Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 1281–1284, February 1993
Medical Sciences

Autoantibodies to the GLUT-2 glucose transporter of β cells in insulin-dependent diabetes mellitus of recent onset

(flow cytometry)

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Contributed by Roger H. Unger, October 29, 1992

ABSTRACT **Purified immunoglobulin G (IgG) from the serum of patients with insulin-dependent diabetes mellitus (IDDM) of recent onset inhibits high-K_m uptake of 3-*O*-methyl-β-D-glucose by rat pancreatic islets. To determine if the inhibition is the result of antibodies against GLUT-2, the high-K_m glucose transporter of β cells, we incubated IDDM sera with rat islet cells and with AtT-20_{ins} cells transfected to express GLUT-2. IDDM sera inhibited glucose uptake in islet cells and in GLUT-2-expressing AtT-20_{ins} cells but not in AtT-20_{ins} cells transfected to express the low-K_m isoform, GLUT-1. In 24 of 30 (77%) patients with newly diagnosed IDDM, IgG binding as measured by immunofluorescence and flow cytometry of the cells transfected to express GLUT-2 was >2 standard deviations from the mean of the nondiabetic population; 29 of 31 (96%) of nondiabetic children were negative ($P < 0.0001$). Increased IgG binding could be removed by absorption with GLUT-2-expressing cells but not with GLUT-1-expressing cells. We conclude that most patients with IDDM of recent onset have autoantibodies to GLUT-2.**

Purified immunoglobulin G (IgG) from patients with insulin-dependent diabetes mellitus (IDDM) of recent onset inhibits high-K_m uptake of 3-*O*-methyl-β-D-glucose (1), consistent with the presence of an autoantibody to the GLUT-2 glucose transporter of β-cells or to some other β-cell surface antigen affecting transport function. To determine if the inhibitory effect is the result of GLUT-2 autoantibodies, we compared the ability of diabetic and nondiabetic IgG to inhibit glucose transport and bind to non-β-cells transfected to express GLUT-2.

METHODS

Study Populations. Sera were obtained in compliance with the guidelines established for this study by the Human Studies Institutional Review Board. We studied sera from 31 patients (2–35 years of age, mean age 11.7 ± 7.2 years; 15 males and 16 females) with IDDM of no more than 2 months known duration and from 30 randomly selected children (1–20 years of age, mean age 8.5 ± 5.2 years; 16 males and 14 females) admitted to Children's Medical Center and free of IDDM, renal, or hepatic disease. IgG was purified from sera for glucose transport measurements (1), while heat-inactivated whole sera were used for immunofluorescence.

Islet Isolation. Islets were isolated and dispersed into a single-cell suspension by using the collagenase digestion–Ficoll gradient purification method described in full detail elsewhere (1).

Cell Lines and Cell Culture Conditions. The AtT-20_{ins} cells were grown in Dulbecco's modified Eagle's medium containing 25 mM glucose; 10% (vol/vol) fetal calf serum; and 50 units of penicillin, 50 μg of streptomycin, and 250 μg of Geneticin (GIBCO) per ml. Stable transfection of AtT-20_{ins} cells with rat GLUT-2 and GLUT-1 cDNA was achieved by electroporation (2). The GLUT-2 transfected cells, designated CGT6 cells, have a K_m of 17 mM and a V_{max} of 17 mmol/min per liter of cell space (2) for 3-*O*-methyl-β-D-glucose transport. These kinetics are very close to those of islet cells (3). The untransfected AtT-20_{ins} cell lines have a K_m of 2 mM and a V_{max} of 0.5 mmol/min per liter of cell space (2). GLUT-1-expressing AtT-20_{ins} cells have a K_m of 4.8 mM and a V_{max} of 5.6 mmol/min per liter of cell space.

