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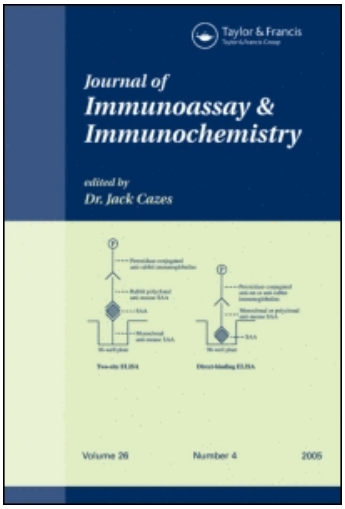

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MEASUREMENT OF GLUCAGON IN HUMAN PLASMA BY ENZYME IMMUNOASSAY

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ABSTRACT

In order to investigate the validity of an enzyme immunoassay for glucagon, the glucagon levels of human plasma were determined by both enzyme immunoassay (EIA) and radioimmunoassay (RIA). After a glucose load, plasma glucagon measured by both EIA and RIA fell in 12 normal subjects. The glucagon levels measured by both assays during glucose tolerance test showed good agreement in a group of 10 patients. After arginine infusion, plasma glucagon increased in 6 normal subjects and 3 patients and glucagon values measured by EIA correlated well with those by RIA. The present study demonstrates correlation between glucagon levles measured by RIA and EIA and indicates the usefulness of EIA for determining glucagon in human plasma. (KEY WORDS: Glucose tolerance test, Arginine test, Radioimmunoassay, Enzyme immunoassay, Plasma glucagon).

INTRODUCTION

Since Unger and his co-workers developed a radioimmunoassay for glucagon in 1959 (1), this has been the method of choice for determination

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of glucagon. Radioimmunoassay has the reproducibility, precision and sensitivity needed for determining plasma levels of glucagon. Recently enzyme immunoassay have been developed for peptides such as human chorionic gonadotropin (2), insulin (3) and TSH (4) and have had increasing clinical application. An enzyme immunoassay for glucagon with limited sensitivity was reported in 1977 by Asano and his co-workers (5). However, the enzyme immunoassay for glucagon has not been reported in the clinical field, because of lack in precision and sensitivity. Recently we have developed a sensitive enzyme immunoassay for glucagon with a detection limit of 1-2 pg (6). The present work describes use of this method with human plasma and a comparison with an established radioimmunoassay (RIA).

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MATERIALS AND METHODS

Plasma was obtained from 18 normal subjects and 13 patients with various diseases. After an overnight fast, 75 g of glucose was given by mouth to 12 normal subjects and 10 patients with diabetes mellitus, fatty liver, chronic hepatitis, obesity or polyp of the colon. Blood samples were obtained from the antecubital vein at 30-min intervals for 3 hours for the normal subjects and 2 hours for the patients (7). In 6 normal subjects, 2 patients with diabetes mellitus and a patient with liver cirrhosis, an arginine test was carried out (8). After the collection of two base line samples 10 min apart, 300 ml of 10 % l-arginine monohydrochloride (Morishita Pharmaceutical Co.,Tokyo, Japan) was infused for 30 min. Blood samples were drawn at 5,10,20,30,40,50,75,90 and 120 min after the arginine infusion. Blood samples were obtained from the antecubital vein with a heparinized syringe.

For glucose determination, one ml of venous blood was added to glass tubes containing approximately 5 mg of NaF. After separating the plasma, glucose was determined by the glucose oxidase method (9). A preliminary study of the effect of heparin on the enzyme immunoassay (EIA) showed

