Nonbinding Inhibitory Antiinsulin Receptor Antibodies

A New Type of Autoantibodies in Human Diabetes

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Abstract

Sera and their IgG from 10/104 diabetic patients (five with insulin-dependent and five with noninsulin-dependent diabetes, NIDDM), contained antibodies that bound ^{125}I-labeled purified human insulin receptors. 9 of these 10 sera failed to inhibit insulin binding (to rat hepatocytes and human placental membranes), did not stimulate glucose oxidation (by isolated rat adipocytes), and did not bind human placental IGF-1 receptors. Only one serum (and its IgG) modestly inhibited insulin binding and stimulated glucose oxidation. We conclude (*a*) that sera from 9/104 diabetics (five insulin-dependent and four noninsulin-dependent) contained a newly identified species of IgG antiinsulin receptor autoantibodies (AIRA), which bound to the insulin receptor at a locus different from the insulin binding site and did not inhibit insulin binding; and (*b*) that only 1/104 diabetic sera contained low-titer "conventional" antiinsulin receptor autoantibodies that bound to the insulin receptor at or near the insulin binding site, inhibited insulin binding and caused a clinical condition, which was difficult to distinguish from typical NIDDM.

Introduction

High-titer, antiinsulin receptor autoantibodies (AIRA),[1] which inhibit insulin binding, cause a distinct clinical syndrome characterized by severe insulin resistance, glucose intolerance or diabetes mellitus, acanthosis nigricans, and other autoimmune disorders (1). These AIRA are considered to be rare, although their prevalence has not been systematically investigated (2). It has been suspected, that high-titer AIRA, which are able to cause extreme insulin resistance, may represent only the "tip of the iceberg" (2) while low-titer AIRA may be more common, and may be responsible for a noninsulin-dependent diabetes (NIDDM)-like syndrome in a substantial number of patients. So far, such low-titer AIRA have not been reported in diabetic patients.

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1. *Abbreviations used in this paper:* AIA, antiinsulin antibodies; AIRA, antiinsulin receptor autoantibodies; IGF-1, insulin like growth factor 1; IRIP, insulin receptor immuneprecipitation; NIDDM, noninsulin-dependent diabetes mellitis; PCO, polycystic ovary syndrome.

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It has been established that insulin binds to the α-subunit of the insulin receptor which appears to be localized entirely on the outside of the cell. Inasmuch as one insulin receptor α-subunit is more than 10 times larger than insulin, it appears likely, that some individuals may have AIRA directed against sites on the receptor which are not involved with insulin binding. Such antibodies have been produced in mice (3) but have not been demonstrated in human subjects. It was the objective of this study to determine in sera from patients with diabetes and certain other disorders (*a*) the prevalence of high-titer and low-titer conventional binding-inhibitory AIRA; and (*b*) to determine whether these sera contain another species of AIRA, which do not inhibit insulin binding.

To search for AIRA which do not inhibit insulin binding, we used a newly developed sensitive insulin receptor immunoprecipitation (IRIP) assay in combination with two insulin-binding assays (to rat hepatocytes and human placental membranes). The IRIP assay determined binding of all types of AIRA to highly purified radiolabeled human placental insulin receptors. The hepatocyte and placental membrane assays determined only AIRA which inhibited insulin binding. We have used both assays to test sera from 104 diabetic patients, from 34 patients with rheumatic disorders and from 8 patients with polycystic ovary syndrome (PCO), disorders in which AIRA have been reported (2). 43 normal subjects served as negative controls and 4 patients with AIRA and type B severe insulin resistance served as positive controls.

Methods

Subjects. Some details of the study population are shown in Table I. Blood was obtained from all subjects after an overnight fast. The 43 normal controls were healthy hospital employees and medical students. Clinical and laboratory data from the four patients with type B severe insulin resistance (patients 1, 2, D.L., and B-10) have been published (4–6). The NIDDM sera were obtained from randomly selected patients from the diabetic clinic at Temple University Hospital. These patients were predominantly female (89%), middle-aged (mean age 58 yr), and obese (81%). Of the 41 treated with insulin, 9 received doses of 100 U/d or more suggesting marked insulin resistance. Mean duration of the disease was 15 yr. The IDDM sera were obtained from the diabetic clinics of St. Christopher's Hospital for Children and Temple University Hospital and from the office of Dr. Arthur Krosnick in Trenton, NJ. The patients with IDDM were younger (mean age 21 yr), and most (82%) were either normal or underweight. 20 of the 51 patients were newly diagnosed and their blood was taken before insulin treatment was started. The rheumatic disease sera were obtained from the rheumatology clinic at Temple University Hospital and from Dr. Leroy Hunninghake of Princeton Rheumatology Associates, Princeton, NJ. 16 of the 34 patients had rheumatoid arthritis, 11 had systemic lupus erythematosis, 2 had scleroderma, and the rest had diverse disorders including polymyositis, polychondritis, Sjogren's syndrome,

