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Insulin Antibodies and Hypoglycemia in Diabetic Patients

Can a Quantitative Analysis of Antibody Binding Predict the Risk of Hypoglycemia?

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We report a noninsulin-dependent diabetes mellitus (NIDDM) patient with spontaneous, severe hypoglycemic reactions and the presence of insulin antibodies. He had a remote antecedent history of beef–pork insulin therapy as well as exposure to hydralazine. Detailed insulin binding kinetic studies were performed in this patient as well as in six other insulin-treated diabetic patients with anti-insulin antibodies (three with and three without an obvious cause of hypoglycemia). Sera from the current patient and five of the six other diabetic patients (one NIDDM, four IDDM) revealed two types of binding sites: high-affinity with low capacity (K_d, 0.4–12.4 $\times 10^{-9}$ mol/L; binding capacity, 0.6–659 mU/L) and low-affinity with high capacity (K_d, 0.3 to 35.7 x 10^{-7} mol/L; binding capacity; 202–113,680 mU/L). One NIDDM patient had only high-affinity antibodies (K_d, 22.9 $\times 10^{-9}$ mol/L; binding capacity of 78 mU/L). Type of diabetes mellitus, insulin antibody titers or their binding capacities, insulin levels (total, bound, or free), and bioavailable insulin were not related to hypoglycemic reactions. Two calculated values by the method described tended to discriminate patients with and without hypoglycemia. The calculated amount of low-affinity antibody bound insulin ranged from 69.4–2090 mU/L vs <4–70.6 mU/L in patients with and without hypoglycemia, respectively. The best discrimination was afforded by the percent saturation of low-affinity binding sites; values were clearly higher in the patients with hypoglycemia (2.5–34.4 %) than in those without hypoglycemia (not detectable, 0.06, 0.15%). Consideration of the possible drug-associated insulin antibody formation in insulin-treated diabetics and the novel quantitative analysis of the antibody binding kinetics should prove helpful in evaluating patients with high insulin antibody titers and assessing the risk of hypoglycemia.

Key Words: Insulin antibodies, hypoglycemia, hydralazine, binding kinetics.

Introduction

The development of insulin antibodies in diabetic patients treated with exogenous insulin can occur shortly after the introduction of insulin therapy *(1)*. High titers of serum insulin antibodies are frequently associated with insulin resistance in insulin-treated diabetic patients *(2,3)*, but also can result on occasion in hypoglycemic reactions caused by the dissociation of insulin from the immune complexes *(4)*. Although insulin antibodies also may affect diabetes control *(5,6)* and, possibly, the complications of this disease *(7)*, the significance of these antibodies in individual patients is not clearly understood. Insulin antibodies also have been associated with hypoglycemia in a number of patients without prior insulin exposure *(8–13)*. With an occasional exception *(14)*, most studies have reported no consistent differences among these antibodies in the number of binding sites, dissociation constants, and binding capacities, or even species specificity *(15–17)*.

A patient with noninsulin dependent diabetes mellitus (NIDDM), who presented with unexplained spontaneous hypoglycemic reactions and a remote prior history of insulin therapy, prompted the current study. Detailed analysis of the patient's serum as well as sera from six other patients with insulin antibodies led us to the conclusion that a new approach to the quantitative analysis of the binding kinetics of insulin antibodies may be helpful in predicting the contribution of these antibodies to the hypoglycemia.

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Table 1
Clinical Characteristics of the Patients with Insulin Antibodies

Patient# sex/age	Type of DM, duration, yr	Daily insulin dosage/type	Insulin antibody % binding	Serum insulin mU/L, total/free	Hypoglycemia
1. M/63	NIDDM (27)	None	76.9	1345/76	Spontaneous hypoglycemia (5–9 times/d)
2. F/62	IDDM (34)	Novolin 44 U (N)/24 U (R)	47.0	2167/24	Unexplained hypoglycemia (3–4 times/wk)
3. F/69	IDDM (45)	Beef-pork 26 U (UL)/16 U (R)	10.6	329/26	Unexplained hypoglycemia (2–3 times/mo)
4. F/44	IDDM (38)	Beef-pork 22 U (N)	19.5	80/10	Unexplained hypoglycemia (2 to 3 times/mo)
5. F/60	NIDDM (10)	Humulin 32 U (N) and oral hypoglycemic agent	69.0	124/45	None
6. F/63	NIDDM (2)	Humulin 15 U (N)	14.8	37/16	None

