Autoimmunity to CD38 and GAD in Type I and Type II diabetes: CD38 and HLA genotypes and clinical phenotypes

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Abstract

Aims/hypothesis. Autoantibodies against *CD38* have been found in some patients with Type II (non-insulin-dependent) diabetes mellitus and have been shown to stimulate insulin secretion by cultured human islets. We tested whether this new form of autoimmunity, (i) overlaps with anti-GAD autoimmunity, (ii) identifies an insulin-deficient phenotype, (iii) is under the influence of genetic factors.

Methods. We screened 496 adults by immuno-blot analysis in the Botnia Study (298 with Type II and 98 with Type I (insulin-dependent) diabetes mellitus, 100 non-diabetic control subjects).

Results. *CD38*-autoantibodies were found in 8.4% of Type II diabetic patients ($p < 0.003$ vs 0% of control subjects), particularly in anti-GAD positive (14% vs 6% of anti-GAD negative, $p = 0.0004$). *CD38ab* were also found in 4% of Type I diabetic patients; in the whole study group, 59% of anti-*CD38* positive had *DQB1**02 compared with 38% of anti-*CD38* negative ($p = 0.04$).

On the OGTT, beta-cell function (as the ratio of insulin-to-glucose areas) was impaired ($p = 0.02$) only in

association with anti-GAD positivity (3.2 ± 3.1 U/mol, mean $\pm$ SD) but not in anti-*CD38* positive patients (5.6 ± 2.9) as compared with patients free of autoimmunity (4.5 ± 4.6 , $p = \text{NS}$).

In 44 Type II diabetic patients (22 negative and 22 positive for anti-*CD38*), no mutations were detected in any of the 8 exons, 5' end of intron 1 or the 5' and 3' untranslated regions of the *CD38* gene. The previously described missense mutation (Arg140Trp) in exon 3 was not found in this cohort. There was no association between the PvUII polymorphism and clinical phenotype.

Conclusion. Anti-*CD38* autoimmunity identifies a clinical phenotype similar to non-autoimmune Type II diabetes, with relative preserved beta-cell function and weak genetic influence. [Diabetologia (2002) 45:1298–1306]

Keywords *CD38* antibodies, *CD38* gene, GAD antibodies, Type II diabetes mellitus, Type I diabetes mellitus, missense mutation, *CD38* polymorphisms, insulin secretion, HLA, LADA.

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Abbreviations: cADPR, Cyclic ADP-ribose; ICA, islet-cell cytoplasmic antigens; GAD, glutamic acid decarboxylase; IA-2, protein tyrosine phosphatase-2; RFLP, restriction-fragment length polymorphism; MBP, *CD38*-maltose binding protein; *CD38ab*, *CD38* autoantibodies; GADab, GAD autoantibodies; SSCP, single stranded conformation polymorphism; OD, immunoblot optical density readings; AUC, area under the OGTT curve; LADA, latent autoimmune diabetes in adults

Human *CD38* (ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase), a Type II transmembrane glycoprotein, is a bi-functional ecto-enzyme catalysing the interconversion of NAD⁺ and cyclic ADP-ribose (cADPR) [1, 2, 3, 4]. cADPR seems to be involved in the regulation of calcium-dependent intracellular calcium release through the interaction with the ryanodine receptor [5, 6]. In immunocompetent cells, activation of *CD38* by specific agonistic antibodies causes cell activation and proliferation, induces cytokine secretion, and modulates apoptosis [7, 8, 9, 10]. *CD38* mRNA and/or protein have been detected in the mammalian brain, spleen, heart, liver, kidney, intestine and

pancreas [11, 12, 13]. In rat pancreatic islets, cADPR induces the release of calcium from microsomes [14]. Moreover, in rat and mouse islets (but not in their microsomes) glucose increases the concentration of cADPR; in turn, cADPR stimulates insulin secretion from digitonin-permeabilised islets [14, 15, 16]. Glucose-induced insulin secretion has been shown to be enhanced in transgenic mice overexpressing *CD38* in pancreatic beta cells [17], and attenuated in *CD38* knockout mice [18]. Taken together, these observations have led to the hypothesis that *CD38* could be involved in insulin secretion through an intracellular calcium mobilisation system alternative to the inositol-3-phosphate pathway [15].

In recent years, the possibility that an autoimmune reaction against pancreatic beta cells, an event extensively shown in Type I (insulin-dependent) diabetes mellitus, could take place also in some patients with typical Type II (non-insulin-dependent) diabetes mellitus has received increasing attention. Autoantibodies to islet-cell cytoplasmic antigens (ICA), glutamic acid decarboxylase (GAD), and protein tyrosine phosphatase-2 (IA-2) have been detected in subsets of patients with Type II diabetes. The presence of these autoantibodies is associated with a distinct clinical phenotype: autoantibody-positive patients with Type II diabetes are younger, leaner and more insulin deficient than their antibody-negative counterparts [19].

