

Autoantibodies to CD38 (ADP-Ribosyl Cyclase/Cyclic ADP-Ribose Hydrolase) in Caucasian Patients With Diabetes

Effects on Insulin Release From Human Islets

Cinzia Pupilli, Stefano Giannini, Piero Marchetti, Roberto Lupi, Alessandro Antonelli, Fabio Malavasi, Shin Takasawa, Hiroshi Okamoto, and Ele Ferrannini

The type II transmembrane glycoprotein CD38 (ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase) has been proposed as a mediator of insulin secretion from pancreatic β -cells and as a candidate for autoimmune reactions in type 2 diabetes. We evaluated the presence of anti-CD38 autoantibodies in Caucasian patients with diabetes and investigated the effect of these antibodies on insulin secretion from isolated human pancreatic islets. The presence of anti-CD38 autoantibodies was evaluated by using Western blot analysis in 236 patients with type 2 diabetes (mean age 63 years), in 160 patients with type 1 diabetes (mean age 38 years), and in 159 nondiabetic subjects. Anti-CD38 autoantibody titers at least 3 SD above the mean value of the control group were found in 9.7% of type 2 diabetic patients and in 13.1% of type 1 diabetic patients ($\chi^2 = 15.9$, $P = 0.0003$ vs. 1.3% of control subjects). No significant differences were observed in sex distribution, current age, age at diabetes onset, BMI, fasting serum glucose, or glycemic control between anti-CD38⁺ and anti-CD38⁻ diabetic patients in either the type 2 or type 1 diabetic groups. The effect of 23 anti-CD38⁻ and 13 anti-CD38⁺ sera on insulin secretion at low (3.3 mmol/l) or high (16.7 mmol/l) medium glucose concentrations was evaluated in isolated human pancreatic islets. Data are medians (interquartile range). The anti-CD38⁺ sera potentiated insulin release both at low [95 (64) vs. 23 (12) μ U/ml of control incubations, respectively, $P < 0.0001$] and high [271 (336) vs. a control of 55 (37) μ U/ml, respectively, $P = 0.001$] medium glucose concentrations, whereas the anti-CD38⁻ sera did not. Furthermore, in the pooled data from all 36 tested sera, insulin levels in the islet incubation medium were directly

related to the anti-CD38 antibody titer. We conclude that autoantibodies to CD38 are associated with both type 1 and type 2 diabetes in Caucasian subjects. These autoantibodies exert a stimulatory effect on insulin secretion by cultured human islets. The role of this autoimmune reaction in the pathogenesis of diabetes remains to be elucidated. *Diabetes* 48:2309–2315, 1999

Human CD38 (ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase) is a single-chain type II transmembrane glycoprotein that is commonly used as a phenotypic marker of differentiation and activation in hematopoietic cells (1). CD38 is a bifunctional ectoenzyme that catalyzes both the conversion of NAD⁺ to cyclic ADP-ribose (cADPR) and the hydrolysis of cADPR back to ADPR (2–4). cADPR appears to be involved in the regulation of Ca²⁺-dependent intracellular Ca²⁺ release through interaction with the ryanodine receptor (5,6). In immunocompetent cells, activation of CD38 by specific agonistic antibodies causes several effects, including cell activation and proliferation, induction of cytokine secretion, and modulation of apoptosis (7–9). CD38 also behaves as a selectin by interacting with endothelial CD31, thereby promoting adhesion of endothelial to CD38⁺ cells (10).

Alternative biological roles for CD38 have been hypothesized because of the presence of its mRNA or protein in other mammalian tissues such as brain, spleen, heart, liver, kidney, intestine, and pancreas (11–14). Experimental evidence indicates that cADPR induces the release of Ca²⁺ from microsomes of rat pancreatic islets (14). Moreover, glucose increases the concentration of cADPR in rat and mouse islets but not in their microsomes; in turn, cADPR stimulates insulin secretion from digitonin-permeabilized islets (14–16). Finally, glucose-induced insulin secretion is enhanced in transgenic mice that overexpress CD38 in pancreatic β -cells (17) and is attenuated in CD38 knockout mice (18). Taken together, these observations have led to the intriguing hypothesis that CD38 may be involved in insulin secretion through an alternative intracellular Ca²⁺ mobilization system to the inositol-3-phosphate pathway (15).

Both insulin resistance and insulin deficiency play a role in the pathogenesis of type 2 diabetes (19). In recent years, the possibility that an autoimmune reaction against pancreatic

From the Department of Internal Medicine and the Metabolism Unit, Consiglio Nazionale delle Ricerche (CNR) Institute of Clinical Physiology (A.A., E.F.), and the Department of Endocrinology (P.M., R.L.), University of Pisa; the Endocrinology Unit, Department of Clinical Pathophysiology (C.P., S.G.), University of Florence; the Institute of Biology and Genetics (F.M.), University of Ancona, Italy; and the Department of Biochemistry (S.T., H.O.), Tohoku University School of Medicine, Sendai, Japan.

Address correspondence and reprint requests to Ele Ferrannini, MD, CNR Institute of Clinical Physiology, Via Savi 8, 56126 Pisa, Italy. E-mail: ferranni@ifc.pi.cnr.it.

Received for publication 19 March 1999 and accepted in revised form 19 August 1999.

cADPR, cyclic ADP-ribose; ICA, islet cell cytoplasmic antigen; IgG, immunoglobulin G; KRBB, Krebs-Ringer bicarbonate buffer; LADA, latent autoimmune diabetes of adults; MBP, maltose binding protein; PBS, phosphate-buffered saline; PVDF, polyvinylidene fluoride.

β -cells (an event extensively demonstrated in type 1 diabetes) may take place in some patients with typical type 2 diabetes has received increasing attention. In fact, autoantibodies to islet cell cytoplasmic antigens (ICA), GAD, and protein tyrosine phosphatase-2 (IA-2) have been detected in subsets of patients with type 2 diabetes; the presence of these autoantibodies has been found to predict the subsequent need for insulin therapy (20). Recent findings have suggested that functional alterations of CD38 may be associated with type 2 diabetes. Thus, a missense mutation in the CD38 gene, which leads to a 50% reduction of in vitro ADPR cyclase and cADPR hydrolase activities, has been identified in Japanese patients with type 2 diabetes (21), and autoantibodies to CD38 have been detected in a sizeable proportion of these patients (22).