Table I. Subjects Studied

Subjects	n	Sex M/F	Age	Obese body wt. > 120%	FPG	Insulin-treated patients	Duration diabetes	AIA
			yr		*mg/dl*		*yr*	
NIDDM	53	6/47	57.8±1.3 (34–76)	43/53	230±12.6	41/53	15.1±1.0	21/49
IDDM	51	30/21	21.2±1.8 (3–66)	9/51	310±26	31/51*	5.7±1.6	13/36
Rheumatic disorders	34	5/29	52.0±2.7 (22–87)	6/34	91±3	0/34	—	0/34
PCO	8	0/8	27.5±4.5 (24–37)	6/8	77±1	0/8	—	0/8
Type B	4	0/4	25.5±2 (19–30)	0/4	200–400	4/4	1.9±0.5	0/4
Normal controls	43	25/18	27.4±1.1 (21–51)	3/43	90±3	—	—	0/43

* Blood was obtained from 20 patients before insulin therapy. Presented are mean±SEM and range in parentheses.

and psoriatic arthritis. The PCO sera were obtained from the obstetric-gynecologic clinic at Temple University Hospital.

The study protocol was approved by the human research committees at Temple University Hospital and at St. Christopher's Hospital for Children.

Preparation and purification of insulin and IGF-1 receptors. Insulin receptors were prepared and purified from human placental membranes as described (7). Briefly, human placental membranes were solubilized in 50 mM Tris-HCl, pH 7.4 containing 2% Triton X-100 and purified 2,400-fold by affinity chromatography first on wheat germ agglutinin (WGA)-Sepharose and then on insulin-Sepharose. Receptors were eluted from insulin-Sepharose under mildly acidic conditions (50 mM acetate, pH 5.0) and without urea in order to preserve their stability. The purified insulin receptors bound 28.5 μg of insulin/mg protein. Kept at −70°C, aliquots of the same receptor preparation could be used for at least 6 mo without showing signs of diminished binding to serum containing high-titer AIRA (from patient 1).

Insulin-like growth factor 1 (IGF-1) receptors were purified from Triton X-100 solubilized human placental membranes by WGA-Sepharose chromatography, followed by immuneaffinity chromatography with α-IR-3, a monoclonal antibody directed against the IGF-1 receptor, as described previously (8).

Iodination of insulin and IGF-1 receptors. The purified insulin and IGF-1 receptors were iodinated with chloramine-T according to Jacobs et al. (9) with modifications. Receptor protein (0.65 μg) dissolved in 50 mM Tris-HCl, pH 7.4, containing 0.1% Triton X-100, was reacted with 0.1 μCi of Na ^{125}I in 0.5 ml of Tris-HCl buffer and with 1 μg of chloramine T. Iodinated receptors were purified first over Sephadex-G 25 columns, equilibrated with Tris-HCl buffer, pH 7.4. The purity of the tracer after gel filtration was usually between 80–90% as determined by precipitability with 12.5% PEG. A second purification over a WGA column, while not significantly increasing PEG precipitability, markedly decreased nonspecific binding. Intactness of the labeled insulin receptor was tested by comparing its ability to bind insulin with that of unlabeled insulin receptor. As seen in Fig. 1, binding of ^{131}I-insulin to the ^{125}I-labeled and the unlabeled receptor as well as displacement of ^{131}I-insulin by unlabeled insulin from the ^{125}I-labeled and unlabeled receptor were similar. Scatchard transformation of the data resulted in two nearly identical curvilinear functions (not shown). This finding was compatible with the presence on the insulin receptor of two independent, noninteracting species of binding sites or alternatively, with negative cooperativity between the same species of binding sites.

Insulin and IGF-1 receptor immuneprecipitation. Serum (5 μl), labeled receptor (50 μl, ~ 5,000 cpm) and Tris-HCl buffer, pH 7.4 (final volume 500 μl) were incubated for 24 h at 4°C. Thereafter, 200 μl of undiluted rabbit anti-human gamma-globulin was added and the incubation continued for another 24 h. The samples were then centrifuged for 30 min at 3,000 rpm, the supernate was aspirated and the pellet washed twice with buffer and counted in an automatic gamma counter.

Preparation and culture of hepatocytes. Hepatocytes were isolated by in situ collagenase digestion of livers from fed Sprague-Dawley rats (200–300 g). Primary cultures of rat hepatocytes were prepared as described previously (10).

Hepatocyte insulin-binding-inhibition assay. Insulin binding to hepatocytes was examined as described (10). Briefly, hepatocytes in monolayer (2×10^6 cells/25 cm^2 flask) were preincubated with sera from the various study groups for 2 h at 37°C, then washed twice with Krebs-Ringer bicarbonate (KRB) buffer. After that, the cells were incubated in 2.5 ml KRB buffer containing 1% BSA, ^{125}I-insulin (0.3 ng) with or without unlabeled insulin. Insulin binding in the presence of 10,000 ng/ml of native insulin was usually < 1.2%, was regarded as nonspecific, and subtracted from total binding.

Preparation of placental membranes. Placental membranes were prepared from normal human placentas obtained within 1 h after delivery as described (7). These crude membranes, suspended in Tris-HCl buffer (0.05 M, pH 7.4) containing 0.1 mM PMSF were kept at −70°C until used for the assay.

