Radioimmunoassay of Insulin A and B Chains in Normal and Diabetic Human Plasma

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SUMMARY

A sensitive radioimmunoassay has been developed for the detection of the insulin chains in the plasma of normal and diabetic human subjects. The S-sulfonated derivatives of bovine insulin A and B chains were purified, iodinated with high specific activity and used for immunoassay by the double antibody technic. Twelve noninsulin-requiring diabetic and nine normal, nonobese subjects were studied before and during a four-hour oral glucose tolerance test by determination of plasma immunoreactive insulin, A chain and B chain. The results of our data indicate that: detectable levels of both insulin A and B chain do exist in human plasma; A chain is present in higher concentration than B chain in both normal and diabetic plasma; and greater than normal amounts of A chain can be detected in diabetic subjects and the concentration increases during a glucose tolerance test in these patients. DIABETES *17*:61-66, February, 1968.

The determination of physiologic levels of the intact insulin molecule in biologic fluids is now feasible by the method of radioimmunoassay, but as yet no procedure has been described for the quantitative determination of the A and B polypeptide chains of insulin. Recent evidence[1] suggests that the insulin chains are capable of exerting biologic activity in in vitro assay systems; in addition an enzyme is known to exist in the liver and pancreas[2,3] which is capable of reducing insulin to form the A and B chains and could serve as a source of the insulin chains in plasma. The present report will describe a method of radioimmunoassay capable of detecting concentrations of both insulin A and B chain at 2 to 200 mμg./ml. and the application of this assay to plasma obtained from normal and diabetic human subjects.

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MATERIALS AND METHODS

Preparation of insulin A and B chains

Bovine insulin (24 I.U./mg.) was cleaved into the A and B chains by sulfitolysis[4] and separated by the method of Dixon and Wardlaw.[5] The S-sulfonated (SSO_3) chains were tested for purity by cellulose acetate electrophoresis and amino acid analysis. As previously described,[1] these preparations were essentially pure and were used as the source of antigen for immunization of guinea pigs by the method of Yagi et al.[6]

Iodination of insulin and SSO_3-chains

Insulin and the SSO_3-chains were iodinated with I-131 by the method of Greenwood et al.,[7] which labeled the insulin molecule and the chain derivatives with 90 to 95 per cent efficiency and resulted in specific activities ranging from 70 to 96 mc./mg. of protein. Purification of the labeled antigens was achieved initially by passage of the reaction mixture through a Sephadex G-25 column to remove inorganic I-131. This was followed by a second gel filtration of the iodinated protein through Sephadex G-75. Similar elution patterns were obtained from each of the three iodinated proteins.

Radioimmunoassay procedure

A standard double antibody technic[8,9] was used as the basis for the radioimmunoassay of insulin and the chain derivatives. In each case specific antibody was added in a concentration to bind between 40 and 60 per cent of the labeled antigen. Figure 1 depicts the representative standard curves obtained for the SSO_3-chains when increasing concentrations (0.1 to 10.0 mμg./50λ) of the unlabeled antigen were added. The resulting standard curves are similar for each of the three labeled proteins and indicate a sharp fall in per cent recovery of labeled antigen-antibody complex as increasing amounts of known unlabeled antigen are added to the assay system. The specificity of these assays was previously established by a series of cross-reactions with varying concentrations of the guinea pig antiserums

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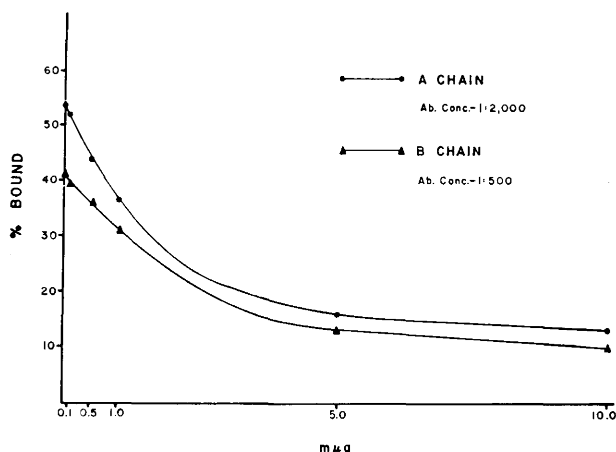

