

Structurally Abnormal Insulin in a Diabetic Patient

Characterization of the Mutant Insulin A3 (Val → Leu) Isolated from the Pancreas

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Abstract

We have recently identified a diabetic patient with marked fasting hyperinsulinemia. Family study revealed that the abnormality was an autosomal dominant trait. High-performance liquid chromatography (HPLC) profile of the patient's serum insulin showed that she had an abnormal insulin in addition to a normal insulin. We have purified her insulin(s) from the specimen of her pancreas, which was biopsied during an operation of cholelithiasis. Insulin was also immunologically purified from the serum of her portal vein. The reverse-phase HPLC analysis revealed that the ratios of normal to abnormal insulin in the pancreas, portal vein, and peripheral vein were 5:4, 4:5, and 1:7, respectively. Radioreceptor assay for insulin using guinea pig kidney membrane revealed that the binding activities of the normal component insulin, the abnormal component insulin and her pancreatic insulin containing both components were 100, 5, and 50% of standard human insulin, respectively. The biological activities of the normal component, the abnormal component and her pancreatic insulin to stimulate glucose oxidation in rat adipocytes were found to be 100, 8, and 60% of standard human insulin, respectively. Analysis of amino acid sequences of the abnormal insulin purified from her pancreas strongly suggested the substitution of leucine for valine at the third position of the A chain, A3 (Val → Leu).

Introduction

Diabetes mellitus is a clinical syndrome induced by various causes, and the possibility had been considered for many years (1-5) that a structurally abnormal insulin might be one cause of diabetes. The first case with a mutant insulin was reported by Tager et al. (6) and Given et al. (7) in 1979, 1980, and since that time, a few cases (8-12) have been described. The point mutation sites of the insulin genes and resulting amino acid substitutions have been determined in three cases; e.g., insulin Chicago B25 (Phe → Leu) (13, 14), insulin Los Angeles B24 (Phe → Ser) (15), and insulin Wakayama A3 (Val → Leu) (12, 16). The abnormality is an autosomal dominant trait and the

families with abnormal insulins are hyperinsulinemic, but not all are diabetic. The biologic activities of the abnormal insulins are low, and hyperinsulinemia is thought to result from feedback compensatory mechanism and/or reduced degradation of the insulins.

We recently identified a new female patient with abnormal insulin causing diabetes (17). During the investigations, she was found to have multiple gallstones. Since she had had a history of severe right hypochondral pain after meals, and one of these stones was found to be nearly dropped to the common bile duct by intravenous cholecystography, a cholecystectomy was performed. At that time, as the informed consent of the patient and her family was obtained, a piece of pancreas (~0.6 g) from the pancreatic tail was biopsied and a venous blood was obtained from the portal vein.

We describe the characteristics of the patient's pancreatic insulin and serum insulin.

Methods

Clinical characteristics of the patient

The clinical description of the patient can be found in detail elsewhere (17). Her clinical characteristics are summarized as follows; (a) Initial fasting plasma glucose was 244 mg/dl and serum insulin was 128 μ U/ml; (b) C-peptide/insulin molar ratio was reduced to ~1.0. (c) disappearance rate of endogenous insulin was decreased; (d) the levels of counter-insulin hormones were normal; (e) antibodies to insulin and insulin-receptor were absent; (f) 125 I-insulin binding to the patient's red blood cells was normal; (g) sensitivity to exogenous insulin was nearly normal; (h) her diabetes was mild and could be treated by oral hypoglycemic drugs.

Family studies

Examination of the patient's family by an oral glucose tolerance test revealed that four members (mother, sister, brother, and daughter) had marked fasting hyperinsulinemia and two (mother and sister) were overtly diabetic. The abnormality thus was thought to be an autosomal dominant trait (17) (Table I).

