Binding of antibodies in sera from Type 1 (insulin-dependent) diabetic patients to glutamate decarboxylase from rat tissues. Evidence for antigenic and non-antigenic forms of the enzyme

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Summary. An islet protein of M_r 64000, identified as the γ -amino butyric acid (GABA)-synthesizing enzyme, glutamate decarboxylase, is a major target for antibodies in Type 1 (insulin-dependent) diabetes mellitus. This enzyme is also expressed in brain and in some other tissues and may exist in multiple forms. The aim of this study was to determine the ability of antibodies from diabetic patients to recognize glutamate decarboxylase from rat islets, brain and other normal rat tissues. Glutamate decarboxylase was detected at high activity levels in brain and at lower levels in islets, kidney, liver, pituitary gland, thyroid gland, adrenal gland, testis and ovary. The ability of antibodies in sera of diabetic patients to immunoprecipitate enzyme activity from detergent extracts of tissues was determined. Antibodies in sera from diabetic pa-

tients were found to bind the enzyme from islet and brain extracts, but bound less than 20% of the activity from other tissues. The ability of antibodies to immunoprecipitate the brain enzyme was significantly correlated with the presence of antibodies to the islet 64 kilodalton antigen. These studies show that the glutamate decarboxylase activity expressed in brain shares antigenic determinants with the islet 64 kilodalton antigen. Isoforms of the enzyme expressed in other non-neuronal tissues may be antigenically distinct and may lack determinants recognized by diabetes-associated antibodies.

Key words: Autoantigens, autoantibodies, glutamic acid decarboxylase, pancreatic islets.

Type 1 (insulin-dependent) diabetes mellitus is the result of the specific destruction of the Beta cells of the pancreatic islet. There is considerable evidence to suggest that the disease is autoimmune in nature [1], and this has led to the search for Beta-cell specific components that may be the targets of an autoimmune response. An islet protein of M_r 64000 (64 kilodalton (kDa) antigen) has been described as a major islet cell protein recognized by antibodies in sera of newly-diagnosed Type 1 diabetic patients [2, 3]. This protein was shown to be expressed by Beta cells of the pancreatic islet but was not detected in other cells within the islet or in several other tissues [4]. By analysing antibody reactivity to proteolytic fragments of the antigen, two distinct antibody specificities have been identified in sera of Type 1 diabetic patients that recognize 50 kDa, or both 37 kDa and 40 kDa fragments of the antigen [5]. More than 90% of recent-onset diabetic patients possess antibodies to at least one of the 64 kDa antigen-derived tryptic fragments, suggesting that the presence of these antibodies may be highly sensitive markers for disease development [5].

The 64 kDa antigen was identified recently as glutamate decarboxylase (GAD) [6]. GAD is the enzyme

responsible for the synthesis of the neurotransmitter, γ -amino butyric acid (GABA) in the GABA-ergic neurons of the central nervous system. Within the pancreatic islet, GAD and GABA expression have been shown to be restricted to the Beta cells [6–8]. GABA synthesis and secretion in islets may play a role in the paracrine regulation of glucagon or somatostatin secretion [9].

In addition to the central nervous system and pancreatic Beta cells, GABA and GAD have also been detected in several other tissues including kidney, liver, adrenal gland, ovary and testis [10–13]. In our previous analysis of tissue specificity, we failed to detect expression of 64 kDa antigen in several of these tissues. This could, potentially, be the result of the low level of expression of the enzyme in these tissues. However, there is evidence that GAD exists as multiple isoforms. Two forms of GAD (GAD₆₅ and GAD₆₇) have been identified in mammalian brain which differ in their antigenic properties and intraneuronal distribution [14]. Furthermore, three kinetically distinct forms of GAD have been purified from brain on the basis of hydrophobic-interaction chromatography [15]. Both GAD₆₅ and GAD₆₇ have been detected in pancreatic islets and both forms are recognized by diabetes-associ-