Recently, the presence of autoantibodies to *CD38* has been described both in Japanese [20] and Caucasian [21, 22] patients with Type II diabetes. These findings raise the question of whether Type II diabetic patients with anti-*CD38* autoantibodies have a clinical phenotype similar to that of anti-GAD positive patients, who share features of latent autoimmune diabetes (LADA). To answer this question, we screened and characterised a large group of Type II diabetic patients from the Botnia study [23]. Furthermore, we investigated their *HLA* genotype, as anti-GAD positive patients often show a genotype similar to that of autoimmune Type I diabetes [19].

Genetic factors also play an important part in the pathogenesis of Type II diabetes. However, the genetic factors are largely unknown except for mutations causing monogenic forms of diabetes, which have been shown in the insulin, insulin receptor, glucokinase and mitochondrial genes together with genes for several transcription factors [24, 25]. Recently, another study [26] that screened for mutations in the *CD38* gene, reported a missense mutation causing an amino acid substitution – Arg140Trp – in exon 3 in 4 out of 31 Type II diabetic Japanese patients but in none of the control subjects. Furthermore, a different study [27] reported a bi-allelic restriction-fragment length polymorphism (RFLP) in intron 1 for the enzyme PvUII in Italian patients. In that study, only three AA homozygous patients were found and two of them had

Type II diabetes. The aims of this study therefore were to evaluate the prevalence of *CD38ab* and of *CD38* gene mutations and polymorphisms in Type I and II diabetic patients.

Subjects and methods

Subjects. From the database of the Botnia Study, which is a cohort-based study of Type II diabetes [23], we selected 298 Caucasian patients with Type II diabetes according to the revised World Health Organisation (WHO) criteria [28, 29], and 100 non-diabetic subjects with no family history of diabetes and normal fasting plasma glucose concentrations (<6.1 mmol/l) (Table 1). By selection, the diabetic group included 100 anti-GAD positive patients and 198 anti-GAD negative patients who were representative of the whole Botnia diabetic cohort with respect to BMI and age at diagnosis of diabetes. Blood samples were obtained after an overnight fast and stored at -20°C until use. In 283 subjects (187 patients and 96 control subjects), an OGTT (75 g) was carried out, with blood sampling at 0, 30, 60 and 120 min after glucose ingestion.

We also studied 98 patients (55 men, 43 women) with Type I diabetes (with a mean fasting C-peptide concentration of 0.02 ± 0.06 nmol/l) extracted from the Diabetes 2000 Registry in Southern Sweden (Table 1). 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* autoantibodies, immunoblotting was carried out using a recombinant *CD38*-maltose binding protein (MBP) fusion protein of 68 kilo-Dalton obtained, as previously described, from a human *CD38* cDNA encoding amino acids 45–300 [30]. Of recombinant *CD38*-MBP, 20 μg were electrophoresed on 10% SDS polyacrylamide gel and electrotransferred to polyvinylidene fluoride membrane (PVDF, Immobilon P, Millipore, Bedford, Mass., USA). After preincubation of the membrane with phosphate buffered saline (PBS) containing 5% non-fat dry milk and 0.15% Tween 20 (blocking solution) for 60 min, the sera were diluted 1:1000 in the same buffer and incubated with the membrane for 60 min using a Screener Blotter Mini 56 (Sampatec, Osaka, Japan). The membranes were washed with PBS-Tween and incubated for 60 min with a rabbit anti-human antibody labelled with horseradish peroxidase (American Quialex, La Mirada, Calif., USA) diluted 1:1600. The signals were revealed using an enhanced chemiluminescence detection system (ECL, Amersham, Arlington, Ill., USA), and quantitated by densitometry. The images were acquired using a Hamamatsu CCD C3077/-01 video-camera (Hamamatsu Photonics KK, Japan) connected to an IQ Base software (Hamamatsu Photonics KK), and analysed using the Image 1.28 software (W. Rasband, National Institute of Health, Research Service Branch, NIMH, Bethesda, Md., USA). The specificity of the signals was assessed by the following tests: (i) 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 [20, 21], and used for immunoblotting. (ii) Six anti-*CD38* positive and six anti-*CD38* negative 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 recombinant expression technique from the yeast, *Pichia pastoris* (1 $\mu\text{g}/\text{lane}$) [31]. (iii) Signals obtained from diabetic or normal