These observations prompted us to search for anti-CD38 autoantibodies in Caucasian subjects and to verify whether their presence is associated with diabetes. We also attempted to identify a role for these autoantibodies in insulin secretion by using the isolated human pancreatic islet model.

RESEARCH DESIGN AND METHODS

Patients. Among the outpatients attending the clinics at the University of Florence and the University of Pisa, we randomly selected 236 Caucasian patients with type 2 diabetes and 160 patients with type 1 diabetes, all of whom were diagnosed according to World Health Organization criteria (23), and 159 nondiabetic subjects with no family history of diabetes and normal fasting plasma glucose levels (<6.1 mmol/l) (Table 1). Blood samples were obtained after an overnight fast and were stored at -70°C until tested. Plasma glucose concentrations were measured by using the glucose oxidase technique, and HbA_{1c} was measured by using high-performance liquid chromatography. Plasma insulin and C-peptide levels were measured via radioimmunoassay (Medgenix, Brussels). Informed consent was obtained from all study subjects; the protocol was approved by the local ethics committee.

Detection of anti-CD38 antibodies. To screen for the presence of anti-CD38 antibodies, immunoblotting was performed by using a recombinant CD38-maltose binding protein (MBP) fusion protein of 68 kDa obtained as previously described from human CD38 cDNA encoding amino acids 45–300 (24). A total of 20 μg of recombinant CD38-MBP were electrophoresed on 10% SDS polyacrylamide gel and were electrotransferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon P; Millipore, Bedford, MA). The membrane was subsequently incubated with a blocking solution of phosphate-buffered saline (PBS) containing 5% nonfat dry milk and 0.15% Tween 20 for 60 min. Sera from diabetic patients and nondiabetic subjects were diluted 1:1,000 in the same buffer and were incubated with the membrane for 60 min by using a Screener Blotter Mini 56 (Sampletec, Osaka, Japan). After washing with PBS containing 0.15% Tween 20, the membranes were incubated for 60 min with a rabbit antihuman antibody labeled with horseradish peroxidase (American Qualex, La Mirada, CA) diluted 1:1,600. The signals were revealed by using an enhanced chemiluminescence (ECL) detection system (Amersham, Arlington, IL) according to the manufacturer's instructions. The autoradiographic signals were quantitated by densitometry; images were acquired with a Hamamatsu CCD C3077/01 videocamera (Hamamatsu Photonics KK, Tokyo) connected to IQ Base software (Hamamatsu Photonics KK) and were analyzed by using Image 1.28 software (courtesy of William Rasband, National Institutes of Health, Bethesda, MD).

The specificity of the signals was evaluated with the following tests. First, in preabsorption experiments, three diabetic sera positive for anti-CD38 antibodies were incubated overnight at 4°C with or without 100 $\mu\text{g}/\text{ml}$ of human recombinant CD38-MBP or LacZ-MBP in blocking solution (22) and were used in immunoblotting as described above. Second, six anti-CD38⁺ and six anti-CD38⁻ diabetic sera diluted 1:1,000 were immunoblotted against the extracellular domain of human CD38 with the putative glycosylation sites eliminated by site-directed mutagenesis obtained by the recombinant expression technique from the yeast, *Pichia pastoris* (1 $\mu\text{g}/\text{lane}$) (25). Third, signals obtained from diabetic or normal sera were compared with signals produced by an antihuman CD38 monoclonal antibody (T16; Cosmo Bio, Tokyo) tested on the same PVDF membrane at a concentration of 5 $\mu\text{g}/\text{ml}$.

Insulin secretion from cultured human pancreatic islets. Effects of sera on insulin secretion from human pancreatic islets were tested in 23 anti-CD38⁻ diabetic patients (4 were on diet therapy, 10 took oral hypoglycemic agents, and 9 took insulin alone or in combination with oral hypoglycemic agents) and 13 anti-CD38⁺ subjects (2 nondiabetic control subjects and 11 diabetic patients, of whom

TABLE 1
Characteristics of the study subjects

	Subjects		
	Control	Type 2 diabetic	Type 1 diabetic
<i>n</i>	159	236	160
Sex (F/M)	76/83	134/102	83/77
Age (years)	52 \pm 19	63 \pm 10	38 \pm 13
BMI (kg/m^2)	27.1 \pm 4.9	28.7 \pm 5.1	23.3 \pm 2.5
Diabetes duration (years)	—	11 \pm 9	16 \pm 10
Age at onset (years)	—	52 \pm 11	21 \pm 10
HbA_{1c} (%)	5.0 \pm 0.4	7.0 \pm 1.4	7.9 \pm 1.3
Fasting plasma glucose (mmol/l)	5.2 \pm 0.8	9.2 \pm 2.6	10.3 \pm 4.0

Data are means \pm SD.

1 was on diet therapy, 5 took oral hypoglycemic agents, and 5 took insulin alone or in combination with oral hypoglycemic agents).

Isolated human pancreatic islets were obtained by collagenase digestion and density-gradient purification (26) from the pancreases of human cadaver donors referred through the local organ procurement organization with the permission of the local ethics committee. At the end of the isolation procedure, islets were resuspended in M199 tissue culture medium supplemented with 10% serum and antibiotics (100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 50 $\mu\text{g}/\text{ml}$ gentamycin, and 0.25 $\mu\text{g}/\text{ml}$ amphotericin B). We loaded $\sim 1,000$ islets in 15 ml of culture medium onto 25- cm^2 uncoated plastic flasks (BioBraun, Milan, Italy), which were then cultured at 37°C in an atmosphere of 95% O_2 and 5% CO_2 . Within 7–12 days of isolation, the insulin secretory function of the islets was assessed in response to varying glucose concentrations by using the batch incubation method as previously described (27). Groups of islets of comparable size (20/tube) were preincubated at 37°C for 45 min in Krebs-Ringer bicarbonate buffer (KRBB) supplemented with 3.3 mmol/l glucose and 0.5% bovine serum albumin. The islets were then washed and incubated at 37°C for 45 min in KRBB containing 3.3 or 16.7 mmol/l glucose, either with or without the addition of 10% serum from patients who were immunoblot positive or negative for the presence of anti-CD38 antibodies. At the end of this incubation period, aliquots of the medium were collected to measure insulin concentrations. For each test serum (or control), two to four clusters of islets were incubated separately.