Glucose Transport Measurements. The uptake of 3-*O*-methyl-β-D-glucose into islet cells, CGT6 cells, and GLUT-1-expressing AtT-20_{ins} cells was measured by the method described previously (1). All cells were incubated with 6.5 mg of IgG per ml for 15 min at 37°C prior to uptake measurements. Measurements of initial velocity of zero-trans uptake were made at 3 sec, 6 sec, and 15 sec in duplicate (1) with 6×10^5 cells. Results are means ± SEM for each time point for all cell types and IgG fractions.

Immunofluorescence/Flow Cytometric Analyses. AtT-20_{ins} cells and CGT6 cells were harvested and washed twice in Dulbecco's phosphate-buffered saline (PBS) (pH 7.6) by sedimentation at 500 × *g* for 30 sec at room temperature. Cells were divided into 1.5-ml Microfuge tubes at a density of ≈10^5 cells per tube, incubated for 1 hr at 4°C in 150 μl of patient sera, washed twice by centrifugation at 500 × *g* for 30 sec in PBS, resuspended in R-phycoerythrin-labeled goat anti-human heavy-chain-specific IgG (R-PEAb) (Fisher Scientific), and incubated for 1 hr at 4°C with occasional shaking. After two washes by centrifugation at 500 × *g* for 30 sec in PBS, cells were resuspended in 500 μl of PBS.

IgG binding was determined by flow cytometry performed on a FACScan (Becton Dickinson) flow cytometer. Forward-scatter threshold was set at 100 by using the E-01 forward-scatter detector. Linear amplifier gains were 6.18 for forward scatter and 1.22 for 90°-angle light scatter with a photomultiplier setting of 274 V. Forward and 90°-angle light scatter were read on linear scale, and fluorescence measurements were made on logarithmic scale. IgG binding was found to depend upon (*i*) the fraction of dead cells in the population, (*ii*) autofluorescence of the cultured cells, and (*iii*) nonspecific IgG binding from control sera to the cultured cells. Since dead cells bind IgG from both control and IDDM sera, propidium iodide was used to show that strongly positive events in the upper left hand region of Fig. 3 *Left* are dead. Therefore, we focused only on the other cell populations of

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Abbreviations: IDDM, insulin-dependent diabetes mellitus; GLUT, glucose transporter; R-PEAb, rhodamine-conjugated phycoerythrin-labeled goat anti-human heavy-chain-specific IgG.

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that histogram. Autofluorescence was corrected by using 530-nm emission to compensate for 575-nm emission with unstained cells. Voltages to the FL1 (530 nm) and FL2 (575 nm) photomultipliers were adjusted so that significant signals were obtained in the first decade of FL2 and compensation (FL1−FL2) was 45.9%. While this maneuver optimizes the R-PEAb signal, the ability to determine absolute amounts of IgG bound to the cells is compromised. Thus, relative display of IgG binding is observed. List mode data from 10^4 events per sample were acquired and stored for later analysis.

Flow Cytometric Analysis. Initially, representative FL2 histograms of all accumulated data for both diabetic and control specimens were compared for histogram morphology. Subsequently axes were defined that split positively stained but dead cells from live unstained cells that also show higher forward scatter. This defines a region that is sensitive to increased cellular FL2 fluorescence and reflects increased IgG bind to the surface of intact cells.

Preabsorption Experiments. For absorption studies, three 150-μl samples from selected patients with IDDM of recent onset or nondiabetic children were incubated with 10^5 CGT6 cells or with 10^5 AtT-20 cells for 1 hr at 4°C. The cells were sedimented at 5,000 × *g* for 60 sec, and the absorbed supernatant serum samples were analyzed for antibody binding to CGT6 cells as described above.

Statistical Analyses. Results of experiments were analyzed with the unpaired two-tailed *t* test except where otherwise indicated.

RESULTS

Effects of IgG from Diabetic Patients and Nondiabetic Subjects on 3-*O*-Methyl-β-D-Glucose Uptake by Islet Cells, CGT6 Cells, and GLUT-1-Expressing AtT-20_{ins} Cells. Purified IgG from seven patients with IDDM of recent onset significantly inhibited initial rates of 3-*O*-methyl-β-D-glucose uptake by rat islet cells by 40% compared with IgG from six nondiabetic controls ($P < 0.05$) (Fig. 1), confirming our earlier report (1) of 50% inhibition.