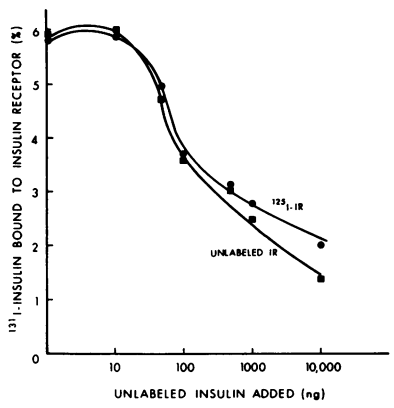

Figure 1. Displacement by unlabeled insulin of ^{131}I-insulin bound to ^{125}I-insulin receptor (*circles*) or to unlabeled insulin receptor (*squares*). Shown are means of duplicate determinations. IR, insulin receptor.

Placental membrane insulin and IGF-1 binding-inhibition assays. Placental membrane suspensions were diluted 1:20 with Tris-HCl buffer (0.05 M, pH 7.4) containing 2 mM $CaCl_2$ and 0.1% BSA. 400 μl of this suspension was preincubated with serum (dilution 10^{-1} to 10^{-5}) for 2 h at 20°C. After that, the membranes were centrifuged, the pellet washed twice, and resuspended in 400 μl of fresh Tris-HCl buffer containing ^{125}I-labeled insulin or IGF-1 (~ 100,000 cpm) with or without unlabeled insulin or IGF-1. At the end of the incubation period, 2 ml of PEG (12.5%) was added, the samples centrifuged at 2,000 *g* for 45 min at 4°C, the supernatant discarded and the pellet counted in an automatic gamma counter. Insulin binding in the presence of 10 μg of native insulin was usually less than 12%, IGF-1 binding in the presence of 500 ng of synthetic IGF-1 less than 13%. Both were regarded as nonspecific binding and subtracted from total binding.

Preparation of isolated adipocytes. Isolated adipocytes were prepared from epididymal fat pads of ad lib.-fed (rat chow; Ralston Purina Co., St. Louis, MO), 180–200 g male Sprague-Dawley rats by the method of Rodbell (11), as modified by Cushman (12). Briefly, epididymal fat pads were removed, minced, and digested at 37°C with crude collagenase (20 mg/6 g fat pad, Worthington Biochemicals) in KRB (10 mM) buffer, pH 7.4, containing 10 mg/ml BSA (bovine serum albumin powder, Fraction V, Reheis Chemical Co.). After 45–60 min of digestion, the liberated cells were washed five times by centrifugation (1 min at 600 *g*) and resuspended in the same buffer.

Glucose oxidation bioassay. Isolated rat adipocytes (~ 5×10^4/flask) were incubated for 2 h at 37°C in 1.5 ml KRB buffer containing 0.1 mM glucose and 0.1 μCi/ml 1-[^{14}C]glucose in rubber capped polyethylene bottles in a metabolic shaker bath. After that, 0.5 ml of 6 N H_2So_4 was added and the incubation continued for another hour. The generated $^{14}CO_2$ was trapped in No. 1 Whatman filter paper, which had been soaked with hyamine hydroxide (0.4 ml) and positioned in wells hung inside the incubation bottles. After 1 h the wells were cut off, submerged in ACS (Amersham Corp., Arlington Heights, IL) scintillation liquid and counted in an automatic scintillation counter. All tests were performed in sextuplicate.

Preparation of IgG. IgG was prepared by affinity chromatography with protein A-Sepharose as described (13).

Analytical procedures. Glucose was determined with a Beckman glucose analyzer (Beckman Instruments, Palo Alto, CA). Free insulin was measured by RIA (14). Plasma antiinsulin antibodies were determined according to Gerbitz and Kemmler (15) using acidified charcoal to strip the sera from bound insulin before determining their insulin binding capacity. Statistical evaluation was performed with the paired or unpaired Student *t* test.

Results

^{125}I-Insulin receptor binding and displacement

Nonspecific binding, i.e., radioactivity immunoprecipitated after incubation of ^{125}I-labeled insulin receptor with buffer alone was 4.1±0.3% (n = 24). ^{125}I-insulin receptor binding after incubation with control sera (dilution 1:100, n = 43) was 3.4±0.3% (after subtraction of nonspecific binding). Intraassay (n = 19) and interassay (n = 6) coefficients of variation were 0.07 and 0.12, respectively. There was no difference in ^{125}I-insulin receptor binding by control sera (dilution 1:100, n = 8) and by the equivalent amounts of IgG extracted from these sera (3.2±0.5% vs. 2.5±0.3%, NS). ^{125}I-insulin receptor binding increased in a concentration dependent fashion after incubation with sera from four patients with type B severe insulin resistance, reaching 20, 31, 41, and 48% at serum dilutions of 1:100 for patients 2, B-10, 1, and D.L., respectively (Fig. 2, left panel). In contrast, ^{125}I-insulin receptor binding after incubation with increasing concentrations of normal sera (n = 14) increased to a maximum of 3.0±0.4%.

