

# Diabetes Due to Secretion of a Structurally Abnormal Insulin (Insulin Wakayama)

## Clinical and Functional Characteristics of [Leu<sup>A3</sup>] Insulin

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### Abstract

We have identified a non-insulin-dependent diabetic patient with fasting hyperinsulinemia (90  $\mu$ U/ml), an elevated insulin:C-peptide molar ratio (1.68; normal, 0.05–0.20), normal insulin counterregulatory hormone levels, and an adequate response to exogenously administered insulin. Insulin-binding antibodies were absent from serum, erythrocyte insulin receptor binding was normal, and >90% of circulating immunoreactive insulin coeluted with <sup>125</sup>I-labeled insulin on gel filtration. The patient's insulin diluted in parallel with a human standard in the insulin radioimmunoassay, confirming close molecular similarity.

The patient's insulin was purified from serum and shown to possess both reduced binding and ability to stimulate glucose uptake and oxidation in vitro. Analysis of the patient's insulin by high-performance liquid chromatography (HPLC) revealed two products: 7.3% of insulin immunoreactivity coeluted with the human standard, while the remaining 92.7% eluted as a single peak with increased hydrophobicity. Family studies confirmed the presence of hyperinsulinemia in four of five relatives in three generations, with secretion of an abnormal insulin documented by HPLC in the three tested. Leukocyte DNA was harvested from the proband and the insulin gene cloned. One allele was normal, but the other displayed a thymine for guanine substitution at nucleotide position 1298 from the putative cap site, resulting in a leucine for valine substitution at position 3 of the insulin A chain. Insulin Wakayama is therefore identified as [Leu<sup>A3</sup>] insulin.

### Introduction

Hyperinsulinemia is a frequent occurrence in nonketotic Type II diabetes, especially in the obese (1, 2). It is generally accompanied by elevated C-peptide levels and a reduction in the hypoglycemic response to exogenous insulin (3, 4). Recently, several patients with hyperinsulinemia, relatively normal C-peptide levels resulting in elevated insulin:C-peptide molar ratios, and with a normal response to exogenous insulin have been identified. These patients have been proven to secrete a structurally abnormal insulin (5–8). Two of these patients have had the specific abnormality localized by comparison with synthesized insulin

analogs using high-performance liquid chromatography (HPLC)<sup>1</sup> following localization of the defect by Mbo II restriction enzyme analysis (7, 9, 10) and subsequently by detailed analysis at the genomic level (11, 12). The study of these mutant insulins has improved our understanding of structural determinants of insulin receptor binding and function.

The proband of the current study was shown to secrete an abnormal insulin of increased hydrophobicity on HPLC (7). However, structural differences could not be identified by restriction enzyme analysis. The current study extends considerably our understanding of this familial mutant insulin. Affected family members possess a normal insulin allele and one which codes for a leucine for valine substitution at position 3 of the insulin A chain. The clinical and biological impact of this autosomal, dominantly inherited defect is described.

### Methods

*Clinical information.* The proband was a 56-yr-old, non-obese (height, 148 cm; weight, 43 kg) Japanese woman who presented in 1980 with polyuria and weight loss. Hyperglycemia (273 mg/dl at fasting) with glucosuria and ketonuria were present. Physical examination was unremarkable, as were routine laboratory and radiographic tests. Highly purified pork or beef insulin was administered for 2 mo, but was discontinued because of allergic reactions to both insulins at the injection site. Subsequently, she has been treated with oral hypoglycemic agents with adequate control. She had no history of obesity or hypoglycemic symptoms. Her younger brother subsequently developed diabetes mellitus following a febrile illness in 1982 at age 52, and has also been treated with oral hypoglycemic agents.

