Nagoya J. med. Sci. 31: 143-153, 1968

STUDIES ON NONPRECIPITATING INSULIN ANTIBODY

RADIOIMMUNOASSAY OF INSULIN BINDING CAPACITY AND ITS RELATIONSHIP TO INSULIN NEUTRALIZING ACTIVITY AND ABILITY TO EVOKE CUTANEOUS ANAPHYLACTIC REACTION

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ABSTRACT

Skom and Talmage have demonstrated a non-precipitating antibody against insulin in the sera of insulin-treated diabetics. But their method of determining the antibody is not quantitative.

In the present study, a titration of the insulin binding activity of anti-insulin was performed more quantitatively using ^{131}I-labeled insulin and antiguinea pig γ-globulin rabbit sera under the condition of insulin excess. Moreover, the insulin binding activity of anti-insulin was correlated with its insulin neutralizing activity and also with its ability to evoke a cutaneous anaphylactic reaction.

A considerably good correlation was observed between the insulin neutralizing activity and insulin binding activity. On the other hand, between the insulin binding activity and the ability to evoke cutaneous anaphylactic reaction was only a poor correlation observed.

INTRODUCTION

Antigenic properties of an exogenous insulin have become an obstacle to its clinical use. Reaction of insulin with its antibody has been reported as a non-precipitating one by many investigators[1)2)3)].

Skom and Talmage[3)4)] have been able to demonstrate a non-precipitating antibody against insulin in the sera of insulin-treated diabetics. They first incubated the diabetic sera with ^{131}I-instulin and then precipitated the complexes of ^{131}I-insulin and its antibody by antihuman γ-globulin sera. As they also pointed out, their method is not quantitative, since the antigen-antibody reaction occurs under a limited condition which the amount of ^{131}I-insulin is constant while the antibody to be titrated is varying, therefore, the relation between the antibody and antibody-bound insulin is not linear. Thus, their method is merely semi-quantitative.

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Received for publication November 27, 1967.

In the present study, attempts were made to develop a more quantitative method to measure the insulin binding activity of the anti insulin serum and to correlate the activity with its insulin neutralizing activity and also with its ability to evoke cutaneous anaphylactic reactions.

MATERIALS AND METHODS

Preparation of anti-insulin guinea pig sera: Adult guinea pigs weighing 300–400 g were used. Twice-recrystallized sperm whale insulin* was used as antigen. An antigen preparation for immunizing injection was made according to a modified method[5] of Moloney and Coval[6]. The saline used throughout the present study was a phosphate-buffered saline prepared by adding 0.15 M-Na_2HPO_4-HCl solution to an equal volume of 0.9% NaCl solution. One volume of anhydrous lanolin was first mixed with 2 volumes of the paraffin oil containing 5 mg/ml of heat killed tubercle bacilli. To this paraffin-lanolin mixture, the saline containing 4 mg/ml of the insulin was added at 3 to 1 ratio in volume. The resulting mixture was thoroughly emulsified in a mortar. The antigen preparation thus prepared contained 1 mg of the insulin per ml. Each guinea pig was given a subcutaneous injection of 0.5 ml of the antigen preparation at each of two separated parts on the back. Total three injections were given to each guinea pig at 4-week-intervals. Blood was collected by cardiac puncture 2 weeks after the last injection. The immune serum was lyophilized and stored in the cold room (4°C) until use.

Preparation of partially purified antibody against guinea pig γ-globulin (anti-guinea pig γ-globulin): Guinea pig γ-globulin was separated from normal guinea pig sera with diethylaminoethyl cellulose column chromatogrsphy according to the method of Sober and Peterson[7] and used as antigen. An antigen preparation was similarly made as described above. One volume of the saline containing 10 mg/ml of guinea pig γ-globulin was added to 3 volumes of the 1 to 2 mixture of anhydrous lanolin and the paraffin oil containing 5 mg/ml of heat-killed tubercle bacilli. The mixture was thoroughly emulsified. Rabbits weighing about 2.5 kg were given a subcutaneous injection of 0.5 ml of the antigen preparation at each of four separated parts on the back. Four weeks later, a booster injection was given to each rabbit. Blood was collected by cardiac puncture 2 weeks after the booster injection. Partially purified antibody was prepared by precipitating the antisera with one third saturation of ammonium sulfate or with 12% sodium sulfate solution[8].