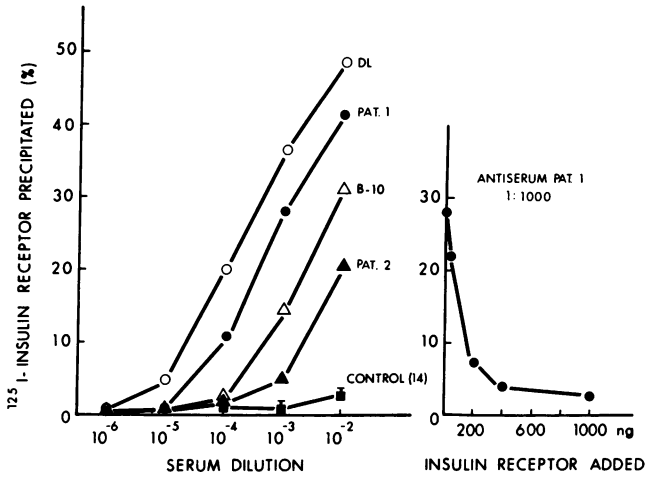

Figure 2. (*Left panel*) Effects of serial dilutions of 4 sera from patients with type B severe insulin resistance. ^{125}I-labeled, purified, human placental insulin receptors were incubated for 24 h at 4°C with serum. Bound ^{125}I-insulin receptor was precipitated with rabbit anti-human gamma-globulin. Number of control sera is indicated in parentheses. (*Right panel*) Displacement of ^{125}I-insulin receptor bound to an AIRA containing serum (from patient 1, dilution 1:1,000) by unlabeled insulin receptor protein. Shown are means of triplicate determinations.

The right panel of Fig. 2 shows displacement of ^{125}I-insulin receptor bound to serum from patient 1 (dilution 1:1000). Addition of unlabeled insulin receptor resulted in a progressive decrease of ^{125}I-insulin receptor binding from 28% (no unlabeled receptor added) to 2.4% with addition of 1,000 ng of receptor protein. Addition of unlabeled insulin or glucagon in doses ranging from 100 to 10,000 ng/ml did not affect insulin receptor binding.

Insulin receptor binding to sera from diabetic, rheumatic, and PCO patients

Insulin receptor binding to sera (dilution 1:100) from one of eight patients with PCO, 1 of 34 patients with rheumatic diseases, 5 of 51 patients with IDDM, 5 of 53 patients with NIDDM, and all 4 patients with type B severe insulin resistance exceeded, in four or more consecutive assays, the normal range defined as the mean±3 SD of 43 normal control sera (Fig. 3). ^{125}I-insulin receptor binding by the IRIP positive sera was concentration dependent (Fig. 4), and was comparable with serum and IgG. As seen in Fig. 5 (right panel) insulin receptor immunoprecipitation with seven IRIP positive sera (dilution 1:1000) and equivalent amounts of IgG prepared from these sera were highly correlated (r = 0.98, P < 0.001). At a 1:100 dilution (left panel) the correlation was weaker (r = 0.73, P < 0.05) and serum bound slightly more receptor than IgG suggesting some nonspecific binding with serum. Clinical characteristics of the 12 IRIP positive patients are presented in Table II.

Inhibition of insulin binding

Cultured rat hepatocytes. Insulin binding to hepatocytes, preincubated with sera (dilution 1:100) from all 8 patients with PCO, all 34 patients with rheumatic diseases, all 51 patients with IDDM and all 53 patients with NIDDM was within the normal range (defined as the mean±3 SD of 25 normal con-

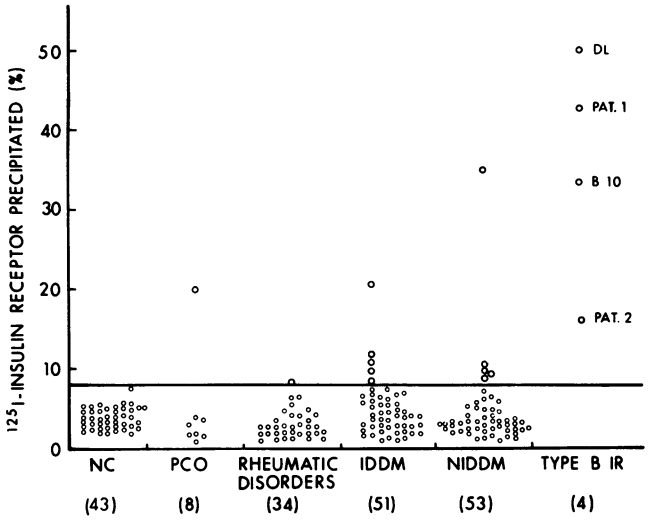

Figure 3. ^{125}I-insulin receptor binding to sera (dil. 1:100) from normal controls (NC, n = 43) and from patients with polycystic ovary syndrome (PCO, n = 8), rheumatic disorders (n = 34), insulin dependent diabetes (IDDM, n = 51), (NIDDM, n = 53), and type B severe insulin resistance (n = 4). The horizontal lines indicate the normal range (mean±3 SD of 43 normal controls).