Circulating insulin counterregulatory hormones were within the normal range (cortisol, 24.5  $\mu$ g/dl; growth hormone, 1.6 ng/ml; glucagon, 56 pg/ml; triiodothyronine, 0.9 ng/ml; thyroxine, 7.9  $\mu$ g/dl), and anti-insulin, anti-receptor, and islet cell surface antibodies were absent. Thyroid anti-microsomal antibodies were positive (1: 102,400) and thyroid biopsy confirmed chronic lymphocytic thyroiditis. Thyroid function tests, however, were normal.

*Clinical studies.* The proband and five of her first degree relatives agreed to be studied. Fasting levels of plasma glucose, C-peptide, and serum insulin were determined in all. The glycemic response to exogenous pure pork insulin (Actrapid; Novo Research Institute, Copenhagen, Denmark), 0.1 U/kg administered intravenously, was determined in the proband on two occasions. The glucose, insulin, and C-peptide responses to 75 g oral glucose were determined in the proband and three other family members.

*Characterization of circulating insulin.* The percent of fasting insulin immunoreactivity corresponding to circulating proinsulin was determined by gel filtration on Biogel P-30 (Bio-rad Laboratories, Richmond, CA) for the proband and all five family members.

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1. *Abbreviations used in this paper:* ACN, acetonitrile; HPLC, high-performance liquid chromatography; IRI, immunoreactive insulin; TFA, trifluoroacetic acid.

Insulin for receptor binding, biological activity studies, and HPLC was purified using octadecyl silica cartridges (Sep-Pak; Waters Associates, Millipore Corp., Milford, MA) (Cohen, R. M., B. D. Given, J. Licino-Paixao, S. A. Provow, P. A. Rue, B. H. Frank, M. A. Root, K. S. Polonsky, H. S. Tager, and A. H. Rubenstein, manuscript submitted for publication). Cartridges were prepared by washing with 5 ml of acetonitrile (ACN, HPLC grade, Fisher Scientific Co., Fairlawn, NJ) followed by 5 ml of water. Plasma drawn into a disposable plastic syringe was loaded undiluted onto the cartridge at a rate of 20 ml/h using a Harvard pump (Harvard Apparatus Co., Inc., S. Natick, MA), with a 0.45- $\mu$ m filter (Millex HA; Millipore Corp., Bedford, MA) interposed between the syringe and cartridge. The syringe was washed with 5 ml of water pumped through the filter and cartridge. The filter and syringe were then discarded and all subsequent steps were performed using glass syringes at the same pump flow rate.

The cartridge was washed sequentially with three different solvent mixtures. The first wash consisted of 5 ml 20% acetonitrile (ACN): 79.2% water: 0.8% trifluoroacetic acid (TFA, Aldrich Chemical Co., Milwaukee, WI), followed by 5 ml of a 99% water: 1% TFA mixture, and 5 ml of an 80% ACN: 20% methylene chloride (Aldrich Chemical Co.) mixture. 3 ml of ACN were applied to the cartridge to remove residual methylene chloride.

Insulin was eluted from the cartridge with 5 ml 40% ACN: 59.4% water: 0.6% TFA and collected into a siliconized scintillation vial. The ACN was evaporated under reduced pressure, after which the remaining solution was quick-frozen in dry ice acetone and lyophilized. The lyophilized powder was completely soluble in as little as 0.1 ml of neutral buffer or dilute acetic acid. Yields by this method were 70–90%.

In vitro receptor binding of insulin extracted from propositus serum was studied using rat adipocytes (13). The biological activity of her insulin was compared with a semisynthetic human insulin standard (Novo Research Institute) and with insulin extracted from a normal control for its ability to stimulate uptake of 2-deoxy-D-[1- $^{14}$ C]glucose (sp act, 50 mCi/mM; New England Nuclear, Boston, MA) and oxidation of [ $^{14}$ C]glucose (sp act, 250 mCi/mM, New England Nuclear) in isolated rat adipocytes (14, 15). Insulin extracted from the plasma of the propositus and three family members was separated by reverse-phase HPLC (7) using a C-18 column (Altex, Inc., Berkeley, CA) and a mobile phase of 30% ACN and 70% TEAP buffer (20 mM triethylamine, 50 mM sodium perchlorate, 100 mM phosphoric acid brought to pH 3.0 with NaOH; all reagents were HPLC grade). The column eluant was collected and assayed for insulin immunoreactivity as described (7). Recoveries of insulin immunoreactive material injected onto the column were 70–90%.