a positive bias at -20°C for 2 months. In contrast, the addition of EDTA in amount of 2.5 mg/ml of blood resulted in no changes at storage (13). Therefore, 5 ml of blood was added to glass tubes containing 2000 U of aprotinin (Trasylol®, Bayer Co.) and 6 mg of EDTA, for the measurement of plasma hormones. Blood specimens were centrifuged at 4°C and separated plasma was kept at -20°C until the assay. Plasma insulin was measured by the double antibody method of Morgan and Lazarow (10). RIA for glucagon was performed using dextran-coated charcoal and anti-glucagon antibody, G21 (11). Pork crystalline glucagon (Lot.GLF 599 A) donated by Dr.Mary Root,Eli Lilly Co.,Indianapolis,U.S.A., was used as standard. The detection limit was 15 pg/ml. EIA for glucagon was carried out by a double antibody solid phase method, as previously reported (6). A fifty µl sample was allowed to incubate at 4°C for three days with 200 µl of phosphate saline buffer, 50 µl (500 U) of aprotinin (Antagosan®,Hoechst Co.West Germany), 100 µl of antiglucagon rabbit serum,N6E, and 100 µl of peptide[21-29]-enzyme conjugate containing 0.2 µl of β-D-galactosidase. The antiserum N6E was produced in a rabbit against synthetic glucagon fragment[15-29] (12).One hundred µl of a suspension of anti-rabbit IgG antibody-coupled to cellulose was added and the mixture shaken at 30°C for 3 hours. After three washings with the buffer, the cellulose was resuspended in 500 µl of substrate reagent containing 10 µg of 4-methyl-umbelliferyl-β-D-galactoside and incubated overnight at room temperature. After terminating the reaction by addition of 3 ml of 0.1 mol/L carbonate buffer (pH 10.5), the fluorescence intensity of the supernatant was measured using a fluorometer with excitation wavelength 365 nm and emission wavelength 450 nm. Pork crystalline glucagon, purchased from Sigma Co. (Lot 78B-0370) was used as standard. The minimum detectable dose was 40 pg/ml or 2 pg/tube (6). The coefficients of intra- and interassay variation were 3.7-14.5 % and 9.0-18.5 %, respectively (6). The determination of plasma IRG by EIA and RIA for the normal subjects were performed simultaneously. In contrast, plasma IRG from patients was measured by EIA one to two months after RIA.

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In the present study, data from the normal subjects are presented as mean values and standard errors of the mean. Individual data are presented for the patients, because of widely differing concentrations. The correlation between the levels of plasma glucagon measured by RIA and EIA are reported for samples from the arginine tests.

RESULTS

Glucose tolerance test

The changes in blood glucose, plasma insulin (IRI), and plasma glucagon (IRG) measured by RIA and EIA during glucose tolerance tests in a group of 12 normal subjects are presented in Fig.1. Blood glucose increased from the base line of 82 ± 1 mg/100 ml to a peak of 127 ± 3 mg/100 ml at 30 min and then decreased. Plasma IRI increased from the base line of 13 ± 2 μU/ml to a peak of 59 ± 8 μU/ml at 30 min and returned to the initial level by 180 min. Plasma IRG measured by RIA using antibody G21 fell from the initial level of 71 ± 7 pg/ml to a nadir of 53 ± 6 pg/ml at 60 min and returned to the base line at 180 min. Plasma IRG measured by EIA with antibody N6E fell from the base line level of 100 ± 4 pg/ml to a nadir of 83 ± 8 pg/ml at 90 min and then returned to the initial level. There was a similar pattern of plasma IRG changes measured by both RIA and EIA. The results of patients are presented in Table 1.

Arginine infusion test

Changes in blood glucose, plasma IRI and plasma IRG during the arginine infusion test in a group of 6 normal subjects are shown in Fig.2. Blood glucose increased from the base line of 84 ± 3 mg/100 ml to 93 ± 5 mg/100 ml at 20 min and then fell to 71 ± 5 mg/100 ml at 60 min. Plasma IRI increased from the base line of 26 ± 4 μU/ml to 99 ± 11 μU/ml at 30 min and returned

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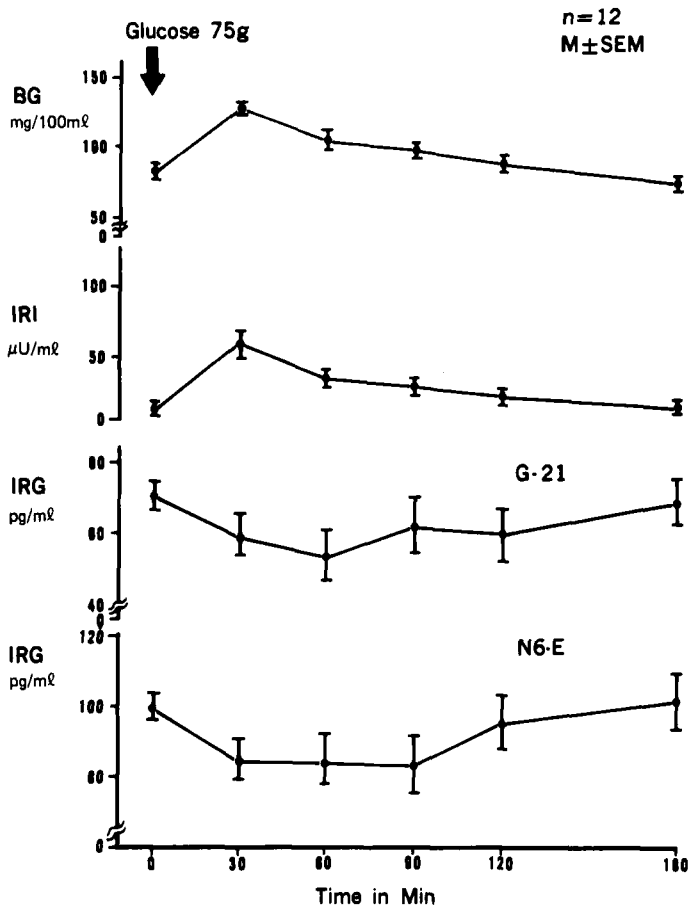