Preparation of radioactive iodinated insulin: The radioactive iodinated insulin (^{131}I-insulin) was prepared according to a modified method of Lowell *et al.*[9]. One ml of 0.75% KIO_3 and 1 ml of the iodide solution that was prepared so

* Shimizu Pharmaceutical Co., Ltd.

as to contain 180 μg of KI and 10 mc of $Na^{131}I$ per ml were added to about 1.5 ml of CCl_4. After extraction of I_2 with CCl_4 by adding 3 drops of 1 N-HCl, the upper aqueous layer was removed and 1 ml of 0.12 N-Na_2CO_3 containing 5 mg of the insulin was added to the CCl_4 layer. The mixture was shaken and allowed to stand for one hour. The upper aqueous layer containing the insulin was dialysed against a diluted HCl solution of pH 3 in the cold room. The HCl solution was renewed several times during dialysis for 3 days. Protein content of the ^{131}I-insulin solution was determined according to the method of Lowry[10].

The ^{131}I-insulin thus prepared had a specific activity of 1 mc per mg of the insulin. Therefore, one atom of the iodine was expected to have combined with one molecule of the insulin on the assumption that the molecular weight of the insulin is 24,000. Before use, the specific activity of the ^{131}I-insulin was adjusted to an appropriate activity for each use by adding the iodinated insulin that was prepared in the same manner as the ^{131}I-insulin except for omission of $Na^{131}I$.

Measurement of radioactivity: Radioactivity of the ^{131}I-insulin was measured using a well type scintillation counter.

Assay of hormonal activity of iodinated insulin: Hormonal activity of native and iodinated insulin was assayed by the mouse convulsion procedure[5)11)]. The used animals were adult male mice of CF#1 strain and they were starved overnight before use.

Antigenicity of iodinated insulin: The antigenicity of the iodinated insulin to the insulin antibody and that of the native insulin were measured by a modified immunochemical procedure of Berson and Yalow[12)13)] and compared to assess the cross-reactivity of the iodinated insulin to the insulin antibody. One mg of lyophilized anti-insulin serum (guinea pig No. 24), was added to the mixture of 0.055 u of the ^{131}I-insulin and a varying amount (0–0.2 u) of the native or iodinated insulin and the resulting mixture was then incubated at 37°C for one hour. Free and antibody-bound ^{131}I-insulins were separated by precipitating the latter with the anti-guinea pig γ-globulin and radioactivities of the supernatant and the precipitate were separately measured. Ratio of the radioactivity found in the precipitate containing the bound insulin to that of the supernatant containing the free insulin (B/F) was plotted against an amount of the native or iodinated insulin added.

Assay of insulin neutralizing activity of anti-insulin sera: The insulin neutralizing activity of the anti-insulin sera was assayed by mouse convulsion procedure[5)11)]. The assay was carried out by injecting a varying amount of the antiinsulin sera together with 0.2 ml of the saline containing 0.25 u insulin which invariably causes convulsion in all mice injected.

Active and passive skin tests for demonstration of ability of anti-insulin to evoke cutaneous anaphylactic reaction: a) Active skin test: The guinea pig previously sensitized with the insulin, was injected intradermally with 0.1 ml of the saline containing 20 μg of the insulin. The erythema with induration appearing at the site of injection reached a maximum 2 to 3 hours after the injection and remained unchanged for about 20 hours, gradually weakening thereafter. The degree of erythema was measured 3 hours after the injection and graded as + for the erythema measuring 5-9 mm in diameter, ++ for 10-19 mm, +++ for more than 20 mm and − for less than 4 mm. No erythema developed in any non-sensitized guinea pigs similary tested. b) Passive skin test: The test was carried out by the method of passive cutaneous anaphylaxis (PCA)[14]. One tenth ml of the anti-insulin sera or its dilutions with saline was injected intradermally into the dorsal skin of normal guinea pigs. Four hours later, an intracardiac injection of 500 μg insulin in 1 ml saline with 10 mg of Evans blue was given. Thirty minutes later, the size of blue spots appearing at the site of the injection of the anti-insulin serum was measured after stripping off the skin. The reactions were similary graded as in the active skin test.

Precipitation of antibody insulin complex by anti-guinea pig γ-globulin: One half ml of a ^{131}I-insulin solution containing 0.2 to 0.5% bovine serum albumin (Armour) was added to 0.5 ml of the saline dissolving 1 mg of lyophilized anti insulin serum and a resulting solution was then incubated at 37°C for one hour. With this solution was a certain amount of anti-guinea pig γ-globulin mixed and the mixture was incubated at 37°C for one hour, then being left overnight in the cold room. The precipitates were sedimented by a centrifugation at 7,000 rpm for 15 minutes and resuspended in 1 ml of an ice-cold saline and were again span down. Resulting sediments (anti-γ-globulin—anti-insulin—insulin complexes) were dissolved in 1 ml of 0.5 N-NaOH. Radioactivities of the supernatant, the washing saline, and the NaOH-dissolved sediment were separately measured.