Table 2
Characteristics of Insulin Binding Kinetics of Insulin Antibodies

	Serum insulin	High-affinity antibody		Low-affinity antibody		Insulin bound to		Saturation[a]
Patient no.	Bound, mU/L	K_d, $10^{-9}M$	Capacity, mU/L	K_d, $10^{-7}M$	Capacity, mU/L	High-affinity, mU/L	Low-affinity, mU/L	Low-affinity antibody, %
1.	1269	7.8	659	10.9	30,400	500	769	2.5
2.	2143	12.3	61	12.6	30,976	53	2090	6.7
3.	303	0.4	0.9	4.5	6686	0.9	302.1	4.5
4.	70	0.6	0.6	0.3	202	0.6	69.4	34.4
5.	78	7.2	32	35.7	113,680	7.4	70.6	0.06
6.	21	22.9	78	N.D.[b]	N.D.	21	N.D.	—
7.	15	12.4	39	6	7902	2.7	12.3	0.15

[a] % Saturation = (insulin bound/binding capacity) × 100.
[b] N.D., none detected.

Results

The clinical characteristics and the insulin binding kinetics data of these patients are shown in Table 1 and Table 2, respectively. There was no association between serum total or free insulin levels or insulin antibody binding or type of diabetes and the presence of hypoglycemic reactions. The Scatchard plots of the insulin-antibody binding data showed bimodal distributions in all patients, except patient #6. The binding kinetics study of the insulin anti-bodies showed two types of binding sites; high-affinity with lower binding capacity (K_d, 0.4 to 12.4 × 10^{-9} mol/L; binding capacity, 0.6–659 mU/L) and low-affinity with higher binding capacity (K_d, 0.3–35.7 × 10^{-7} mol/L; binding capacity; 202–113,680 mU/L). One NIDDM patient (#6) had only high-affinity site antibodies (K_d, 22.9 × 10^{-9} mol/L) with a binding capacity of 78 mU/L.

There was a similar lack of association between the binding capacities of these antibodies and the presence of hypoglycemic reactions. Therefore, we hypothesized that the insulin loosely bound to the low-affinity binding sites might provide a basis to explain the hypoglycemic events. Accordingly, we proceeded to calculate the actual amount of insulin bound to each class of antibodies from the Scatchard's analysis data as described in the Appendix. Serum total bound insulin was calculated in each patient as the total minus the free insulin measured. The results are shown in Fig. 1. The actual amount of low-affinity antibody bound insulin tended to be higher in patients with unexplained hypoglycemia than in patients without hypoglycemia but there was a slight overlap (compare patient #5 without hypoglycemia to patient #4 with hypoglycemia). Calculation of the hypothetical bioavailable insulin (low-affinity antibody-bound plus free insulin) between the two patients made the discrimination even worse (Fig. 2). A

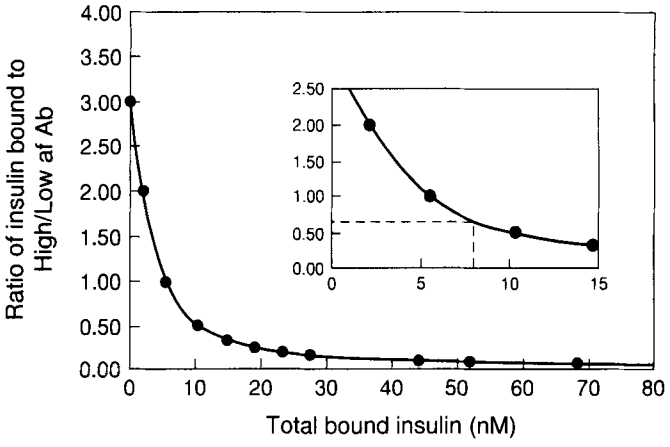

Fig. 1. Relationship between total bound insulin and the ratio of insulin bound to high-affinity sites to that of low-affinity sites (patient #1). Insert: Broken lines indicate the corresponding values for total bound insulin and the ratio of insulin bound to high-affinity sites to that of low-affinity sites in this patient.

