

The Diabetic Antigen Glutamic Acid Decarboxylase (GAD 65) in the Human Peripheral Blood

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Key Words

Glutamic acid decarboxylase · GAD 65 · Diabetes mellitus type 1 · Autoimmunity · Fluorescence correlation spectroscopy · Molecular visualization

Abstract

Background: Glutamic acid decarboxylase (GAD 65) is a diabetes-associated antigen which is generally considered to be strictly intracellular. In order to better understand autoimmunity, this study demonstrates the appearance of GAD 65 in the peripheral human blood and presents implications for the diagnosis and therapy of some autoimmune diseases.

Methods: The GAD 65 molecules are detected by their interaction with monoclonal antibodies labeled with dyes in an experimental setup with fluorescence correlation spectroscopy (FCS). These interactions result in changes in Brownian motion measured as fluorescence fluctuations. Sera from 153 patients with diabetes mellitus type 1 and controls were investigated. To enable the representation of the molecule as a model for further discussions, we present structural visualizations of its hydrophobic properties, leading to possible interactions with the cell membrane lipids and epitope locations. **Results:** The GAD 65 antigen could be measured with a sensitivity of 2.65 µg/ml in 'clean systems' resulting from spiking experiments and human sera. The GAD 65 antigen could be identified in 8 patient sera: 4 children with diabetes mellitus type 1 and 4 adults initially taken as controls but who

retrospectively showed signs of autoimmunity. **Conclusion:** We conclude that these findings are of significance for the concept of autoimmunity, i.e. in an initial step the immune system is primed by its accessibility to GAD 65. Our experimental results may also be important for the therapy of diabetes mellitus type 1 and other autoimmune diseases by the passive administration of GAD 65 antibodies.

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Introduction

Autoimmunity affects about 5–10% of the Caucasian population. Diabetes mellitus type 1 (insulindependent diabetes mellitus type 1; IDDM) represents a major problem among these autoimmune diseases since complications are debilitating and life threatening, causing a major burden in all respects [1–3]. If the present trends continue, a doubling of new cases of IDDM in European children younger than 5 years is expected until 2020, and the prevalence of cases younger than 15 years will rise by 70% [4]. Therefore, early diagnosis is desirable for the prevention and perhaps eradication of some autoimmune diseases.

Glutamic acid decarboxylase (GAD) catalyzes the irreversible α -decarboxylation of L-glutamate to γ -aminobutyrate (GABA). Among other enzymes, GABA and GAD are consistently present in the pancreatic β -cells.

GAD 65, the 65-kDa human isoform of GAD, is a typical autoantigen, involved in IDDM [5, 6]. In autoimmune diabetes, a cell-mediated immune reaction to the pancreatic β -cells leads to their complete destruction, resulting in the inability of the body to produce the required insulin. Therefore, the patient who suffers from IDDM needs an external supply of insulin. Hitherto, the GAD 65 antigen has been considered to be strictly intracytoplasmic. This is a misconception in immunology since an intracellular antigen would never have access to the immune system.

In this presentation, we show – to our knowledge for the first time – the appearance of the diabetes-associated antigen GAD 65 in the peripheral blood. Once accessible to the immune system, GAD 65 may activate macrophages and lymphocytes as the initial step of autoimmunity. Speculatively, and as shown in other immune disorders such as rhesus disease, this initiation of autoimmunity should be preventable by the passive administration of a specific antibody, since early autoantibodies may be a signal of insufficient regulation. On the other hand, GAD 65 may be a potential candidate for the development of vaccines.