Table 1 Clinical characteristics

<i>n</i>	Control subjects 100	Type I diabetic patients 98	Type II diabetic patients 298	<i>p</i>
Sex (men/women)	50/50	55/43	131/167	ns
Age (years)	53±14	32±12	67±11	<0.0001
BMI (kg·m ⁻²) ^a	25.0±3.5	23.8±3.5	28.4±4.6	<0.0001
WHR (cm·cm ⁻¹) ^b	0.87±0.08	—	0.92±0.08	<0.0001
Age of onset (years)	—	22±12	60±12	<0.0001
Fasting plasma glucose (mmol/l)	5.5±0.6	15.6±5.9	9.6±3.3	<0.0001
Fasting plasma insulin (μU/ml)	4.6±3.0	—	12.4±13.5	<0.0001
HbA _{1c} (%)	5.4±0.4	7.6±1.7	7.2±2.1	<0.0001
Serum LDL cholesterol (mmol/l)	4.2±1.3	2.8±0.7	4.2±1.0	<0.001
Serum HDL cholesterol (mmol/l)	1.4±0.3	1.4±0.4	1.2±0.3	<0.0001
Serum triglycerides (mmol/l)	1.3±0.5	1.1±0.6	1.9±1.4	<0.0001
Systolic BP (mmHg) ^c	128±18	124±14	149±20	<0.0001
Diastolic BP (mmHg) ^c	78±8	74±9	74±9	<0.0001
Anti-hypertensive Txt (%)	14	—	56	<0.0001 ^d
Anti-GAD positive (%)	3.0	64.1	33.4	
Anti-CD38 positive (%)	0	4	8.4	<0.006 ^d

^a BMI = body mass index; ^b WHR = waist-to-hip ratio; ^c BP = blood pressure; ^d χ^2 test

sera were compared with those produced by an anti-human-CD38 monoclonal antibody (T16, Cosmo Bio Co., Tokyo, Japan) tested on the same PVDF membrane at the concentration of 5 μg/ml. (iv) Serum samples positive for the presence of anti-CD38 antibodies were immunoblotted with a recombinant LacZ-MBP fusion protein to exclude the presence of antibodies directed against MBP. The inter-assay variation coefficient of anti-CD38 autoantibodies by immunoblotting was 8%.

Analytical measurements. Plasma glucose concentrations were measured with a hexokinase method (Boehringer Mannheim, Mannheim, Germany). Serum insulin concentrations were measured by radioimmunoassay (Pharmacia, Uppsala, Sweden) with an inter-assay CV of 5%. Fasting serum C-peptide concentrations were measured by a radioimmunoassay with an inter-assay CV of 9% (Human C-peptide RIA Kit, Linco Research, Mo., USA). HbA_{1c} concentration was measured by a high-performance liquid chromatography, with a reference value of 5 to 7%. The plasma concentrations of cholesterol and triglycerides and separate lipoprotein fractions were measured by enzymatic methods using commercial kits (Boehringer Mannheim, Mannheim, Germany). GAD autoantibodies (GADab) were measured from frozen serum samples by a radioimmunoprecipitation assay using recombinant human ³⁵S-GAD₆₅ produced by in vitro transcription/translation [19]. The results were expressed as relative units [RU = (sample cpm–mean cpm of three negative controls)/(cpm of a positive internal references serum–mean cpm of three negative controls)]. Antibody concentrations exceeding 5 RU, which represents the mean + 3 SD of 296 Finnish healthy control subjects, were considered positive. In the Combined Autoantibody Workshop, the specificity of the assay was 99% and the sensitivity was 75% [32]. A GADab level of 5 RU in our study corresponds to 32 IU/ml according to the WHO standard [33].

HLA DQB1 genotypes. HLA DQB1 genotyping was carried out by polymerase chain reaction (PCR) for the second exon followed by dot-blot hybridisation with sequence-specific oligonucleotide (SSO) probes labelled with digoxigenin (DIG Oligonucleotide 3'-End Labelling Kit, Boehringer Mannheim, Mannheim, Germany) [19]. Three DQB1 probes were used to

distinguish DQB1 alleles 0201 or 0202 (02), 0302, and 0602 or 0603 (03) alleles. The genotypes were shown as 02/0302, 0302/X, 02/X, 0602(3)/X, 0302/0602(3), 02/0602(3) and X/X, where X could mean either a homozygous allele or any allele other than 02, 0302 or 0602(3). The 02/0302, 0302/X or 02/X genotypes were considered DQB1-risk genotypes.

