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EVIDENCE FOR ANALOGUES OF INSULIN IN HUMAN SERUM

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Abstract

The serum samples taken at 60 minutes after 50-gram oral glucose load from 6 cases of adult onset diabetic patients, a borderline case of diabetes mellitus and four healthy adult volunteers were subjected to extraction with acid-alcohol and gel filtration on the Bio-Gel P-30 column (1.6×90 cm). The fraction sizes of 2.0 ml and 4.05 ml were employed. Each of the fractions was radioimmunoassayed for immunoreactive insulin (IRI) after lyophilization and following results were obtained. 1) There are two groups of insulin in human serum, and each group could be separated more than two subgroups. 2) Adult onset diabetic subjects on diettherapy show the higher level of Peak I and Peak II than normal subjects. 3) Existence of these analogues of insulin in human serum are reported firstly, but it's physio-pathological significances remained to be verified.

INTRODUCTION

Since the development of radioimmunoassay of insulin[1], the concentration of insulin in human serum has studied extensively. However the chemical and biological nature of the circulating hormone in vivo is not entirely clear. Haën *et al.*[2], Kimmel *et al.*[3] and Elliott *et al.*[4] suggested the possibility of an abnormal insulin in human serum. The present study was undertaken to clarify analogues of insulin in human serum.

MATERIALS AND METHODS

50-gram oral glucose tolerance tests were given to four healthy adults (age: 19-23 years old), three adult onset diabetic patients (age: 64-71 years old), a borderline case of diabetes mellitus (age: 70 years old) and the other three adult onset diabetic subjects (age: 47-71 years old). The serum samples

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taken at 60 minutes after glucose load were stored at −20°C until used. Extraction and gel filtration of the sera were carried out with the modification[5] of the reported methods of Davoren[6] and Melani *et al.*[7] within three months after sampling. Serum was mixed with water (1 volume serum to 2 volumes water) and 7.5 ml of the cold acid-ethanol was added for each ml of the original serum. The acid-ethanol consisted of 500 ml of 99.5% ethanol and 10 ml of concentrated hydrochloric acid. The tube was allowed to stand at 4°C for 20 hours and after centrifugation (2800 r.p.m., 30 min., 4°C), pH of the supernatant was adjusted to 8.3 with ammonium hydroxide. The precipitate was removed by centrifugation at 2800 r.p.m. for 20 minutes at 4°C. After the addition of 0.025 ml of 2 M ammonium acetate for each ml of the supernatant, pH of the solution was readjusted to 5.3 with hydrochloric acid. Then 15 ml of cold 99.5% ethanol and 50 ml of ethyl ether were added slowly for each 10 ml of the extract, and the solution was kept at 4°C for 20 hours. The precipitate was collected by centrifugation at 1500 r.p.m. for 60 minutes at 4°C, dried with N_2 gas and dissolved with 3 ml of 1M acetic acid. After centrifugation, the clear supernatant was applied to the Bio-Gel P-30 column (100-200 mesh, 1.6×90 cm) equilibrated with 1M acetic acid at 4°C with the same elution buffer. The fraction size was 4.05 ml in seven cases which included four healthy volunteers and three adult onset diabetic patients and 2.0 ml in other four cases which included a borderline case of diabetes mellitus and three adult onset diabetics, respectively. After lyophilization, each of the fractions was dissolved with 0.6 ml of 0.1M Tris-HCl buffer, pH 7.6 (containing 0.5 % BSA) and assayed for IRI. The radioimmunoassay of insulin was performed by the reported method of Horino *et al.*[8], utilizing anti-pork insulin guinea pig serum (M 8309) and porcine monocomponent insulin (Lot. No. 834098) as standard. Single component porcine insulin (Lot. No. 615-1082B-108-I) was used as labeled after iodination with ^{125}I.[9] The results were expressed as mean ± S.E.M.