In this study, sera from 153 patients (27 juveniles and 126 adults) with early IDDM and controls from normal admissions were used for the investigations. The GAD 65 molecules are detected by binding with their unique and specific antibodies (lock and key principle: this enables it to pick the GAD 65 molecules in a mixture of different molecules). Because we expected very small concentrations of GAD 65 circulating in human sera, we selected an ultrasensitive technique for the detection of GAD 65, i.e. fluorescence correlation spectroscopy (FCS), which is based on autocorrelation and cross-correlation analysis of fluorescence fluctuations. With FCS, the GAD 65 molecules and their interaction with the corresponding monoclonal antibodies are measured by determining their diffusion times in a tiny volume element. For this purpose the antibody molecules are labeled with fluorochromes and excited by laser light. The diffusion of the molecules through the volume element, due to their Brownian motion, is measured as fluctuations of emitted fluorescence light. The characteristic diffusion times of the molecules is derived from the autocorrelation and cross-correlation analysis of the fluorescence signal. Conclusions on the binding of GAD 65 to the corresponding antibody may be drawn from the changes in the diffusion times. The diffusion time of the labeled molecules depends on their size; therefore, antigen-antibody compounds, which are larger, show a slower diffusion

time than the separated individual molecules. Modifications of the diffusion times provide the required information on the existence and concentration of GAD 65 in the specimens.

First, the sensitivity of FCS for detecting GAD 65 was determined in clean and spiking experiments. FCS is a very sensitive method that is able to detect single molecules in 'clean systems' (this means in distilled water). For GAD 65, we detected the lowest precisely detectable amount of 2.65 $\mu\text{g/ml}$ in such clean systems. For this reason, we did not look for smaller amounts in the search for GAD 65 in peripheral blood. To verify the minimal precisely detectable amount in sera, we added the same amount of GAD 65 to healthy control sera as to the clean systems. In human sera, other molecules such as bilirubin, albumin and other proteins cause interference with the GAD 65 molecules. Because the relative fluctuations of the fluorescent signal become weaker with increasing concentrations of molecules (due to statistical averaging), it is important to minimize the concentration of molecules in the focal volume. Therefore, for the detection of GAD 65 by FCS, the patients' sera had to be diluted to obtain a sufficiently 'clean system'. GAD 65 could thus be detected in 8 patient serum samples. The patients were: 4 children with IDDM and 4 adults (initially taken as controls) but who retrospectively showed signs of autoimmunity and inflammation.

For a basic understanding of the mechanism enabling the primary intracellular GAD 65 to diffuse into serum, we have carried out an analysis of its hydrophobic/hydrophilic properties based on a structural model of the molecule. Regions with higher hydrophobic values at the protein surface can potentially interact with hydrophobic membrane lipids, leading to a reversible anchoring of the molecule to the cell membrane. Additionally, the locations of some epitopes on the GAD 65 structure were visualized, and might serve as a model for the discussion, planning and development of active and passive immunotherapy, based on the GAD 65 antigen. These structural visualizations might help the reader to better follow the text by enabling him or her to imagine the different parts of the molecule for further discussions.

The concentration of 2.65 $\mu\text{g/ml}$ detected in patient sera is higher than expected. As the measurement procedure of the ultrasensitive technique of FCS is rather time-consuming and requires a sufficiently 'clean system', the enzyme-linked immunoabsorbent assay (ELISA) seems to be more suitable for routine diagnosis, and has been proposed by us for further investigation. We believe that GAD 65 may occur in small concentrations in the periph-

eral blood under normal conditions and that GAD 65 antibodies are involved in neutralization and regulation processes, and hence may be useful for passive suppression of incipient IDDM. The neutralization of GAD 65 by specific antibodies in the early stage would be crucial for successful therapy.