FIG. 1. Standard curves of A and B chain radioimmunoassay. The per cent binding of labeled antigen is plotted against increasing amounts of unlabeled standard solutions of A or B chain. The dilution of each specific antiserum is listed. Following a seventy-two-hour incubation at 4° C. the labeled antigen-antibody complex was precipitated by the addition of rabbit antiguinea pig serum and collected by centrifugation after twenty-four-hour incubation at 4° C.

with the labeled insulin and chain antigens. The binding of labeled A chain by its homologous antiserum was specific. Although high concentrations of antiserum to B chain will cross-react with insulin, there was no significant binding (less than 5 per cent) of insulin by B chain antibody at the concentrations of antiserum employed in the immunoassay (table 1).

Selection of patients

Plasma samples were collected from a total of twelve mild, maturity-onset diabetic subjects, all of whom had two-hour blood sugar levels in excess of 120 mg. per 100 ml. following the oral ingestion of 100 gm. of glucose. None of these subjects had received insulin treatment in the past. In addition, blood samples were obtained from nine normal, nonobese, nondiabetic subjects. The samples were collected in the fasting state and hourly for four hours following the oral glucose tolerance test, and were frozen prior to assay. All plasma samples were diluted 1:1 with 0.02 M disodium ethylene diamine tetraacetate to inactivate a plasma inhibitor described by Morgan et al.[10]

TABLE 1

Cross-reaction of I-131-labeled insulin and S-sulfonated chains with guinea pig antiserum as determined by the double antibody immunoprecipitation method

Labeled antigen	Antibody (dilution) A (1:2,000)	B (1:500)	I (1:10,000)
A	48.6*	0.3	1.1
B	1.6	42.0	3.3
I	2.0	4.5	52.4

*Percentage of labeled antigen bound to antiserum

RESULTS

The radioimmunoassay determination for both insulin A and B chain displays a sensitivity equal to that of the intact insulin molecule; however, it initially was noted that the sensitivity of these assays diminished with time following iodination of the protein. This resulted from a diminution of the maximum per cent binding (40 to 60 per cent) of the labeled antigen with its antibody and an undesirable flattening of the standard curves. This problem was found to be due to the formation of immunologically inactive protein of higher molecular weight than the starting material and was reflected in the progressive increase in the first protein peak and reciprocal diminution of the second peak in the Sephadex G-75 separation step of purification (figure 2). The formation of this initial protein

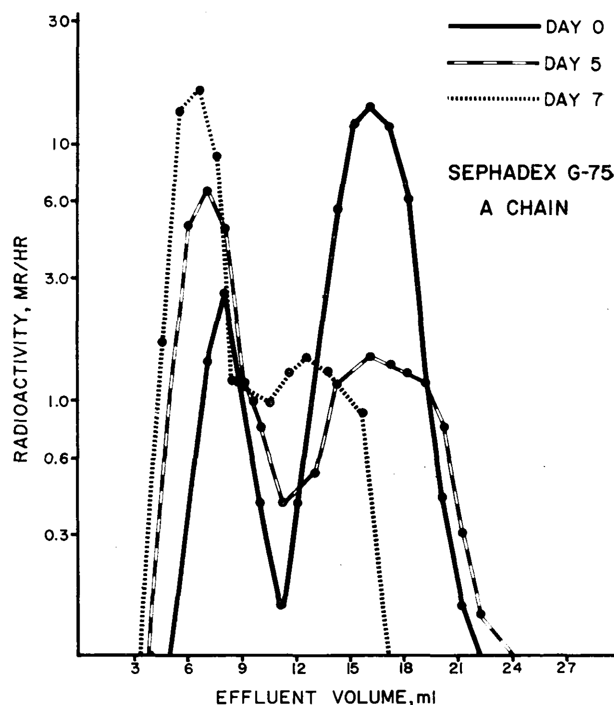

FIG. 2. Purification of I-131-labeled A chain by gel filtration immediately following iodination, five and seven days later. Column size: 1.2 × 36 cm.; sample size: 0.5 ml. Immunologically active iodinated protein is found in the second radioactive peak.

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peak was more active for the iodinated chains than the parent insulin molecule, and it was assumed that this material represented the formation of polymers. It was determined that if the Sephadex G-75 separation was performed immediately prior to each assay, the iodinated protein in the second peak retained a uniform amount of immunologic activity, and the resulting standard curves were similar and reproducible over a two-week period of time.