RESULTS

The serum of four normal adults and three adult onset diabetic patients was analysed with 4.05 ml fraction size. The elution patterns of these samples are shown in Fig. 1., where upper panel(A) is from a normal adult and lower panel(B) from an adult onset diabetic patient. In lower panel, two groups (called Peak I and Peak II, respectively in this paper) of insulin were obtained but Peak I is not clearly demonstrable in upper panel. From these data summarized in the Tab., total insulin (Peak I plus Peak II) in adult onset diabetic patients (maximum blood sugar level after 50-gram oral glucose tolerance test:

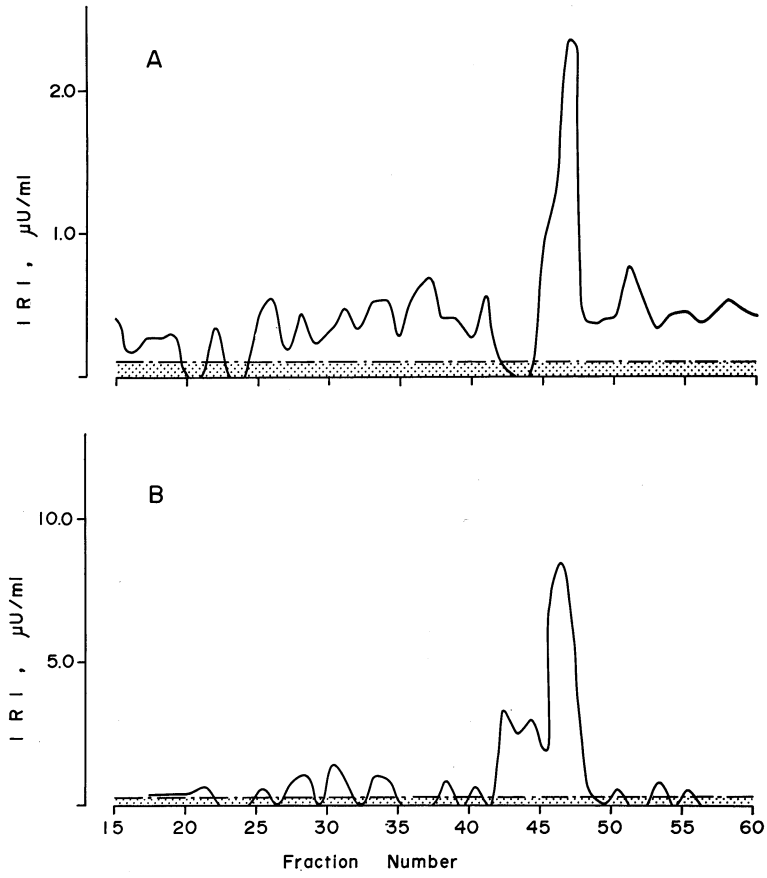

Fig. 1. Elution profiles of extracted human serum insulin on the Bio-Gel (P-30) column with 4.05 ml fraction size. Sera were obtained 60 min after glucose load from a healthy and an adult onset diabetic subjects.
A: A normal adult. (I.N., 20 y.o., F.)
B: An adult onset diabetic patient. (M.W., 68 y.o., F.)
Dotted area: Lower limits of sensitivity.

248.3 ± 30.9 mg/dl) was higher than that of normal adults (maximum blood sugar level after 50-gram oral glucose tolerance test: 136.8 ± 12.7 mg/dl) at 60 minutes after 50-gram oral glucose load. And Peak I and Peak II are also increased in adult onset diabetic subjects, 0.15 ± 0.03 and 0.19 ± 0.01 pM/ml, when compare to those in normal controls (0.03 ± 0.02 and 0.08 ± 0.03 pM/ml), respectively. These two groups of insulin were not demonstrated previously and their structures and functions were unknown.