Insulin isolation procedure from the pancreas (18)

The specimen of pancreas (0.6 g) from the patient was excised at the time of the operation of cholecystectomy, and a 0.43-g specimen was used for insulin isolation after the specimen for histological studies was removed. Before surgery, the purpose and procedures of the pancreas biopsy were explained to the patient and her family in detail, and the consent was obtained. As a control specimen, we used the pancreas from the other patient, who had advanced gastric cancer and had a total gastrectomy plus partial pancreatectomy. First, the specimens were frozen with dry ice and sliced into very thin pieces, which were dissolved in 1.3 ml of 80% (vol/vol) ethanol and were adjusted to pH 3.0 with phosphatidic acid. The solution was mixed extensively for 60 min and the tissue was extracted after centrifugation at 3,000 rpm for 15 min. The tissue residue was reextracted with the same procedure. The combined extracts were brought to pH 8.0 with ammonium hydroxide, mixed for 5 min, and

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Table I. Levels of Fasting Plasma Glucose (FPG) and Serum IRI, and Oral Glucose Tolerance Test (O-GTT) Patterns in the Patient and Her Family Members

Subject no.	Age	Sex	Relationship	FPG	IRI	O-GTT pattern
				mg/dl	$\mu\text{U/ml}$	
1	70	M	Father	98	8	Impaired
2	69	F	Mother	227	314	DM
3	49	F	Sister	180	249	DM
4	47	M	Brother	(not examined)		
5	44	F	Propositus	244	129	DM
6	37	M	Brother	90	237	Normal
7	34	M	Brother	84	11	Normal
8	13	F	Daughter	93	10	Normal
9	11	F	Daughter	104	8	Normal
10	8	F	Daughter	94	111	Normal

FPG, IRI, and O-GTT pattern obtained in the patient's husband were normal.

then centrifuged at 3,000 rpm for 15 min. 9 vol of acid-acetone were added to the supernatant and allowed to stand for overnight after mixture for 10 min. The resulting precipitate was collected by centrifugation at 3,000 rpm for 15 min, and dissolved in 10 ml of ether. Finally, we obtained precipitate including insulin after centrifugation at 3,000 rpm for 15 min and dried under vacuum.

Insulin isolation procedure from the serum

Step 1 (Immunoaffinity chromatography). The antiinsulin serum raised in a guinea pig was immobilized on a cyanogen bromide-activated Sepharose 6B (Pharmacia Fine Chemicals, Piscataway, NJ). The conjugate was packed in a column (1 × 12 cm) and was equilibrated with 10 mM Tris-HCl (pH 8.2) buffer. This column could bind >20 mU of insulin. 3 ml of the patient's serum was applied to the affinity column and the column was washed with the buffer for 3 h. Insulin bound to the column was eluted with 0.01 N HCl and dried under vacuum.

Step 2 (Gel filtration chromatography). Crude purified insulin by the above method contained high molecular weight substances, probably because the antiinsulin serum included polyclonal antibodies. We therefore removed high molecular weight substances by gel chromatography. Samples obtained by step 1 were dissolved in 0.5 ml of 3 M acetic acid and gel filtered on a column (1 × 55 cm) of Bio-Gel P-30 (100–200 mesh, Bio-Rad Laboratories, Richmond, CA), which had been equilibrated with the same solvent. The eluates were collected in tubes (2 ml) and dried under vacuum. We also performed gel filtration chromatography for evaluation of molecular weight of immunoreactive insulin derived from the patient's serum. We used the same column described above, but equilibrated with borate buffer. 0.9 ml of fractions was collected, and the content of insulin of each fraction was measured by radioimmunoassay. Human proinsulin (from Dr. Ronald Chance of Eli Lilly and Co., Indianapolis, IN) and semisynthetic human insulin (Novo Research, Copenhagen, Denmark) were used as standards.

High performance liquid chromatography (HPLC)¹

The partially purified vacuum-dried samples, prepared as described in the insulin isolation procedure from the pancreas and from the serum, were dissolved in 500 μl of 0.1% trifluoroacetate (TFA), and then applied to the HPLC column. The reverse-phase column ($\mu\text{Bondapak C18}$, 3.9

1. *Abbreviations used in this paper:* DPTU, diphenyl thiourea; DTT, dithiothreitol; HPLC, high performance liquid chromatography; IRI, immunoreactive insulin; KRB, Krebs-Ringer bicarbonate; NIDDM, noninsulin-dependent diabetes mellitus; PTH, phenylthiohydantoin; RRA, radioreceptor assay; TFA, trifluoroacetate.