**Characterization of insulin gene structure.** Genomic DNA was isolated from circulating leukocytes obtained from the propositus as described previously (9). The two alleles of the insulin gene were then cloned using a modification of the  $\pi$ VX system developed by Seed (16) and will be described in detail elsewhere (Chan, S. J., Y. Lu, K. Nanjo, T. Sanke, M. Miyano, A. H. Rubenstein, and D. F. Steiner, manuscript in preparation). Briefly, plasmid  $\pi$ HIns was constructed from  $\pi$ VX. It contains a unique 1,400 basepair Eco RI-Xho I DNA fragment derived from the 5' flanking region of the human insulin gene as well as the *Escherichia coli* suppressor F gene and was transformed into *E. coli* strain MC1061 (p3) (16). Subsequently, high molecular weight leukocyte DNA isolated from the propositus was digested to completion with restriction nucleases Eco RI and Sal I, ligated to  $\lambda$ gtWES-Eco RI arms, and packaged into recombinant phage in vitro. This recombinant phage "library" was then amplified on *E. coli* MC1061 (p3,  $\pi$ HIns). During amplification, however, only the recombinant phage containing the human gene sequence would acquire the suppressor F gene due to homologous recombination in vivo with  $\pi$ HIns. These plaques were subsequently isolated by growth on a suppressor minus *E. coli* strain LG75 and positively identified by plaque hybridization to  $^{32}$ P-labeled cloned human insulin gene DNA fragment available in the laboratory (12). To obtain cloned DNA for each allelic insulin gene, advantage was taken of the finding that the two alleles contained a slight difference in the size of the 5' flanking polymorphic region (17). Restriction mapping and DNA sequence analysis of the insulin genes were performed as described previously (12).

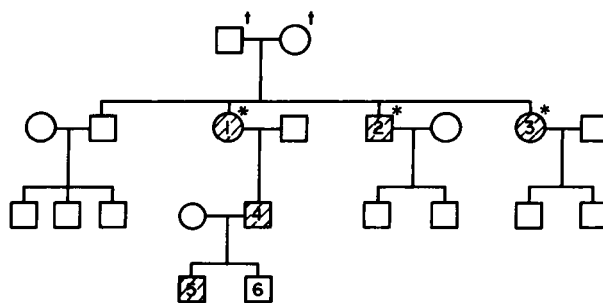
**Assay techniques.** Plasma glucose was measured by the glucose oxidase method, using a glucose analyzer (Model 23, Yellow Springs Instrument Co., Yellow Springs, OH). Serum insulin (18), growth hormone (19), and plasma C-peptide (20) and glucagon (21) concentrations were measured by radioimmunoassay, and cortisol levels were determined by a competitive-binding assay (22). Insulin antibodies were measured as previously described (23). Insulin binding to erythrocyte insulin receptors from the propositus was examined using the method of Gambhir et al. (24). Data is expressed as mean  $\pm$  standard error of the mean. All studies were approved by the institutional review board and written informed consent was obtained.

## Results

**Screening for insulin resistance.** At the time of presentation, the propositus was found to have hyperinsulinemia (Fig. 1). This apparent insulin resistance was not due to elevated levels of counterregulatory hormones or the presence of circulating anti-insulin or anti-insulin receptor antibodies. The patient's erythrocyte insulin receptors, when compared with 20 normal controls, displayed similar specific binding (8.85 vs.  $8.21 \pm 0.2\%$ ) and dissociation constants ( $K_D$ ) ( $0.82 \times 10^9$  M vs.  $0.92 \pm 0.05 \times 10^{-9}$  M) at the high affinity site. The fact that she was not severely insulin-resistant was confirmed by demonstrating a 39% fall in plasma glucose in response to 0.1 U/kg intravenous insulin (from 185 mg/dl baseline to 116 mg/dl at 60 min).