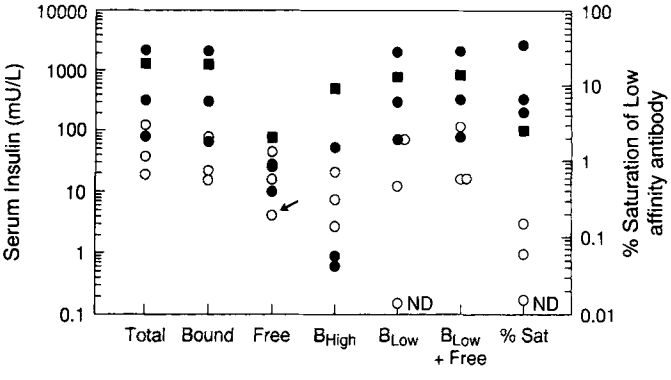

Fig. 2. Total, antibody-bound, free, high-affinity antibody bound (B_{High}), low affinity antibody bound (B_{Low}), bioavailable (B_{Low} + Free) insulin levels, and the percent saturation of low-affinity antibody in patients with hypoglycemia (closed circles) and without hypoglycemia (open circles). Index case is shown as the closed squares (arrow indicates an insulin value below the assay detection limit).

discriminating feature, however, was the degree of saturation (%) of low-affinity binding sites, which was clearly greater (2.5–34.4%) in the patients with frequent hypoglycemia compared with the patients without hypoglycemia (not detectable, 0.06, 0.15%).

Discussion

Two situations have been described in which insulin antibodies cause hypoglycemia; autoimmune insulin antibodies in patients without previous insulin exposure (autoimmune insulin syndrome) and insulin-induced antibodies in insulin-treated diabetic patients. Since the first description of the autoimmune insulin syndrome by Hirata et al. in 1972 *(18)*, about 140 cases have been reported, the majority (120 cases) from Japan, where autoimmune hypoglycemia is the third leading cause of hypoglycemia *(19,20)*. The remaining 20 cases are among Caucasians *(21,22)*. The higher frequency in Japan may be explained by the association with certain HLA types (HLA DR4 DQw3) *(23)*. Autoimmune insulin antibodies can also appear in insulin-dependent diabetics before any treatment with insulin *(24,25)*, in nondiabetics after a viral infection *(24)*, and in patients with other autoimmune diseases *(26)*. Nearly 30% of the Japanese cases are associated with the drugs methimazole or mercaptopropionylglycine *(19,20)*. Also, associations with pyrinitol *(22)*, hydralazine *(13)*, andprocainamide in drug-induced systemic lupus erythematosus *(18)* have been reported.

Many studies have tried to differentiate autoimmune insulin antibodies from insulin-induced antibodies. However, the poly- or monoclonal origin of the immunoglobulins or the class of immunoglobulins themselves cannot differentiate endogenous from exogenous sources of antigenic stimulation reliably *(21)*. In one study, the autoimmune antibodies were most often polyclonal *(16)*, but in several other studies, these were monoclonal *(20,27,28)*. Both autoimmune and insulin-induced insulin antibodies are composed of two types, which can be distinguished by their binding affinity and capacity. The association constants of the autoimmune antibodies are also not different from those of insulin-induced antibodies *(16)*.

In consideration of all these facts, the present case is peculiar in that both autoimmune and insulin-induced mechanisms could have played a role in the production of insulin antibodies. The notion of an autoimmune basis, possibly related to hydralazine in this patient, is supported by his allergic diathesis to many drugs and the associated autoimmune condition of lymphocytic myocarditis. In fact, every time he presented with hypoglycemic reactions, he was on hydralazine. Moreover, the failure of insulin antibodies to disappear long after exogenous insulin was stopped, favors an autoimmune mechanism of insulin antibody production. However, the development of hypoglycemia on restarting insulin after a 7-yr hiatus points toward an insulin-induced mechanism of antibody production.