In an additional series of experiments, the effect of an anti-human CD38 monoclonal antibody (T16) was evaluated at concentrations of 0.5 and 2 $\mu\text{g}/\text{ml}$. This antibody was the same one used for specificity testing.

To document that the effects of human sera on insulin secretion from isolated human islets were because of immunoglobulins, four anti-CD38⁺ sera were adsorbed to protein A, which binds to the Fc portion of immunoglobulin G (IgG) antibodies. Sera were diluted 1:2 in KRBB without HEPES, albumin, or glucose and were incubated in a rotating agitator overnight at 4°C with protein A sepharose 6MB (Pharmacia Biotech, Uppsala, Sweden) (2 ml/ml of serum) previously equilibrated in the same buffer. After centrifugation (3 min at 3,000g), sera were recovered, and the incubation with protein A was repeated for 2 h under the same conditions. Aliquots of sera were incubated with sepharose alone as a control.

Detection of CD38 protein in human pancreatic islets. The presence of CD38 protein in human pancreatic islets was tested by using Western blot analysis. Human pancreatic islets isolated as described above were lysed in a lysis buffer (20 mmol/l Tris, 150 mmol/l NaCl, 1 mmol/l phenylmethylsulfonyl fluoride, 0.25% NP-40). A total of 70 μg of protein were electrophoresed on 10% SDS polyacrylamide gel under denaturing conditions and were electrotransferred onto a PVDF membrane. Nonspecific protein binding was blocked by incubating the membranes with a blocking solution (1 \times PBS, 0.15% Tween 20, 5% nonfat dried milk) for 1 h at room temperature. A monoclonal antibody specific for human CD38 (clone IB₃) (5 $\mu\text{g}/\text{ml}$) was applied onto the membrane for 60 min at room temperature (28). After rinsing with washing buffer (1 \times PBS, 0.15% Tween 20), peroxidase-conjugated antimouse IgG antibody (Sigma, St. Louis, MO) diluted 1:4,000 was applied to the membrane for 60 min at room temperature. The detection of specific signals was performed by using the ECL detection system previously described.

Determination of anti-GAD autoantibodies. Anti-GAD autoantibodies were measured in a subgroup of 98 type 2 diabetic patients. In vitro transcribed and translated recombinant human GAD labeled with [^{35}S]methionine was used as an antigen in a radioimmunoprecipitation assay. Human islet GAD65 cDNA in the vector pB 1882 (courtesy of Dr. Thomas Dyrberg, Novo Nordisk, Copenhagen) was

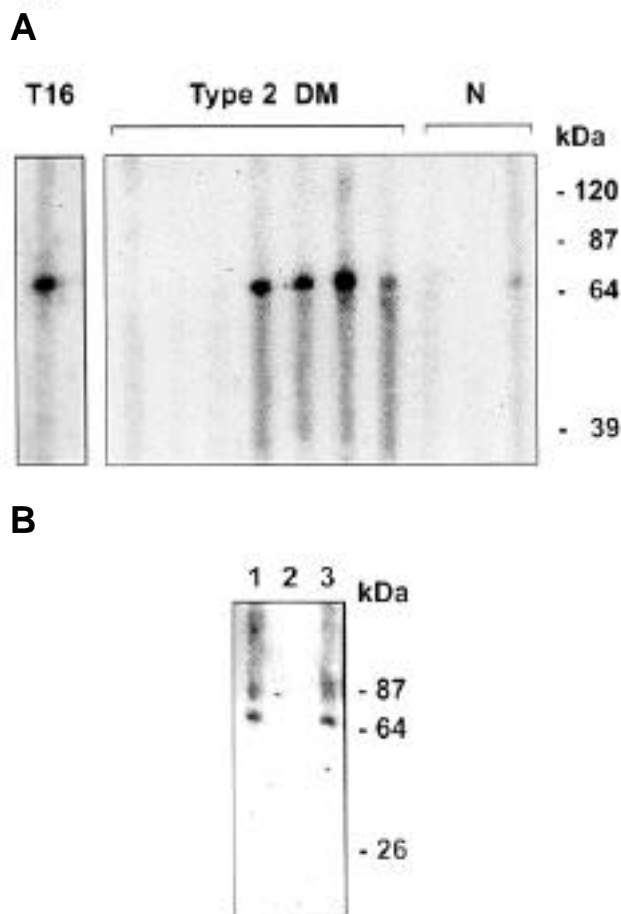


FIG. 1. A: Detection of anti-CD38 autoantibodies in type 2 diabetic (Type 2 DM) patients and nondiabetic subjects (N). Sera were diluted 1:1,000 and were tested against the human recombinant CD38-MBP fusion protein by immunoblotting (see RESEARCH DESIGN AND METHODS). For a positive control, a human anti-CD38 monoclonal antibody (T16) was tested on a portion of the same membrane. **B:** In a representative preabsorption experiment, an anti-CD38⁺ serum was incubated overnight at 4°C alone (lane 1), with 100 µg/ml of human recombinant CD38-MBP (lane 2), or with LacZ-MBP (lane 3) and was tested by immunoblotting. The signal is abolished by an excess of CD38-MBP but not the carrier protein alone.

used to transcribe and translate the protein according to the manufacturer's instructions (Promega, Madison, WI). For the immunoprecipitation and isotyping assay, 50 µl of [³⁵S]methionine-labeled GAD (50,000 cpm) were incubated with 2 µl of serum overnight at 4°C (1:25 dilution) in a 96-well millipore plate. The immunocomplexes were isolated with 1 mg protein A sepharose and were counted as previously described (29). All samples were tested in duplicate, including positive and negative control standard sera. Each assay for anti-GAD antibodies included serially diluted sera from a patient with stiff-man syndrome to further evaluate the cutoff level for positivity for GAD; values at least 3 SD above the control population were considered positive.

Data analysis. Immunoblot optical density readings were corrected for background and were standardized against an internal control sample by calculating the ratio of the unknown to the control (multiplied × 100). For islet incubation data, calculations were based either on the insulin values of the individual incubation aliquots or on the mean insulin values of 2–4 aliquots for each test serum (or control).