Since proinsulin and its 9,000-mol-wt intermediates cross-react in the insulin assay (25) and elevated serum immunoreactive insulin (IRI) can arise from elevations in proinsulin (26, 27), serum from the propositus and her five available family members was subjected to size exclusion gel chromatography. The peak coeluting with  $^{125}$ I-insulin accounted for >90% of serum IRI in all six subjects (data not shown).

**Clinical studies.** To determine if hyperinsulinemia was present in other family members, fasting insulin and C-peptide levels were determined in the five additional family members willing to be studied. As shown in Fig. 1, hyperinsulinemia was present



Subject	Age/Sex	Plasma Glucose (mg/dl)	IRI* (pmol/ml)	CPR (pmol/ml)	IRI:CPR ratio
1	56 F	272	0.62 (90)	0.37	1.68
2	49 M	81	0.36 (52)	0.38	0.95
3	54 F	93	0.50 (73)	0.49	1.02
4	28 M	69	0.55 (80)	0.66	0.83
5	4 M	72	0.23 (33)	0.29	0.79
6	2 M	70	0.02 (3)	0.28	0.07

**Figure 1.** Pedigree of the propositus (subject 1) and her family. Numbered subjects were studied. Family members with fasting hyperinsulinemia (diagonal lines) and/or diabetes (\*) are indicated. CPR, C-peptide immunoreactivity. †, deceased; #,  $\mu$ U/ml in parentheses.

in four of the five, although only the propositus (subject 1) displayed fasting hyperglycemia. Oral glucose tolerance tests performed after an overnight fast are shown in Fig. 2 for the propositus and three others. As can be seen, in addition to the propositus, the brother (subject 2) and sister (subject 3) both met the criteria for the diagnosis of diabetes. Notably, while the propositus and her brother, who had the worst glucose tolerance, had only marginal increases in serum insulin in response to oral glucose, the son (subject 4) and sister had substantial increases in serum insulin and relatively normal glucose tolerance (Fig. 2).

**Characterization of circulating insulin.** Following the initial identification of hyperinsulinemia in the propositus in 1982, HPLC analysis was performed. It identified the presence in serum of an abnormal insulin species of increased hydrophobicity (7). To characterize this patient's circulating insulin further, a number of studies were performed.

Serum insulin from the propositus was assayed in multiple dilutions by radioimmunoassay. As shown in Fig. 3, serum insulin diluted in parallel with semisynthetic human insulin standard, indicating close immunologic similarity. However, insulin purified from propositus serum by Sep-pak extraction showed reduced binding to rat adipocytes. As shown in Fig. 4, the patient's insulin competed with  $^{125}\text{I}$ -labeled insulin for binding to adipocyte receptors only 19.8% as well as the semisynthetic human insulin standard. This difference was not an artifact of the extraction procedure, as demonstrated by the normal binding of insulin extracted from the serum of a normal subject, using the same procedure (Fig. 4).

As would be anticipated from the receptor binding studies, the patient's insulin demonstrated decreased biological activity in vitro as well. Fig. 5 *A* documents decreased uptake of 2-deoxy-D-[1- $^{14}\text{C}$ ]glucose in rat adipocytes in response to the patient's insulin. Glucose oxidation stimulated by the patient's insulin was also reduced as measured by the metabolism of [ $^{14}\text{C}$ ]glucose to  $^{14}\text{CO}_2$  (Fig. 5 *B*). Again, insulin extracted from a normal subject by the same procedure stimulated glucose uptake and metabolism normally (Fig. 5). Insufficient material was available to construct full binding or dose-response curves for insulin from the propositus or normal subject.