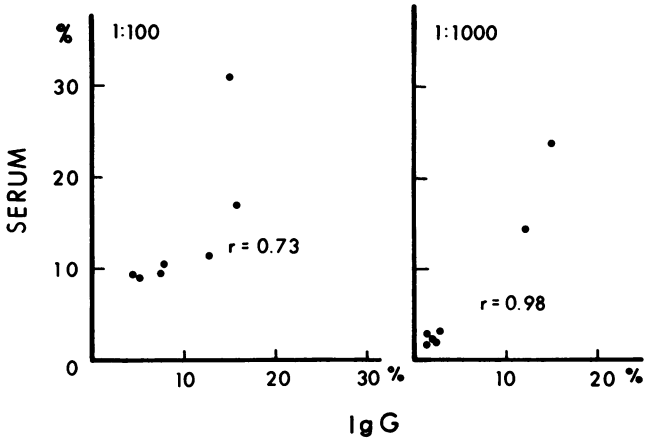

Figure 5. Correlation between ^{125}I-insulin receptor binding to 7 AIRA containing sera (vertical axis) and equivalent amounts of IgG extracted from these sera (horizontal axis). The left panel shows 1:100 dilution, the right panel 1:1,000 dilution of serum and IgG.

trols). Three of four type B sera (patients 1, B-10, and D.L.) inhibited insulin binding to 36, 29, and 51% of normal, respectively, while patient 2 serum was not inhibitory at this dilution.

This latter finding indicated that the hepatocyte assay was less sensitive than the IRIP assay. We, therefore, retested all 12 IRIP positive sera and the 4 type B sera at a tenfold higher concentration (1:10, Table III, left panel). At this dilution, all four type B sera were strongly inhibitory. Serum and IgG from one NIDDM patient (E.T.) significantly inhibited insulin binding (by 24%) while the other 11 IRIP positive sera remained noninhibitory.

Human placental membranes. Next, we examined the possibility that the failure of 11 of the 12 IRIP positive sera to inhibit insulin binding may have been due to insulin receptor species differences (the hepatocytes were of rat, the sera of human origin). However, when we tested the effects of these sera on insulin binding to human placental membranes, the results were comparable to those obtained with hepatocytes (Table III). Type B sera were all strongly inhibitory, while all IRIP positive sera were not inhibitory. Serum from patient E.T. inhibited insulin binding by 14% but this effect was not statistically significant.

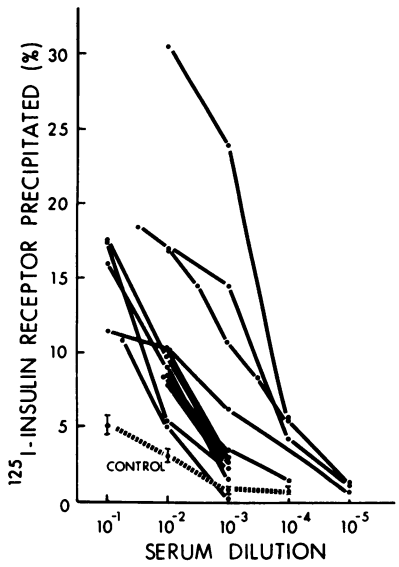

Figure 4. Effects of serial dilution of 12 IRIP positive sera and 14 control sera on ^{125}I-insulin receptor binding. Because of their scarcity, some sera were not tested at higher concentrations (1:10).

IGF-1 receptor binding

Insulin and IGF-1 are closely related peptides with a high degree of amino acid sequence similarity. In addition, insulin and IGF-1 receptors are similar in size and structure (16), and insulin receptors have some affinity for IGF-1 and vice versa (17). It was conceivable, therefore, that antibodies against the IGF-1, rather than against the insulin receptor, were responsible for the ^{125}I-insulin receptor binding of the 12 IRIP positive sera. To explore this possibility, we determined binding of these sera to pure, human placental ^{125}I-labeled IGF-1 receptors. As seen in Fig. 6 (left panel), IGF-1 receptor binding by 10 of the 12 sera fell within the normal range (mean±3 SD of normal controls). IGF-1 receptor binding by two (J.K. and R.C.) slightly exceeded the normal range but only at the highest serum concentration (1:10). Moreover, with one exception (R.C.), where insulin and IGF-1 receptor binding were similar, all sera bound less IGF-1 receptor than insulin receptor (compare Figs. 4 and 6). In addition, none of the 10 sera tested (two were no longer available) inhibited ^{125}I-IGF-1 binding to human placental membranes (Table IV). Together, these findings indicated, that the IRIP positive sera did not contain antibodies against the IGF-1 receptor and that antibodies against the insulin receptor were responsible for the increased insulin receptor binding.

IGF-1 receptor binding by two of the four type B sera exceeded the normal range. One of those two (patient 1) bound considerably less IGF-1 receptor than insulin receptor (compare Fig. 6, right panel, with Fig. 2, left panel). Serum from the other (patient D.L.) exhibited comparable binding affinity to both receptors. The capacity of these sera to bind IGF-1 receptors correlated well with their ability to inhibit IGF-1 bind-

