Insulin Antibodies in Insulin-Dependent Diabetics Before Insulin Treatment

Abstract. *A sensitive assay was used to measure the binding of iodine-125–labeled insulin in serum obtained from 112 newly diagnosed insulin-dependent diabetics before insulin treatment was initiated. Two groups of nondiabetics served as controls: children with a variety of diseases other than diabetes and nondiabetic siblings of insulin-dependent diabetics. Eighteen of the diabetics were found to have elevated binding and 36 were above the 95th percentile of control values. The insulin-binding protein is precipitated by antibody to human immunoglobulin G, has a displacement curve that is parallel and over the same concentration range as serum from long-standing insulin-dependent diabetics, and elutes from a Sephacryl S-300 column at the position of gamma globulin. These insulin antibodies are present in a large percentage of newly diagnosed, untreated diabetics and may be an immune marker of B-cell damage.*

Data from animal models and insulin-dependent diabetics (IDD's) suggest that autoimmunity is involved in the pathogenesis of type 1 diabetes. Islet cell antibodies (ICA's) reacting with antigens in the cytoplasm or on the surface of islet cells are present in a large proportion of IDD's at diagnosis (*1*), and the cell-surface ICA is toxic to B cells (*2*). Islet cell antibodies have been found in individuals years before the onset of clinical diabetes and probably serve as a marker for the B-cell destructive process (*3*). Other less specific immunological abnormalities, such as lymphocytic infiltration of the islets, leukocyte migration inhibition, and raised K-cell levels are also common in IDD's and support the autoimmune hypothesis (*4*).

The development of antibodies to insulin is an accepted consequence of insulin therapy; their presence in hypoglycemic patients never treated with insulin is rare but well documented (*5*). Insulin antibodies have not been considered to be part of the autoimmune process in patients with type 1 diabetes (*6*). We used a sensitive assay for detecting insulin binding in serum and report that, before insulin therapy was initiated, at least 18 percent of a group of IDD's had proteins in their serum that bound insulin and were probably insulin antibodies.

Three groups of subjects were evaluated: 101 children hospitalized for a variety of conditions besides diabetes, 55 nondiabetic siblings of IDD's, and 112 newly diagnosed IDD's. Inclusion criteria for the diabetics were classical, acute-onset, type 1 diabetes with ketonuria or ketonemia, age between 5 and 20 years, body weight less than 105 percent of ideal, and no prior insulin therapy. Serum was tested for insulin binding by using a slight modification of the method of Kurtz *et al.* (*7*). Serum (20 μl) was incubated for 24 hours at 4°C with 200 μl of 0.04*M* phosphate buffer (*p*H 7.5) containing 0.5 percent bovine serum albumin, 0.25 percent bovine gamma globulin, 0.9 percent sodium chloride, and ^{125}I-labeled insulin diluted to approximately 20,000 count/min per tube. Bound tracer was precipitated by adding 1.5 ml of ice-cold 15 percent polyethylene glycol prepared in 0.05*M* Veronal buffer (*p*H 8.6) with 0.1 percent Tween 20 and centrifuging at 2500 rev/min for 30 minutes at 4°C. The pellet was washed once with 12.5 percent polyethylene glycol, centrifuged again, and counted. The mean binding of labeled insulin by 20 μl of serum from the nondiabetic children and the nondiabetic siblings was 1.3 percent (range, 0.9 to 2.7 percent) and 1.7 percent (range, 0.8 to 2.9 percent), respectively (Fig. 1). Many of the diabetics had binding within the normal range, but if upper range (2.9 percent) or mean plus 5 standard deviations (2.8 percent) in the controls are considered as maximum normal levels, then 18 of the diabetics had elevated insulin binding. Thirty-six showed binding above the 95th percentile of the control subjects.

We tested the ability of unlabeled insulin to displace the insulin tracer. High-purity porcine insulin (0.01 to 10.0 ng) was added to the assay. Eleven of the newly diagnosed, untreated IDD's with the highest binding were compared to 12 other diabetics who had been treated with insulin for 1 year and who had had normal insulin binding at diagnosis (Fig. 2). Increasing amounts of unlabeled insulin displaced tracer from the serum of both groups in parallel and over the same concentration range, suggesting that the binding proteins in the newly diagnosed diabetics are specific for insulin and similar to classical insulin antibodies in their