To determine if this inhibition is the result of an antibody against GLUT-2, the studies were repeated in CGT6 cells and AtT-20 cells transfected to overexpress GLUT-1. IgG from the same IDDM patients reduced the initial rate of glucose transport in GLUT-2-expressing CGT6 cells from 15.5 mmol of 3-*O*-methyl-β-D-glucose per min per liter of cell space to 6.2 mmol/min per liter of cell space ($P < 0.05$), a reduction of 60%.

Glucose uptake in GLUT-1-transfected AtT-20_{ins} cells treated with IgG from patients with IDDM of recent onset was identical to cells treated with IgG from nondiabetic individuals. Thus inhibition occurred only in GLUT-2-expressing cells.

Flow Cytometric Analysis of GLUT-2-Expressing CGT6 Cells After Treatment with Sera from Patients with IDDM and Nondiabetic Individuals. To explain GLUT-2 transport inhibition by diabetic IgG, flow cytometric analysis of IgG binding to intact CGT6 cells was carried out. Incubation of CGT6 cells with serum of patients with IDDM of recent onset produced a clear increase in R-PEAb binding, indicative of increased IgG binding to the surface of the intact cell. Some binding of IgG from nondiabetic patient sera also occurred (Fig. 2 *Top*).

Preabsorption of these sera with intact GLUT-2-expressing AtT-20_{ins} cells abolished the increase in R-PEAb fluorescence, while preabsorption with untransfected AtT-20 cells had no effect (Fig. 2). The fluorescence characteristics of R-PEAb-labeled CGT6 cells treated with sera from nondiabetic patients was unaffected by preincubation with either cell line (Fig. 2).

Specificity of Interactions of Sera from Patients with IDDM of Recent Onset and Nondiabetic Patients for CGT6 Cells. Because of binding of nondiabetic IgG to CGT6 cells, a regional analysis of fluorescent cell densities was performed to define the population of cells that reflected increased IgG binding from the sera of IDDM patients (Fig. 3, R_2 region). Because the R_2 region also includes dead cells, the shifts measured in these experiments are minimized because of the background contribution by dead cells. The R_2 region contained ≈37% of the CGT6 cells treated with sera from nondiabetic patients and 57% of the cells treated with sera from patients with IDDM of recent onset (R_2 region, Fig. 3).

To establish the specificity of IgG binding to intact CGT6 cells, analyses of IgG binding to the parental AtT-20_{ins} cells were performed. There was no difference in the fraction of cells found in R_2 when IDDM patient sera and nondiabetic patient sera were incubated with the nontransfected AtT-20_{ins} cell line (not shown).

Subtraction of the percentage of cells found in R_2 when using the nontransfected AtT-20_{ins} cell line from the percentage of cells found in R_2 when using the CGT6 cell line would be expected to reflect the specific binding of IgG to GLUT-2. Fig. 4 shows the results of such an analysis for each individual serum. A positive result was defined as an increase in IgG binding that was >2 standard deviations from the mean observed in the nondiabetic patient population. Twenty-nine of 31 (94%) of the nondiabetic population were negative for IgG binding to GLUT-2, while 23 of 30 (77%) of sera from