Data are means ± SD. Because of their skewed distribution, insulin concentration values are medians (interquartile ranges) and were transformed into their natural logarithms for use in all statistical analyses. Proportions were compared by using the χ^2 test; mean group values were compared by using the Mann-Whitney *U* test (two groups) or the Kruskal-Wallis test (three groups); post hoc comparisons were carried out by using the Bonferroni-Dunn test.

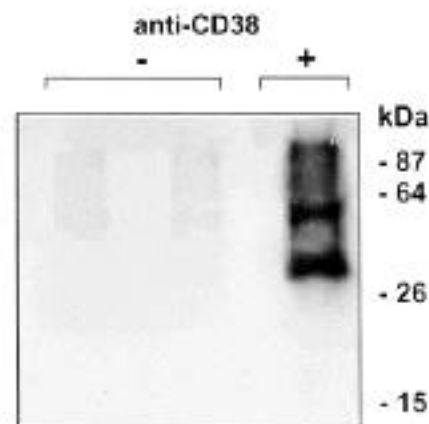


FIG. 2. A representative immunoblot showing the signals obtained by testing two anti-CD38⁻ sera and one anti-CD38⁺ serum against 1 µg/ml of human CD38 from *P. pastoris*, which consists of the extracellular domain with the putative glycosylation sites eliminated by site-directed mutagenesis.

RESULTS

Detection of anti-CD38 antibodies in human sera. As shown in Fig. 1A, a band of ~68 kDa was observed in the sera of some diabetic patients and nondiabetic subjects. The autoradiographic signals were abolished by preincubating anti-CD38⁺ sera with recombinant CD38, although these signals were still detectable when the sera were preincubated with LacZ-MBP (Fig. 1B). When anti-CD38⁺ and anti-CD38⁻ sera were screened against recombinant CD38 from *P. pastoris*, a band of ~30 kDa corresponding to soluble CD38 and a higher molecular mass species (likely due to self-aggregation [25]) were observed for all six anti-CD38⁺ sera, whereas no signal was detected with six anti-CD38⁻ sera (Fig. 2).

In the entire study population, the distributions of optical density values for the diabetic groups clearly diverged from that of the nondiabetic group at >90th percentile (Fig. 3). In the control subjects, optical density was not related to sex ($r = 0.24$, $P = 0.62$), age ($r = 0.05$, $P = 0.56$), or BMI ($r = 0.06$, $P = 0.46$). By defining anti-CD38 positivity as a standardized optical density value of at least 3 SD above the mean value for the control group, 23 of 236 type 2 diabetic patients (9.7%) and 21 of 160 type 1 diabetic patients (13.1%) were positive for anti-CD38 antibodies versus 2 out of 159 nondiabetic subjects (1.3%, $\chi^2 = 15.9$, $P = 0.0003$). Although each diabetic group differed from the control group ($\chi^2 = 11.5$, $P = 0.0007$ for type 2 diabetic subjects; $\chi^2 = 16.8$, $P < 0.0001$ for type 1 diabetic subjects), the prevalence of anti-CD38 positivity did not differ between type 1 and type 2 diabetic patients ($\chi^2 = 1.1$, $P = 0.29$).

No statistically significant differences in sex distribution, age, duration of diabetes, age at onset of diabetes, BMI, fasting plasma glucose concentrations, insulin concentrations, C-peptide concentrations, and HbA_{1c} levels were found between anti-CD38⁺ and anti-CD38⁻ patients in either the type 2 or type 1 diabetic groups (Tables 2 and 3). Among 98 type 2 diabetic patients, 10% were also positive for anti-GAD autoantibodies, but anti-GAD positivity was not significantly different between anti-CD38⁺ and anti-CD38⁻ patients (11 vs. 10%, respectively). However, anti-CD38 and anti-GAD titers were significantly, albeit weakly, related to one another ($r =$

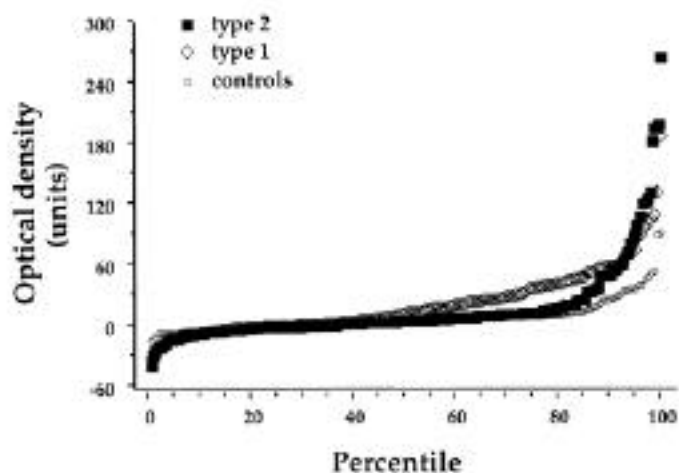


FIG. 3. Distribution of the optical density signals from immunoblots of anti-CD38 autoantibodies in 236 patients with type 2 diabetes, 160 patients with type 1 diabetes, and 159 nondiabetic subjects.

0.25, $P = 0.01$ for the log-transformed values). Anti-GAD⁺ type 2 diabetic patients had significantly lower BMI and higher fasting plasma glucose and HbA_{1c} concentrations and were more often taking insulin than anti-GAD⁻ patients (Table 4).