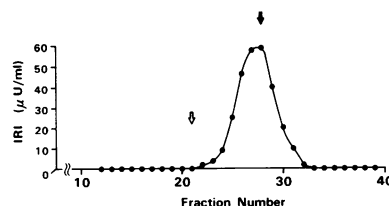


Figure 1. Gel filtration profile of the patient's serum insulin. The patient's serum was chromatographed on a Bio-Gel P-30 column (1 × 55 cm) which had been equilibrated with borate buffer. Insulin content of each fraction (0.9 ml) was measured by radioimmunoassay. The left (open) and the right (closed) arrows denote the position taken by human proinsulin and human insulin, respectively.

mm × 30 cm, 10 μm particle size, Waters Associates, Milford MA) was used. An isocratic mobile phase was composed of 31% (vol/vol) of acetonitrile containing 0.1% TFA. The flow rate was kept constant at 1 ml/

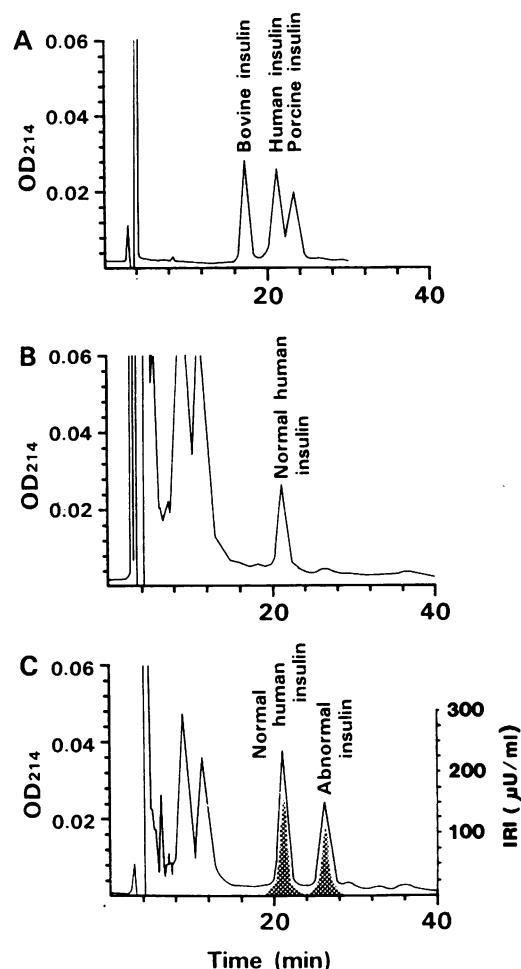


Figure 2. Separation of insulins by reverse-phase HPLC. (A) Standard insulins; a mixture of bovine insulin (Novo), semisynthetic human insulin (Novo) and porcine insulin (Novo) ($\sim 2 \mu\text{g}$ each) was injected. (B) Insulin-containing extracts from the other patient's pancreas obtained by partial pancreatectomy and total gastrectomy. (C) Insulin-containing extracts from the patient's pancreas obtained by pancreas biopsy during the operation of cholecystectomy. HPLC column and the procedures were described in Methods. Absorbance at 214 nm was measured in each eluted fraction. Only peaks indicated by name contain immunoreactive insulin. In (C) IRI of each fraction was also measured and was indicated by shaded area.

min, and fractions (0.5 ml) were collected at 28°C, and dried under vacuum. The residues were dissolved in 0.01 N HCl and borate buffer, and each fraction was measured by radioimmunoassay for insulin. The apparatus used for HPLC consisted of model 441 Waters liquid chromatograph equipped with model 510 solvent delivery system (Waters Associates), and Toyo Soda CP8000 chromato processor (Toyo Soda, Tokyo, Japan).

Radioreceptor assay (RRA)

Suzuki et al. (19) reported that kidney of guinea pig has many insulin receptors on the plasma membrane and can be used for radioreceptor assay for unextracted serum insulin. We examined the receptor binding activity of the patient's insulin according to their methods with a slight modification (17).

The kidneys from five male guinea pigs (200–250 g) were removed under nembutal anesthesia, rinsed with saline, trimmed of connective tissue, divided into small pieces weighing ~11 mg, and homogenized extensively in 100 ml of 0.3 M sucrose solution. Then the homogenate was centrifuged at 10,000 *g* for 20 min and the resulting supernatant was centrifuged at 10⁵ *g* for 90 min. The precipitate was suspended in 5 ml of 25 mM Tris-HCl buffer and homogenized strongly and kept frozen at -40°C. ¹²⁵I-insulin binding to the insulin receptors on the kidney membranes was examined in 400 µl of buffer (50 mM Tris-HCl, 0.1% bovine serum albumin (BSA), 10 mM CaCl₂, 2 mM N-ethylmaleimide pH 7.6) containing kidney pellets (100 µg of protein), ¹²⁵I-insulin (50 µl) and various concentration of either standard insulin or samples (50 µl). Samples included sera from the patient's peripheral and portal vein, sera from her family members, partially purified pancreatic insulin prepared as described above, and normal or abnormal component insulin of patient's pancreas purified by HPLC. When samples such as purified pancreatic insulin were assayed, they were dissolved in insulin-free serum and then the incubation for RRA was carried out. After an overnight incubation, 1 ml of the buffer was added and centrifuged at 3,000 rpm for 20 min and the radioactivity of the precipitate was counted.