Table 1. Glutamate decarboxylase activity of rat tissues

Tissue	Glutamate decarboxylase activity ($\mu\text{mol} \cdot 16 \text{ h}^{-1} \cdot \text{g protein}^{-1}$)
Brain	104.26 $\pm$ 1.51
Islets	9.52 $\pm$ 0.16
Kidney	6.55 $\pm$ 0.26
Liver	3.73 $\pm$ 0.34
Pituitary gland	3.58 $\pm$ 1.80
Thyroid gland	2.75 $\pm$ 0.35
Adrenal gland	1.89 $\pm$ 0.77
Testis	1.46 $\pm$ 0.12
Ovary	0.92 $\pm$ 0.09
Spleen	< 0.5
Thymus	< 0.5

The activity of glutamate decarboxylase in tissue homogenates was determined by measuring the enzyme-catalysed release of $^{14}\text{CO}_2$ from [$1\text{-}^{14}\text{C}$] glutamic acid. Results are expressed as the means $\pm$ SEM of three independent observations

ated antibodies [16, 17]. However, the identities of isoforms of GAD expressed by other non-neuronal tissues have yet to be resolved and it is possible that isoforms of GAD expressed by some tissues may lack antigenic determinants recognized by the diabetes-associated antibodies. The aim of the present study was to determine the ability of antibodies in sera of Type 1 diabetic patients to bind GAD from different normal rat tissues.

Subjects, materials and methods

Subjects

Antibodies to GAD were analysed in sera from diabetic and non-diabetic individuals. Sera from 25 Type 1 diabetic children (aged 6–17 years), obtained within 10 days of diagnosis and previously characterized for antibodies to the 64 kDa antigen [18] and tryptic fragments of the antigen [5] were provided by Dr. D. Daneman (The Hospital for Sick Children, Toronto, Ontario, Canada). Sera from 30 healthy individuals (aged 21–70 years) with no known family history of diabetes were included as control subjects. Serum from a diabetic patient previously used as a reference standard in 64k antibody assays [3, 5, 18] was included as a positive control in GAD-antibody determinations.

Assay for glutamate decarboxylase in tissue extracts

Whole organs (brain, kidney, liver, pituitary gland, thyroid gland, adrenal gland, testis, ovary, spleen and thymus) were dissected from adult male or female Wistar rats. Pancreatic islets were isolated from pancreases of neonatal Wistar rats by collagenase digestion and Percoll density gradient centrifugation as previously described [19]. Tissues were homogenized with a Teflon-glass homogenizer in five times their weight of 1 mmol/l 2-aminoethyl isothiuronium bromide, 0.2 mmol/l pyridoxyl phosphate, 1 mmol/l EDTA, 1 mmol/l benzamidine, 25 mmol/l potassium phosphate, pH 7.0 (homogenization buffer). For the assay of GAD, aliquots of homogenates (2–25 μl) were incubated for 16 h at 37°C with 25 μl of 5 mmol/l L-glutamic acid and 0.125 μCi [$1\text{-}^{14}\text{C}$] L-glutamic acid in homogenization buffer. $^{14}\text{CO}_2$ released during the reaction was absorbed onto filter papers soaked with 50 μl of 1 mol/l hyamine hydroxide in methanol and quantified by liquid scintillation spectrometry.

Immunoprecipitation of glutamate decarboxylase from tissue extracts by sera

The detergent, Nonidet P40, was added to homogenates of tissues to a final concentration of 1% (weight/volume, w/v) and extracted for 2 h on ice. The soluble tissue extract was separated from insoluble material by centrifugation at 100,000 g for 30 min. Tissue extracts were diluted to a GAD activity of $4 \times 10^{-8} \text{ mol} \cdot \text{ml}^{-1} \cdot 16 \text{ h}^{-1}$ in homogenization buffer containing 1% Nonidet P40 and 50 μl aliquots of the extract incubated with 12.5 μl of test serum for 5 h on ice. Immune complexes were isolated on 25 μl of protein A Sepharose and washed four times with 1 ml of 10 mmol/l Hepes (pH 7.4), 155 mmol/l NaCl, 10 mmol/l benzamidine, 0.5% (w/v) Triton X-114, 0.5 mg/ml bovine serum albumin, 1 mmol/l 2-aminoethylisothiuronium bromide, 0.2 mmol/l pyridoxyl phosphate and once in 1 ml of homogenization buffer. GAD activity associated with immunoprecipitates was assayed as described above.