Renal failure and/or hemodialysis was considered as a possible etiologic factor in the development of hypoglycemia in our patient *(29,30)*. The observations that the initial episode of hypoglycemia antedated the renal failure by a number of years and that the hypoglycemia recurred only after the reintroduction of insulin in the presence of high levels of insulin antibodies make the association with renal failure unlikely in our view. It has been difficult to predict or explain all hypoglycemic reactions in insulin- treated diabetic patients. Also, elevated free insulin levels and impaired responses of counterregulatory hormones cannot explain all such episodes of hypoglycemia. In these circumstances, insulin antibodies should be considered as one of the possible causes of hypoglycemia. However, it has proven difficult to determine the exact significance of the titer of antibodies or the serum insulin level (either total

or free) in a given patient. Understanding the contribution of insulin antibodies to the clinical situation may be crucial to patient management.

In agreement with other studies *(15,16,31,32)*, most patients had two types of antibodies. In the exceptional patient (#6), the antibodies may have been monoclonal. The ranges of dissociation constants and binding capacities were similar to previous reports and could not be differentiated according to autoimmune or insulin-induced origin. The mechanism of insulin-antibody-mediated hypoglycemia has been discussed widely *(15,21,25,27,33)*. Ultimately, the most promising hypothesis attributes the hypoglycemia to a liberation of biologically active insulin bound to antibodies, particularly of the low-affinity class *(16,21)*. Indeed, most investigators have emphasized the huge binding capacity of low-affinity antibodies as a cause of hypoglycemia *(15,16,31)*. However, in the present study, patients #3 and 4 had hypoglycemic reactions despite relatively small binding capacities of their low-affinity antibodies, whereas patient #5 and #7 had no hypoglycemia despite large binding capacities of their low-affinity antibodies (Table 2). In order to clarify the significance of the low-affinity antibodies, we calculated the degree of saturation and the actual amount of insulin bound to each class of antibodies. Our results suggest that not only the amount of insulin bound, but also the degree of saturation of low-affinity antibodies may play an important role in the development of hypoglyemia.

Notwithstanding the fact that the in vitro studies reported herein may not be an exact reflection of the in vivo state, the data point to the conclusion that circulating antibody-bound insulin might play a crucial role in hypoglycemic reactions. However, one NIDDM patient without hypoglycemia (patient #5) had a comparable amount of insulin bound to low-affinity antibodies to the patient with hypoglycemia (patient #4). To reconcile this apparent paradox, one can postulate a relatively greater degree of insulin resistance in patient #5 vs patient #4.

Ultimately, the absolute ability to predict hypoglycemia in patients with insulin antibodies may still prove difficult from a quantitative point of view, since other factors, such as nutritional status, insulin sensitivity/resistance, counterregulatory mechanisms, dosage/timing of exogenous insulin (in those patients remaining on insulin), and clearance rate of insulin are critically important in modulating the blood glucose response. On the other hand, the observation that low-affinity antibody sites were 17- to 570-fold more saturated in hypoglycemic patients than in those without hypoglycemia lends strong support to the relevance of low-affinity antibodies in precipitating hypoglycemic reactions.

Our patient's response to immunosuppression with prednisone and plasmapheresis was swift, despite no apparent change in the titer of insulin antibodies. Regrettably, no serum was saved to study the distribution of free and antibody-bound insulin in detail at this time. In any event, glucocorticoids are well known to enhance hepatic glucose output and to antagonize the action of insulin on target tissues at a postreceptor level *(34–36)*.

The prognosis of the autoimmune insulin syndrome is usually favorable, because spontaneous (or after withdrawal of the offending agent) remission of hypoglycemia occurs and the antibodies disappear in 4–12 mo *(19)*. Therefore, it is important to detect and withdraw possible immunogenic drugs in such patients, even in those with a past or present history of insulin therapy.

In conclusion, the novel quantitative analysis of binding kinetics described herein, in terms of determining the actual amount of insulin bound to and the percent saturation of the two classes (high- and low-affinity) of antibodies, should prove helpful in evaluating and managing such patients with insulin antibodies. Prospective large-scale studies may help to fine-tune the predictive ability of such analyses.