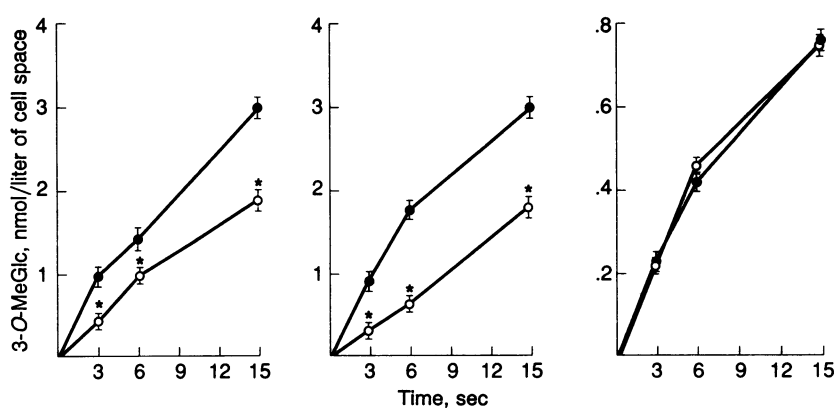

FIG. 1. Effects of purified IgG from nondiabetic subjects (●) and patients with IDDM of recent onset (○) on the uptake of 3-*O*-methyl-β-D-glucose (3-*O*-MeGlc) by dispersed rat islet cells (*Left*), CGT6 cells expressing GLUT-2 (*Center*), and GLUT-1-expressing AtT-20_{ins} cells (*Right*). Data points for islet cells and CGT6 cells are the means ± SEM uptake of 3-*O*-methyl-β-D-glucose by each cell type after incubation with purified IgG from six nondiabetic human sera and seven patients with recent-onset IDDM. Data for GLUT-1-expressing AtT-20_{ins} cells were from five nondiabetic individuals and six patients with recent-onset IDDM. The rate difference between curves with IgG from nondiabetic sera and sera from IDDM patients in islet cells and CGT6 cells are significant at $P < 0.05$.

