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INVESTIGATION ON ANTI-GLUTAMIC ACID DECARBOXYLASE ANTIBODIES IN TYPE I DIABETES MELLITUS

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ABSTRACT

Antibodies directed against the enzyme glutamic acid decarboxylase (GAD) are believed to be the main cause of destruction of pancreatic islet cells in type I (insulin dependent) diabetes mellitus. The enzyme was found both in the brain and pancreatic beta cells. Although similarities in identity of GAD in human and rat brain have been demonstrated, little is known about the interaction between the enzyme and antiserum in type 1 diabetic patients. In the present study GAD was partially purified from rat brain homogenate. The four-step procedure involves, sequentially, an ultracentrifugation, DEAE-cellulose, hydroxyapatite resin, and Sephadex G-200 gel filtration chromatography. The enzyme activity was assayed either manometrically or fluorimetrically. The results showed a positive correlation between the rates of CO_2 production with the changes of fluorescence intensities of the enzyme after addition of glutamate. The collected fraction from the gel filtration chromatography showed approximately 140-fold purification of the enzyme with a 15% yield. The specific activity of the enzyme of brain supernatant and the partially purified enzyme collected from every chromatographic step was measured upon addition of the serum samples from type I diabetes (n=11). A marked decrease in the rate of CO_2 production or the change of fluorescence intensities of the enzyme was observed, indicating an interaction between the enzyme and the patients' sera. However, serum samples from healthy control individuals had little effect on the enzyme activity of the partially purified GAD. The results suggested that rat brain GAD might be used as an *in vitro* reagent for screening of type I diabetes, using an enzyme inhibition assay.

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INTRODUCTION

Type I diabetes mellitus is well documented as an autoimmune disease in which pancreatic beta cells are progressively destroyed.[1-4] Autoantibodies directed against a specific protein of the pancreatic beta cells are believed to be the main cause of the destruction. A membrane-bound protein expressed by the beta cells was reported to be a major autoantigen in type I diabetic patients. This protein was demonstrated to be the GABA synthesizing enzyme, glutamic acid decarboxylase (GAD).[5-7] Both GABA and GAD were found not only in

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the brain GABA-ergic system but also in the pancreatic beta cells.[8,9] The immune response to GAD in type I diabetic patients suggests that anti-GAD antibody may be considered as an autoimmune marker.[10-12] The most common methods for identification of anti-GAD antibody involved the immunoprecipitation of radioligand assay (RIA) and enzyme-linked immunosorbent assay (ELISA), in which *in vitro* translated GAD has been used as an antigen.[13]

However, the production and /or availability of translated GAD appear to be limited. It is therefore of interest to look for other sources of the enzyme for screening of type I diabetes in a large population. Because the expression of GAD in human and rat brain is reported to be similar,[14-15] the present study was undertaken to investigate the possibility of applying rat brain GAD for identification of type I diabetic patients.

MATERIAL AND METHODS

Materials

DEAE-cellulose, Sephadex G-200, hydroxyapatite, phenyl methyl sulfonylfluoride (PMSF), dithio-trathiol (DTT), aprotonin, pyridoxal phosphate (PLP) and bovine serum albumin were obtained from Aldrich Chemical Company, Dorset, U.K. All other reagents used were unless stated otherwise of analar grade (or the highest available) and made up in double distilled water.

Serum samples from 11 diagnosed patients with type I insulin depended diabetes were examined. The office guide to diagnosis and classification of insulin dependent diabetes mellitus, outlined by the American Diabetic Association[16] was used for diagnosis of the patients. Serum samples from apparently healthy subjects (age and sex were matched), with no familiar background of diabetes were assayed as control.

GAD Preparation

In each experiment, five rats (male Wistar, 200-250g) were killed by decapitation and brains were removed onto an ice-cold glass plate over crushed ice and chopped into the consistency of mince, which was rapidly transferred into 50mL buffer (pH 7) containing 25 mM potassium phosphate, 0.2 mM pyridoxal -5- phosphate, 1mM EDTA, 0.1 mM phenyl methyl sulfonylfluoride, 5mM dithiotrathiol and 1% aprotonin and homogenized on ice. Purification of GAD was essentially carried out as described by Nathan et al.[17] The homogenate was centrifuged at 70,000 for 60 min at 4°C (LS-50 Beckman) and the supernatant was poured into a column of DEAE-cellulose (1.5cm×40cm) and eluted with a linear gradient of phosphate buffer (pH 7) from 0.03 to 0.30 M containing 5 mM DTT. The protein fractions were detected by spectrophotometer at 280 nm and the activity of the enzyme was assayed.

The active GAD fractions were combined and chromatographed on a 0.5cm×30cm hydroxyapatite resin column, which was eluted as above with the same gradient of phosphate buffer. The active fractions were then collected and pooled for Sephadex G-200 gel filtration chromatography.

Enzyme assays

The activity of GAD in the rat brain preparations was measured by two different methods; a Warburg manometeric method,[18] and a fluorimeteric method.[19] In the manometeric method 1 mL aliquots of the brain supernatant or eluted partially purified enzyme were adjusted to pH 7.4 and incubated at 37°C for 5 min. The reaction was then started by addition of 100 μL glutamate solution (10 mM) and CO_2 production was measured for 15 min. The results are expressed as μL CO_2 produced/min/mg protein. In the fluorimetric method 1 mL of the

Table I: Partial purification of glutamic acid decarboxylase from rat forebrain.