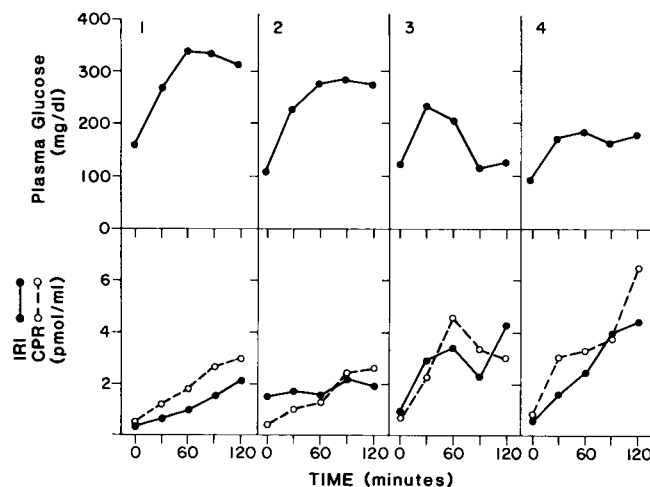


Figure 2. Changes in the levels of plasma glucose (top), IRI, and C-peptide (CPR) (bottom) after administration of 75 g of glucose orally to the propositus (1), brother (2), sister (3), and son (4).

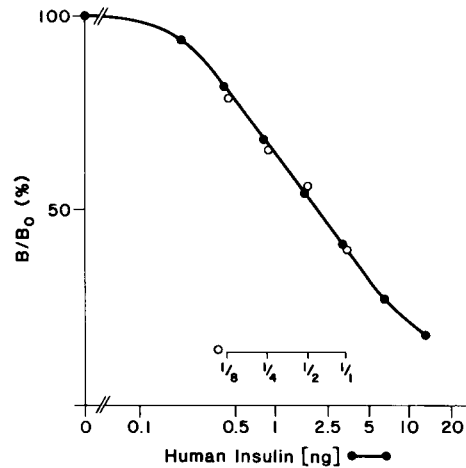


Figure 3. Radioimmunoassay dilution curves of the propositus' serum insulin (○) and semisynthetic human insulin (●). The dilutions performed for propositus' serum are indicated in the figure.

Having demonstrated hyperinsulinemia in several first degree relatives, serum insulin from the propositus and three hyperinsulinemic family members was subjected to HPLC. As shown in Fig. 6, 7.3% of the propositus' insulin coeluted with the human insulin standard (peak *b* in Fig. 6). However, the remaining 92.7% eluted as a single peak of significantly greater hydrophobicity (peak *d* in Fig. 6). This finding is in agreement with our original report (7). The hyperinsulinemia in the family members was also due to high circulating levels of the mutant insulin (Fig. 6). Importantly, human insulin, when added to a patient sample, eluted at its expected position, confirming the absence of a separation artifact (data not shown).

**Characterization of genomic DNA.** The ability to clone and sequence leukocyte DNA has allowed the molecular abnormality in two previous cases (insulins Chicago and Los Angeles) to be confirmed at the genomic level.

Both alleles of the insulin gene of the propositus were cloned and sequenced. One allele was entirely normal. However, in the other allele, a thymine for guanine substitution was found in the codon for position 3 of the A chain, resulting in a leucine for valine substitution (Table I). The cloning procedure will be