Table II. Clinical Characteristics of 12 Patients with AIRA

Patients	Sex	Age	Height/Weight	IRI	Insulin antibodies	Duration of diabetes	Insulin requirements	Comments
		yr	*cm/kg*	*μU/ml*		*yr*	*U/d*	
NIDDM								
E.T.	F	61	165/106	95	Yes	32	200	AN, IR, Vitiligo, ↑Sed. rate
H.E.	F	58	157/123	15	No	12	0	HT, Osteoarthritis
W.H.	M	70	178/75	136	ND	3	0	AN, IR
R.M.	F	63	161/73	20	Yes	30	40	+ Syph. serology
R.C.	F	71	168/108	48	Yes	16	82	AN, HT, cholesterol
IDDM								
K.W.	F	40	169/82	10	Yes	37	40	
J.K.	M	16	160/55	25	Yes	8	45	Hashimoto's thyroiditis
C.H.	F	9	137/38	16	No	0	35	Hashimoto's thyroiditis Hypopituitarism
M.S.	M	31	196/95	75	Yes	1	33	
A.R.	F	45	165/62	24	Yes	25	32	Hashimoto's thyroiditis Hypoglycemia
Rheumatic disease								
E.M.	F	81	157/73	22	No	—	—	Rheumatoid arthritis
PCO								
I.B.	F	37	124/64	15	No	—	—	Multiple sclerosis

AN, acanthosis nigricans; HT, hypertension; IR, insulin resistance.

Table III. Effects of Sera on Insulin Binding

Patients	Cultured rat hepatocytes		Crude human placental membranes
	Serum (1:10)	IgG	Serum (1:10)
NIDDM			
E.T.	76%*‡	76%‡	86%
R.C.	84%	94%	104%
R.M.	93%	96%	92%
W.H.	102%		—
H.E.	100%		90%
IDDM			
K.W.	102%		111%
C.H.	99%		104%
J.K.	103%		90%
M.S.	103%		91%
A.R.	102%		91%
Rheumatic disease			
E.M.	103%		95%
PCO			
I.B.	98%		92%
Type B			
Patient 1	6%§	5%§	15%§
D.L.	5%§		11%§
Patient 2	52%§		65%§
B-10	5%§		—

* Expressed as percent ^{125}I-insulin binding after preincubation with control sera.
‡ $P < 0.05$.
§ $P < 0.01$.

ing to human placental membranes (Table IV). Serum from patient D.L. (dilution 1:10) was the most potent (71% inhibition) followed by serum from patient 1 (46% inhibition). These data suggested that serum D.L. and probably also serum from patient 1 contained binding-inhibitory antibodies against the insulin as well as the IGF-1 receptor (18).

Glucose oxidation

Stimulation of glucose oxidation (1-[^{14}C]glucose to $^{14}CO_2$) by insulin (100 ng/ml) was 380±27% (range 300–495%) of buffer controls (Fig. 7). Stimulation of glucose oxidation by control sera (dilution 1:50, $n = 8$) was 14.2±11.9% (mean±SD) of the effect of 100 ng/ml insulin. 9 of 10 IRIP positive sera (two were no longer available) did not stimulate glucose oxidation significantly more than control sera. In contrast, stimulation by serum E.T., which also inhibited insulin binding to hepatocytes, was significantly elevated (85±28%) and comparable to that of the two type B sera tested (D.L. and patient 1, 85±24% and 91±20%, respectively).

AIA and insulin concentrations

5 of 10 IRIP positive sera tested contained AIA, i.e., they bound more insulin than 25 control sera (2.9±0.48%, range 0–9%). Insulin binding, however, did not correlate with insulin receptor binding ($r = 0.35$, NS).

Six of the IRIP positive sera had elevated serum insulin concentrations (range 16–95 μU/ml). Serum insulin also did not correlate with insulin receptor binding ($r = 0.06$, NS).

Discussion

To test for AIRA, which do not inhibit insulin binding, we have developed a new immunoprecipitation assay. This assay

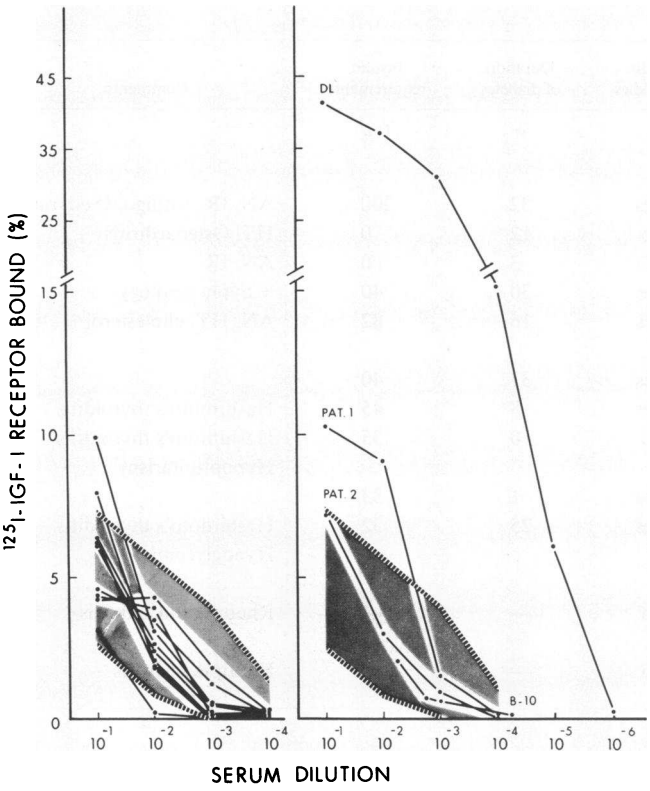

Figure 6. Effects of serial dilutions of 10 IRIP positive sera (left panel) and of 4 type B severe insulin resistance sera (right panel) on ^{125}I-IGF-1 receptor binding. The shaded area indicates the normal range (mean±3 SD of 10 normal controls).