Effects of anti-CD38 sera on insulin secretion from human pancreatic islets. In control islet preparations (no serum added), medium insulin concentrations were approximately twofold higher when the medium glucose concentration was 16.7 mmol/l compared with 3.3 mmol/l [55 (37) vs. 23 (12) μ U/ml, respectively, $n = 8$, $P < 0.0001$] (Fig. 4). The 13 anti-CD38⁺ sera potentiated insulin release both at low [95 (64) μ U/ml, $P < 0.0001$ by analysis of variance] and high medium glucose concentrations [271 (336) μ U/ml, $P = 0.001$], whereas the 23 anti-CD38⁻ sera did not [29 (28) and 56 (112) μ U/ml, respectively, $P = 0.06$ and 0.33, respectively, vs. control]. In post hoc testing, the insulin values in islets incubated with anti-CD38⁺ sera were significantly higher than those of both control incubations and anti-CD38⁻ sera (Fig. 4). In the pooled data of all 36 tested sera, the intensity of the optical signals for CD38

TABLE 2
Clinical characteristics of the type 2 diabetic patients according to anti-CD38 antibody status

	Anti-CD38 ⁺	Anti-CD38 ⁻	<i>P</i>
<i>n</i>	23	213	—
Sex (F/M)	15/8	119/94	NS
Age (years)	63 ± 14	62 ± 11	NS
BMI (kg/m ²)	30.8 ± 6.4	28.4 ± 4.9	NS
Diabetes duration (years)	11 ± 10	11 ± 8	NS
Age at onset (years)	51 ± 13	52 ± 11	NS
Fasting plasma glucose (mmol/l)	9.6 ± 3.1	9.2 ± 2.6	NS
Fasting plasma C-peptide (ng/ml)	3.2 ± 2.6	2.6 ± 1.5	NS
Fasting plasma insulin (μ U/ml)	14 ± 6	15 ± 15	NS
HbA _{1c} (%)	7.1 ± 1.5	7.0 ± 1.4	NS
Treatment (diet/oral hypoglycemic agent/insulin*) (%)	13/52/35	18/56/26	NS

Data are means ± SD. *P* values refer to mean group comparisons by the Mann-Whitney *U* test or χ^2 test. *Insulin alone or in combination with oral hypoglycemic agents.

TABLE 3
Clinical characteristics of the type 1 diabetic patients according to anti-CD38 antibody status

	Anti-CD38 ⁺	Anti-CD38 ⁻	<i>P</i>
<i>n</i>	21	139	—
Sex (F/M)	11/10	71/68	NS
Age (years)	34 ± 12	40 ± 12	NS
BMI (kg/m ²)	24.2 ± 2.1	23.2 ± 2.5	NS
Diabetes duration (years)	16 ± 9	16 ± 10	NS
Age at onset (years)	19 ± 8	21 ± 10	NS
Fasting plasma glucose (mmol/l)	9.6 ± 3.1	9.2 ± 2.6	NS
HbA _{1c} (%)	8.1 ± 0.8	7.8 ± 1.3	NS

Data are means ± SD. *P* values refer to mean group comparisons by the Mann-Whitney *U* test or χ^2 test.

autoantibodies was directly related to medium insulin levels at low and high glucose exposures (Fig. 5).

The T16 anti-CD38 monoclonal antibody was added at concentrations of 0.5 or 2.0 μ g/ml to three clusters of islets each for the low and high medium glucose concentrations. At 3.3 mmol/l glucose, neither antibody concentration affected insulin release [20 (15) and 22 (14) vs. 21 (11) μ U/ml of 25 batches of control islets, $P = 0.83$]; however, at 16.7 mmol/l glucose, the antibody stimulated insulin secretion in a dose-dependent manner to 81 (69) and 137 (97) vs. 50 (39) μ U/ml of control islets ($P = 0.003$).

When anti-CD38⁺ sera were adsorbed to protein A, their ability to enhance insulin secretion at 16.7 mmol/l glucose was virtually abolished (Fig. 6).

In the same preparation used for the insulin secretion studies (i.e., isolated human pancreatic islets), Western blot analysis of the lysate against the monoclonal antibody IB₄ demonstrated a single band of ~46 kDa (Fig. 7).

DISCUSSION

This study provides evidence that high-titer autoantibodies reacting with human recombinant CD38 are present in the sera of some Caucasian subjects. The specificity tests documented that CD38 is indeed the protein recognized by the native autoantibodies. Anti-CD38 antibodies were found in significant excess in the sera of patients with either type 1 or

TABLE 4
Clinical characteristics of the type 2 diabetic patients according to anti-GAD antibody status

	Anti-GAD ⁺	Anti-GAD ⁻	<i>P</i>
<i>n</i>	10	88	—
Sex (F/M)	7/3	46/42	NS
Age (years)	70 ± 7	65 ± 10	NS
BMI (kg/m ²)	24.4 ± 3.4	28.8 ± 5.0	0.002
Diabetes duration (years)	13 ± 13	11 ± 9	NS
Age at onset (years)	56 ± 12	53 ± 12	NS
Fasting plasma glucose (mmol/l)	10.1 ± 3.8	8.8 ± 2.4	0.1
HbA _{1c} (%)	7.8 ± 1.2	6.9 ± 1.3	0.03
Insulin treatment (%)	90	33	0.0006

Data are means ± SD. *P* values refer to mean group comparisons by the Mann-Whitney *U* test or χ^2 test.

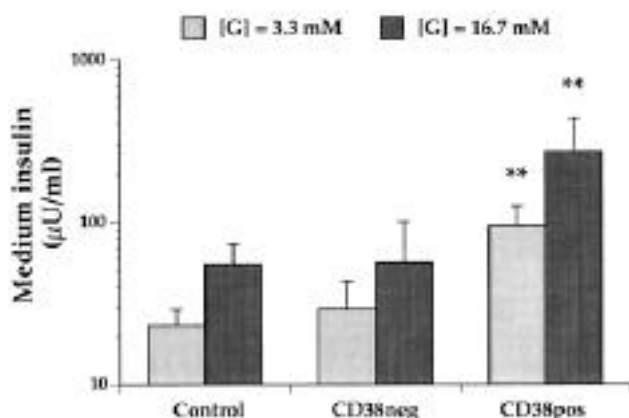


FIG. 4. Insulin response to low (3.3 mmol/l) and high (16.7 mmol/l) medium glucose concentrations by isolated human islets incubated with no sera (Control), anti-CD38⁺ sera (CD38pos) ($n = 13$), or anti-CD38⁻ sera (CD38neg) ($n = 23$). Each value is the mean of two to four individual incubation aliquots. Data are medians (interquartile ranges). ** $P < 0.001$ vs. control and anti-CD38⁻ sera.

type 2 diabetes compared with a nondiabetic reference group. Thus, anti-CD38 antibodies are a new marker of autoimmunity in human diabetes.