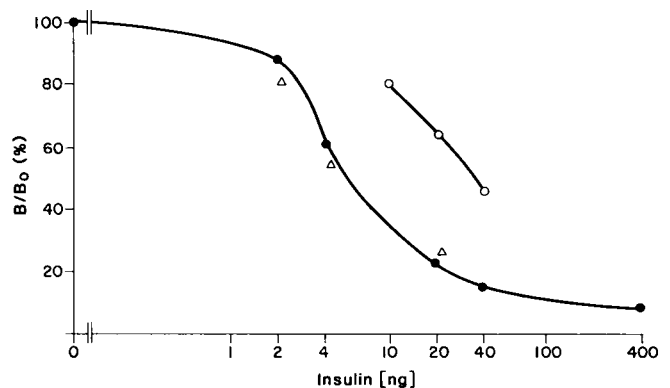


Figure 4. Binding of  $^{125}\text{I}$ -insulin to rat adipose tissue cells in the presence of varying amounts of semisynthetic human insulin standard (●) and IRI isolated from the serum of the propositus (○) and a normal subject (Δ). Results are expressed as the percentage of  $^{125}\text{I}$ -insulin specifically bound per  $1 \times 10^5$  rat adipocytes.

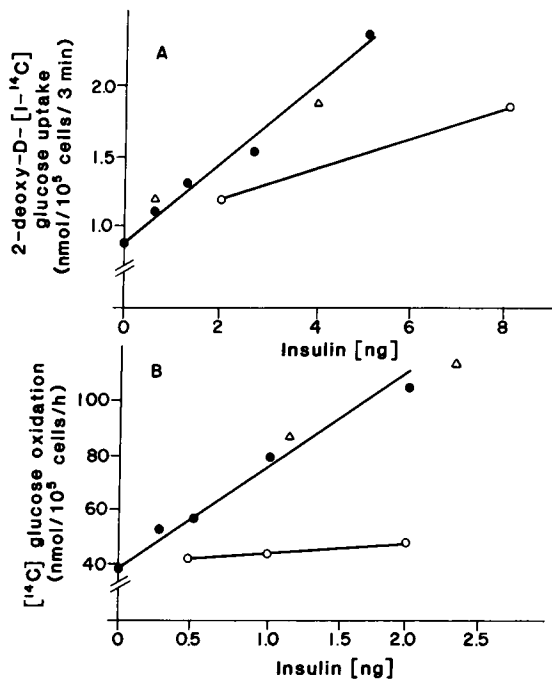


Figure 5. Stimulation of 2-deoxy-D-[1-<sup>14</sup>C]glucose uptake (A) and [<sup>14</sup>C]glucose oxidation (B) in rat adipocytes by varying amounts of human insulin standard (●) and insulins purified from serum of the propositus (○) and a normal subject (Δ).

described in detail elsewhere (Chan, S. J., Y. Lu, K. Nanjo, T. Sanke, M. Miyano, A. H. Rubenstein, and D. F. Steiner, manuscript in preparation).

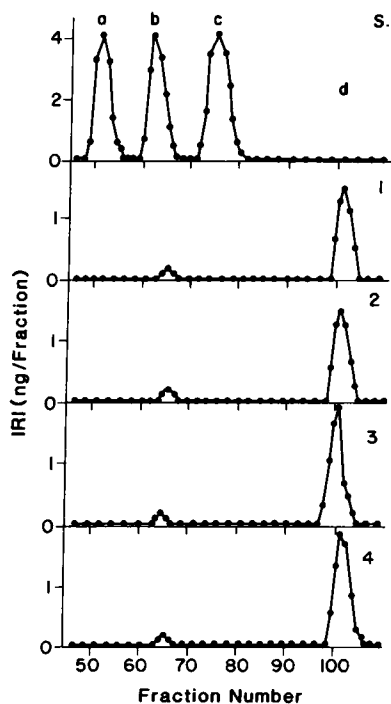


Figure 6. HPLC analysis of serum insulin from the propositus (1), brother (2), sister (3), and son (4). Chromatography of a mixture of bovine (a), human (b), and porcine (c) insulins is shown in the upper panel (S).