The activity of antibodies to brain GAD in sera from control and diabetic sera were quantified by determining the enzyme activity immunoprecipitated by sera from a cytosolic extract of rat brain. Brains were homogenized in a 10-fold volume of homogenization buffer and centrifuged at 100,000 g for 30 min. The supernatant was diluted to a GAD activity of $4 \times 10^{-8} \text{ mol} \cdot \text{ml}^{-1} \cdot 16 \text{ h}^{-1}$ in homogenization buffer and immunoprecipitated with sera as described above. Enzyme activity in immunoprecipitates was calculated relative to that immunoprecipitated by a standard antibody-positive reference serum included in each assay. Test sera were regarded as positive if the relative antibody activity exceeded 2 SD of the activity in sera from a group of control individuals.

Assay for antibodies to the islet 64 kDa antigen

The serum activity of antibodies to the 64 kDa antigen in detergent-solubilized extracts of islets (64k-antibodies), and antibodies to 50 kDa, 40 kDa and 37 kDa polypeptides in trypsin-solubilized extracts of islets (50 k-, 40 k- and 37 k-antibodies, respectively), were determined as previously described [5].

Protein determinations

The protein content of tissues was assayed by the Biuret Reaction [20].

Statistical analysis

The degree of association between antibody activities was tested by linear regression analysis.

Results

Activity of glutamate decarboxylase in tissue extracts

The results of an analysis of GAD activity in tissue homogenates of normal adult rat tissues are presented in Table 1. The highest GAD activity was found in brain. Enzyme activity was also detected in islets, kidney, liver, pituitary gland, thyroid gland, adrenal gland, testis, and ovary. GAD activity of spleen and thymus was below the detection limit of the assay.

Immunoprecipitation of glutamate decarboxylase by antibodies in sera of diabetic patients

In all tissues, GAD was detected in particulate and cytosolic forms. In order to investigate whether GAD in each tissue was recognized by antibodies in diabetic patients'

Fig. 1. Recognition of glutamate decarboxylase (GAD) by antibodies in sera from Type 1 (insulin-dependent) diabetic or control individuals. Nonidet P40-solubilized extracts of rat tissues were adjusted to the same GAD activity and incubated with sera from 64k-antibody-positive diabetic patients (first three bars for each tissue), with serum from a 64k-antibody-negative diabetic patient (fourth bar) or a 64k-antibody-negative control serum (fifth bar). The activity of GAD immunoprecipitated was calculated as a proportion of the total activity added. The results are the means $\pm$ SEM of 2–4 independent observations

sera, tissue homogenates were extracted in Nonidet P40 to solubilize the membrane-associated enzyme. The activities recovered from pituitary gland, thyroid gland, spleen and thymus were too low for these tissues to be included in further analyses. Detergent extraction solubilized greater than 85% of the total GAD activity from other tissues without inhibiting enzyme activity. Detergent extracts from these tissues were adjusted to an equivalent GAD activity and incubated with sera previously determined to be either strongly positive or negative for antibodies to the islet 64 kDa antigen. Immune complexes were adsorbed onto protein A Sepharose and the activity of GAD associated with immunoprecipitates determined. The 64k-antibody-positive sera immunoprecipitated greater than 63% of the GAD activity from islets or brain, but bound less than 20% of the activity from all other tissues (Fig. 1).