Material and Methods

Fluorescence Correlation Spectroscopy

Principle. FCS is a correlation analysis of fluctuations of fluorescence radiation. The method enables the direct measurement of the interaction of antigen molecules with specific monoclonal antibodies [7–15]. Specific antibodies are used for the detection of the GAD 65 antigen. For this purpose, the behavior of the GAD 65 molecules and their interactions with antibodies labeled with fluorescent dyes are observed in a volume element. The Brownian motion of the labeled antibody molecules is a function of the reaction with the GAD 65 molecule (the Brownian motion of the GAD 65-antibody complex is slower than the motion of single antibodies). The movement of the molecules is measured in a tiny virtual volume element formed by a laser beam crossing the sample (GAD 65 and labeled antibodies) in the FCS setup. Only a sufficiently small volume element allows the observation of fluctuations due to the kinetics of the single molecules (in a large volume, the macroscopic physical parameters are statistically averaged by a large number of molecules and fluctuations related to microscopic kinetics cannot be observed). The volume element is an open measurement volume in which the molecules can freely diffuse inside and outside of the volume. The fluorescent dyes are excited by the laser light and emit a fluorescence radiation. When a fluorescent molecule enters the tiny illumination volume element the average fluorescence intensity increases. When the molecule leaves the volume element the average fluorescent intensity decreases. The characteristic diffusion time of the molecules in the volume element is then detected as fluctuations of the emitted fluorescence light intensity (the diffusion time is proportional to the reciprocal of the average diffusion speed of the molecules). Interactions of the GAD 65 antigen with the corresponding antibodies result in changes in diffusion time and thus of fluorescent light fluctuations. The correlation analysis provides parameters of the physical model under the fluctuations. By time correlation of the measured fluorescence light, the mean diffusion time and therefore the mass of the molecules can be calculated. Since the relative fluctuations become smaller with increasing numbers of measured molecules, it is important to minimize the number of molecules in the focal volume.

Experimental Setup. The optical unit of the FCS setup consists of a ConfoCor 2 (Zeiss) spectrometer and a confocal Zeiss Axiovert 100 microscope (fig. 1). An argon ion laser (458, 488 or 514 nm) and 2 helium neon lasers (543, 633 nm) are used for the excitation of the fluorescent molecules. From the laser unit, the laser beams are directed to the microscope (Axiovert 100 M) via the main beam splitter (dichroic mirror). The laser light is concentrated by a C-Apochromat objective (×40/1.2 W corr.) and passes through the GAD/anti-GAD immune complexes, pipetted into a borosilicate chamber (Nalge Nunc, Lab Tek. chambered cover

glass, German borosilicate cover glass, 8-chamber version), located on top of the inverted microscope (fig. 1). The laser beams are focused on small spots (approximately 0.5 μm in diameter) in the sample solution (tiny virtual volume elements).

The molecules in the sample labeled with fluorescent dyes are excited by the laser light. The emitted fluorescence light is collected by the high numerical aperture objective and passes through the main beam splitter (fig. 1). The beam splitter allows the separation of fluorescence light and excitation light by transmitting the emitted fluorescence light to the spectrometer (ConfoCor 2) and reflecting the excitation light back to the objective. The transmitted fluorescence light then passes through a pinhole which blocks scattered excitation light and fluorescence light from outside the focal spot. The intensity of the filtered light is measured by photo diodes (APD: avalanche photo diode).

Measurements. After band-pass filtering of the light, the fluorescence intensity fluctuations are recorded over time and the autocorrelation is measured. The measured fluorescence signal is compared with itself at different time steps by autocorrelation analysis at a later time. This enables the detection of recurring patterns. Fluctuations in the fluorescence signal are quantified by temporal autocorrelation of the recorded intensity signal. The detected fluorescence signal $I(t)$, at time step t , fluctuates around the mean value $\langle I(t) \rangle$. By assuming constant excitation power, the fluctuations of the emitted fluorescence signal are defined as the deviations from the temporal average of the signal:

$$\delta I(t) = I(t) - \langle I(t) \rangle$$

The mean value of the fluorescence signal $I(t)$ is given by:

$$\langle I(t) \rangle = \frac{1}{T} \int_0^T I(t) dt$$

The autocorrelation function $G(\tau)$ combines the average fluctuation of the molecule at time step t with its fluctuation at a later time step $(t + \tau)$. The normalized autocorrelation function is defined as:

$$G(\tau) = \frac{\langle \delta I(t) \cdot \delta I(t + \tau) \rangle}{\langle I(t) \rangle^2}$$

The intensity signal is analyzed with respect to its self-similarity after the lag time τ (correlation time). The autocorrelation function enables the detection of very subtle changes in fluorescence intensity. The autocorrelation amplitude $G(0)$ is the normalized variance of the fluctuating fluorescence signal. Every process producing fluctuations in fluorescence intensity has a characteristic correlation time.