Materials and Methods

Subjects

We studied sera from a diabetic patient (NIDDM) with spontaneous hypoglycemia and from six insulin-treated diabetic patients (two NIDDM and four IDDM) with positive insulin antibody binding (insulin binding > 5%). The sera from the six latter patients were obtained retrospectively from samples sent to the endocrinology laboratory for insulin antibody and insulin (free and total) analysis. We attempted to match patients with and without hypoglycemia by their insulin antibody (% binding) values (*see* Table 1). All six patients were currently receiving insulin therapy and the former one was no longer receiving it. Among the six patients on insulin treatment, three had unexplained hypoglycemic reactions, and the other three had none (Table 1). The presence of unexplained hypoglycemia (excluding obvious causes, such as insulin, oral hypoglycemic agents or drugs known to cause hypoglycemia) was derived retrospectively from carefully documented chart records.

Free and Total Insulin Measurement

Anti-insulin antibody levels were measured by determining the amount of radioactive porcine insulin (Diagnostic Products Corp., Los Angeles, CA) bound to insulin-free serum *(37)*. Free insulin was separated by polyethylene glycol precipitation of immunoglobulins (antibody-bound insulin) using equal volumes of serum and ice-cold 25% polyethylene glycol (PEG 8000; Sigma Chemical Co., St. Louis, MO). Total insulin was determined by preincubating the serum with 1/5 volume of 1 N HCl for 1 h (stripping insulin from antibody-binding sites) and neutralizing with one-fifth the original serum vol of 1 *N* NaOH before adding 25% polyethylene glycol *(38)*. The supernatant total insulin

was separated by centrifugation at 2500*g* at 4°C for 1 h and analyzed for its immunoreactive insulin content by the monoclonal antibody based immunoenzymometric method (IEMA) using an automated immunoassay analyzer, AIA 1200 (TOSOH Medics Inc., San Francisco, CA).

In Vitro *Saturation Kinetics of Anti-Insulin Antibodies*

Free and antibody-bound insulin were removed from serum as described above. Briefly, serum was first heated at 56°C for 10 min to inactivate complement. All reagents were diluted by, and the immunoglobulin precipitates were reconstituted in barbital-albumin buffer (7 mmol/L sodium barbital, 12 mmol/L sodium acetate, 130 mmol/L sodium chloride, 0.5% bovine serum albumin, pH 7.5). The insulin-free preparation of immunoglobulin was obtained by acidifying the serum to release bound insulin and then precipitating the immunoglobulin with polyethylene glycol at a final concentration of 12.5%. This immunoglobulin preparation (50 µL, final dilution of 1:10) was incubated in duplicate with 100 µL of [^{125}I] monoiodinated (Tyr^{14}A or Tyr^{26}B) human insulin alone to determine maximal [^{125}I]-insulin binding, which was diluted to a final concentration of 50,000 cpm/100 µL (Amersham, Arlington Heights, IL, 2000 Ci/mmol, 2.7×10^{-11} mol/L), and with 100 µL of increasing quantities of unlabeled recombinant human insulin (concentrations from 0.01 nmol/L to 1 µmol/L; from *Escherichia coli,* Sigma Chemical Co., St. Louis, MO). The reactions were equilibrated overnight at 4°C. Specific binding was determined by precipitating bound complexes with 500 µL of 25% ice-cold polyethylene glycol after the addition of 250 µL of ice-cold carrier (0.36% bovine serum γ-globulin; Sigma Chemical Co., St. Louis, MO) *(15)*. The precipitation was performed by centrifugation at 1000*g* for 1 h at 4°C. The supernatants were decanted, and the pellets were counted in an automated γ-counter.