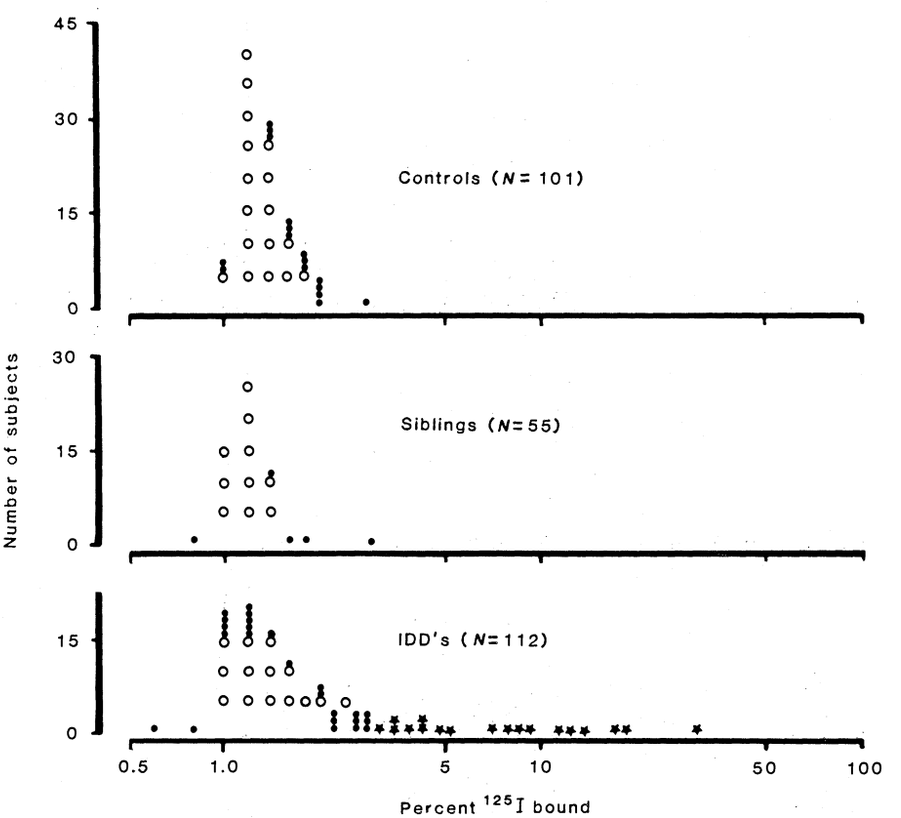

Fig. 1. Binding of ^{125}I-labeled insulin from serum of nondiabetic controls, nondiabetic siblings of insulin-dependent diabetics, and newly diagnosed, untreated, insulin-dependent diabetics. For subjects with normal binding, (○) indicates five individuals and (●) single individuals; (★) represents subjects with elevated binding (greater than 5 standard deviations above the mean value for nondiabetics).

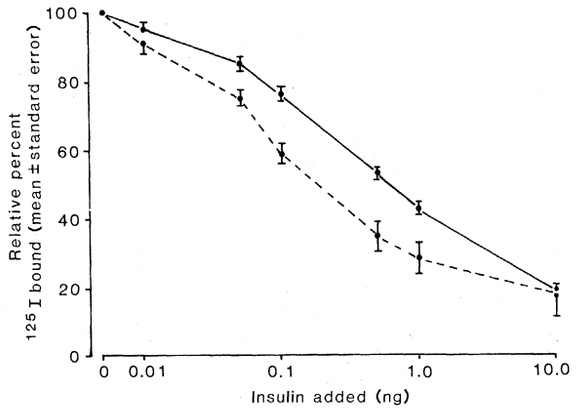

Fig. 2. Displacement of ^{125}I-labeled insulin from serum by increasing concentrations of unlabeled insulin. Eleven insulin-dependent diabetics treated with insulin for 12 months (continuous line) are compared with 11 newly diagnosed, untreated, insulin-dependent diabetics known to have elevated binding (dashed line).

binding characteristics. This competition between tracer and unlabeled insulin was similar to that seen in standard insulin radioimmunoassays, also suggesting that the binding proteins in our diabetics are in fact antibodies.

One milliliter of serum from one of the newly diagnosed diabetics was applied to a Sephacryl S-300 column (0.7 by 3.0 cm). The insulin-binding protein eluted in the same position as gamma globulin, suggesting that in this patient the protein had a molecular weight similar to that expected for insulin antibodies.