measured the ability of AIRA of any type to bind ^{125}I-labeled, highly purified, human placental insulin receptors. Its validity was supported by the demonstration, that sera from four patients with type B severe insulin resistance (known to contain AIRA), but not normal sera, increased ^{125}I-insulin receptor binding in a dose dependent manner. Moreover, ^{125}I-insulin receptor bound to AIRA could be displaced by nonlabeled insulin receptor protein, but not by insulin or glucagon in concentrations up to 10^{-6} M. Other immunoprecipitation assays have been reported which, instead of ^{125}I-insulin receptor, used ^{125}I-insulin bound (19) or crosslinked (20) to insulin receptors on crude human placental membrane fractions. Since ^{125}I-insulin was attached to the receptor in these assays, insulin antibodies could also be bound and had to be removed before AIRA could be determined.

Table IV. Effects of Sera on IGF-1 Binding to Human Placental Membranes

Patients	Serum* (1:10)	IgG (1:5)
NIDDM		
E.T.	89%	
R.C.	95%	
R.M.	86%	
H.E.	91%	
IDDM		
K.W.	88%	
C.H.	91%	
J.K.	96%	
M.S.	97%	
PCO		
J.B.	92%	
Type B insulin resistance		
Patient 1	54%§	28%§
D.L.	29%§	27%§
Patient 2	95%	75%‡

* Expressed as percent ^{125}I-insulin binding after preincubation with control sera.
‡ $P < 0.05$.
§ $P < 0.01$.

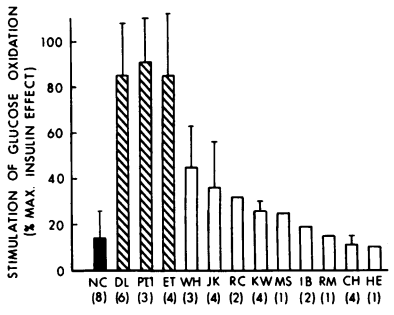

Figure 7. Effects of sera (dilution 1:50) from 8 normal controls (*black columns*), 3 patients with binding-inhibitory AIRA (*hatched columns*), and 9 patients with nonbinding-inhibitory AIRA (*open columns*) on $^{14}CO_2$ production from 1-[^{14}C]-glucose from isolated rat adipocytes. Data (mean±1 SD) are presented in percent maximal insulin effect (100 ng/ml) during a 2-h incubation at 37°C. Sera from D.L., patient 1, and E.T. stimulated significantly more $^{14}CO_2$ production than controls ($P < 0.01$).

Using the new IRIP assay, we found that 12 sera and their IgGs (5/51 patients with IDDM, 5/53 with NIDDM, 1/8 with PCO and 1/34 with rheumatic diseases) were positive, i.e., ^{125}I-insulin receptor binding after preincubation with these sera (dilution 1:100) or equivalent amounts of IgG exceeded the normal range defined as the mean±3 SD of 43 control sera. This suggested the presence of AIRA of the IgG subclass in these 12 sera. 11 of these 12 positive sera (all except one NIDDM serum), failed to inhibit insulin binding to cultured hepatocytes, even when used at high concentrations (dilution 1:10). The possibility was considered, that their failure to inhibit insulin binding was caused by species specificity. Chemical and immunological differences in insulin receptors, prepared from different species, have been reported (21, 22). We found, however, that the IRIP assay positive, hepatocyte assay negative sera also did not inhibit insulin binding to human placental membranes. In addition, it appeared unlikely, that the greater sensitivity of the IRIP assay resulted in detection of low-titer AIRA only in the IRIP but not in the hepatocyte or placental membrane assays. Three of the 11 sera reacted stronger in the IRIP assay than serum from a patient with type B severe insulin resistance (patient 2, Fig. 4), yet were negative in both binding-inhibition assays, while patient 2 serum was inhibitory in both (Table III).

Lastly, the close chemical and immunological similarities between the insulin and IGF-1 receptors made it necessary to exclude the possibility that some or all of the sera contained antibodies against the IGF-1 rather than against the insulin receptor. Using ^{125}I-labeled, highly purified IGF-1 receptors in an immunoprecipitation assay, we could show, however, that only 2/12 IRIP positive sera had low titers of antibodies against the IGF-1 receptor, or alternatively, had AIRA which cross-reacted with the IGF-1 receptor.