Experiments were performed to determine whether there is a direct association between antibodies to brain GAD and antibodies to the islet 64 kDa antigen. Sera from 25 recent-onset diabetic children, previously characterized for antibodies to the 64 kDa antigen [5, 18], and 30 healthy control individuals, were analysed for antibodies to the rat brain enzyme. The mean GAD activity ($\pm$ SD) immunoprecipitated by antibodies in sera of the 30 control sera was $6.17 \pm 3.44\%$ of that precipitated by a reference serum included in each assay. Sera were regarded as positive if the GAD activity in immunoprecipitates exceeded 2 SD of the mean activity of the control subjects' sera. Sixteen of the diabetic patients (64%) were determined to be positive for antibodies to brain GAD. Antibodies to intact islet 64 kDa antigen and antibodies to 50 kDa, 40 kDa and 37 kDa tryptic fragments of 64 kDa antigen were present in 20 (80%), 20 (80%), 19 (78%), and 19 (78%) of the 25 diabetic patients respectively. A strong positive correlation was found between antibodies to brain GAD and islet 64 kDa antigen (Fig. 2a; $r = 0.91$,

$p < 0.001$). A weaker, but significant, correlation was observed between GAD-antibodies and antibodies to the 50 kDa tryptic fragment (Fig. 2b; $r = 0.70$, $p < 0.001$). GAD-antibodies were not associated with antibodies to the 37 kDa or 40 kDa tryptic fragments (Fig. 2c and d).

Discussion

Baekkeskov et al. [6] have provided evidence that at least part of the diabetes-associated islet 64 kDa antigen is the GABA-synthesizing enzyme, glutamate decarboxylase. In particular, antibodies in sera of Type 1 diabetic patients were found to immunoprecipitate a GAD activity from brain and islet extracts. The authors did not demonstrate a direct association between antibodies to GAD and antibodies to the islet 64 kDa protein. In the results presented here, a strong positive correlation between antibodies to brain GAD and 64k-antibodies was observed, providing convincing evidence that the islet antigen shares antigenic properties with brain GAD. Fewer sera from recent-onset diabetic patients were positive in the GAD-antibody assay (64%) than were positive for 64k-antibodies (80%). It is possible that inhibitory effects of antibody binding on enzyme activity may reduce the number of individuals positive for GAD-antibodies in this assay. All 64k-antibody-positive patients who were negative for GAD-antibodies had low 64k-antibody levels. Thus, under the conditions described, the GAD-antibody assay has a lower sensitivity of antibody detection than the 64k-antibody assay. However, the relative simplicity of the GAD-antibody assay procedure, particularly in the preparation of the antigen extract, makes this assay more attractive for the further analysis of antibodies to the antigen in specific populations.

Both brain GAD and the islet 64 kDa antigen migrate as two components on sodium dodecyl sulphate-polyacrylamide gel electrophoresis [6] and may therefore be complexes of more than one polypeptide chain. There is evidence that individual components of brain GAD may differ immunochemically [21]. By analysing antibodies to tryptic fragments of the 64 kDa antigen, we have identified two distinct antibody specificities in sera of Type 1 diabetic patients [5]. Determinants recognized by these antibodies may be localized on different polypeptide chains [5]. A significant association between antibodies to brain GAD and antibodies to a 50 kDa trypsin fragment was observed in this study, suggesting that determinants recognized by GAD-antibodies are present on this fragment. The degree of association was weaker than the association with antibodies to intact antigen. Trypsin treatment may alter the conformation of some determinants on the native antigen, impeding or enhancing recognition by autoantibodies. No association was observed between GAD-antibodies and antibodies to either the 37 kDa or 40 kDa tryptic fragments. Some sera which did not immunoprecipitate a GAD activity were strongly positive for antibodies to these fragments. We have previously suggested that antibodies to the 37 kDa and 40 kDa polypeptides recognize determinants that are hidden on the native 64 kDa antigen, but that are exposed on

Fig. 2. Associations of antibodies to rat brain glutamate decarboxylase (GAD) with antibodies to islet 64 kDa antigen. The ability of antibodies in sera from Type 1 (insulin-dependent) diabetic patients to immunoprecipitate GAD from brain extracts was compared with the ability of antibodies to immunoprecipitate 64 kDa antigen from detergent extracts of islets (**a**), or 50 kDa (**b**), 37 kDa (**c**) and 40 kDa (**d**) fragments of antigen from trypsin-treated particulate fractions of islets. Data are expressed as a percentage of the antibody in a 64k-antibody-positive reference serum and are the means of the least two independent observations

trypsin cleavage [5]. The failure of 37k- and 40k-antibodies to bind a GAD activity in non-protease-treated brain extracts is consistent with this proposal. However, it is also possible that these antibodies bind a protein that is distinct from the GAD enzyme.

