechaunism: evidence for a defect intrinsic to the receptor. *J Clin Endocrinol Metab* 55:1108–13, 1982
9. Taylor SI, Leventhal S: Defect in cooperativity in insulin receptors from a patient with a congenital form of extreme insulin resistance. *J Clin Invest* 71:1676–85, 1983
10. Taylor SI, Marcus-Samuels B, Ryan-Young J, Leventhal S, Elders MJ: Genetics of the insulin receptor defect in a patient with extreme insulin resistance. *J Clin Endocrinol Metab* 62:1130–35, 1986
11. Whittaker J, Zick Y, Roth J, Taylor SI: Insulin stimulated receptor phosphorylation appears normal in cultured Epstein-Barr virus-transformed lymphocyte cell lines derived from patients with extreme insulin resistance *J Clin Endocrinol Metab* 60:381–86, 1985
12. Cama A, Taylor SI: Tyrosine kinase activity of insulin receptors from an insulin-resistant patient with leprechaunism. *Diabetologia* 30:631–37, 1987
13. Kobayashi M, Olefsky J, Elders MJ, Mako ME, Given BD, Schedewie HK, Fiser RH, Hintz RL, Horner JA, Rubenstein AH: Insulin resistance due to a defect distal to the insulin receptor: demonstration in a patient with leprechaunism. *Proc Natl Acad Sci USA* 75:3469–73, 1978
14. Elders MJ, Schedewie HK, Olefsky J, Givens B, Char F, Bier DM, Baldwin D, Fiser RH, Seyedabadi S: Endocrine-metabolic relationships in patients with leprechaunism. *J Natl Med Assoc* 74:1195–210, 1982
15. Elsas LJ, Endo F, Strumlauf E, Elders J, Priest JH: Leprechaunism: an inherited defect in a high-affinity insulin receptor. *Am J Hum Genet* 37:73–88, 1985
16. Taylor SI, Samuels B, Roth J, Kasuga M, Hedo JA, Gorden P, Brasel DE, Pokora T, Engle RR: Decreased insulin binding in cultured lymphocytes from two patients with extreme insulin resistance. *J Clin Endocrinol Metab* 54:919–30, 1982
17. Roth RA, Cassell DJ, Wong KY, Maddux BA, Goldfine ID: Monoclonal antibodies to the human insulin receptor block insulin binding and inhibit insulin action. *Proc Natl Acad Sci USA* 79:7312–16, 1982
18. Forsayeth JR, Montemurro A, Maddux BA, De Pirro R, Goldfine ID: Effect of monoclonal antibodies on human insulin receptor autophosphorylation, negative cooperativity, and down-regulation. *J Biol Chem* 262:4134–40, 1987
19. Taylor SI, Marcus-Samuels B: Anti-receptor antibodies mimic the effect of insulin to down-regulate insulin receptors in cultured human lymphoblastoid (IM-9) cells. *J Clin Endocrinol Metab* 58:182–86, 1986
20. Layne E: Spectrophotometric and turbidimetric methods for measuring proteins. *Methods Enzymol* 3:447–54, 1957
21. Ullrich A, Bell JR, Chen EY, Herrera R, Petruzzelli LM, Dull TJ, Gray A, Coussens L, Liao YC, Tsubokawa M, Mason A, Seeburg PH, Grunfeld C, Rosen OM, Ramachandran J: Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. *Nature* (Lond) 313:756–61, 1985
22. Taylor SI, Underhill LH, Marcus-Samuels B: Assay of antibodies directed against cell surface receptors. *Methods Enzymol* 109:656–67, 1986
23. Pilch PF, Czech M: Interaction of cross-linking agents with the insulin effector system of isolated fat cells. *J Biol Chem* 254:3375–81, 1979
24. Taylor SI, Schroer JA, Marcus-Samuels B, McElduff A, Bender TP: Binding of insulin to its receptor impairs recognition by monoclonal anti-insulin antibodies. *Diabetes* 33:778–84, 1984
25. Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 76:4350–54, 1979
26. Burnette WN: "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Anal Biochem* 112:195–203, 1981
27. Bar RJ, Muggeo M, Kahn CR, Gorden P, Roth J: Characterization of insulin resistance and acanthosis nigricans. *Diabetologia* 18:209–16, 1980
28. Taylor SI, Underhill LH, Hedo JA, Roth J, Serrano Rios M, Blizzard RM: Decreased insulin binding to cultured cells from a patient with the Rabson-Mendenhall syndrome: dichotomy between studies with cultured lymphocytes and cultured fibroblasts. *J Clin Endocrinol Metab* 56:856–61, 1983
29. Hedo JA, Moncada VY, Taylor SI: Insulin receptor biosynthesis in cultured lymphocytes from insulin-resistant patients. *J Clin Invest* 76:2355–61, 1986

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/37/7/982/356350/37-7-982.pdf by guest on 24 June 2024