Biological activity

We measured the biological activity of the patient's insulin by glucose oxidation. Male Wistar rats (150 g, fed ad lib.) were killed by decapitation, and the epididymal adipose tissues were removed. Isolated adipocytes were prepared by the use of collagenase (Worthington Biochemical Corp., Freehold, NJ) according to the method of Rodbell (20). Stimulation of glucose oxidation was measured by means of incubating isolated rat adipocytes at 37°C with [1-¹⁴C]glucose in Krebs-Ringer bicarbonate

(KRB) buffer containing various concentrations of either standard insulin or samples. Samples included the patient's pancreatic insulin prepared as described above, and normal or abnormal component insulin of patient's pancreas purified by HPLC. They were dissolved in KRB buffer supplemented with 1% BSA, and the incubation was carried out. After 1 h of incubation the generated ¹⁴CO₂ was collected and counted in a liquid scintillation counter as described previously by Kasuga et al. (21).

Determination of amino acid sequence of the patient's insulin

Amino acid sequences of both normal and abnormal component insulins of the patient's pancreas purified by HPLC were then determined using a model 470A protein sequencer equipped with a model 120A phenylthiohydantoin (PTH) analyzer (both from Applied Biosystems, Foster City, CA) and a model SP 4200 computing integrator (Spectra-Physics Inc., Mountain View, CA). Since we analyzed purified insulins (normal and abnormal components) without dividing into A- and B-chains, we could detect two N-terminal amino acids at one cycle.

Results

Gel filtration chromatography. The patient's serum was gel-filtered on Bio-Gel P-30 to yield the profile shown in Fig. 1. Most of insulin immunoreactivity was eluted at the position of insulin, indicating that the hyperinsulinemia of the patient is not due to hyperproinsulinemia.

HPLC. The patient's insulin purified from her pancreas, portal venous blood, and peripheral venous blood, and those purified from peripheral venous blood of her family members were applied to HPLC column. The results are shown in Fig. 2 and Fig. 3. The elution profiles of the insulins from the patient and family members demonstrated two peaks of immunoreactive insulins. One peak appeared at the position of normal human insulin. The insulin peak eluted later was thought to be a structurally abnormal insulin that was more hydrophobic than normal human insulin. The ratios of the immunoreactivity of normal insulin to abnormal insulin were 5:4 in insulin from the patient's pancreas (Fig. 2 C), 4:5 in the patient's serum from the portal vein (Fig. 3 B), 1:7 in the patient's serum from the peripheral vein (Fig. 3 A), and 1:4–1:7 in her family member's serum from the peripheral vein (Fig. 3 C–F). The ratio of normal to abnormal