We selected type 2 diabetic subjects who were aged >40 years to reduce the representation of patients with latent autoimmune diabetes of adults (LADA) (30). In fact, the aver-

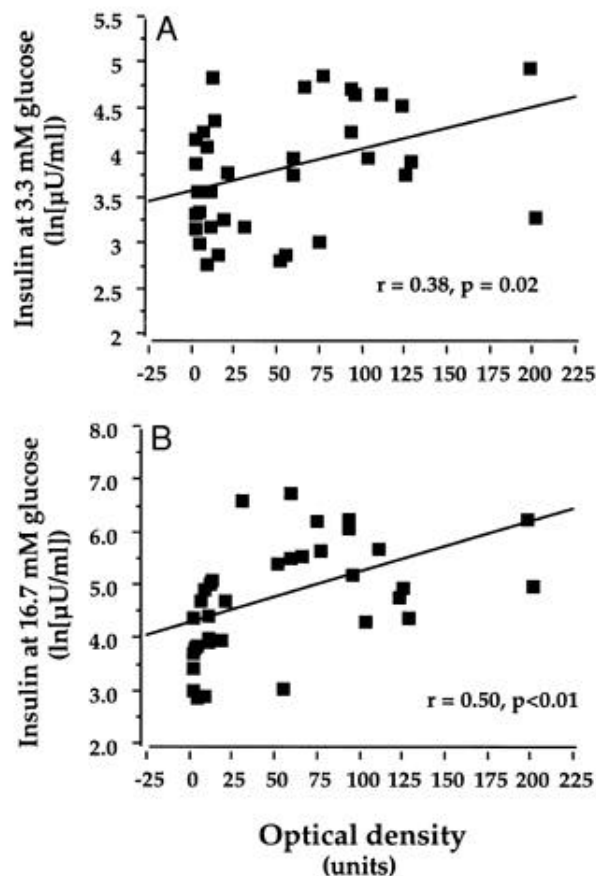


FIG. 5. Relationship between anti-CD38 antibody titer and the insulin response to low (A) and high (B) medium glucose concentrations by isolated human islets incubated with 13 anti-CD38⁺ and 23 anti-CD38⁻ sera.

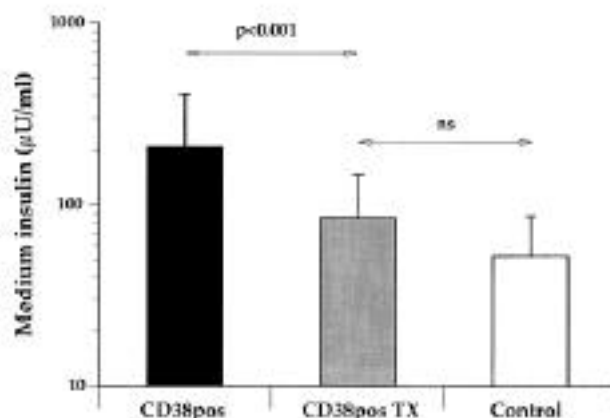


FIG. 6. Insulin response to high (16.7 mmol/l) medium glucose concentrations by isolated human islets incubated with no sera (Control) ($n = 26$ incubation aliquots), anti-CD38⁺ sera (CD38pos) ($n = 26$), or anti-CD38⁺ sera preabsorbed to protein A (CD38pos TX) ($n = 8$). Data are medians (interquartile ranges).

age phenotype of our type 2 diabetic patients (according to age, BMI, disease duration, and habitual glycemic control) was that of classic type 2 diabetes (Table 1). The prevalence of anti-CD38⁺ sera (9.7%) was comparable to the prevalence of anti-GAD autoantibodies in our series (Table 4) and to that reported in previous studies of type 2 diabetes (20,30). Although anti-GAD positivity was low among anti-CD38⁺ subjects, the serum titers of the two autoantibodies were significantly, albeit weakly, correlated to one another. Our findings in this Caucasian population closely agree with observations made in Japanese type 2 diabetic patients (22). The fact that autoantibody prevalence and diabetic phenotype were remarkably similar between Japanese and Caucasian patients is particularly important in the light of the large interethnic differences in autoimmunity (31).

Autoimmunity is recognized with increasing frequency in type 2 diabetic patients. In the U.K. Prospective Diabetes Study, which involved a large cohort of newly diagnosed patients, the presence of autoantibodies to ICA and/or GAD identified subsets of patients characterized by a lower BMI, higher HbA_{1c} concentrations, and a higher probability to require insulin therapy than age-matched patients without such autoantibodies (20). In a recent population-based study from Finland (32), anti-GAD⁺ type 2 diabetic patients

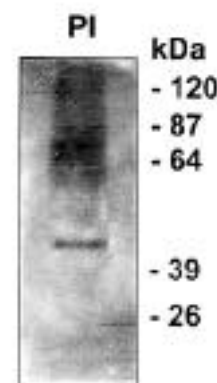


FIG. 7. Western blot analysis for CD38 protein in a lysate of isolated human pancreatic islets. The specific monoclonal antibody IB₄ detects a single band of ~ 46 kDa.

had somewhat more severely impaired β -cell function but fewer of the other metabolic abnormalities of type 2 diabetes compared with anti-GAD⁻ patients, although the frequency of susceptibility genotypes was intermediate between that of type 2 and type 1 diabetes. Unlike the U.K. patients, however, BMI and age at onset did not differ between anti-GAD⁺ and anti-GAD⁻ patients. In our type 2 diabetic patients, who had a mean age (63 years) similar to that in the Finnish study (68 years), anti-GAD positivity still identified an intermediate, LADA-like phenotype. In contrast, anti-CD38 positivity was not associated with a lower BMI, a younger age at onset, or more marked insulin deficiency; this was also the case for the type 1 diabetic patients. These results suggest that the adult autoimmune diabetic phenotype may be more heterogeneous than previously thought depending on type of autoimmune marker (GAD or CD38) and the ethnic background of the population (e.g., northern or southern European, Japanese). The full clinical and genetic characterization of anti-CD38 positivity in diabetes remains to be studied.