To further establish that the insulin-binding proteins in the newly diagnosed diabetics were insulin antibodies, we precipitated the proteins with goat antiserum to human immunoglobulin G (IgG). The assay was performed exactly as described above, except that 60 μl of goat antiserum to human gamma globulin (Antibodies, Inc.) plus 3 percent polyethylene glycol in 0.04*M* phosphate buffer was used instead of the 15 percent polyethylene glycol. A single wash with 3 percent polyethylene glycol in 0.04*M* phosphate buffer was performed. Binding of ^{125}I-labeled insulin in ten of the diabetics previously found to have elevated binding was 33.4, 26.2, 11.1, 8.8, 8.5, 6.6, 4.9, 4.9, 4.7, and 4.5 percent, whereas binding in ten control children remained low (1.1 ± 0.04 percent). Control runs with goat antiserum to rabbit IgG in place of the antiserum to human IgG gave similarly low values in controls and diabetics.

A possible explanation for our detection of insulin antibodies before exposure to insulin and the failure of others to do so involves the ability of our assay to detect small amounts of excess binding. If 5 percent binding were arbitrarily considered to be the detection limit of the assay, the limit of sensitivity would be 2×10^{-3} μU bound per milliliter of insulin. In contrast, the assays that have been employed by others are less sensitive (*8–13*). The nonspecific binding or binding of tracer by the serum of nondiabetics in our assay is very low (mean, 1.2 percent). There are probably two major reasons for this. The first is our use of the assay methodology of Kurtz *et al.* (*7*). Previous assays measured nonspecific binding at about 7 percent (*14*), 6 percent (*12*), 5 to 15 percent (*15*), and 3 percent (*13*). With low nonspecific binding it becomes possible to distinguish diabetics with a small amount of insulin-binding protein from nondiabetics. The second reason is the use of a high-purity tracer of high specific activity. We used A^{14} insulin tracer (*16*), which is virtually all mono-iodinated insulin and has a bioactivity like that of native insulin and a specific activity several times greater than that used in many previous studies. Since the insulin tracer (specific activity, ~ 360 μCi/μg) is contaminated with very little unlabeled insulin, the competition for binding between added tracer and unlabeled insulin is markedly reduced. The A^{14} insulin tracer has lower nonspecific binding than other tracers in bioassays, and this same superiority may apply to the insulin-binding assay (*16*). Other potentially important assay differences include incubation at 4°C, the use of unextracted serum, and the low volume of serum tested.

There are additional reasons why insulin antibodies or other insulin-binding proteins have not been thought to be commonly present before insulin treatment. The presence of insulin-binding antibody in newly diagnosed diabetics may have been underestimated because the interval during which such antibodies can theoretically be detected may be very short. It is possible that circulating insulin antibody can be detected only when B-cell destruction is nearly complete. A similar mechanism has been proposed in glomerulonephritis, in which glomerulobasement membrane antibody becomes detectable in the serum only after removal of the kidneys (*17*). Very shortly after severe B-cell destruction the patients require insulin treatment and develop antibodies to the injected insulin. Also, if the diabetics have insulin in their serum when the sample is drawn, there is competition between this endogenous insulin and the tracer, lessening the chance of detecting binding in the diabetics. The displacement experiment suggests that the presence of about 5 to 10 μU per milliliter of tracer is sufficient to prevent detection of increased tracer binding in some patients. Ten nanograms of added insulin on the displacement curve is equivalent to approximately 1.25 μU/ml. Levels of insulin in this range or higher have been found in newly diagnosed IDD's (*18*). Furthermore, since the improvement in antibody assays, no one has, to our knowledge, studied a large number of untreated IDD's and nondiabetic controls.

Although most investigators have concluded that insulin antibodies are not present in untreated diabetics, others claim to have identified apparent autoantibodies in such patients. Sebriakova and Little (*12*) reported several untreated diabetics with binding above the sensitivity limit of their assay, and in one patient, bound tracer insulin was displaced by increasing concentrations of unlabeled insulin. Using a complement-consumption technique, Pav *et al.* (*19*) found apparent insulin antibodies in serum from diabetics who had never received insulin therapy. In addition, insulin-induced lymphocyte transformation was found in six of ten newly diagnosed IDD's, four of whom had never received insulin, suggesting lymphocyte sensitization to insulin or an endogenous insulin precursor (*20*).

These insulin antibodies may have been induced as a consequence of the B-cell destructive process. IDD's develop insulin antibodies when treated with human insulin (*21*), presumably because aggregation or some change in tertiary structure resulting from the manufacturing process makes the insulin immunogenic. A similar alteration in insulin structure may take place during B-cell destruction, with consequent development of insulin antibodies. Support for this hypothesis was recently provided by the observation that reovirus type 1–induced diabetes in mice is associated with insulin antibodies (*22*).