Correction of gross radioactivity of precipitate for nonspecific adsorption into precipitate: Gross radioactivity measured on the precipitate was corrected for the nonspecific adsorption of radioactivity into the precipitate (anti-γ-globulin—anti-insulin—insulin complex) according to a standard curve which was obtained by using normal guinea pig serum in place of anti-insulin serum.

RESULTS

I) Hormonal activity and antigenic properties of iodinated insulin

a) The hormonal activity—The hormonal activity of the iodinated insulin was assayed by mouse convulsion procedures. As shown in Table 1, the

TABLE 1. Comparison of Hormonal Activity of Iodinated Insulin and that of Native Insulin

Insulin injected		No. of convulsive mice / No. of mice tested
0.25 u	iodinated	10/10
	native	9/10
0.15 u	iodinated	5/10
	native	5/10

iodinated insulin showed the hormonal activity to the same degree as the native insulin.

b) The antigenic properties—The antigenicity of the iodinated insulin and that of the native insulin were compared by assessing the cross reactivity of the iodinated insulin to the insulin antibody. The results are shown in Fig. 1. As indicated in the figure, the B/F 1.0 corresponds to 0.050 units when the iodinated insulin was employed, while the same B/F corresponds to 0.045 units when the native insulin was employed. This indicates that 0.050 units of the iodinated insulin have the same reactivity to the insulin antibody as have 0.045 units of the native insulin. Therefore, the relative antigenicity of the iodinated insulin to the native one is found to be 90%.

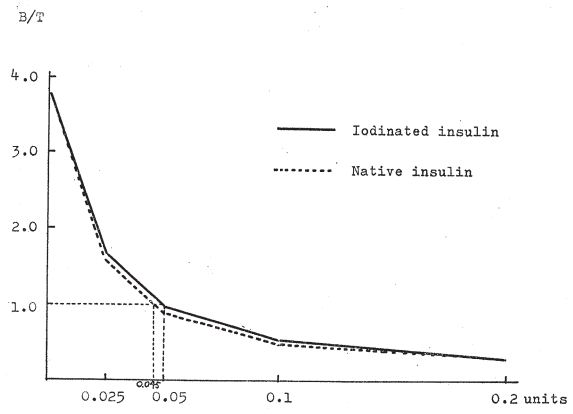

FIG. 1. Comparison of reactivities of native and iodinated insulins to anti-insulin sera.

II) Factors influencing radioimmunoassay of insulin binding capacity of insulin antibody

a) Incubation period—Prior to addition of 6 mg of anti-guinea pig γ-globulin, the mixture of 0.02 u of ^{131}I-insulin, 2 mg of bovine serum albumin (Armour),

TABLE 2. Influence of the Incubation Period to B/T

Incubation period (min)	Radioactivity (cpm) of			
	Sediment	Supernatant	Washing saline	B/T
1/2	37,112	12,594	659	0.736
4	37,672	12,307	623	0.743
8	37,926	12,116	921	0.745
15	37,342	12,865	891	0.732
30	37,692	12,952	693	0.733
60	36,742	14,131	925	0.709
120	37,592	13,187	1,152	0.724

and 1 mg of a lyophilized anti-insulin serum guinea pig No. 28 were incubated for various periods ranging from 1/2 to 120 minutes. Radioactivities of the supernatant, sediment, and washing saline were measured. As seen in Table 2, the incubation period had no influence at all.

b) Influence of anti-guinea pig γ-globulin on insulin binding capacity of anti-insulin sera—In order to see an influence of the anti-guinea pig γ-globulin on insulin binding capacity of the anti-insulin sera, the solution containing 0.02 u of ^{131}I-insulin and 0.2 to 0.5% bovine serum albumin was first incubated with 0.5 ml of the saline dissolving 1 mg of the lyophilized anti-insulin serum (guina pig No. 24) at 37°C for one hour, and to this mixture was a varying amount of the anti-guinea pig γ-globulin added. The resulting mixture was incubated at 37°C for one hour and then left overnight in the cold room. Radioactivity of the precipitate was measured and plotted against an amount of the anti-guinea pig γ-globulin added. As shown in Fig. 2, 6 mg of the anti-guinea pig γ-globulin precipitated antibody-insulin complexes at maximum. The results seem to indicate that, when less than 6 mg of the anti-guinea pig γ-globulin