Regarding the activity of anti-CD38 autoantibodies, our results in human pancreatic islets were interesting. First, CD38 was identified in lysates of the same preparation of human pancreatic islets that was used for insulin secretion studies. Second, when coincubated with human islets, anti-CD38⁺ sera generally potentiated insulin release in a dose-dependent manner (Figs. 4 and 5). This secretory enhancement was glucose independent because it occurred both at low and high medium glucose concentrations, whereas the T16 anti-CD38 monoclonal antibody only potentiated insulin release in response to high glucose concentrations. Finally, when antibodies were removed from anti-CD38⁺ sera, they lost their ability to stimulate insulin release from human islets. Thus, human anti-CD38 antibodies are mostly stimulatory for islet function. The presence of autoantibodies stimulating the secretion of insulin both in islet cell cultures and in rats in vivo has been previously reported in patients with spontaneous hyperinsulinemic hypoglycemia and type 1 diabetes (33). Interestingly, our results with human islets are the opposite of those obtained in rat pancreatic islets (22) in which both anti-CD38⁺ sera from Japanese diabetic patients and T16 monoclonal antibodies inhibited insulin release. In rat islets, however, only sera cross-reacting with rat CD38 (a minority among anti-CD38⁺ human sera) could be used. Therefore, stimulatory and inhibitory autoantibodies that recognize different epitopes of the CD38 protein may both occur in different ratios in any given diabetic population. Moreover, in rat islets, the blocking effect of anti-CD38⁺ sera on insulin secretion was associated with reciprocal changes in islet cADPR cyclase and cADPR hydrolase activity (22). Conceivably, in human pancreatic islets, modulation of the enzymatic activities of CD38 may be different and may eventually lead to accumulation of cADPR and stimulation of insulin secretion. The observation that the CD38 gene is polymorphic, with at least two alleles identified in the Caucasian population, suggests further complexity (34). Association or linkage may exist between diabetes and selected allele products. These and other important aspects of CD38 autoimmunity (cellular and subcellular localization of the protein, epitopic mapping [35], cellular immune responses to CD38, interaction with intracellular Ca²⁺ metabolism) await further study.

As with anti-GAD immunity (36), the potential pathogenic significance of anti-CD38 autoantibodies is multifaceted. Anti-CD38 autoantibodies may merely reflect immunostimulation by sequestered antigens released from damaged cells. Alternatively, CD38 in islets or another nonself antigen with a sequence homologous with CD38 (molecular mimicry) may activate an autoimmune process that leads to β -cell damage. That anti-CD38 autoantibodies are stimulatory to human islets justifies the speculation that β -cell exhaustion (17) may be a mechanism involved in the insulin secretory failure of human diabetes.

ACKNOWLEDGMENTS

Partial financial support for this study was provided by grants from the Italian Ministry of University and Scientific Research.

We thank Dr. Mohammed Hawa for the assay of anti-GAD antibodies. We also thank Nadia Misciglia for her excellent technical assistance.

The results of this study were presented in a preliminary form at the Annual Meeting of the American Diabetes Association, Chicago, June 1998.

REFERENCES

- Metha K, Shahid U, Malavasi F: Human CD38, a cell-surface protein with multiple functions. *FASEB J* 10:1408-1417, 1996
- Howard M, Grimaldi JC, Bazan F, Lund FE, Santos-Argumedo L, Parkhouse RM, Walseth TF, Lee HC: Formation and hydrolysis of cyclic ADP-ribose by lymphocyte antigen CD38. *Science* 262:1056-1059, 1993
- Zocchi E, Franco L, Guida L, Benatti U, Bargellesi A, Malavasi F, Lee HC, DeFlora A: Single protein immunologically identified as CD38 displays NAD⁺ glycohydrolase and cyclic ADP-ribose hydrolase activities at the outer surface of human erythrocytes. *Biochem Biophys Res Commun* 196:1459-1465, 1993
- Takasawa S, Tohgo A, Noguchi N, Koguchi T, Nata K, Sugimoto T, Yonekura H, Okamoto H: Synthesis and hydrolysis of cyclic ADP-ribose by human leukocyte antigen CD38 and inhibition of the hydrolysis by ATP. *J Biol Chem* 268:26052-26054, 1993
- Lee HC, Aarhus R: ADP-ribosyl cyclase: an enzyme that cyclizes NAD⁺ into a calcium-mobilizing metabolite. *Cell Regul* 2:193-202, 1991
- Sitsapesan R, McGarry SJ, Williams AJ: Cyclic ADP-ribose, the ryanodine receptor and Ca²⁺ release. *Trend Pharmacol Sci* 16:386-391, 1995
- Funaro A, Spagnoli GC, Ausiello CM, Alessio M, Roggero S, Delia D, Zaccolo M, Malavasi F: Involvement of the multilineage CD38 molecule in a unique pathway of cell activation and proliferation. *J Immunol* 145:2390-2396, 1990
- Ausiello CM, La Sala A, Ramoni C, Urbani F, Funaro A, Malavasi F: Secretion of IFN- γ , IL-6, granulocyte-macrophage colony-stimulating factor and IL-10 cytokines after activation of human purified T lymphocytes upon CD38 ligation. *Cell Immunol* 173:192-197, 1996
- Zupo S, Rugari E, Dono M, Tamborelli G, Malavasi F, Ferrarini M: CD38 signalling by agonistic monoclonal antibody prevents apoptosis of human germinal center B cells. *Eur J Immunol* 24:1218-1222, 1994
- Deaglio S, Morra M, Mallone R, Ausiello CM, Prager E, Garbarino G, Dianzani U, Stockinger H, Malavasi F: Human CD38 (ADP-ribosyl cyclase) is a counter-receptor of CD31, an Ig superfamily member. *J Immunol* 160:395-402, 1998
- Mizuguchi M, Otsuka N, Sato M, Ishii Y, Shin-ichiro K, Yamada M, Nishina H, Katada T, Ikeda K: Neuronal localization of CD38 antigen in the human brain. *Brain Res* 697:235-240, 1995
- Koguma T, Takasawa S, Tohgo A, Karasawa T, Furuya Y, Yonekura H, Okamoto T: Cloning and characterization of cDNA encoding rat ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase (homologue to human CD38) from islets of Langerhans. *Biochem Biophys Acta* 1223:160-162, 1994
- Fernandez JE, Deaglio S, Donati D, Svoboda Beusan I, Corno F, Aranega A, Forni M, Falini B, Malavasi F: Analysis of the distribution of human CD38 and its ligand CD31 in normal tissues. *J Biol Regul Homeost Agents* 12:81-91, 1998
- Takasawa S, Nata S, Yonekura H, Okamoto H: Cyclic ADP-ribose in insulin secretion from pancreatic cells. *Science* 259:370-373, 1993
- Takasawa S, Akiyama T, Nata K, Kuriki M, Tohgo A, Noguchi N, Kobayashi K, Kato I, Katada T, Okamoto H: Cyclic ADP-ribose and inositol 1,4,5-trisphosphate as alternate second messengers for intracellular Ca²⁺ mobilization in normal and diabetic β -cells. *J Biol Chem* 273:2497-2500, 1998