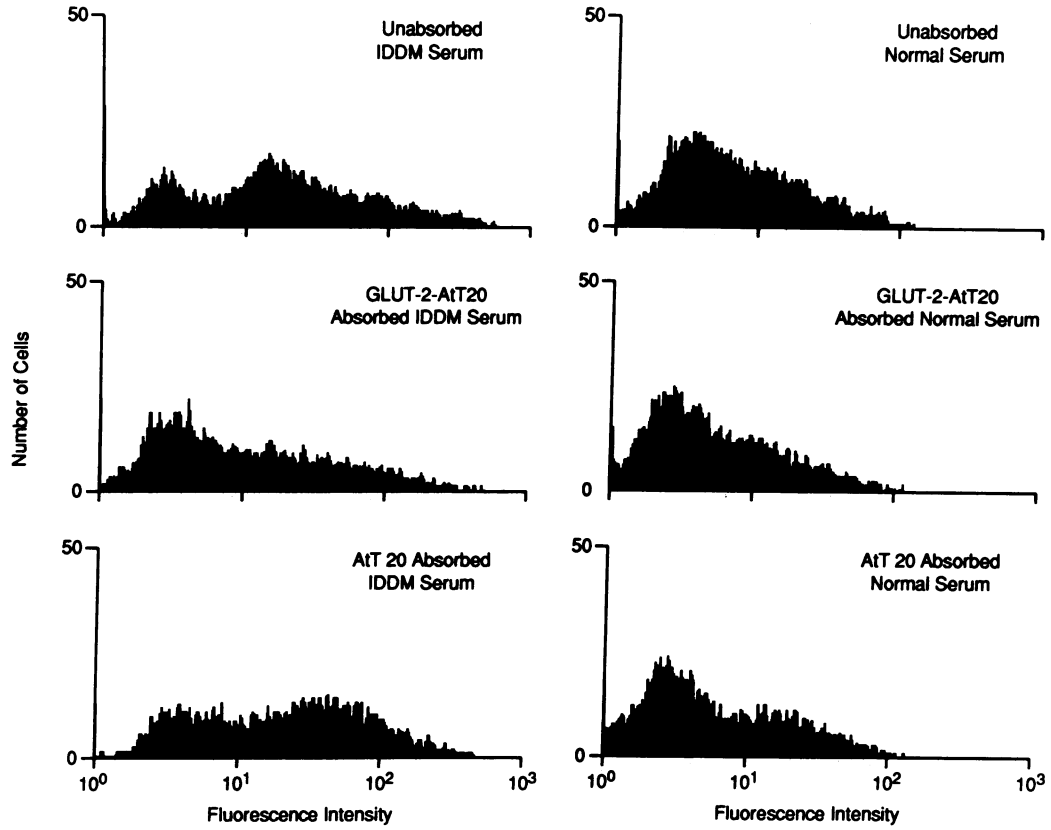

FIG. 2. Effect of preincubation of serum samples from patients with IDDM of recent onset (*Left*) or nondiabetic individuals (*Right*) with no additions (*Top*), intact CGT6 cells (*Middle*), or intact nontransfected AtT-20_{ins} cells (*Bottom*). Absorption of serum samples was accomplished by incubation of sera for 1 hr at 4°C with the appropriate cell type as described in text.

IDDM patients were positive. Thus, 81% of negative results were from nondiabetic patients and 92% of positive results were from patients with IDDM. The Youden index of these results gave $J = 0.73$, and the level of significance of the separation between the two populations was $P < 0.0001$.

DISCUSSION

Autoimmune diabetes is associated at its onset with autoantibodies to several β-cell antigens, including cytoplasmic islet cell antibodies (ICA-Cyt) (4), insulin autoantibodies (5), and antibodies to glutamic acid decarboxylase (GAD) (6). The idea of autoantibodies directed against the glucose transporter in IDDM was first suggested by the clinical observation that loss of glucose-stimulated insulin secretion preceded loss of other β-cell functions—an observation that was confirmed in BB/Wor rats (7, 8). The demonstration that partially purified IgG from patients with IDDM of recent onset could inhibit glucose-stimulated insulin secretion in perifused rat islets (9) taken together with our observation that IgG from patients with IDDM of recent onset inhibited high K_m glucose uptake by rat islets (1) raised the possibility of autoantibodies to GLUT-2. However, because antibodies from IDDM patients recognize multiple proteins from islet

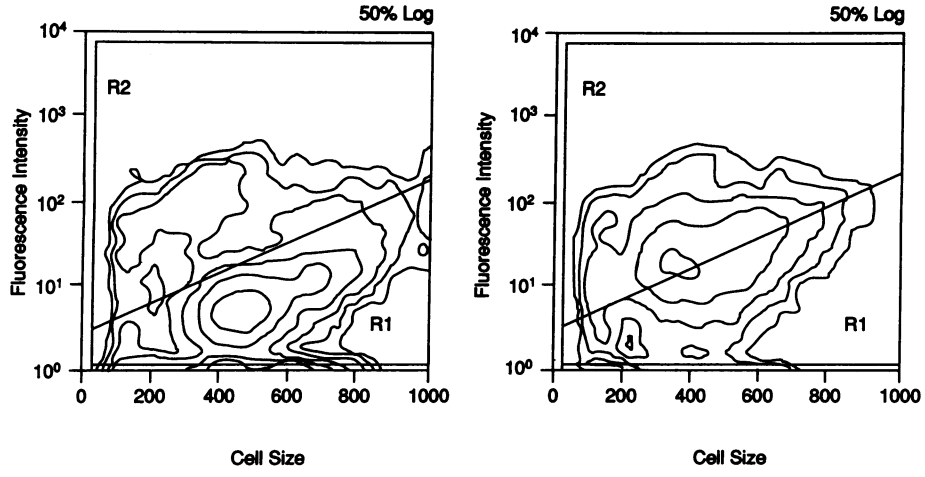

FIG. 3. Contour analysis of CGT6 cells after treatment with sera from nondiabetic individuals (*Left*) and patients with IDDM of recent onset (*Right*). Regional parameters R_1 and R_2 are defined by the diagonal line which is set parallel to the axis of the third 50% logarithmic density of labeled CGT6 cells after treatment with sera from nondiabetic individuals as described in text.