If all fluctuations arise from changes in the local concentration of the molecules within the volume element of the focal spot, the variations in fluorescence intensity can be written as:

$$\delta I(t) = \int_V W(r) \delta(\eta C(r, t)) dV$$

The term $\delta(\eta C(r, t))$ determines the dynamics of the molecules: $\delta C(r, t)$ describes the fluctuations in the local molecule concentration at time t and the parameter η determines photon count rate per detected molecule per second. The function $W(r)$ describes the spatial distribution of the emitted fluorescence light. Substituting the expression for the intensity fluctuations in the autocorrelation function yields:

$$G(\tau) = \frac{\int \int W(r) W(r') \langle \delta(\eta C(r, t)) \delta(\eta C(r', t + \tau)) \rangle dV dV'}{\left(\int W(r) \delta(\eta C(r, t)) dV \right)^2}$$

The autocorrelation function $G(\tau)$ is characterized by the correlation time τ and the correlation amplitude $G(0)$. These parameters can be easily understood and interpreted by considering the underlying physical models.

If the model of free diffusion is used, the fluctuation signal is characterized by the diffusion time (in-out motion of the molecules in the volume element) and the average number of molecules in the volume element. This implies that the molecules are freely diffusing in three dimensions with a diffusion coefficient D . The larger the mass of the molecule, the longer the diffusion time and, as a consequence, the fluctuation is getting slower. By approximating the function $W(r)$ by a three-dimensional Gaussian distribution (which is decayed to e^{-2} at r_0 in lateral direction and for $z = z_0$ in axial direction), the autocorrelation function can be calculated analytically, for the model of free diffusion, as:

$$G(\tau) = G(0) \frac{1}{\left(1 + \frac{\tau}{\tau_D}\right)} \frac{1}{\sqrt{1 + \left(\frac{r_0}{z_0}\right)^2 \left(\frac{\tau}{\tau_D}\right)^2}}$$

τ_D is the diffusion time. This is the mean transit time of the molecule in the volume element (reciprocal of the average diffusion speed of the molecules). The autocorrelation amplitude $G(0)$ is proportional to the reciprocal of the average number of molecules $\langle N \rangle$ in the volume element:

$$G(0) = \frac{1}{\langle N \rangle}$$

By assuming a three-dimensional Gaussian distribution of the emitted fluorescence light ($W(r)$), the effective focal volume is calculated by:

$$V_{\text{eff}} = \pi^{\frac{3}{2}} r_0^2 z_0$$

The dimensions r_0 and z_0 are determined by calibration measurements. Then, the local concentration of the fluorescent molecules in the volume element can be determined from the amplitude $G(0)$ of the autocorrelation function:

$$\langle C \rangle = \frac{\langle N \rangle}{V_{\text{eff}}}$$

$$\langle C \rangle = \frac{1}{V_{\text{eff}} G(0)}$$

The relationship between the diffusion time τ_D and the diffusion coefficient D is given by:

$$\tau_D = \frac{r_0^2}{4D}$$

The decay of the autocorrelation function is determined by the diffusion time (fig. 1). The diffusion time τ_D is determined as fit parameter from the autocorrelation curve. Every augmentation of the mass of a biomolecule resulting from the interaction with a second molecule can be determined from the increase in diffusion time. Molecular interactions are followed up by recording successive autocorrelation curves with the shortest possible inte-

gration time. From those autocorrelation curves, changes in diffusion time are determined (fig. 1).

In autocorrelation analysis, the measured fluorescence signal is compared with itself at a later time. Cross-correlation analysis is a generalization of autocorrelation analysis, whereby two different signals are correlated to detect common features in both independently measured signals. This provides much higher detection specificity and removes unwanted measurement artifacts such as intensity fluctuations of the laser source and 'afterpulsing' of the detector. In dual-color cross-correlation analysis, two different fluorescent dyes are excited in the volume element by use of two overlapping laser beams. The resulting fluorescence signals are detected by separate photo diodes.