The combined evidence suggested that 11 of the 12 IRIP positive sera contained antibodies that bound to the insulin receptor (giving the positive IRIP results) but at a locus different from the insulin binding site (giving the negative hepatocyte or placental membrane binding-inhibition results). To our knowledge, such antibodies have not previously been reported in human subjects. In mice, Kull et al., however, have produced a monoclonal AIRA that specifically precipitated ^{125}I-insulin receptor complexes but did not inhibit insulin binding, indicating, that it interacted with the receptor at a site different from the insulin binding site (3). Furthermore, evidence for the presence of different species of AIRA has recently been provided by De Pirro et al. (23). They have shown, that IgG obtained from a patient with type B severe insulin resistance could be fractionated into several protein populations which differed in their abilities to inhibit insulin binding and to exert insulin-like activities. Presence of different populations of AIRA has also been postulated by Kahn et al. (24) based on their observation that serum from one patient with type B severe insulin resistance (B-3) stimulated glucose oxidation maximally but inhibited insulin binding weakly whereas serum from another patient (B-2) was equally potent in inhibiting insulin binding and stimulating glucose oxidation.

Of interest was the frequent association of the nonbinding-inhibitory AIRA with autoimmune diseases (Table II). The patient with polycystic ovary syndrome and AIRA had multiple sclerosis. Of the five patients with IDDM and AIRA, two had Hashimoto's thyroiditis, a third had Hashimoto's thyroiditis and idiopathic hypopituitarism. These findings are in accord with the well recognized high prevalence of autoimmune phenomena in IDDM (25). Also of note was that 4 of our 53 NIDDM sera contained AIRA of the nonbinding-inhibitory type and that 2 of these 4 patients had acanthosis nigricans. Autoantibodies are known to occur in NIDDM, albeit less commonly than in IDDM. For instance, as reviewed by Kaldany (26), 54/851 patients with NIDDM (6.3%) had circulating islet cell antibodies. In addition, one animal model for NIDDM, the New Zealand obese mouse, develops obesity, glucose intolerance, insulin resistance and low titers of antiinsulin receptor antibodies (27).

The similar incidence, in this study, of non-binding-inhibitory AIRA in patients with IDDM and NIDDM, 2 diseases with apparently different pathogenesis suggested, that the antibodies may have been a consequence rather than the cause of the metabolic alterations. It is possible, for instance, that insulin receptors may be altered in diabetes and give rise to the formation of antibodies.

The functional significance of these antibodies remained uncertain. In vitro, unlike binding-inhibitory AIRA (24), nonbinding-inhibitory AIRA did not inhibit insulin binding, nor did they stimulate glucose oxidation. As far as in vivo activities are concerned, it is noteworthy that none of the five IDDM patients appeared to be insulin resistant (as judged by their daily insulin requirements), while only one of four NIDDM patients had hyperinsulinemia (not associated with obesity) which was probably indicative of insulin resistance (Table II). Except for this one patient (W.H.) there was no evidence, that these antibodies interfered with the action of insulin, nor that they activated the insulin effector system analogous to the way by which some plant lectins, such as concanavalin-A and WGA, mimic insulin actions (28, 29). Insulin requirements and insulin levels, however, are rather crude parameters to assess insulin resistance and more in vitro and in vivo studies are needed to evaluate possible biological effects of these antibodies.

A second objective of the study was to determine the prevalence of conventional binding-inhibitory AIRA in the diabetic population. While the number of patients studied was not large enough to define their precise prevalence our data suggested, nevertheless, that these AIRA appeared to be rare. We were able to find only 1 among 104 diabetic sera, which bound insulin receptors, inhibited insulin binding to hepatocytes and exerted insulin-like activity (stimulation of glucose oxidation) to a significantly greater extent than normal control sera. Since all these effects could be reproduced with the patient's IgG, the evidence indicated, that this serum contained low titers of IgG binding-inhibitory AIRA. Flier et al., in their original report describing the syndrome of severe insulin resistance caused by AIRA, were the first who also studied the effects of 13 diabetic sera on insulin binding to various target cells including IM-9 lymphocytes (1). Inspection of Figure 2 of their paper suggested that none of the diabetic sera were inhibitory, whereas one of five SLE sera may have modestly inhibited insulin binding. A somewhat higher prevalence than observed in this study was recently reported by Ludwig et al. who found, that 3/29 newly diagnosed children with diabetes had low titers of IgG binding-inhibitory AIRA (30). These investigators used 2 SD above and below the mean to define the normal range. Inspection of their data suggested, that none of their sera would have been positive, had they used 3 SD (as we did). On the other hand, it is, of course, possible that we may have underestimated the prevalence of low-titer binding-inhibitory AIRA by using a wider normal range.

In addition, Maron et al. reported AIRA of the IgM class in 10 of 22 young, untreated IDDM patients, none of whom had stigmata of the type B syndrome (31). These investigators defined AIRA by the sera's ability to stimulate lipogenesis in rat adipocytes. Since many substances other than AIRA can exert insulin-like bioactivities, it is not certain, that their sera contained AIRA.

Our patient with binding-inhibitory AIRA was a 61-yr-old obese (233 lbs., 65 in.) female with acanthosis nigricans (periorbital and elbows), vitiligo and insulin resistant NIDDM (her blood sugar concentration remained > 200 mg/dl on 200 U of insulin and 750 mg of chlorpropamide per d). This patient illustrated, that there are individuals with low titers of AIRA who, on clinical grounds alone, could not be distinguished from the large number of similar appearing patients with idiopathic NIDDM.

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