FIGURE 1. Changes in blood glucose (BG), plasma insulin (IRI) and plasma glucagon (IRG) during glucose tolerance test in a group of 12 normal subjects. IRG was measured by radioimmunoassay using antiserum G-21 and by enzyme immunoassay using antiserum N6-E.

to the initial level at 60 min. Plasma IRG measured by RIA (G21) increased from the base line of 109 ± 7 pg/ml to 273 ± 59 pg/ml at 30 min and returned to the initial level at 120 min. Plasma IRG determined by EIA (N6E) increased from the base line of 130 ± 13 pg/ml to a peak of 379 ± 60 pg/ml and fell to the initial level at 120 min. The changes in blood glucose and plasma IRG during the arginine test in 3 patients are presented in Table 2. In order

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TABLE 1

Comparison of Plasma Glucagon Measured by EIA and RIA during Glucose Tolerance Test in a Group of 10 Patients with Various Diseases

Patients Diagnosis	Measurements*	Minutes after Glucose				
		Fasting	30	60	90	120
U.Y.	BG	168	301	354	362	305
Diabetes	RIA	85	75	50	25	80
Mellitus	EIA	51	68	62	60	54
S.T.	BG	142	233	311	357	372
Diabetes	RIA	80	80	50	45	30
Mellitus	EIA	77	89	58	45	59
S.Ss.	BG	88	166	270	291	320
Diabetes	RIA	95	60	65	45	40
Mellitus	EIA	87	54	50	47	43
S.Se.	BG	108	195	228	225	218
Diabetes	RIA	90	60	60	105	60
Mellitus	EIA	113	95	99	96	100
H.W.	BG	165	238	368	368	342
Diabetes	RIA	55	80	50	45	80
Mellitus	EIA	39	48	37	36	31
T.O.**	BG	89	195	167	153	133
Diabetes	RIA	70	50	53	45	50
Mellitus	EIA	55	40	40	41	62
A.K.	BG	69	131	152	178	187
Obesity	RIA	70	10	35	70	25
	EIA	94	65	59	60	70
K.H.	BG	70	120	117	76	72
Fatty	RIA	60	60	55	65	45
Liver	EIA	49	46	46	43	53
K.I.	BG	103	141	203	136	126
Chronic	RIA	50	40	20	40	50
Hepatitis	EIA	46	34	34	37	43
T.M.	BG	128	144	180	169	154
Colon	RIA	70	30	10	20	15
Polyp	EIA	58	61	45	47	41

* BG: Venous plasma glucose,mg/100 ml. RIA: Glucagon measured by radioimmunoassay; pg/ml and EIA: Glucagon measured by enzyme immunoassay; pg/ml