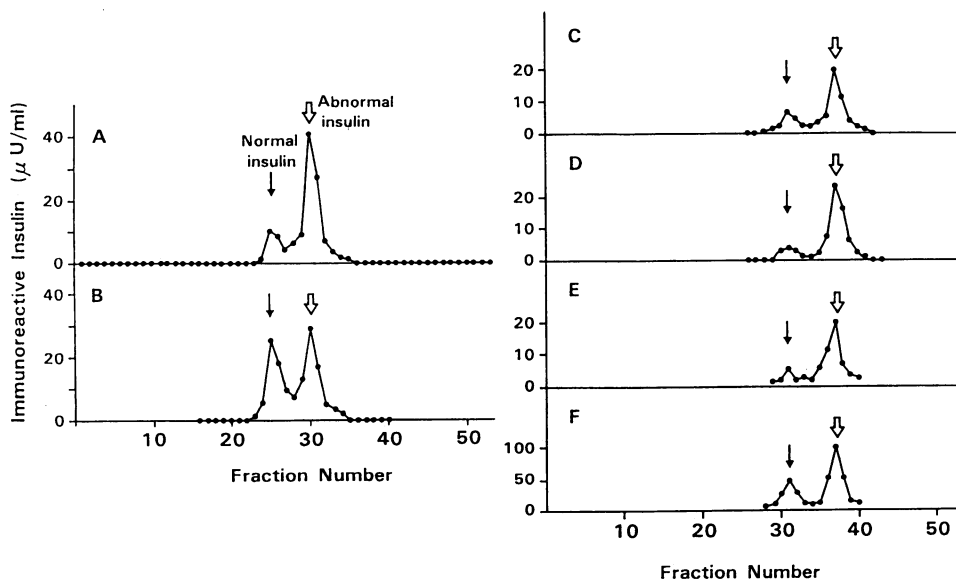


Figure 3. Separation of immuno-purified serum insulins by reverse-phase HPLC. (A) Insulin from patient's peripheral vein. (B) Insulin from patient's portal vein. (C–F) Insulins from peripheral vein of family members with hyperinsulinemia. HPLC column and the procedures were described in Methods. Immunoreactive insulin was measured in each eluted fraction. Closed and open vertical arrows indicate the position of normal and abnormal insulin, respectively. Note that the positions of the insulin peaks between A, B, and C–F are slightly different. Since HPLC analyses of these two groups were carried out at different times, the difference may be caused by the slightly different experimental conditions such as the composition of mobile phase or temperature of the column.

insulin from the patient's pancreas (5:4) determined by the absorbance at 214 nm was almost identical to that determined by immunoreactive insulin, suggesting that there was no difference in the immunoreactivity between the normal and the abnormal insulins (Fig. 2 C).

Radioreceptor assay. We performed RRA of the insulins obtained from the pancreas and portal vein of the patient, from the peripheral vein of both the patient and her families. Further, normal and abnormal insulins separated by HPLC from her pancreatic insulin were also assayed by RRA. As shown in Fig. 4, the binding activities of insulins, which contained abnormal insulin, were all decreased. Compared with standard human insulin, the binding activities were 50% for the patient's pancreatic insulin, 24% for the patient's serum insulin of the portal vein, 12% for insulins from the peripheral vein of both the patient and her family members with abnormal insulinemia, and ~5%

for the abnormal insulin component purified by HPLC from her pancreatic insulin. In contrast, displacement curves of the insulin from the normal component purified by HPLC from her pancreatic insulin and the serum of a family member without hyperinsulinemia (subject 7 in Table I) were completely superimposed on that of standard human insulin.

Biological activity. Fig. 5 shows the results of the ability of the patient's pancreatic insulin (mixture of normal and abnormal insulin), purified insulins (normal component and abnormal component) by HPLC, and standard human insulin to stimulate glucose oxidation in isolated rat adipocytes. The biological potency of the pancreatic insulin was reduced to 60%, and that of the abnormal component insulin was reduced to as little as 8% of the standard insulin, while the biological potency of the normal component insulin of the patient was identical to that of the standard insulin. However, maximally stimulated levels of glu-