Fraction	Total protein (mg)(SD)	μL CO_2 production / min / mg protein(SD)	ΔF/min/mg protein (SD)
Brain supernatant	640.0(15.60)	0.05(0.00)	0.23(0.01)
DEAE-cellulose eluate yield	61.5(7.33)	0.25(0.01)	1.36(0.19)
[%]		56	54
Purity [fold]		5.0	5.2
Hydroxyapatite eluate yield	3.7(0.19)	2.25(0.17)	10.51(0.85)
[%]		26	26
Purity [fold]		45	46
Gel filtration eluate yield	0.67(0.09)	7.06(0.42)	32.50(2.04)
[%]		15	15
Purity [fold]		141	140

Table II: Effect of serum of type I diabetes on rat brain GAD activity.

Serum sample	Manometric assay (% of inhibition)	Fluorimetric assay (% of inhibition)
Type I diabetes	59(9)*	66(11)*
Non-diabetes	7(6)	7(5)

The activity of the partially purified rat brain glutamic acid decarboxylase was measured by both manometric and fluorimetric methods in the presence of serum samples from either type I diabetic patients (n=11) or non-diabetic subjects (n=11). Values are mean with SD in round brackets. Statistically significant differences between the diabetic and non-diabetic groups were determined by Student's t-test. $*p<0.005$.

enzyme preparations (pH 7.4) was pre-incubated at 37°C and transferred to a cuvet containing 100 µL glutamate solution (10mM). The cuvet was then placed into a LSE spectrophotofluorimeter (Perkin-Elmer, Norwalk, CT) with the excitation wavelength of 495 nm and the emission wavelength of 540nm, and increasing of fluorescence intensities (ΔF) was monitored for 15 min against a blank containing all components except glutamate. The results are expressed as ΔF/min/mg protein. The rate of glutamate decarboxylation or change of fluorescence intensities were linear with times up to more than 30 min. The determination of the protein concentration was performed by the method of Lowry et al.[20]

Enzyme Inhibition Assay

In order to examine the possible interaction between the partially purified rat brain GAD (final step) and the serum samples taken from type I diabetic patients, the activity of the enzyme was measured after the addition of 100 µL of the serum samples into the assay mixture. The experiment was carried out as above and CO_2 production or the changes of fluorescence intensities were measured either by manometric technique or fluorimeteric assay respectively. The results were expressed as percentage inhibition of the enzyme.

RESULTS

The specific activity of GAD in rat brain supernatant and the fractions eluted from each chromatographic step as measured by both manometric and fluorimetric assays is summarized in Table I. A positive correlation was found between the rate of CO_2 formation and the changes of fluorescence intensities in the different steps of purification (r=0.98). Of 640 mg brain supernatant protein applied to the above described chromatographic purification steps, 0.67 mg enzyme protein was obtained, which its specific enzyme activity increased approximately 140 folds of that in the supernatant. As can be seen in Table I the values of yield percentage and the purification folds of the enzyme as recorded by the two different assay procedures were quite similar. The values are in good agreement with the results previously reported.[17,21]

Pooled enzyme eluted from the gel filtration step was used to assess the interaction between GAD and serum samples from type I diabetic patients. The results are summarized in Table II. Based on the enzymatic assays, the patient's serum samples caused a 59% inhibition of the CO_2 production and a 66 % reduction in the enhancement of the fluorescence intensities of the enzyme /substrate complex. However, the percentage of inhibition as measured by the manometric method was not significantly different to that of fluorimetric assays.

The rate of CO_2 production or the changes of fluorescence intensities in each step were measured by manometric or fluorimetric methods respectively. The results are mean of 6 separate experiments with SD in round brackets. In each experiment 5 forebrains were processed as described in the *Methods* section.

DISCUSSION

The results reported in this paper demonstrated that the release of CO_2 and the changes of the fluorescence intensities of the assay mixture increased markedly as purification proceeded (Table I). The rate of CO_2 production by the rat brain supernatant and the eluted fractions from the three chromatographic steps, positively correlated with the increasing rate of the fluorescence intensities (r=99%) of the enzyme preparations. It is very well know that the changes of fluorescence intensities of an enzyme with PLP prosthetic group in the presence of its substrate can be taken as a measure for the enzyme activity.[19,22,23] The increasing fluorescence intensities of the rat brain GAD upon addition of glutamic acid is interpreted as being consistent with the enzyme/substrate complex formation.[19,22,23] In addition, the similar yield percentages and purification folds of the enzyme preparations indicated that the fluorimeteric technique is well suited for the assay of GAD.

The inhibition of the rat brain GAD activity by addition of the serum samples from type I diabetic patients may be considered as another important finding of the present study (Table II). The identical rate of inhibition of the enzyme as measured by manometric and fluorimetric assays may indicate that there is probably a factor in the patient's serum samples which interacts with the enzyme/substrate complex. Because GAD is reported as an autoantigen in type I diabetes,[5-7] it is logical to assume that the enzyme inhibition may have resulted from an antigen/antibody reaction.

However, the interaction between rat brain GAD and the human antibody may be considered particularly with the view of the protein structure of GAD. It has been demonstrated that there are many similarities in identity of rat brain GAD 65 Kda with the human GAD 67 Kda.[15]

It is concluded that rat brain GAD might be used as an *in vitro* reagent for screening of type 1 diabetes, using an enzyme inhibition assay. However, a major question that remains to be answered is whether rat brain GAD can be used as a reagent for identification of type I diabetes in human. The inhibition of the enzymatic activity of the rat brain GAD by the serum samples of the clinically diagnosed type I diabetic patients (Table II) may address this question, by suggesting that rat brain GAD may be inhibited by anti-GAD antibodies of the patient's sera.

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