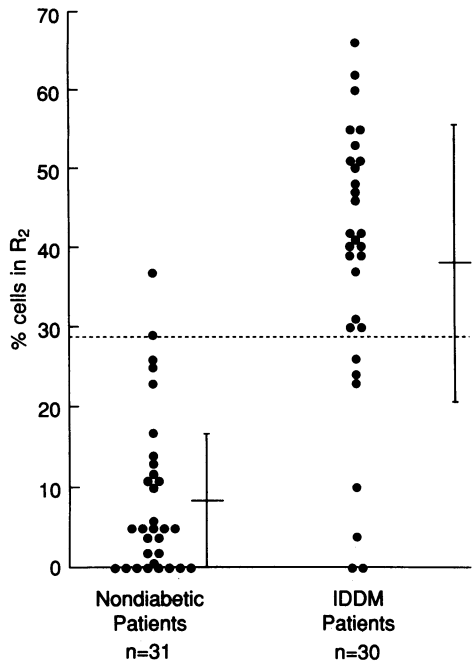

FIG. 4. Specific IgG binding to CGT6 cells determined by subtracting the percentage of cells found in R_2 when using nontransfected AtT-20_{ins} cells from cells found in R_2 when using CGT6 cells. Separation of the population of IDDM patients from the nondiabetic population is significant at $P < 0.0001$.

cells (4–6), it was possible that inhibition of glucose transport was secondary to antigen–antibody interactions other than GLUT-2.

The present report confirms the inhibitory action of IgG from patients with IDDM of recent onset upon glucose uptake by normal rat islets and describes comparable inhibition of glucose transport in non-islet-cells transfected to express GLUT-2. Since these CGT6 cells are not known to express any β-cell surface antigens other than GLUT-2 and because no effect was observed on glucose transport into AtT-20_{ins} cells overexpressing GLUT-1, these data strongly imply that antigen–antibody interaction with an epitope of GLUT-2 interferes with high K_m glucose transport.

The use of immunofluorescence and flow cytometry provided clear-cut evidence of increased IgG binding to GLUT-2-expressing AtT-20_{ins} cells by sera from patients with IDDM when compared with sera from nondiabetic subjects. Subtraction of variable nonspecific IgG binding to nontransfected AtT-20_{ins} cells enhanced the observed differences in binding to CGT6 cells. The increased binding of IgG of IDDM patients could be abolished by preincubation with CGT6 cells but not by untransfected AtT-20_{ins} cells.

Of the 30 sera tested from patients with recent-onset IDDM, 77% showed IgG binding that was >2 standard deviations from the mean of 31 control sera (Fig. 4). Of the sera that were >2 standard deviations from the mean of controls, 92% were from individuals with IDDM, and 81% of all sera that shifted <2 standard deviations were from nondiabetic individuals. The level of significance of the difference between the two patient populations is $P < 0.0001$, and the Youden index of this test is $J = 0.73$.

The discriminatory capability of the methodology used here is somewhat better than the ICA-cyt (10) antibody test, which is widely used for tracking prediabetics, and about the same as anti-GAD antibody test (11). Whether or not the addition of GLUT-2 to the list of β-cell autoantigens in autoimmune diabetes will enhance the ability to predict the disease can be determined only after specimens collected prior to onset of IDDM are studied. In the only prediabetic sample thus far tested, binding to GLUT-2-transfected cells was greater before than after the onset of overt diabetes. It is hoped that this test will prove useful in predicting the development of autoimmune destruction of β cells.

Although we assume that autoantibodies to GLUT-2 are epiphenomenal rather than pathogenic, as is believed to be the case with other autoantibodies to β-cell antigens, it is possible that inhibition of GLUT-2 function could impair glucose sensitivity in β cells.

We are indebted to Ms. Marilyn Alford and Dr. Brett Giroir for providing sera from nondiabetic children as well as their clinical histories. This work was supported by grants from the Department of Veterans Affairs (Institutional Research Support Grant 549-8000 and Merit Review 821-103) and the National Institutes of Health (DK-PO1-42582 and DK-02700-31).

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