Our finding of insulin-binding proteins in IDD's before insulin treatment prompts many additional questions. Is there any relation between these insulin-binding proteins and classical ICA's? Does the development of insulin antibodies before insulin therapy depend in part on the degree of residual B-cell secre-

tion? Is the antibody response to exogenous insulin, especially human insulin, different in patients depending on the presence or absence of antibodies before diagnosis? Since the immune response to exogenous insulin may vary depending on type of histocompatibility antigen (HLA), is there any association between the presence of insulin antibodies before insulin treatment and either of the HLA types associated with type 1 diabetes, Dr3 or Dr4?

In summary, we have found insulin antibodies in at least 18 percent of a group of untreated IDD's. The antibodies may be present in a larger percentage of diabetics, but with the current assay their values overlap with normal values. This measurement may prove superior to ICA's as a marker of B-cell damage, since cytoplasmic ICA is not specific for B cells (*23*), cell-surface ICA is present in a high percentage of nondiabetics (*24*), and both ICA measurements are difficult to perform and require subjective interpretation. The ease of the insulin-binding assay may make it useful for large-scale studies.

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Biotinated Probe Containing a Long-Terminal Repeat Hybridized to a Mouse Colon Tumor and Normal Tissue

Abstract. *The cloned complementary DNA pMCT-1, which contains an intracisternal A particle long-terminal repeat, is more highly expressed in a mouse colon tumor than in the normal mouse colon. In situ hybridization of biotin-substituted pMCT-1 to fixed frozen sections shows that expression of pMCT-1 is seen throughout the tumor and is highly heterogeneous on a cellular basis, while expression is undetectable in any cell in the normal colonic mucosa.*

The complementary DNA clone pMCT-1 (*1, 2*) contains a mouse highly repetitive intracisternal A particle long-terminal repeat (*3*). This clone hybridizes to a population of transcripts that are heterogeneous in size and that show 50-fold higher expression in the dimethylhydrazine-induced transplantable mouse colon tumor from which it was derived (*4*) than in the normal mouse colonic mucosa (*2*). Expression is also high in several leukemia cell lines (*2, 5*). In the colon tumor RNA molecules that hybridize to pMCT-1 comprise approximately 0.8 percent of the polyadenylated RNA population.

As in all experiments that detect changes in gene expression in complex tissues, the question arises as to whether the differences seen between the colon tumor and normal colon represent alterations in the distribution of cell types. In situ hybridization of biotin-substituted pMCT-1 to frozen sections of tumor and normal tissues demonstrates that not only is expression undetectable in any cell in the normal colonic mucosa, but that expression is highly heterogeneous throughout the transplantable tumor. The results presented here illustrate the value of complementing investigations on gene expression with methods in which the architecture of the tissue and spatial relations between cells are preserved.

Frozen sections of freshly dissected colon tumor 36 grown in male BALB/c mice (*4*), normal mouse colon, and liver were hybridized to biotin-substituted pMCT-1 and pBR322, as described in the legend to Fig. 1. The photographs in Fig. 1 show sections fixed for 5 minutes in cold Carnoy's B fixative (60 percent ethanol, 30 percent chloroform, and 10 percent acetic acid) and briefly incubated at room temperature with autodigested Pronase (Calbiochem). In our experiments the use of 4 percent paraformaldehyde (*6, 7*) or ethanol and acetic acid (3:1 by volume) (*8*) as fixatives did not preserve morphology as well in frozen sections, but recent results show that 4 percent paraformaldehyde for 30 minutes at room temperature is the fixative of choice for Friend erythroleukemia cells in culture. Brief Pronase digestion is necessary to obtain optimum hybridization and hence signal, but without careful monitoring during this step, overdigestion and destruction of tissue can result. Detection of hybridization was primarily by indirect immunofluorescence.

The morphology of the cells and crypts of the normal mouse colon was well preserved (Fig. 1A). In 11 experiments with 37 sections from 7 normal mouse colons, no signal was ever detected with biotin-substituted pMCT-1. These results are not shown since they yield completely black photographs due to the necessity of filtering out all but the emitted light to record the signal. In contrast, hybridization of biotin-substituted pMCT-1 to the colon tumor always resulted in fluorescence throughout the tumor (Fig. 1C). A similar signal was seen in 13 sections from 8 different tumors. In addition, however, localized bright areas were usually seen, and in