Mutation screening of the CD38 gene. The gene encoding for CD38, located on the short arm of chromosome 4, contains eight exons separated by seven introns [34, 35]. Genomic DNA of a randomly selected sample of 44 Type II diabetic patients (22 negative and 22 positive for CD38ab) was screened for mutations in the CD38 gene with PCR and single stranded conformation polymorphism (SSCP). The fragments included all 8 exons, 648 bp of intron 1340 bp of the 3' untranslated region as well as 200 bp of the 5' untranslated region, including the GA-binding protein binding motif, two Sp1 sites and the retinoic acid binding site.

PCR conditions. 25 ng of genomic DNA was amplified in a final volume of 20 μl, containing 15 pmol of each primer [exon 1, Forward primer (F): GCACAGGAGCCTATAGTTGTT, reverse primer (R): GTATCCTGGTCTGATCCTC-G; exon 2, F: GAACTCGCAGATGCAGGGTG, R: GTCAACTTCTAA-AACCGAGACA-T; exon 3, F: GGCATATAATAGATGCTTC-C, R: TGGACCTATGAATTGTTACC; exon 4, F: TCCACTA-TGACTGAACAGCC, R: AGCACTGACTGAGTAACGTC; exon 5, F: CTTAACCAGCTATTGCTAAG, R: ACTGTGATA-TTGTCAACAGG, exon 6, F: TCTGCCTGCTGGTTGTGA-G, R: TCCTGAGTCAATTTGTTCCC; exon 7, F: CCTTGT-CCAGGGCGTGCTAC, R: AAGCTCAGAGGAGGCTAAG-G; exon 8, F: AGCGAATTGGACGACAGATG, R: CATTGA-CCTTATTGTGGAGG; intron 1, F: GAACTCGCAGATGCA-GGGTG, R: GTCAACTTCTAAAACCGAGACAT; 3'UTR region, F: GAGGGTCCTCCACAATAAGG, R: TGACTATCTG-TAGGAAGGTAC], 10 mmol/l Tris-HCL (pH 8.4), 50 mmol/l KCl, 1.5 mmol/l MgCl₂, 200 nmol/l of dNTP, 1 μCi ³²P-dCTP and 0.5 U Taq polymerase (Pharmacia-Biotech, Uppsala, Sweden), in a DNA Thermal Cycler 9600 (Perkin-Elmer Corporation, Norwalk, Conn., USA). The PCR protocol included denaturation at 94 °C for 5 min, followed by 30 cycles of denatur-

ation at 94 °C, for 60 s, annealing between 56 to 62 °C, for 60 s and extension at 72 °C, for 45 s followed by a final extension of 10 min at 72 °C. After denaturation with 3 µl of a stop solution (95% formaldehyde, 20 mmol/l EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol) at 95 °C for 5 min, 6 µl of each amplicon was electrophoresed on non-denaturing 5% polyacrylamide (Acrylamide/Bisacrylamide 49:1) gels with 5% glycerol at room temperature (8 Watt overnight), and without glycerol at 4 °C (35 Watt for 4 h). Following electrophoresis, gels were dried in a gel dryer and then exposed on X-rays films overnight.

Genotyping the known single nucleotide polymorphisms in intron 1 and exon 3. We typed two published polymorphisms in all Type II diabetic and control subjects ($n=398$). The C→T polymorphism at codon 140 of exon 3 (Arg140Trp) was typed according to another study [26]. Briefly, 12 µl of the PCR fragments were digested at 65 °C for 3 h with 0.05 U of BseNI. The fragments were separated on a 3.5% agarose gel stained with ethidium bromide. To type the C→G PvUII polymorphism in intron 1 [27], 25 ng of genomic DNA was amplified in a final volume of 15 µl, containing 10 pmol of each primer, 10 mmol/l Tris-HCL (pH 8.4), 50 mmol/l KCl, 1.5 mmol/l MgCl₂, 200 nmol/l dNTP, 1% formamide and 0.5 U Amlitaq Gold Polymerase (Pharmacia-Biotech, Uppsala, Sweden), in a DNA Thermal Cycler 9600 (Perkin-Elmer, Norwalk, Conn., USA). The PCR protocol included denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 60 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s followed by a final extension of 10 min at 72 °C. After digestion with 1 U of PvUII (New England Biolabs), the digestion product was mixed 1:1 with BPB-formamide and 1 µl was separated on a non-denaturing 5% polyacrylamide (Acrylamide:Bisacrylamide 49:1) gel using GelScan2000 Ultra-thin gel analyser (Corbett Research, Sydney, Australia). The fragments were visualized with ethidium bromide using GelPro software (Corbett Research, Sydney, Australia), and genotypes were named AA (homozygous for the undigested 185 bp allele), AB (heterozygous), BB (homozygous for the digested allele, 100+85 bp).