The plasma immunoreactive insulin (IRI) levels were determined (figure 3) and no significant difference ($p > .05$) existed in the mean fasting levels of IRI between two groups (69 ± 18.6 μU./ml. in the diabetics vs 53 ± 19.7 μU./ml. in the normals); however, following the ingestion of glucose the diabetic patients responded with a delayed and prolonged rise in plasma IRI as has been reported by others.[11]

The results of the immunoassay for plasma insulin chains are depicted in figure 4. The mean values of twelve diabetic and nine normal humans indicated that there was no significant difference between the two groups in insulin B chain concentration. The values ranged between 4.4 ± 0.6 and 7.8 ± 0.6 mμg./ml. and did not change following glucose ingestion. In contrast, there was a distinct difference ($p < 0.1$) between the fasting levels of insulin A chain in the diabetic (47 ± 6.5 mμg./ml.) and nondiabetic (22 ± 2.3 mμg./ml.) groups. In addition, the plasma insulin A chain in the diabetic group rose to a peak concentration of 68 ± 16.6 mμg./ml. at two hours following the administration of oral glucose, whereas the peak response (25 ± 1.5 mμg./ml.) in the nondiabetic, normal control group was not statistically different from the fasting level.

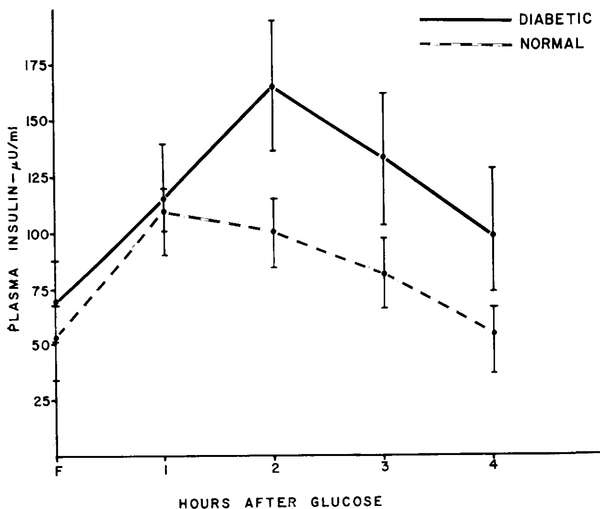

FIG. 3. Plasma immunoreactive insulin (IRI) concentrations before and during a glucose tolerance test in nine normal and twelve maturity-onset diabetic subjects. Each value represents the mean concentration ± S.E.M.

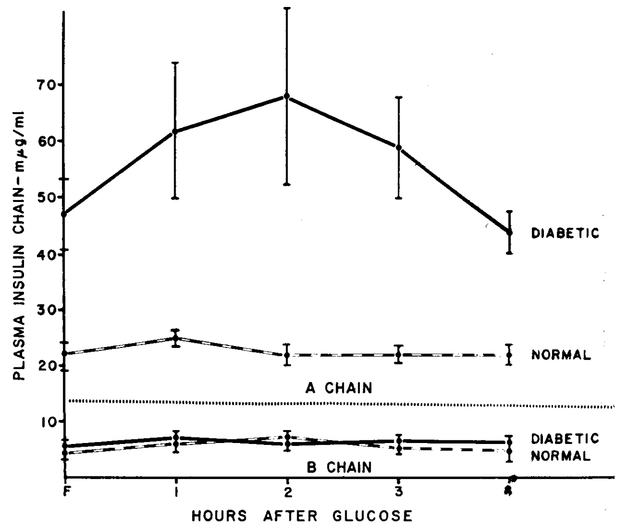

FIG. 4. Plasma insulin A and B chain concentrations before and during a 100 gm. oral glucose tolerance test in normal and maturity-onset diabetic subjects. Each value represents the mean concentration ± S.E.M.

Since a significant difference between normal and diabetic patients was noted in the A chain immunoassay, the specificity of this procedure was examined by a series of in vitro experiments in which either human insulin or porcine A chain (SSO_3) standards were added to the bovine A chain labeled antigen-antibody reactions (table 2). The results indicate that porcine A chain cross-reacts equally well with bovine A chain and that human insulin at physiologic concentrations does not bind with bovine A chain antibody. Since porcine A chain is structurally identical to human A chain, it is probable that the bovine A chain radioimmunoassay is capable of detecting human A chain in plasma.