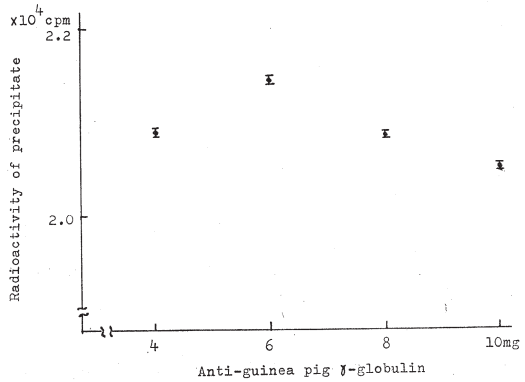

FIG. 2. Relation between amounts of anti-guinea pig γ-globulin and radioactivity of precipitated antibody-bound insulin.

were used in this particular system, some antibody insulin complexes were left in the supernatant, and that when more than 6 mg were used, the ^{131}I-insulin, once coupled with the insulin antibody, was released to the supernatant. Therefore, on radioimmunoassay of the insulin or insulin antibody, the amount of the anti-guinea pig γ-globulin must be appropriate to obtain a maximum radioactivity in the precipitate.

c) *Effect of total insulin in assay system of amount of antibody-bound insulin*— Table 3 and Fig. 3 show a typical example of precipitation of antibody ^{131}I-insulin complexes by the anti-guinea pig γ-globulin. In this experiment, 1 mg of the lyophilized anti-insulin serum (guinea pig No. 24) and, as a control, the same amount of a normal guinea pig serum were used. In Fig. 3, the radioactivity of the antibody-bound insulin (B) was plotted against the total

TABLE 3. A Typical Example of Precipitation Experiments and Calculation of B/T

	Insulin added (units)	Radioactivity (cpm)					
		Sediment	B*	Super-natant	Washing saline	T**	B/T
Anti-insulin serum (guinea pig No. 24)	0.2	8,695	7,805	25,741	463	34,899	0.223
	0.1	6,821	6,501	10,181	172	17,174	0.378
	0.05	6,273	6,203	2,472	40	8,785	0.699
	0.025	4,302	4,292	213	0	4,515	0.945
Control serum (nonimmune guinea pig)	0.2	1,199		33,777	625	35,601	
	0.1	564		16,737	295	17,596	
	0.05	270		8,560	136	8,966	
	0.025	131		4,350	75	4,556	

* B: Obtained by subtracting the radioactivity due to nonspecific adsorption from that of sediment.

** T: Total radioactivity calculated by summing the radioactivities of sediment, supernatant, and washing saline.

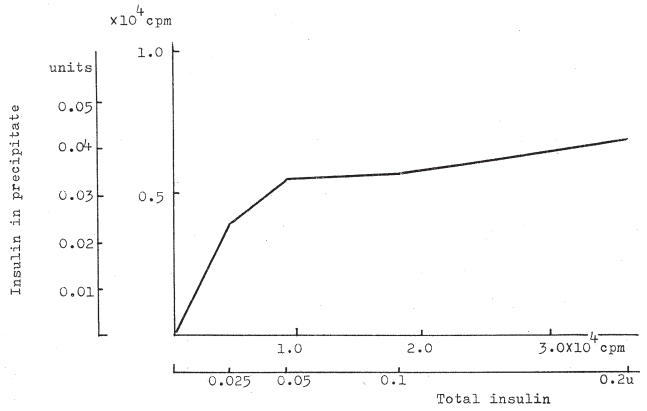

FIG. 3. Relation between total insulin and antibody-bound insulin.

radioactivity which was calculated by summing up those of the sediment, supernatant and washing saline. Radioactivities of the bound as well as total insulin were converted into biological units of the insulin. Amounts of the antibody-bound insulin seemed to approach to a maximum value at around 0.05 u of the total insulin and to remain almost unchanged. The result seems to indicate that all the insulin antibody present in the system was saturated with insulin when an amount of the added insulin exceeded 0.05 u.

d) Choice of B/T most appropriate for calculation of insulin antibody titer— In Fig. 4, T was plotted on the abscissa by logarythmic coordinate, B/T on the spindle. As shown in the preceeding paragraph, more than 0.5 u of the total insulin is necessary to saturate all the insulin antibody present in the assay system. Therefore, it can be seen from Fig. 4 that B/T must be less than 0.7. As seen in the figure, the relation between B/T and T is linear only at the range from 0.4 to 0.8 of B/T. Therefore, in this study, a titer of the insulin binding antibody was expressed by the B that is obtained by multipling T at 0.6 of B/T by 0.6. For an example, in Fig. 4, the T at 0.6 of B/T corresponds to 0.06 u and the titer of the insulin binding antibody is 0.036 u.