Data analysis. Immunoblot optical density readings (OD) were corrected for background, and standardised against an internal control sample by calculating the ratio of the unknown to the control (multiplied by 100). Glucose and insulin area under the OGTT curve (AUC) were calculated by the trapezium rule. All values are given as means $\pm$ SD. Because of their skewed distribution, insulin concentration and AUC values, and anti-CD38 and anti-GAD antibody concentrations were transformed into their natural logarithms for use in statistical analysis. Proportions were compared by the Chi square test; mean group values were compared by Mann-Whitney U test (two groups) or Kruskal-Wallis test (three groups); 2-way ANOVA was used to test the separate influence of anti-GAD and anti-CD38 positivity, and their interaction, on the phenotypic and metabolic variables.

Results

Detection of anti-CD38 antibodies in human serum. A band of about 68 kilo-Dalton was observed in the sera of some diabetic patients and normal subjects. The autoradiographic signals were abolished by preincubating anti-CD38 positive sera with recombinant CD38 but

Fig. 1 Distribution of the concentrations of anti-CD38 (OD) and anti-GAD (RU) autoantibodies in 298 patients with Type II diabetes and 100 non-diabetic subjects

not with LacZ-MBP [21, 22]. When anti-CD38 positive and anti-CD38 negative sera were screened against recombinant CD38 from *P. pastoris*, a band of approximately 30 kilo-Dalton, corresponding to the soluble CD38, and a higher molecular mass species (probably due to self-aggregation) were observed for all six anti-CD38 positive sera, whereas no signal was detected with six anti-CD38 negative sera.

In the whole study group, the distribution of optical density values of the diabetic group clearly diverged from that of the non-diabetic group above the 90th percentile (Fig. 1). In the control subjects, optical density was not related to sex ($r^2=0.005$, $p=NS$), age ($r^2=0.08$, $p=NS$), or BMI ($r^2=0.10$, $p=NS$). By defining anti-CD38 positivity as a standardised optical density value at least 3 SDs above the mean value of the control group (i.e., >2.01 OD), 25 of 298 Type II diabetic patients (8.4%) (Table 1) were positive for anti-CD38 antibodies compared with 4 out of 100 Type I diabetic patients and none of 100 control subjects ($\chi^2=10.3$, $p<0.006$). By selection, the diabetic group was enriched with anti-GAD positive patients (33.4%, Table 1). Positivity for anti-CD38 antibodies was associated with positivity for anti-GAD antibodies: 14% of the GADab positive and 5.6% of the GADab negative Type II diabetic patients had CD38ab ($\chi^2=12.6$, $p=0.0004$); the respective antibody titres were not, however, related to one another ($p=NS$). Anti-CD8

Table 2 Clinical characteristics of Type II diabetic patients according to anti-GAD (GAD⁺) or anti-CD38 (CD38⁺) autoantibody positivity

	GAD ⁻ /CD38 ⁻	GAD ⁺ /CD38 ⁻	GAD ⁺ /CD38 ⁺	GAD ⁻ /CD38 ⁺	<i>p</i> ^a	
<i>n</i>	187	86	14	11	GAD ⁻	CD38 ⁻
Sex (men/women)	85/102	38/48	7/7	1/10	NS	NS
Age (years)	68±11	66±13	65±10	74±10	0.03	NS
BMI (kg·m ⁻²)	28.7±4.2	27.6±4.9	27.6±5.9	30.1±7.4	0.02	NS
WHR (cm·cm ⁻¹)	0.9±0.1	0.9±0.1	0.9±0.1	0.9±0.1	NS	NS
Age of onset (years)	60±11	58±12	55±12	66±10	0.03	NS
Fasting [G] (mmol/l)	9.2±2.7	10.7±4.2	9.7±3.5	8.7±3.3	0.001	NS
Fasting [I] (μU/ml)	12±12	13±17	11±7	13±14	NS	NS
HbA _{1c} (%)	7.2±1.5	7.1±3.1	7.3±2.7	7.4±1.6	NS	NS
LDL cholesterol (mmol/l)	4.3±1.0	4.0±0.9	4.2±1.0	4.7±1.0	0.02	NS
HDL cholesterol (mmol/l)	1.2±0.3	1.3±0.3	1.3±0.5	1.3±0.3	0.02	NS
Triglycerides (mmol/l)	2.1±1.6	1.6±0.8	1.6±0.9	1.6±0.5	0.003	NS
Systolic BP (mmHg)	148±18	140±19	146±12	153±7	0.01	NS
Diastolic BP (mmHg)	84±11	80±8	81±12	79±6	0.04	NS
Anti-GAD (RU)	1.1±1.6	40.3±43.2	27.0±33.5	1.2±2.1	0.0001	NS
Anti-CD38 (OD U)	0.1±0.6	0.1±0.7	3.3±0.8	3.5±1.6	NS	0.0001