TABLE 2

Cross-reaction of human insulin and porcine A chain standards in the bovine A chain radioimmunoassay

	Concentration of standard (mμg.)				
	0	0.25	0.5	1.0	2.0
Human insulin	66.7*	67.1	67.0	68.9	65.6
Porcine A chain (SSO_3)	67.0	62.6	58.6	50.4	40.3
Bovine A chain (SSO_3)	67.0	64.1	60.1	54.2	41.2

*Percentage of labeled antigen bound to antiserum

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Detection of reduced and alkylated bovine A chain by the A-SO_3 radioimmunoassay

The antigen used in the present immunoassay is the S-sulfonated derivative of bovine A chain (A-SSO_3). The ability of the corresponding antiserum to react with other forms of bovine A chain was examined in the following series of experiments.

Purified bovine A-SSO_3 chain was reduced by β mercaptoethanol at *p*H 8.6 for one hour at room temperature followed by alkylation with iodoacetamide, iodoacetic acid or ethylenimine for thirty minutes. The reaction mixture was diluted in 0.25 per cent bovine serum albumin and phosphosaline (BSA-PS) to give a final A chain concentration of 50 mμg./ml. These dilutions were assayed for A chain concentration and compared to similar concentrations of unreduced or reduced and nonalkylated A-SSO_3 chain.

Crystalline bovine insulin (Mann Laboratories) was reduced and alkylated by the method of Crestfield, Moore and Stein.[18] Final dilutions were made in both BSA-PS and nondiabetic human serum and the concentration adjusted to give 50 mμg./ml. A chain assuming complete reduction of the insulin. Similar concentrations of unreduced and reduced, nonalkylated insulin were included in the radioimmunoassay for A chain. Buffer solutions with and without reactants served as controls.

The results of the A chain radioimmunoassay are listed in table 3. The A chain concentrations of the reduced or alkylated derivatives of insulin and S-sulfonated A chain were compared to the level obtained from the assay of a similar dilution of untreated A-SSO_3 chain. The maximum recovery (30 per cent) of reduced or alkylated A-SSO_3 chain is in agreement with results reported by Yagi et al.[6] and indicates that the alteration of the cysteine residues of A chain does affect the determinant region reacting with the antiserum to A-SSO_3 chain. Since there is no significant cross reaction of insulin or B chain with A chain antiserum, it is assumed that the A chain concentrations obtained by reduction or alkylation of insulin are due to the formation of A chain: When the sulfitolysis derivatives of insulin were added to BSA-PS diluent, the concentrations of A chain compared favorably to those obtained by similar treatment of A-SSO_3 chain. The increased recoveries observed when these products were added to human serum (53 per cent by the free sulfhydryl derivative) suggest that favorable conformational changes in the A chain molecule have occurred which allow detection by the antiserum to A-SSO_3 chain. Since the free sulfhydryl form of the reduced insulin gives the greater A chain response, the possibility exists that covalent attachment to serum protein has occurred.

DISCUSSION

The development of the radioimmunoassay has provided a specific method for the detection of small concentrations of hormones present in human plasma.

TABLE 3

Detection of A chain after reduction of S-sulfonated bovine A chain (A-SSO_3) and bovine insulin

			A Chain concentration			
			BSA-PS* diluent		Human serum diluent	
Test substance	Reduction	Alkylation	mμg./ml.	per cent recovery	mμg./ml.	per cent recovery
A-SSO_3	–	—	51.0	(100)	—	—
A-SSO_3	+	—	13.0	(26)	—	—
A-SSO_3	+	acetamide	15.2	(30)	—	—
A-SSO_3	+	acetate	13.0	(26)	—	—
A-SSO_3	+	ethylamine	0.5	(1)	—	—
Insulin	–	—	0.8	(2)	27.5	(5)
Insulin	+	—	14.0	(28)	52.0	(53)
Insulin	+	acetamide	11.4	(22)	47.0	(43)
Insulin	+	acetate	11.2	(22)	45.0	(39)
Insulin	+	ethylamine	4.2	(8)	32.5	(15)
Tris†	–	—	0.30	—	25.0	—
Tris	+	acetamide	0.35	—	25.2	—

*BSA-PS: 0.25 per cent bovine serum albumin in 0.1 M phosphate, 0.9 per cent saline, *p*H 7.6
†Tris: 0.55 M tris—HCl, *p*H 8.6

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The major requisites for the immunoassay are a sensitive system for the measurement of the antigen-antibody reaction at low antigen concentrations and purity of the labeled known antigen. The double antibody system employed in these studies has been successfully applied to the determination of plasma insulin and other hormones.[12] The extension of this technic to the measurement of insulin A and B chains with sufficient sensitivity to detect circulating levels in human plasma offers no serious obstacle. It is necessary, however, to purify the iodinated insulin chains by gel filtration immediately prior to each assay in order to remove non-immunoreactive chain polymers.