## Discussion

The propositus presented with glucose intolerance, hyperinsulinemia, and an elevated insulin:C-peptide molar ratio. Obesity and counterregulatory hormone excess were absent, as were other less likely causes of insulin resistance: circulating anti-insulin or anti-insulin receptor antibodies, or alterations in receptor binding. Since this presentation resembled the two previously reported cases manifesting structurally abnormal insulin molecules, exogenous insulin was administered to test *in vivo* insulin sensitivity. The adequate drop in blood glucose noted supported the likelihood that the circulating IRI was not comprised entirely of normal insulin. Gel filtration of serum excluded hyperproinsulinemia and documented that the circulating IRI in this patient (and five of her family members) coeluted with <sup>125</sup>I-insulin. Based on these findings, HPLC analysis of propositus insulin was performed that documented a molecular abnormality (7). Further characterization of this mutant insulin species followed.

The patient's circulating IRI diluted in parallel with the standard in the insulin assay (Fig. 3), again demonstrating the inability of polyclonal antisera to detect molecular differences across insulin species (mutant or animal) (6). However, fundamental differences in bioactivity were apparent. The patient's insulin competed with <sup>125</sup>I-labeled insulin for binding to rat adipocytes only about 20% as well as a human insulin standard (Fig. 4). The patient's insulin also showed markedly reduced potency in its ability to stimulate glucose uptake and oxidation in rat adipocytes (Fig. 5). Insufficient material was available to produce a complete dose-response curve, which made exact quantitation difficult, and the relative contributions of the normal and mutant insulins present in propositus serum with respect to binding and biological activity could not be assessed.

Having documented that the propositus secreted a mutant insulin, family members willing or able to cooperate were screened. As shown in Fig. 1, four of the five individuals from three generations displayed fasting hyperinsulinemia and increased insulin:C-peptide molar ratios. To demonstrate the presence of abnormal insulin in these family members with hyperinsulinemia, plasma was extracted and HPLC analysis performed on samples from the three affected adults. All produced the abnormal insulin, as predicted from peripheral insulin and C-peptide levels (Fig. 6). It is important to note, however, that the insulin gene has not, as yet, been cloned in these family members, and the definitive conclusion that they synthesize the identical mutant insulin as the propositus must await this result.

Mbo II restriction enzyme analysis of DNA from two previous patients secreting abnormal insulin had localized the defect to one or the other of the paired phenylalanine residues at positions 24 and 25 of the B chain, allowing the peptide defect to be identified by comparison to semisynthesized analogs on HPLC (7, 10). Subsequent (11) or simultaneous (12) cloning and sequencing of leukocyte DNA had identified the point mutations responsible. Mbo II restriction enzyme analysis of DNA extracted from the propositus revealed no abnormality, thus requiring the cloning and sequencing of leukocyte DNA for determination of peptide structure. A thymine for guanine substitution at position 1298 of the insulin gene in one allele was documented, resulting in a leucine for valine substitution at position 3 of the insulin A chain (Table I). The other allele was normal; Insulin Wakayama (7) can thus be identified as human [Leu<sup>A3</sup>] insulin. This finding was unexpected, since the valine residue at A3 has not been thought to be important in the active biological site of the

Table 1. Gene Sequence of Insulin Wakayama

		A chain position									
		1	2	3	4	5	6	7	8	9	10
Normal	Gly GGC	Ile ATT	Val GTG	Glu GAA	Gln CAA	Cys TGC	Cys TGT	Thr ACC	Ser AGC	Ile ATC	
Mutant	Gly GGC	Ile ATT	Leu TTG	Glu GAA	Gln CAA	Cys TGC	Cys TGT	Thr ACC	Ser AGC	Ile ATC	
	↑ 1,292		↑ 1,300							↑ 1,320	

Comparison of nucleotide and amino acid sequences of the two alleles in the region of the point mutation. Numbers above the amino acid sequence indicate position of amino acid residues of the insulin A chain. Numbers below the nucleotide sequence indicate the position of nucleotides from the putative cap site. A single G to T transversion at the codon for valine at position A3 resulted in a leucine for valine substitution.

insulin molecule (28–32). It is therefore likely that this neutral for neutral amino acid substitution produced subtle conformational changes. These changes were insufficient to alter immunogenicity (Fig. 3), but markedly affected receptor binding, biological activity (Figs. 4, 5), and surface hydrophobicity as assessed by HPLC (Fig. 6). Knowledge of the structure of this mutant insulin will permit its synthesis. Availability of larger quantities of this insulin variant will then allow more definitive studies of its binding and glucose utilization characteristics to be carried out in the presence and absence of normal insulin.