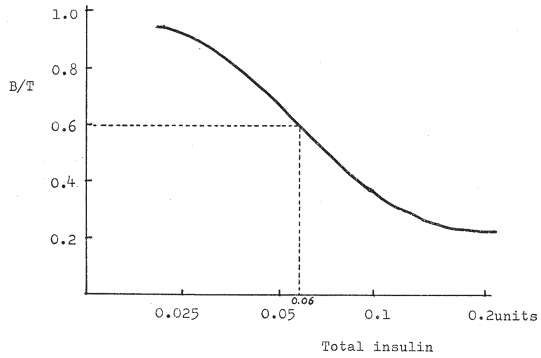

FIG. 4. Relationship between B/T and total insulin.

III) Comparison of insulin binding activity of anti-insulin sera with its insulin neutralizing activity and ability to evoke cutaneous anaphylactic reaction

Table 4 shows anti-insulin activities meassured by three different methods of 26 guinea pigs. The insulin binding activity measured by radioimmunoassay and the neutralizing activity measured by mouse convulsion procedure were expressed by an amount of the bound insulin and of the neutralized insulin in milli units per mg of a lyophilized anti-insulin serum. The ability to evoke

TABLE 4. Comparison of three Activities of 27 Anti-insulin Sera

Guinea pig No.	Insulin binding activity (mu/mg)	Insulin neutralizing activity (mu/mg)	Skin reaction	
			active.	passive.
3	7.5	7.1-14.3	+	+
4	8.1	7.1-14.3	++	−
7	3.5	< 7.1	+	+
8	6.3	< 7.1	+++	
9	not done	> 3.6	++	+
10	3.3	> 7.1	++	++
11	2.0	< 3.6	−	−
12	4.3	> 7.1	++	++
13	1.6	< 3.6	−	++
14	2.5	3.6- 7.1	++	+++
19	3.2	7.1-14.3	+	++
21	9.0	>14.3	+	+++
22	4.3	7.1-14.3	++	+
23	34	>14.3	+	+++
24	34	>28.6	++	+++
25	11	>14.3	++	+++
26	19	21.4-28.6	+++	+++
27	34	>21.4	+	+++
28	30	7.1-14.3	+	
30	16	14.3-28.6	+	++
31	0.9	< 3.6	++	−
32	5.4	7.1-14.3	+	++
33	0.8	< 3.6	−	−
34	4.6	7.1-14.3	+	+
35	25	>28.6	++	+++
37	7.8	7.1-14.3	++	+++
38	0.5	< 3.6	−	−

cutaneous anaphylactic reaction was expressed by the intensity of a skin reaction.

As seen in the Table, the insulin binding activity of a given serum fell within the range of the neutralizing activity of the serum in 14 of the 26 cases, while, in other 10 cases, the former was lower than the latter and this relation was reverse in one case (guinea pig. No. 28). The result indicates a considerable correlation between the insulin binding activity and the neutralizing activity, though the former appeared to be lower than the latter.

The ability to evoke cutaneous anaphylactic reaction, however, showed a far less correlation with either the insulin binding activity or the neutralizing activity.

DISCUSSION

The present study clearly shows that the reaction between insulin and its antibody occurs as a nonprecipitating reaction under the regulation by the law of mass action and that an amount of insulin in an assay system must be so controlled as to be in a state of "antigen excess". In the present experiments, the amount of insulin in the assay system to create a state of

"antigen excess" was determined according to such a B/T that not only is small enough in order for the insulin antibody to be saturated with insulin, but also lies within a range where the relation between the amount of insulin and B/T is linear.

The quantitative character of the present method is supported by a relatively good correlation between the insulin binding acitivty measured by the method and the insulin neutralizing activity, although the latter appeared to be higher than the former. This discrepancy may be due to a semi-quantitative character of the mouse convulsion procedure used in the present study to measure the insulin neutralizing activity.

On the other hand, a poor correlation between the insulin binding activity and the ability to evoke cutaneous anaphylactic reaction is understandable, for the former is purely effected by a humoral factor while the latter is largely mediated by cellular contributions, thus involving many other factors.

SUMMARY

A titration of the insulin binding activity of anti-insulin was performed using ^{131}I-insulin and anti-guinea pig γ-globulin rabbit sera.

A considerably good correlation was observed between the insulin neutralizing activity and insulin binding activity, thus supporting a quantitative character of the present method. On the other hand, between the insulin binding activity and the ability to evoke cutaneous anaphylactic reaction was only a poor correlation observed.

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