The lack of significant in vitro cross-reaction between labeled antigen and antibody for insulin and each chain establishes the specificity of each of the three assays in relationship to the other two. Therefore it is possible to determine physiologic levels of the insulin chains in the presence of the intact insulin molecule. The results of our data from human normal and diabetic subjects indicate that: (a) detectable levels of both insulin A and B chain do exist in human plasma, (b) A chain is present in higher concentration than B chain in both normal and diabetic plasma, and (c) greater than normal amounts of A chain can be detected in diabetic subjects and the concentration increases during a glucose tolerance test in these patients.

It should be noted that the average concentration of A chain detected in normal human plasma is ten times greater than that observed for insulin on a weight basis (22 mμg./ml. vs 2.2 mμg./ml.). The possibility that such a level of A chain could exert biologic activity should be considered. Recently we investigated the biologic activity of purified insulin A and B chains upon muscle and adipose tissue.[1] A chain, but not B chain, was capable of stimulating the conversion of labeled glucose to carbon dioxide in the rat epididymal fat pad, thus A chain simulated the action of the intact insulin molecule upon adipose tissue. Both A and B chain inhibited the basal uptake of glucose by the rat hemidiaphragm, but neither chain prevented the expected stimulation of glucose uptake in the presence of unmodified insulin. Thus, the dual ability of A chain to stimulate glucose uptake by adipose tissue yet inhibit glucose uptake in muscle when additional insulin is absent might indicate that this portion of the insulin molecule could be regarded as an insulin antagonist. In view of the recent developments suggesting biologic modifications of insulin in the diabetic state,[13,14] it is possible that the increased levels of A chain in the diabetic subjects are of pathologic importance. Present investigations further suggest that certain young obese individuals with a normal blood sugar response to glucose ingestion also have abnormally high plasma A chain levels.[15]

The origin of the circulating levels of the insulin chains is not apparent from the present work. It is now thought that the insulin molecule is primarily degraded by the glutathione-insulin transhydrogenase enzyme in the liver, which acts to cleave the insulin molecule into its A and B chains.[16] An additional source of the plasma insulin chains could be the islet cells of the pancreas since evidence has recently been presented that in the biosynthesis of insulin the two chains are first synthesized as separate polypeptide units and then combined to form the intact insulin molecule.[17] This finding suggests that a situation might exist in diabetes mellitus in which there is an asynchronous production of the insulin polypeptide chains, resulting in the observed differences in plasma concentrations of the A and B chains.

Further investigation will be necessary before a physiologic role can be given to the circulating levels of the insulin chains in human plasma. Nevertheless the demonstration of detectable concentrations of the chains in both normal and diabetic plasma, and the increased levels of A chain in the latter, suggests that a defect in the formation or degradation of the insulin molecule exists in patients with diabetes mellitus.

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Relationship of Coronary Arteriosclerosis to Blood Pressure

In the search for factors which increase the incidence of coronary atherosclerosis, hemodynamic factors are sometimes overlooked. Although biochemical and nutritional factors do influence the rate of atherosclerosis, these alone apparently do not determine the degree or rapidity with which these lesions develop.

In a brief note, G. E. Burch and N. P. DePasquale (Amer. Heart J. 63:720, 1962), described the case of a patient who had a congenital anomaly of the coronary vessels. In this instance, the left coronary artery arose from the aorta and the right coronary artery from the pulmonary artery. This patient survived to ninety years of age and, at autopsy, was found to have severe arteriosclerotic lesions of the left and none of the right coronary artery. The authors cited the reports of others which confirmed this observation.

The obvious conclusion, according to the authors, is that low intravascular pressure in the coronary arteries seems to protect against arteriosclerosis, even though the lipid content of the blood circulating within is undoubtedly similar to that within high pressure arterial systems.

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