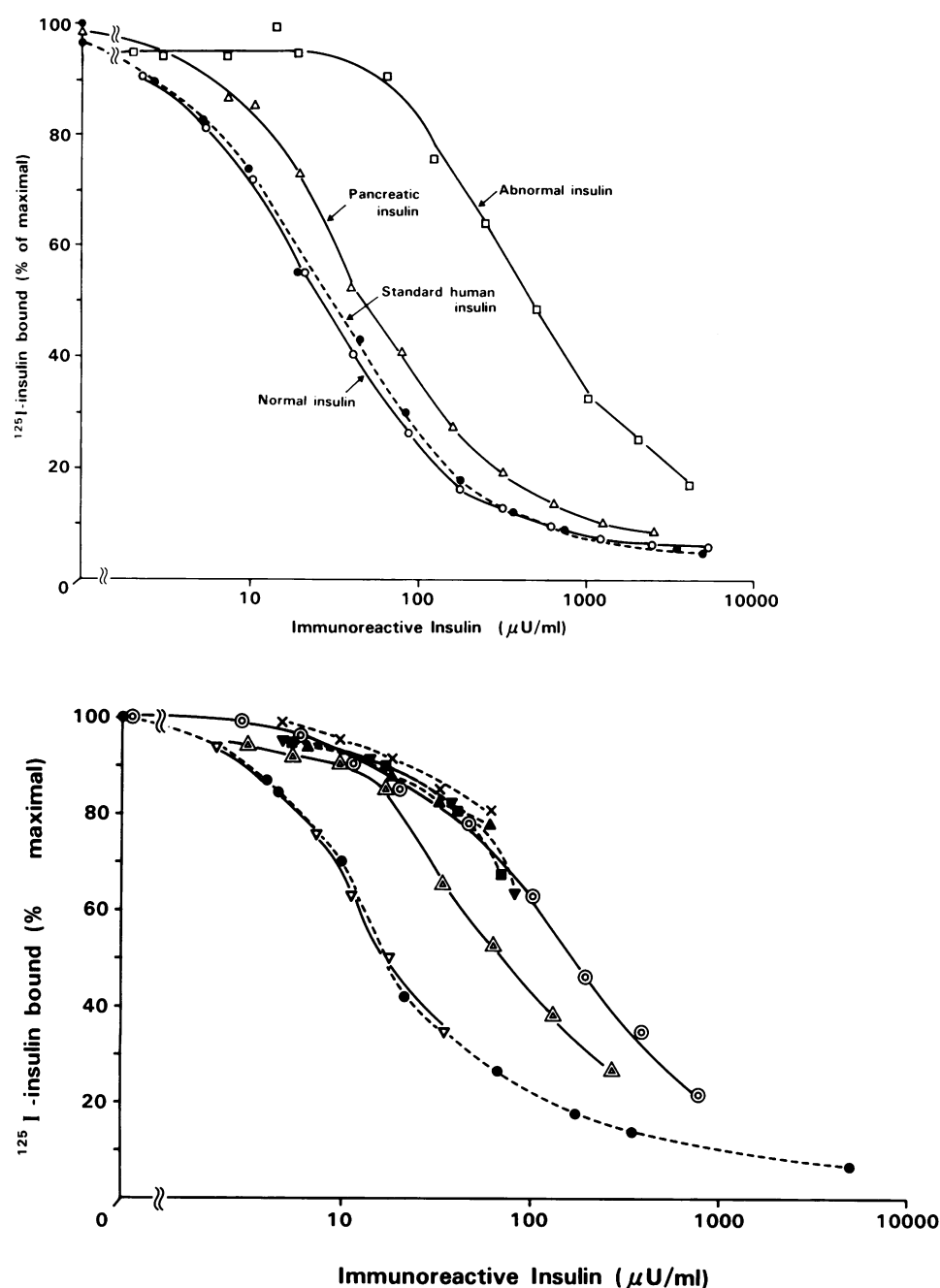


Figure 4. Ability of various insulins to inhibit ^{125}I -labeled porcine insulin binding to guinea pig kidney membrane. (upper) Standard human insulin (●); the insulin isolated from patient's pancreas (mixture of normal and abnormal insulins (Δ); normal (○) and abnormal component insulin (□) purified by HPLC from patient's pancreas. Details of purification procedure of each sample was described in text. (lower) Standard human insulin (●); the insulin isolated from sera of the patient (●), sera of family members with hyperinsulinemia (subject 2: ■, subject 3: ▲, subject 6: ▼, subject 10: ×), and sera of family members without hyperinsulinemia (subject 7) (∇); and the immunopurified insulin isolated from patient's portal vein serum (Δ).