^a 2-way ANOVA for the independent effect of anti-GAD and anti-CD38 positivity

Table 3 Plasma glucose and insulin responses to oral glucose in non-diabetic subjects and in Type II diabetic patients stratified by anti-GAD and anti-CD38 positivity

	GAD ⁻ /CD38 ⁻	GAD ⁺ /CD38 ⁻	GAD ⁺ /CD38 ⁺	GAD ⁻ /CD38 ⁺	Control subjects	<i>p</i> ^a	
						GAD ⁻	CD38 ⁻
0' [G] (mmol/l)	9.2±2.7 ^b	10.7±4.3 ^b	9.7±3.5 ^b	8.7±3.3 ^b	5.5±0.5	0.001	–
30' [G] (mmol/l)	13.6±3.2 ^b	14.1±3.7 ^b	13.9±3.7 ^b	12.1±3.9 ^b	8.3±1.6	–	–
60' [G] (mmol/l)	15.9±4.2 ^b	16.5±5.1 ^b	16.1±4.7 ^b	12.5±5.4 ^b	7.4±2.0	–	–
120' [G] (mmol/l)	15.8±5.8 ^b	16.5±7.0 ^b	16.1±6.2 ^b	10.6±4.6 ^b	5.6±1.2	–	–
AUC _G (mol/l·2 h)	1.7±0.5 ^b	1.8±0.6 ^b	1.8±0.5 ^b	1.4±0.6 ^b	0.8±0.1	–	–
0' [I] (μU/ml)	12±12 ^b	13±17 ^b	11±7 ^b	13±14 ^b	5±3	–	–
30' [I] (μU/ml)	46±44	35±31 ^b	30±18	44±22	52±39	0.02	–
60' [I] (μU/ml)	66±60	44±48	44±35	71±39	53±41	0.01	–
120' [I] (μU/ml)	70±75 ^b	55±68 ^b	51±46	49±42	27±22	0.02	–
AUC _I (mU/l·2 h)	6.6±5.5	4.9±4.6	4.5±3.4	6.6±3.0	4.8±3.2	0.007	–
AUC _I /AUC _G (U/mol)	4.5±4.6 ^b	3.2±3.1 ^b	3.0±2.5 ^b	5.6±2.9	5.7±3.7	0.02	–

^a 2-way ANOVA for the independent effect of anti-GAD and anti-CD38 positivity

^b *p*<0.005 or less for the comparison with controls by Bonferroni-Dunn test

G, plasma glucose; I, plasma insulin concentration; AUC_G, glucose area; AUC_I insulin area

concentrations were weakly related to the duration of the disease in Type II diabetic patients (*r*=0.15; *p*=0.01).

The clinical characteristics of Type II diabetic patients stratified by anti-GAD as well as anti-CD38 positivity is shown (Table 2). Anti-GAD positive patients had a definite phenotype, consisting of a younger age and an earlier age of disease onset, higher fasting plasma glucose concentrations, and a better lipid and blood pressure profile as compared with patients who were negative for both antibodies. In contrast, the clinical phenotype of anti-CD38 positive patients was similar to that of antibody-negative patients. When both autoantibodies occurred, the dominant phenotype

was imposed by anti-GAD positivity. When this analysis was repeated by pooling Type II and Type I patients together, the results were very similar.

The plasma glucose and insulin responses to oral glucose administration are given (Table 3) for the Type II diabetic patients subgrouped according to anti-GAD and anti-CD38 positivity. The plasma glucose response, as the glucose area, did not differ by either antibody positivity and was higher than in control subjects for all diabetic subgroups. The plasma insulin response, on the other hand, was lower in association with anti-GAD, but not anti-CD38, positivity. Consequently, the insulin-to-glucose area ratio (an empirical index of the glucose sensitivity of the beta cell) was

Table 4 Frequency of Type I diabetes susceptibility *HLA DQB1*-genotypes in control subjects and in Type I and II diabetic patients according to positivity for anti-CD38 and anti-GAD autoantibodies

HLA-DQB1	Type II diabetic patients								Type I diabetic patients				Control subjects	
	GADab ⁺				GADab ⁻									
	CD38Ab ⁺		CD38Ab ⁻		CD38Ab ⁺		CD38Ab ⁻		CD38Ab ⁺		CD38Ab ⁻		CD38Ab ⁻	
	N	%	N	%	N	%	N	%	N	%	N	%	N	%
Total	14	100	86	100	11	100	187	100	4	100	93	100	100	100
02/0302	2	14	10	12	0	0	8	4	1	25	27	29	2	2
02/X ^a	4	29	12	14	3	27	30	16	3	75	21	23	16	16
0302/X	2	14	19	22	0	0	24	13	0	0	30	32	12	12
Other ^b	6	43	45	52	8	73	125	67	0	0	15	16	70	70
02 ⁺	7	50	32	37	6	55	61	33	4	100	49	53	34	34
02 ⁻	7	50	54	63	5	45	126	69	0	0	44	47	66	66