Precipitated counts represent the total bound insulin (B_T). Aliquots of the [^{125}I]-insulin stock solution provided a measure of the total cpm (T) added to each tube. Specific binding (B_{SP}) was defined as [^{125}I]-insulin precipitated in the presence of immunoglobulin and carrier (B_T) minus that precipitated with carrier alone (B_{NS}). T minus B_T represents free insulin (F). The concentration of bound insulin was calculated from the equation

$$B\ (mol/L) = [B_{SP}(cpm)/T(cpm)] \cdot T\ (mol/L) \quad (1)$$

where T (mol/L) represents the sum of the concentrations of [^{125}I] insulin and cold insulin added to each tube. The binding data were plotted according to Scatchard's equation *(39)*:

$$B_{SP} / F = -K_a(B) + K_a B_{max} = -(1/K_d)(B) + (1/K_d)B_{max} \quad (2)$$

where K_a is the association constant, K_d is the dissociation constant, and B_{max} is the binding capacity of the insulin antibodies. The two linear components of the Scatchard plot obtained in the insulin-antibody binding experiments were fitted by a computerized nonlinear least-squares curve-fitting program, RSTRIP (polyexponential curve stripping, least-square parameter estimation, Version 5.0; MicroMath Inc., Salt Lake City, UT). Binding in the range of bound insulin concentrations corresponding to the high-affinity binding site is corrected in this method by subtracting the contribution of the low-affinity site. The resultant binding data were replotted according to Scatchard's equation. Dissociation constants and binding capacities for each class of insulin antibodies were calculated from the slopes and *x*-axis intercepts of the corresponding plots, respectively.

Details of the calculation of insulin bound to each class of antibody are documented in the Appendix.

Case (Patient #1)

A 63-yr-old man, diabetic (NIDDM) for the last 27 yr, presented with a 5-yr history of anxiety, confusion, weakness, and sweating in 1993. He had received no oral hypoglycemics or insulin injections since early 1988. By the time of presentation, the frequency and severity of the hypoglycemia had increased (5–9 episodes/d). He had initially been treated with chlorpropamide in 1967, which was later switched to phenformin and continued until the medication was taken off the market in 1977. He was then started on tolazamide, which was later discontinued because of inadequate glycemic control and then put on insulin (beef–pork insulin, 50 U of NPH and 20 U of Regular daily). Insulin was stopped around 1980, and he was restarted on tolazamide, which was continued until 1987. It was discontinued again for inadequate control, and he was restarted on insulin (human insulin, 70/30, 25 U; which was tapered to 10–12 U daily). Insulin was discontinued entirely owing to recurrent hypoglycemia in 1988. He was known to have a drug sensitivity to sulfonamides. He also had had hypertension since 1978, which was difficult to control, despite multiple medication trials, including α-methyl DOPA, hydralazine, and thiazide diuretics. In April 1991, he was admitted with generalized edema, and a renal scan detected chronic renal parenchymal disease (BUN 27 mg/dL, creatinine 2.6 mg/dL). In May 1992, he was diagnosed as having lymphocytic myocarditis by myocardial biopsy. The antinucleic acid antibody (ANA) test was negative. Tests for antithyroid microsomal antibody, antismooth muscle antibody, and antimitochondrial antibody were negative. He developed progressive renal disease (BUN 119 mg/dL, creatinine 5.2 mg/dL) and was started on hemodialysis in July 1993. The range of the fasting blood glucose levels was 33–155 mg/dL at that time.

At the most current admission (1993), the lowest blood glucose was 27 mg/dL, with concurrent total insulin 1345 mU/L, free insulin 76 mU/L, and insulin antibody binding (76.9%). The serum TSH was 2.0 μU/mL (normal; 0.4–5.5 μU/mL), and a Cosyntropin stimulation test for adrenal cortical function was normal (basal serum cortisol, 18.1 μg/dL, 1 hr peak after stimulation, 47.7 μg/dL). On this occasion, the ANA test was positive, with a titer of 1:80 that showed a homogeneous pattern.

A diagnosis of insulin-antibody-induced hypoglycemia was made, and he was started on prednisone 30 mg daily and plasmapheresis (4 times). His blood glucose level rose to 519 mg/dL on the day after prednisone administration and plasmapheresis. After the second plasmapheresis, serum total insulin was 1003 mU/L, free insulin was 50.6 mU/L, and the insulin antibody binding was 76.5%. Prednisone was decreased to 15 mg daily without the return of hypoglycemia (range of blood glucose, 102–208 mg/dL), and he was discharged. He later reported that when he decreased the dose of prednisone further, hypoglycemic reactions returned. He was lost to follow-up and died in 1994 of unknown causes.