In this study, the interaction of GAD 65 with antibodies is determined and the autocorrelation and cross-correlation functions are based on the model of free diffusion of molecules in a solution. Analyses were made with 5–30 measurements, each of 20 s; the autocorrelation and cross-correlation were determined and the diffusion time defined as mean value. The emissions were recorded with wavelengths between 505 and 550 nm and above 650 nm.

Specimens

153 patients (27 juveniles and 126 adults) were investigated. The sera from the pediatric patients came from a serum bank, the adult sera were fresh.

All children between 5 and 12 years were investigated after the onset of IDDM and already had autoantibodies.

126 sera of adults from normal admissions to the clinic were also investigated and served as controls (instead of healthy blood donors as usual controls). Although they came to the clinic for various reasons, retrospectively 4 of those subjects showed signs of autoimmunity and inflammation. The sera had not been checked before for IDDM autoantibodies.

In these patients, we searched for GAD 65, believing in a multiparenchymal and therefore pancreatic participation in the general inflammatory and autoimmune process.

Reagents

GAD 65 Antigen. The GAD 65 antigen was kindly provided by Phadia (formerly Pharmacia), Sweden-Diagnostics, Freiburg/Breisgau, Germany. The recombinant antigen was used in all 'clean' and 'spiking experiments'. The GAD 65 antigen was produced in Sf9 insect cells in the Baculovirus systems. These recombinant antigens are expressed as His-Tag proteins and purified on immobilized metal affinity chromatography (IMAC) columns. Based on sodium dodecyl sulfate (SDS) electrophoresis, the molecular weight of the GAD 65 antigen is 60 kDa.

Antibodies. Monoclonal antibody 1 and antibody 2, also kindly provided by Phadia, were used for the detection of GAD 65. Antibody 1 and antibody 2 react with different epitopes of the GAD 65 compound. It was prepared from hybridoma cell cultures (Z2 – 15042 and Z3 – 09042), respectively (Phadia). Supernatants were purified by affinity chromatography on protein-A Sepharose FF (Amersham Biosciences), IgG1(Z3) under high salt concentration conditions. Fluorescence labeling was done at the Technical University of Graz. After many unsuccessful attempts with commercial sources of Cy5, we had to prepare our own reagents with Alexa 647 and Alexa 488. This was necessary to increase labeling efficiency, reduce the free dye, and minimize the background fluorescence and the denatured proteins.