^a X denotes any other allele than 02, 0302 or 0602(3)

^b Includes subjects with 02/0602(3)

lower in anti-GAD positive patients. Furthermore, this ratio was lower than in non-diabetic subjects in all subgroups except for the patients with sole anti-CD38 positivity.

There was no association between CD38 positivity and the *HLA-DQB1* genotypes 0201/0302 or 0302/X, which confer increased risk for Type I diabetes (Table 4). However, apart from the presence of GADab, 52% (13/25) of the CD38ab positive Type II diabetic patients had the *HLA-DQB1**02 allele compared with 34% (93/273) of the CD38ab negative patients ($p=0.08$). All four CD38ab positive Type I diabetic patients had *DQB1**02. Thus, in the whole study group, 17 of 29 (59%) CD38ab positive subjects had *DQB1**02 compared with 176 of 466 (38%) CD38ab negative subjects ($p=0.04$). By PCR-SSCP in 44 Type II diabetic patients, no mutations were found in any of the eight exons, the 5' end of intron 1 or the 5' and 3' untranslated regions of the *CD38* gene. The missense mutation found by another study [26] in exon 3 was also screened for in all Type II diabetic and control subjects ($n=398$), and was found in none. The allele frequency of the PvUII genotype was 18.4% for the undigested allele A and 81.6% for the digested allele B. There was no association between the PvUII polymorphism genotypes and either CD38ab positivity or diabetic status among the subjects studied. The genotype frequency (AA-AB-BB) was 1.1%–34.4%–64.5% in control subjects, 3.7%–28.9%–67.4% in GADab negative Type II diabetic patients, and 4.3%–29.8%–66.0% in GADab positive Type II diabetic patients ($p=NS$). There was no association between the PvUII genotypes and clinical phenotype, except for a slightly higher mean systolic blood pressure in those Type II diabetic patients carrying allele A (AA genotype: 157 mmHg, AB genotype: 149 mmHg) compared with those homozygous for allele B (141 mmHg; $p=0.04$).

Discussion

This study provides evidence that high-level autoantibodies reacting with human recombinant CD38 are in the sera of patients with Type II diabetes from the Botnia cohort as well as in patients with Type I diabetes from the Diabetes 2000 Registry in Southern Sweden. Specificity tests confirmed that CD38 is indeed the protein recognised by the native autoantibodies. In Type II diabetic patients, positivity for CD38 antibodies was associated with positivity for anti-GAD antibodies. In the whole study group, positivity for anti-CD38 antibodies was associated with *DQB1**02.

The prevalence of anti-CD38 antibodies in this Type II diabetic cohort was 8.4%, which is slightly lower than that observed in Japanese [20] or Italian Type II diabetic patients [21, 22]. Given the relatively large sample size in these studies, this result probably refers to the inter-ethnic variability of autoimmune phenomena. In previous studies [20, 21, 22], no association was found between CD38ab and GADab positivity, although in the Italian study [21] the serum concentrations of the two autoantibodies were weakly correlated. In our study, the prevalence of CD38ab positivity was higher in GADab positive than GADab negative Type II diabetic patients (14% vs 6%, respectively) but the titres of the two autoantibodies were not related to one another. The association of GADab positivity with the presence of other autoantibodies (against thyroid, adrenal gland, or gastric mucosa) in Type II diabetic patients has been reported in an Italian clinic-based cohort [36]. These associations support the view that LADA is one phenotypic expression of a complex autoimmune polyendocrine syndrome, of which the presence of anti-CD38 and anti-GAD autoimmunity in these series could be regarded as a new manifestation.