Appendix

Calculation of Insulin Bound to Each Class of Antibody

Assuming that insulin binding to antibodies after overnight fasting reaches equilibrium, from the Scatchard's plot:

$$B_{sp}\,(cpm)/F\,(cpm) = [B_{sp\,(high)}\,(cpm) + B_{sp\,(low)}(cpm)\,]/\,F(cpm) \tag{3}$$

where $B_{sp\,(high)}$ is [^{125}I] insulin bound to high-affinity antibody, $B_{sp\,(low)}$ is [^{125}I] insulin bound to low-affinity antibody, and F is free [^{125}I] insulin.

From (1), the ratio of the amount of insulin bound to high-affinity antibody to the amount bound to low-affinity antibody can be expressed as follows:

$$\begin{aligned} &B_{high}\,(mol/L)/B_{low}\,(mol/L) \\ &\{[B_{sp\,(high)}(cpm)/T(cpm)\,] \cdot T\,(mol/L)/ \\ &\quad [B_{sp\,(low)}(cpm)/T(cpm)] \cdot T\,(mol/L)\} \\ &= B_{sp\,(high)}\,(cpm)/B_{sp\,(low)}\,(cpm) \\ &[B_{sp\,(high)}\,(cpm)/F\,(cpm)/B_{sp\,(low)}\,(cpm)/F\,(cpm) \\ &(-K_{a\,(high)} \cdot B_{high} + K_{a\,(high)} \cdot B_{max\,(high)}/ \\ &\quad -K_{a\,(low)} \cdot B_{low} + K_{a\,(low)} \cdot B_{max\,(low)}) \end{aligned} \tag{4}$$

where $K_{a\,(high)}$ is the association constant of high-affinity antibodies, $K_{a\,(low)}$ is the association constant of low-affinity antibodies, B_{high} is the concentration of insulin bound to high-affinity antibodies, B_{low} is the concentration of insulin bound to low-affinity antibodies, $B_{max\,(high)}$ is the binding capacity of the high-affinity antibodies, and $B_{max\,(low)}$ is the binding capacity of low-affinity antibodies.

If the ratio of the amount of insulin bound to high-affinity antibodies to that of low-affinity antibodies at a given concentration of total antibody-bound insulin is known, the actual amount of insulin bound to each antibody site can be calculated. To determine the relationship between the ratio of insulin bound to high-affinity antibodies vs insulin bound to low-affinity antibodies and total bound insulin, we chose several arbitrary values of the ratio, and calculated the corresponding amount of total bound insulin and plotted each point (Fig. 1). The graph revealed a close correlation between the ratio of insulin bound to high-affinity antibodies vs low-affinity antibodies and total bound insulin. The ratio of insulin bound to high-affinity antibodies vs. low-affinity antibodies at the concentration of bound insulin of the patient's serum was inferred from the graph for each patient, and the actual amount of insulin bound to each class of antibodies was calculated.

For patient #1, the equations of the two linear components of the Scatchard plot were as follows:

$$\begin{aligned} B_{sp\,(high)}\,(cpm)/Free\,(cpm) &= -0.1279\,B_{(high)} + 0.527 \\ B_{sp\,(low)}\,(cpm)/Free\,(cpm) &= -0.00092\,B_{(low)} + 0.175 \end{aligned} \tag{5}$$

If $B_{(high)} = B_{(low)}$ ($B_{(high)}/B_{(low)} = 1$), from the Eq. (4)

$$\begin{aligned} 1 &= (-0.1279 \cdot B_{(high)} + 0.527/-0.00092 \cdot B_{(low)} + 0.175) \\ 1 &= (-0.1279 \cdot B_{(high)} + 0.527/-0.00092 \cdot B_{(high)} + 0.175 \end{aligned}$$

$$B_{(high)} = 2.76\,(nmol/L),\ B_{(total)} = 5.52\,(nmol/L) \tag{6}$$

In the same way, according to $B_{(high)}/B_{(low)}$, the corresponding $B_{(total)}$ was calculated and plotted.

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