16. Okamoto H, Takasawa S, Nata K: The CD38-cyclic ADP-ribose signalling system in insulin secretion: molecular basis and clinical implication. *Diabetologia* 40:1485-1491, 1997
17. Kato I, Takasawa S, Akabane A, Tanaka O, Abe H, Takamura T, Suzuki Y, Nata K, Yonekura H, Yoshimoto T, Okamoto H: Regulatory role of CD38 (ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase) in insulin secretion in pancreatic cells: enhanced insulin secretion in CD38-expressing transgenic mice. *J Biol Chem* 270:30045-30050, 1995
18. Kato I, Yamamoto Y, Fujimura M, Noguchi N, Takasawa S, Okamoto H: CD38 disruption impairs glucose-induced increases in cyclic ADP-ribose, $[Ca^{2+}]_i$ and insulin secretion. *J Biol Chem* 274:1869-1972, 1999
19. Ferrannini E: Insulin resistance versus insulin deficiency in non-insulin-dependent diabetes mellitus: problems and prospects. *Endocr Rev* 19:477-490, 1998
20. Turner R, Stratton I, Horton V, Manley S, Zimmet P, Mackay IR, Shattock M, Bottazzo GF, Holman R for the UK Prospective Diabetes Study (UKPDS) Group: UKPDS 25: autoantibodies to islet-cell cytoplasm and glutamic acid decarboxylase for prediction of insulin requirement in type 2 diabetes. *Lancet* 350:1288-1293, 1997
21. Yagui K, Shimada F, Mimura M, Hashimoto N, Suzuki Y, Tokuyama Y, Nata K, Tohgo A, Ikehata F, Takasawa S, Okamoto H, Makino H, Saito Y, Kanatsuka A: A missense mutation in the CD38 gene, a novel factor for insulin secretion: association with type II diabetes mellitus in Japanese subjects and evidence of abnormal function when expressed in vitro. *Diabetologia* 41:1024-1028, 1998
22. Ikehata F, Sato J, Nata K, Tohgo A, Nakazawa T, Kato I, Kobayashi S, Akiyama T, Takasawa S, Toyota T, Okamoto H: Autoantibodies against CD38 (ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase) that impair glucose induced insulin secretion in noninsulin-dependent diabetes patients. *J Clin Invest* 102:395-401, 1998
23. World Health Organization: *Diabetes Mellitus: Report of a WHO Study Group*. Geneva, World Health Org., 1985, (Tech. Rep. Ser., no. 727)
24. Tohgo A, Munakata H, Takasawa S, Nata K, Akiyama T, Hayashi N, Okamoto H: Lysine 129 of CD38 (ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase) participates in the binding of ATP to inhibit the cyclic ADP-ribose hydrolase. *J Biol Chem* 272:3879-3882, 1997
25. Munshi CB, Fryxell KB, Lee HC, Branton WD: Large-scale production of human CD38 in yeast by fermentation. *Method Enzymol* 280:318-330, 1997
26. Marchetti P, Giannarelli R, Cosimi S, Masiello P, Coppelli A, Viacava P, Navalesi R: Massive isolation, morphological and functional characterization, and xenotransplantation of bovine pancreatic islets. *Diabetes* 44:375-381, 1995
27. Giusti L, Marchetti P, Trincavelli L, Lupi R, Martini C, Lucacchini A, Del Guerra S, Tellini C, Carmellini M, Navalesi R: Peripheral benzodiazepine receptors in isolated human pancreatic islets. *J Cell Biochem* 64:273-277, 1997
28. Malavasi F, Caligaris-Cappio F, Dellabona P, Richiardi P, Carbonara AO: Characterization of a murine monoclonal antibody specific for human early lymphopoietic cells. *Hum Immunol* 9:9-20, 1984
29. Schmidli RS, Colman PG, Bonifacio E, participating laboratories: Disease sensitivity and specificity of 52 assays for glutamic acid decarboxylase antibodies. *Diabetes* 44:636-640, 1995
30. Zimmet PZ, Tuomi T, Mackay IR, Rowley MJ, Knowles W, Cohen M, Lang DA: Latent autoimmune diabetes mellitus in adults (LADA): the role of antibodies to glutamic acid decarboxylase in diagnosis and prediction of insulin dependency. *Diabet Med* 11:299-303, 1994
31. Verge CF, Eisenbarth GS: Natural history of autoimmunity in type I diabetes mellitus. In *Diabetes Mellitus*. LeRoith D, Taylor SI Olefsky, Eds. Philadelphia, Lippincott-Raven, 1996, p. 287-307
32. Tuomi T, Carlsson A-L, Li H, Isomaa B, Miettinen A, Nilsson A, Nissén M, Ehrnström B-O, Forsén B, Snickars B, Lahti K, Forsblom C, Saloranta C, Taskinen M-R, Groop LC: Clinical and genetic characteristics of type 2 diabetes with and without GAD antibodies. *Diabetes* 48:150-157, 1999
33. Wilkin TJ, Hammonds P, Mirza I, Bone AJ, Webster K: Graves' disease of the cell: glucose dysregulation due to islet-cell stimulating antibodies. *Lancet* ii:1155-1158, 1988
34. Ferrero E, Saccucci F, Malavasi F: The human CD38 gene: polymorphism, CpG island and linkage to the CD157 (BST-1) gene. *Immunogenetics* 49:597-604, 1999
35. Hoshino S, Kukimoto I, Kontani K, Inoue S, Kaneda Y, Malavasi F, Katada T: Mapping of the catalytic and epitopic sites of human CD38/NAD⁺ to a functional domain in the carboxyl terminus. *J Immunol* 158:741-747, 1997
36. Leslie RDG, Atkinson MA, Notkins AL: Autoantigens IA-2 and GAD in type 1 (insulin-dependent) diabetes. *Diabetologia* 42:3-15, 1999