By selection, the Type II diabetic group included 100 anti-GAD positive patients and 198 anti-GAD

negative patients who were representative of the whole Botnia diabetic cohort with respect to BMI and age at diagnosis. The GADab positive patients had an earlier age of onset, higher fasting plasma glucose concentrations and a more favourable cardiovascular risk factor profile as compared with GADab negative patients, as expected [19]. In contrast, CD38ab positivity did not identify a specific phenotype either with respect to clinical characteristics or to metabolic and cardiovascular profile. On pooling Type II and Type I patients together, again anti-GAD positivity clustered with younger age, earlier onset, and better cardiovascular risk profile; the concomitance of anti-CD38 positivity only slightly attenuated the LADA features of anti-GAD positive patients, showing that the latter autoimmunity dominates over anti-CD38 autoimmunity. In line with the differential phenotype were the OGTT results. For similar plasma glucose responses, the plasma insulin response was lower in anti-GAD positive patients irrespective of their anti-CD38 positivity. When beta-cell function was indexed as the integral ratio of insulin to glucose concentrations, beta-cell dysfunction was worst in association with anti-GAD positivity, and, in comparison with non-diabetic subjects, was only preserved in patients positive only for anti-CD38. These findings indicate that the clinical and pathophysiological features of autoimmunity in adult-onset diabetes can be heterogeneous, ranging from a Type I or Type II intermediate phenotype (e.g., anti-GAD positivity or LADA) to a classic Type II phenotype (i.e., antibody negative). More importantly, the OGTT results show that anti-CD38 autoantibodies per se are not likely to further impair in vivo insulin secretion in patients with established disease. This is in accord with in vitro results showing a stimulatory effect of anti-CD38 autoantibodies on cultured human islets [21] and in dispersed human islet cells [22]. The question, whether prolonged challenge of beta-cell function by stimulating autoantibodies could precipitate overt hyperglycaemia in pre-diabetic individuals, remains open as information on the natural history of anti-CD38 autoimmunity is not available. The finding, that the anti-CD38 titres were related to the duration of the disease in Type II diabetic patients, could suggest that anti-CD38 autoantibodies are associated with a slower progression of the disease.

Positivity for anti-GAD autoantibodies has been linked with *DQB1*0302* in patients with Type II diabetes in the Botnia study [19]. In this cohort (combined Type I diabetic and Type II diabetic groups), anti-CD38 positivity was weakly associated with *HLA-DQB1*02*, a genotype that has been found to be associated with GADab positivity in patients with Type I diabetes [37].

LADA is a familial disease involving autoimmune beta-cell destruction and slow progression towards insulin deficiency [38]. Associations with high-risk

HLA genotypes and autoimmune phenomena (GAD, IA2, ICA) show similarities with Type I diabetes but the phenotypical characteristics of these patients do not allow a clear differentiation from non-autoimmune Type II diabetes without screening of anti-GAD autoantibodies [39]. Early identification of LADA patients could be of clinical importance to prevent or delay beta-cell loss by immune intervention. In contrast, the identification of anti-CD38 autoimmunity might indicate a relative protection against beta-cell failure and a lower risk of insulin requirement. In anti-GAD positive patients, the presence of anti-CD38 antibodies could be associated with an attenuation of their risk of beta-cell failure.

With regard to genetic factors, another study [26] screened for mutations in the *CD38* gene and reported a missense mutation causing an Arg140 to Trp amino-acid substitution in exon 3 in 4 of 31 Japanese Type II diabetic subjects who had first-degree and/or second-degree relatives with Type II diabetes (but in none of the 90 Japanese control subjects). CD38 enzyme activity studies using COS-7 cells expressing the Arg140Trp mutation showed reductions in ADP-ribosyl cyclase and cADPR hydrolase activities of about 50%. It was therefore hypothesised that the Arg140Trp mutation could contribute to the development of Type II diabetes via an impairment of glucose-induced insulin secretion. In the same study, a silent polymorphism was found in exon 2 in the codon for Ile168 from ATA to ATC in 16% of Type II diabetic patients and control subjects. In our study, none of the 298 subjects carried the Arg140Trp mutation; likewise, we were not able to find any SSCP variants in exon 2. Therefore, in our cohort the Arg140Trp mutation does not seem to contribute to the development of Type II diabetes either through the impairment of glucose-induced insulin secretion or by inducing the appearance of anti-CD38 autoantibodies.

Regarding the bi-allelic RFLP in intron 1 for the enzyme PvUII, the observed allele frequency in the whole study cohort was 18.4% for allele A and 81.6% for allele B, similar to those reported in healthy Italian subjects (14% vs 86% [27]). In that study, only three AA homozygous subjects were found and two of them had Type II diabetes. However, no association between Type II diabetes and the PvUII polymorphism was seen in our study.

In conclusion, our study confirms the occurrence of anti-CD38 autoimmunity in both Type II and Type I diabetes in a previously well characterised ethnic group. Despite some degree of association between anti-CD38 and anti-GAD autoimmunity, the clinical phenotypes identified by the two markers were distinct, particularly with regard to in vivo beta-cell function. Other than an association with *DQB1*02*, no other genetic association was found when extensively exploring the *CD38* gene or analysing the PvUII polymorphism.

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