** Glucose tolerance test was performed after treatment with sulfonylurea.

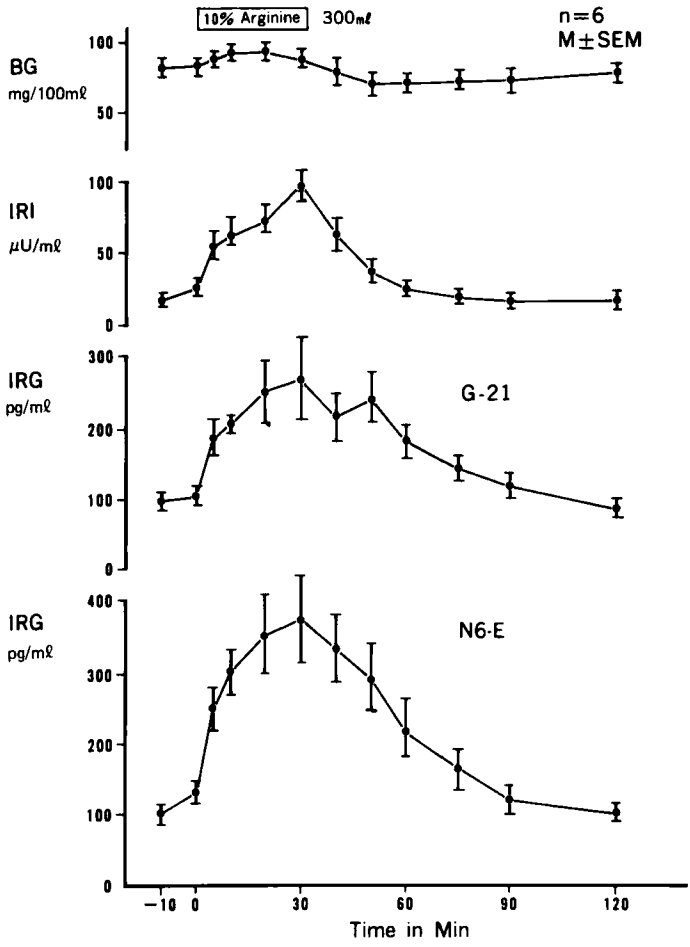

FIGURE 2. Changes in blood glucose (BG), plasma insulin (IRI) and plasma glucagon (IRG) during arginine infusion test in a group of 6 normal subjects. IRG was measured by radioimmunoassay using antiserum G-21 and by enzyme immunoassay using antiserum N6-E.

to investigate the validity of EIA, the correlation between plasma levels of IRG measured by these two assay methods was calculated. The correlation coefficient in the arginine test for the normal subjects was 0.93, and the regression equation Y = 1.54 X - 43.5 while that in the group of the patients was 0.98, with the regression equation Y = 0.89 X -31.3, where Y was plasma IRG measured by EIA and X that measured by RIA.

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TABLE 2

Comparison of Plasma Glucagon Measured by EIA and RIA during Arginine Infusion Test in Three Patients

Patients	Measurements*	Minutes after Arginine Infusion											
		-10	0	5	10	20	30	40	50	60	75	90	120
K.K.	BG	215	208	211	216	221	219	229	253	269	267	251	236
Diabetes	RIA	65	60	155	305	400	445	325		120	145	100	100
Mellitus	EIA	19	20	122	102	214	183	133		81	53	64	56
M.W.	BG	159	167	171	169	178	192	200	213	217	223	221	223
Diabetes	RIA**	10	10	70	65	50	70	50	65	35	10	10	10
Mellitus	EIA	11	14	29	51	17	61	84	83	55	45	26	32
M.S.	BG	124	121	123	123	115	115	115	111	111	110	107	111
Liver	RIA	480	370	825	1650	1522	1400	1200	1375	1200	725	975	875
Cirrhosis	EIA	369	306	891	1080	1340	1260	1030	1170	1150	580	871	823

* BG: Venous plasma glucose; mg/10 ml, RIA: Glucagon measured by radioimmunoassay; pg/ml, and EIA: Glucagon measured by enzyme immunoassay; pg/ml.

** The value 10 represents the level less than the minimal detectable standard.

DISCUSSION

In the present study, plasma IRG determined both by EIA and by RIA decreased in reponse to oral glucose load in the normal subjects, but the correlation between these two methods was poor at the low concentration of plasma glucagon in this test. By contrast, good correlations were found at the higher concentrations following arginine infusion.

An earlier report of this EIA (13) yielded higher values for IRG than with RIA. In this study, different anti-glucagon sera were used: G21 for RIA and N6E for EIA. There is a small difference in the immunoreactivity of these two antisera. As described previously (12), the main immunological determinant for G21 is the C-terminal peptide of glucagon [21-29], whereas for N6E it is a larger C-terminal fragment [15-29]. However, both antisera do not react with gut glucagon-like immunoreactive material and no remarkable difference was observed between the plasma levels of IRG measured by these two different antisera (12). Therefore, the difference observed in plasma IRG measured by RIA and EIA could not be attributed to the different antisera used.

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In the present study, plasma IRG was determined separately in two laboratories, where different standards of pork crystalline glucagon were used. Therefore, a comparison of these two standards was performed by EIA, indicating the correlation coefficient of 0.99 with regression equation $Y = 1.39\ X + 50.8$, where Y was Sigma standard and X Lilly standard. Consequently, the difference calculated in the assay using these different standards ranged 50 to 200 pg/ml below 500 pg/ml of plasma IRG (13). Therefore, the higher value determined by EIA could be due to use of different standards. In addition, as described in method, Plasma IRG from patients was measured by EIA one to two months after RIA. This might also contribute to the difference between IRG levels measured by EIA and RIA.

The present study demonstrates that plasma IRG in human subject can be measured by EIA. A good correlation exists between plasma IRG measured

by RIA and EIA at the plasma concentration seen in the arginine infusion test.

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