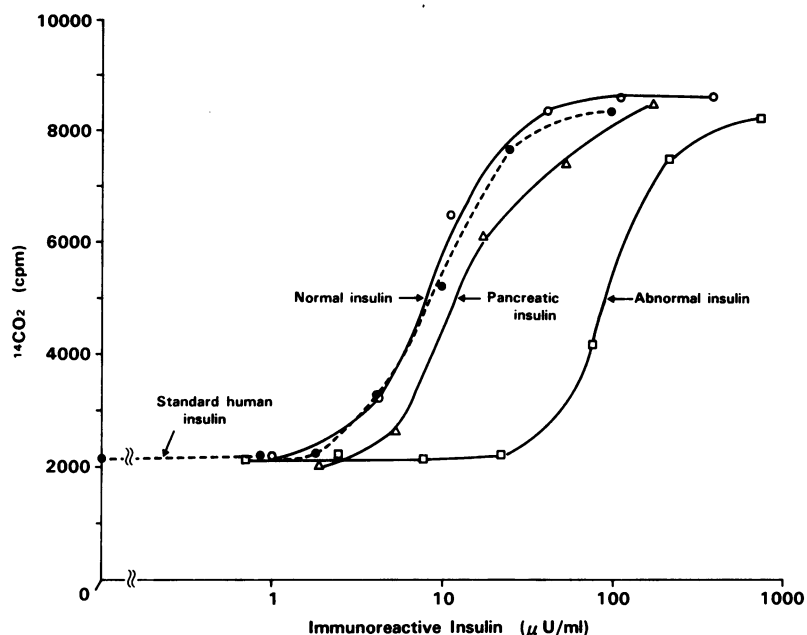


Figure 5. Ability of various insulins to stimulate glucose oxidation in isolated rat adipocytes. Standard human insulin (●); the insulin isolated from patient's pancreas (mixture of normal and abnormal insulins) (Δ); normal (○) and abnormal insulin (□) purified by HPLC from patient's pancreas. Details of purification procedure of each sample was described in text.

cose oxidation were observed in the presence of higher concentrations of the abnormal insulin ($>500 \mu\text{U/ml}$).

Amino acid sequence. We obtained ~ 500 pmol of normal and 400 pmol of abnormal insulin by HPLC from 0.2 mg of extract from the patient's pancreas. We performed amino acid sequencing both normal and abnormal insulin. As shown in Fig. 6, we found that the third amino acids from N-terminal of the abnormal insulin consisted of leucine and asparagine, although that position of the normal insulin were occupied with valine and asparagine. Since we could not find other differences between normal and abnormal insulin (Fig. 7), we concluded that the abnormal insulin of this patient contained a leucine for valine substitution at position 3 of the A-chain.

Discussion

We recently found a patient whose insulin was a substitution of leucine for valine at the third position of the A-chain. This phe-

notype is identical to insulin Wakayama (9, 12, 16), but we could not trace any relationship between two families. Two families have lived far apart, in the western and eastern parts of Japan. Since this substitution is not fatal and induces only (maturity-onset) noninsulin dependent diabetes mellitus (NIDDM), there is a possibility that this mutant insulin may have existed for a long time and is widely scattered in Japan. Alternatively, this mutation may have occurred entirely independently in different parts of Japan. However, we must consider that a different genotype can produce the same phenotype. The DNA sequence that corresponds to valine is GTG, and if one point mutation induces substitution of leucine for valine, two DNA sequences (e.g., TTG and CTG) can be candidates, and insulin Wakayama was a former one (12, 16). DNA sequence of our family is now under investigation.

The abilities to bind to the insulin receptors and to stimulate glucose oxidation of the patient's abnormal insulin purified by HPLC from her pancreatic insulin were 5% and 8% that of nor-

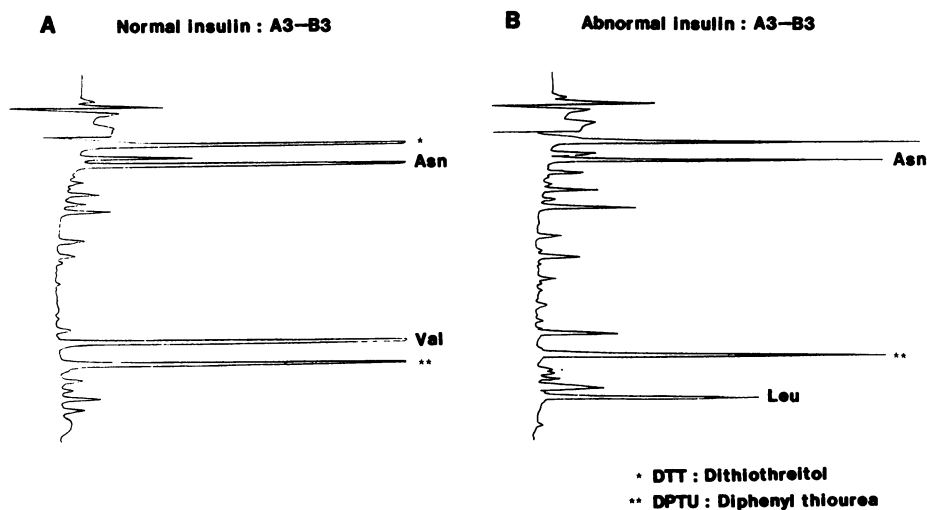


Figure 6. HPLC profiles of the PTH amino acids obtained by the Edman degradation procedure (3rd cycle) of normal (A) and abnormal (B) insulin. These insulins were purified from the patient's pancreas by the insulin isolation procedures and reverse-phase HPLC as described in Methods. Third position of the A and B chains of normal insulin consisted of asparagine (Asn) and valine (Val), and that of abnormal insulin asparagine (Asn) and leucine (Leu), indicating the substitution of leucine for valine at the A3 position. Peaks by DTT (*) and DPTU (**) were detected as internal standards at each cycle of HPLC analysis of PTH amino acids.