In addition to pancreatic Beta cells, GAD has been detected in a number of other non-neuronal tissues, either by measurement of enzyme activity or by immunocytochemistry with antibodies raised to the brain enzyme [10–13]. Consistent with these reports, we were able to detect GAD activity in kidney, liver, pituitary gland, adrenal gland, thyroid gland, testis and ovary. However, in contrast to the enzyme in islets and brain, GAD extracted from most of these tissues was not recognized by antibodies in sera of 64k-antibody-positive diabetic patients. In an earlier study, diabetes-associated antibodies failed to immunoprecipitate a 64 kDa protein from a number of non-islet tissues, including kidney, liver, adrenal and pituitary glands [4]. The GAD activity expressed by these tissues appears to be immunochemically distinct from the enzyme in brain and islets, lacking antigenic determinants recognized by diabetes-associated 64k-antibodies. Despite failure of autoantibodies to bind GAD from several non-neuronal tissues, GAD expressed by these tissues may have structural features in common with the brain enzyme. Polyclonal antibodies raised to the brain enzyme recognize a GAD immunoreactivity on sections of both ovary and testis [12, 13]. Furthermore, cDNA for brain

GAD₆₇ has been shown to hybridize with mRNA isolated from testis [13]. The major testicular mRNA species recognized was 2.5 kilobases, distinct from the 3.7 kilobase mRNA predominant in brain. These results indicate that GAD in brain and testis are encoded by different mRNA species, but that these molecules have considerable sequence homology and the proteins may share some antigenic determinants recognized by GAD antisera. Recent studies have shown that cDNA for GAD₆₅, which hybridizes predominantly with a 5.6 kilobase mRNA in islets and brain, also recognizes a 2.5 kilobase transcript in human islets, which may be related to the major testicular species [22]. Comparison of nucleotide sequences of cDNA for antigenic and non-antigenic forms of the GAD molecule may help to identify domains recognized by autoantibodies in diabetes.

It is unclear whether the distinct GAD activities expressed in different tissues have similar functions. It is evident that GABA synthesis in brain has a signalling role, GABA being the major inhibitory neurotransmitter in the nervous system [23]. There is evidence that GABA has a signalling function in islets as well. In pancreatic Beta cells, GABA and GAD co-localize with microvesicles that have similar properties to the GABA storage vesicles in the nervous system [24]. Furthermore, GABA receptors are expressed by pancreatic Alpha cells, and GABA, or GABA agonists, have been shown to influence the secretion of glucagon and somatostatin release from

pancreatic islets [9, 25]. GABA may therefore be a paracrine regulator of hormone release in the islet. In other tissues, a signalling function for GABA is less clear. In kidney, GAD may have a metabolic role, particularly in the catabolism of glutamine [10]. In spermatozoa, GAD-like immunoreactivity was detected predominantly in the mid-piece region, which is rich in mitochondria and responsible for energy generation, again suggestive of a metabolic function. Thus, GAD enzymes expressed in kidney, testis and possibly other tissues may be functionally, as well as antigenically distinct from the enzymes expressed in brain and islets.

In conclusion, the results of this study show that, despite the rather widespread tissue distribution of GAD enzyme activity, GAD isoforms recognized by autoantibodies in diabetes may be restricted to the brain, an immunologically privileged site, and pancreatic Beta cells. Further characterization of the structures and patterns of expression of the GAD isoforms may help determine why some forms of GAD are autoantigens in diabetes whereas others are not.

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