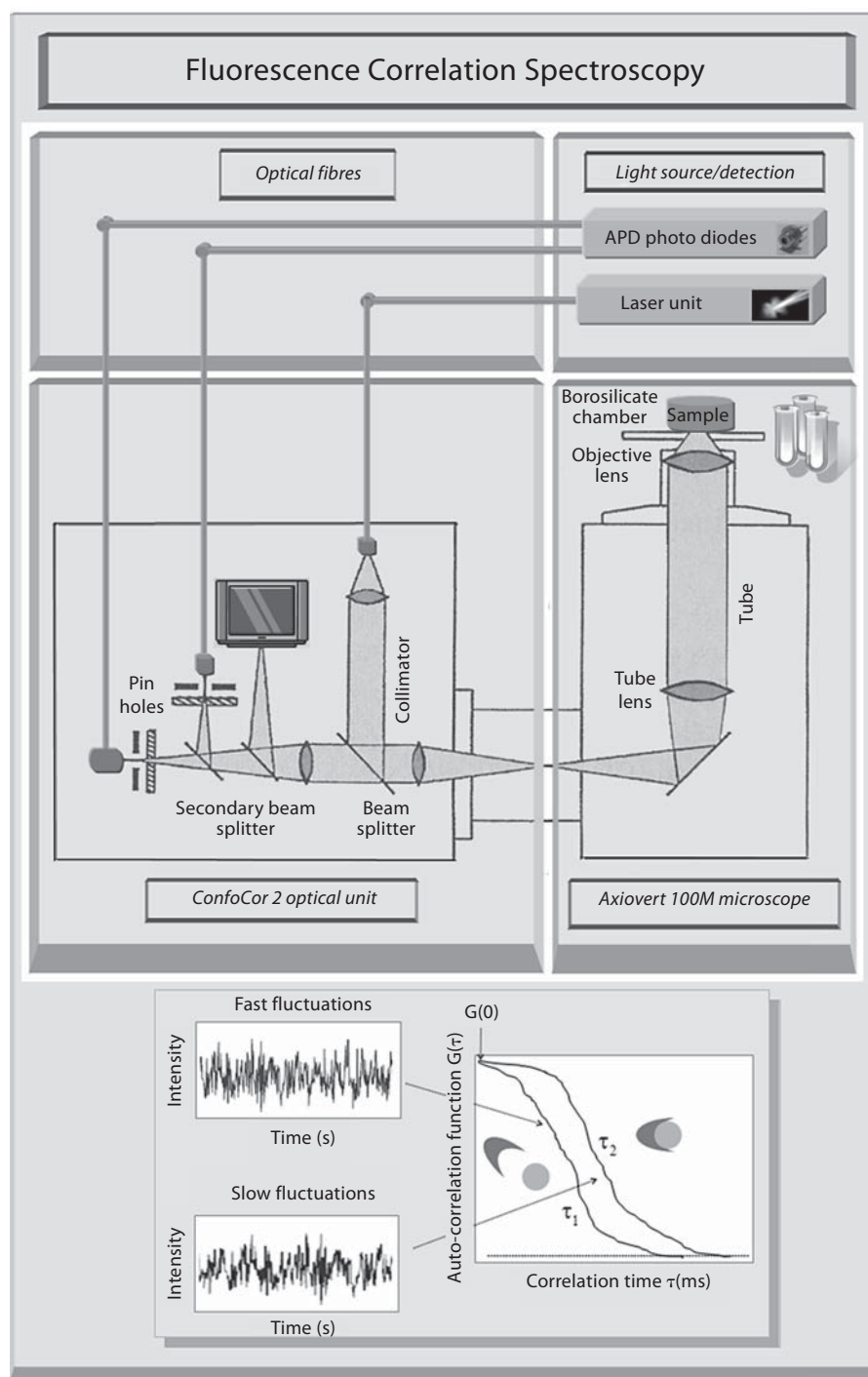


Fig. 1. Principle of FCS. The figure shows the experimental setup and the relations between the measured quantities and the autocorrelation analysis, as discussed in the text.

Specification of Monoclonal Antibody 1. Anti-GAD M67 IA11G1F ('Z2'), lot: 15042, subclass: IgG2a was used as one component in all experiments. The antibody recognizes a confirmatory epitope, hence reacts successfully in the ELISA, and furthermore precipitates the GAD from Sf 9 cells. Anti-GAD M67 ('Z2') was buffered in PBS pH 7.5 and 0.05% NaN₃ (= 7.8 mM) was added. Labeling was done with Alexa fluor 647 carboxylic acid suc-

cinimidylester (Molecular Probes). 120 μ l (2 mg/ml) were labeled, the degree of labeling was 1.7 (1.7 moles was fluorophor/mole antibody). The conjugates were controlled by thin layer chromatography (TLC). Methanol was used as medium for TLC and no free dye could be detected. After chessboard titration, this antibody was used at a dilution of 1:1,000.

Table 1. Diffusion times of the individual antibodies and the GAD 65-antibody complexes (means of 5 measurements)

Reagents	Diffusion time, ms
Antibody 1, labeled with Alexa 647	469
Antibody 2, labeled with Alexa 488	230
Antibody 1 in compound with GAD 65	985
Antibody 2 in compound with GAD 65	630

Table 2. Detectable GAD 65 in peripheral blood of patients

Patient No.	Age	Sex	Diagnosis
1	6	m	IDDM
2	7	m	IDDM
3	8	f	IDDM
4	12	m	metabolic syndrome, hyperIgE ¹
5	38	m	IDDM
6	55	m	Hashimoto's disease, rheumatoid arthritis ¹
7	63	m	IDDM
8	60	f	IDDM, rheumatoid arthritis

Synopsis of the 8/153 patients with the circulating GAD 65 antigen in their peripheral blood. Illustrated above, cross-correlation with diffusion >900 or >600 ms. In accordance with our spiking experiments, positivity was defined as a diffusion time of the antigen-antibody complex at least twice that of the antibody alone.