Sample \ cycle	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Human A chain	G	I	V	E	Q	C	C	T	S	I	C	S	L	Y	Q	L	E	N	Y	C	N
insulin B chain	F	V	N	Q	H	L	C	G	S	H	L	V	E	A	L	Y	L	V	C	G	E
Normal insulin	G	I	V	E	Q			T	S	I		S	L	Y	Q	L	E	N	Y		N
	F	V	N	Q	H	L		G			H	L	V	E	A	L	Y	L	V		G
Abnormal insulin	G	I	L	E	Q			T	S	I		S	L	Y	Q	L	E	N	Y		N
	F	V	N	Q	H	L		G			H	L	V	E	A	L	Y	L	V		G
Sample \ cycle	22	23	24	25	26	27	28	29	30												
Human B chain	R	G	F	F	Y	T	P	K	T												
Normal insulin	R	G	F	F	Y	T	P	K	T												
Abnormal insulin	R	G	F	F	Y	T	P	K	T												

Figure 7. Total amino acid sequences of normal and abnormal insulins purified from the patient's pancreas by the insulin isolation procedures and reverse-phase HPLC as described in Methods. Each amino acid is expressed as a single letter. Since cys (C) can not be detected in this analysis, several cycles (cycle 6, 7, 11, 19, and 20) remained to be blank.

mal human insulin, respectively. The results indicate that there is a good parallelism between binding activity and biological activity of the abnormal insulin in the patient. Tager et al. (22) and Olefsky et al. (23) reported that the biological activity of insulin Chicago was much lower than its binding activity and suggested that the abnormal insulin was an active antagonist of insulin action. Keefer et al. (24) and Kobayashi et al. (25), however, found no antagonistic effect in those analogues. Furthermore, Kobayashi et al. (26) clarified that the biological activity of insulin Los Angeles was proportional to its receptor binding activity. In concert with their results (24–26) the biological activity of the abnormal insulin of this patient was reduced to almost the same extent as the binding activity.

Shoelson et al. (9) described that the variant insulin represented 57% and the normal insulin 43% of the patient's stored pancreatic insulin in the case of insulin Chicago. Our results show that the ratio of abnormal to normal insulin in the pancreas is 4:5, and support the idea that both normal and abnormal insulins are secreted in almost equal proportions, indicating the codominant expression of both alleles of the insulin gene. However, we cannot insist that the expression of both alleles is exactly the same, because it was known that the quantities of HbS heterozygotes, which also have a point mutation of an allele like an abnormal insulin gene, are only 20–50% of all hemoglobins (27).

The reason why the ratio of an abnormal insulin to a normal insulin in the peripheral vein is higher than that in the pancreas is probably due to a decreased metabolism of an abnormal insulin. The ratio of an abnormal insulin to a normal insulin in the portal vein was found to be slightly lower than that in the pancreas as expected.

N-terminal and C-terminal of A-chain and C-terminal of B-chain (especially B23–26) are thought to constitute the receptor binding site which is important for the action of insulin (28), and the amino acid sequences in these parts are conserved through evolution. Substitution of A3 amino acid is therefore supposed to influence the conformation of the receptor binding site like B24 or B25 substitution, and to induce the decreased binding activity to the insulin receptor, which may result in

decreased bioactivity and decreased metabolism of the abnormal insulin.

It is interesting to consider the reasons why patients with mutant insulin have diabetes. Family study shows that not all of those with a mutant insulin are diabetic. Noninsulin-dependent diabetes develops most frequently after middle age. It seems that in this family diabetes develops with age. There is a discussion that diabetes may develop only when β -cell secretion is no longer able to compensate the reduced bioactivity of its secretory products (8), but in the present family, immunoreactive insulin (IRI) level of the propositus' mother who is overtly diabetic is highest and that of the propositus' daughter whose GTT pattern is normal is lowest among the members with hyperinsulinemia. There were no differences in the results of HPLC profiles and RRA between those with and without diabetes. We therefore suppose that insulin insensitivity related to the post-receptor factors may contribute to the development of diabetes at least in part of this family. The analysis of development of diabetes by a mutant insulin will provide valuable informations also for the understanding of the pathogenesis of NIDDM in general.

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