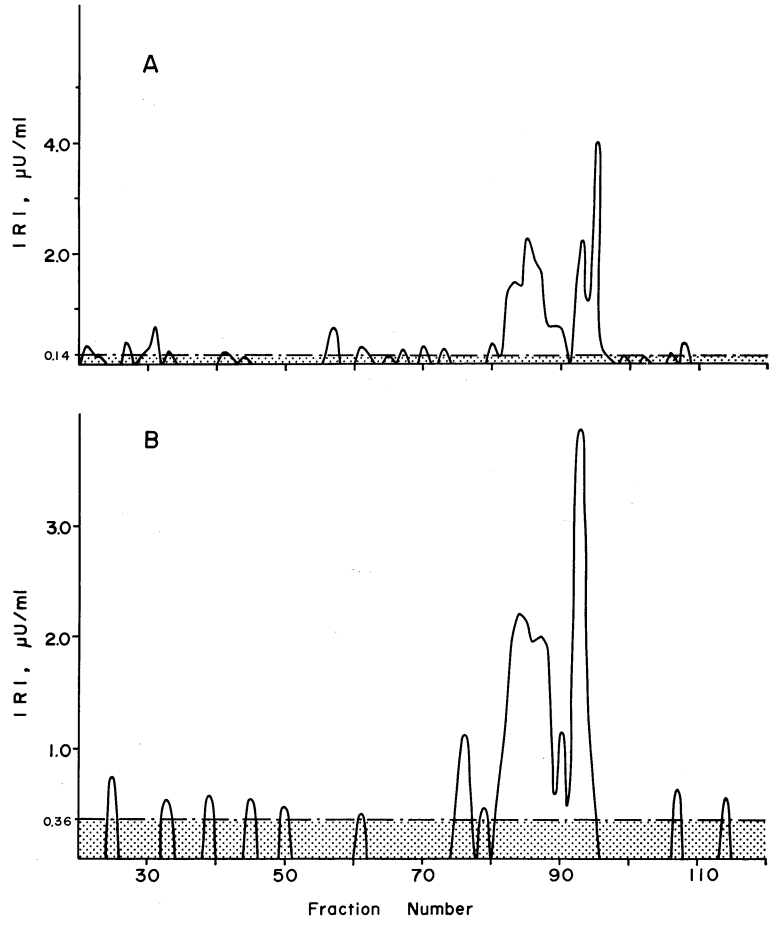

Fig. 2. Elution profiles of extracted human serum insulin on the Bio-Gel (P-30) column with 2.0 ml fraction size. Sera were obtained 60 min after glucose load from two adult onset diabetic patients.
A: An adult onset diabetic patient. (M.K., 47 y.o., M.)
B: An adult onset diabetic patient. (K.A., 71 y.o., F.)
Dotted area: Lower limits of sensitivity.

In order to clarify these peaks, serum samples of other four adults, a borderline case of diabetes mellitus (age: 70 years old) and three adult onset diabetic patients (age: 55–71 years old), were analysed by gel filtration with 2.0 ml fraction size. Other conditions for extraction and assay procedures were the same as above mentioned. The elution patterns of these samples are shown in Fig. 2., where these panels (A and B) are from adult onset diabetic

patients. Two groups of insulin were able to be fractionized in these cases, and each of these two groups of insulin might be separated more than two subgroups.

TABLE. Two Peaks of Serum Insulin (60 min. after 50 g O-GTT)

Case	Maximum BS Level on 50 g O-GTT. (mg/dl)	Peaks (pM/ml)	
		Peak I	Peak II
Normal (n=4)	136.8 ± 12.7*	0.03 ± 0.02**	0.08 ± 0.03***
D.M. (n=3)	248.3 ± 30.9*	0.15 ± 0.03**	0.19 ± 0.01***

mean ± S.E.M.
* There was significant difference ($P < 0.025$).
** There was significant difference ($P < 0.01$).
*** There was significant difference ($P < 0.025$).
These values of peak I and peak II were corrected with the recovery rates of extraction (0.833) and gel filtration (0.647), respectively.

DISCUSSION

Utilizing gel filtration, isolation of proinsulin and insulin from human serum was reported by Melani *et al.*[7] The existence of abnormal insulin in circulation were suggested by Kimmel *et al.*[3] and Elliott *et al.*[4] Recently, Haën *et al.*[2] described that the heterogeneity of proinsulin-size IRI in circulation was more prominent than that of inuslin-size materials. The present studies were undertaken to determine whether these abnormalities of insulin were present in human serum.

Insulin-size IRI were fractionized into two groups as shown in Fig. 1. and Fig. 2., and these fractionized Peak I and Peak II were increased in adult onset diabetic subjects, when compared to those in normal controls. And also total insulin (Peak I plus Peak II) in adult onset diabetic patients was higher than that of normal adults at 60 minutes after 50-gram oral glucose load. This fact is compatible with the reported results of Yalow and Berson.[10] These higher levels of Peak I and Peak II, especially Peak I, in adult onset diabetic subjects might be related to pathogenesis of adult onset diabetes mellitus.

Acknowledgments

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