Finally, returning to the clinical syndrome, all of the affected family members studied displayed fasting hyperinsulinemia in the presence of normal or mildly elevated C-peptide levels, resulting in an elevated insulin:C-peptide molar ratio. Only the propositus underwent an insulin tolerance test, with a resultant modest fall in blood sugar. A second insulin tolerance test, at 2 yr after initiation of treatment with oral hypoglycemic agents, was normal with a 78% fall in plasma glucose at 45 min. Thus, although a normal response to exogenous insulin or a normal euglycemic insulin clamp has been proposed as one of the diagnostic criteria for patients with this syndrome (8), some degree of secondary insulin resistance in those patients with marked glucose intolerance may be seen. It has been proposed that insulin sensitivity improves with normalization of blood glucose in diabetic patients (33), and this may explain the improvement in the propositus' response to exogenous insulin. Thus, a greater than 50% fall in plasma glucose in response to 0.1 U/kg of intravenous insulin may not always be seen in this syndrome. Another variable in this syndrome is the presence or absence of glucose intolerance. Fig. 2 shows the results of 2-h oral glucose tolerance tests on several family members. While fasting hyperglycemia was present only in the propositus, several members displayed peak plasma glucose levels above 200 mg/dl. In another family that was well characterized with oral glucose tolerance tests and with glucose and insulin clamp studies in the propositus (8), variable glucose intolerance was also found and it was hypothesized that diabetes may occur when there is an additional defect in beta cell secretion, such that the sum of normal and abnormal insulin molecules secreted is inadequate to maintain euglycemia (8, 34). Nevertheless, it is obvious that further studies will be required to determine why individuals who secrete the mutant insulin show varying patterns of glucose tolerance. This will include detailed quantitative studies of basal and stimulated levels of the circulating normal and abnormal insulin species,

the biological activity of the mixture of circulating insulins, and the peripheral sensitivity to insulin in vivo in each affected family member. These investigations are now under way.

Since it is likely that most or all patients with a structurally abnormal insulin molecule will be heterozygous, with one normal insulin allele, and since removal of up to 90% of the pancreas can still result in normal glucose tolerance (35), it seems that for glucose intolerance to develop in these patients, an additional diabetogenic insult will need to be present (6, 34). An alternative explanation would involve repression of the normal allele, as suggested by the recent report of two additional cases of abnormal insulin secretion by Seino et al. (36). No normal human insulin was demonstrated in serum in either of their cases on HPLC analysis. However, normal insulin was not added to their putative abnormal insulin samples, and the exact relationship of their findings to those we have described is uncertain.

This represents the second abnormal insulin in which the familial mode of inheritance could be examined. In the initial family studied, the father of the propositus was the earliest identifiable case. Five of six subsequent offsprings were affected (8). In the family reported here, only 6 of 14 potentially affected members could be screened. However, five of those secrete the abnormal species. While a dominant mode of transmission is obvious in both cases, it seems necessary to propose some positive genetic pressure leading to a greater than expected occurrence of the mutant gene in affected families.

The prevalence of genetic mutations affecting the structure of the insulin molecule in the general population is unknown. Up to the present, only those patients manifesting the mutant insulin syndrome (5–8, 36) with unusual or familial Type II diabetes have been screened and discovered. Thus, mutant insulin species with normal or relatively well-preserved binding and biological activity characteristics, and therefore normal metabolic clearances, are unlikely to be discovered by this approach since hyperinsulinemia will be absent or subtle. Future screening techniques using HPLC and/or gene analyses are thus being developed.

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