¹ With early IDDM.

Specification of Monoclonal Antibody 2. Anti-GAD M65 7G5D12G8 ('Z23'), lot: 09042, subclass: IgG1, also reacting with denatured antigen, being successfully tested by ELISA, immunoprecipitation and denatured antigen in a Western blot. This antibody was in buffered PBS pH 7.5 with 0.05% NaN₃ corresponding to a 7.8 mM dilution. 1,400 µl with 430 µg/ml antibody per ml were used. The degree of labeling was 2.8, equal to 2.8 mol fluorophore/mol antibody. The conjugate was controlled by TLC as described above, methanol was used as a medium and no free dye could be observed. After chessboard titration, the antibody was used at a dilution of 1:100 in all experiments.

Measurements in Clean and Spiking Experiments

Clean System. In order to avoid disturbing autofluorescence for standardization, initial antigen detection was carried out in bidistilled water. Purified GAD 65 was diluted in Tris-HCl 0.1 M pH 7.4 at the following concentrations for chessboard titration done with the 2 different labeled monoclonal antibodies, starting with 1,060 µg/ml. Dilutions from 1:100 to 1:1,600 were used according to concentrations of 10.6, 5.3, 2.65 µg/ml and below.

Human Sera. Initial detection of standardized GAD 65 was done in sera from normal healthy controls by adding the same

amount of GAD 65 as to the clean systems. This enabled us to define the detection limit in sera. The sera of the healthy controls were diluted 1:4 in PBS as described above and in spiking experiments. Finally, and based on these experiments, all measurements were carried out under the same conditions by adding the antibodies diluted 1:100 in 10-µl amounts to 10 µl Tris buffer as indicated.

Titration. Antibodies 2 diluted 1:100 were used after chessboard titration with GAD 65. 10 µl of each antibody, 10 µl of Tris-HCl buffer and 10 µl of serum were pipetted into Eppendorf siliconized microcentrifuge tubes, mixed, incubated at 37°C for 30 min in the dark and thereafter pipetted into the borosilicate chamber. This chamber was placed on an inverted Axiovert 100 microscope (Zeiss). Measuring was carried out 50 times and results averaged.

Measurements in Patient Sera

As the minimal detectable amount of GAD 65 had been determined in the previous spiking experiments, we did not go beyond in the patient sera. Measurements in the patient sera were done by diffusion time at the lowest detectable concentrations and above. Sera with such a diffusion time were considered positive since the added antibodies reacted with the GAD 65 antigen forming a larger complex.

Molecular Analysis and Visualization

The Swiss-Pdb Viewer was used for molecular analysis and visualization [16]. As input, the program uses coordinate files resulting from experimental determination of the protein structure [17]. The atomic coordinates are then converted into a view of the protein. For special problem-based representations, proprietary programs (written in a script language) can be read in, facilitating otherwise complex and time consuming calculations on the protein structure. The viewer program runs on a PC with Microsoft Windows.

Results

The measured diffusion times for the considered reagents are listed in table 1. Clean and spiking experiments allowed detection of the added GAD 65 at the concentration of 2.65 µg/ml (lowest precisely detectable amount). Under these conditions, and with such sensitivity, the serum dilution is sufficient and avoids interference by autofluorescence of albumin and other proteins in the FCS system. GAD 65 could be found in 8 patient sera with inflammatory thyroiditis, diabetic polyneuropathy, chronic polyarthritis and diabetes, hyperIgE and metabolic syndrome and in patients with early-onset juvenile diabetes (table 2). Table 2 shows the strongly positive patients with GAD 65 concentrations >2.65 µg/ml.

An additional negative control consisted in testing a biologically inert labeled IgG with GAD 65 behaving as a free protein whose diffusion time is then 3 times faster as compared with the GAD 65-antibody complex.

The method based on the different diffusion times of small versus larger molecules has been shown to be reliable and reproducible. In our system, two different fluorescent-labeled antibodies reacting with GAD 65 were initially used. Measurements were carried out at least 5 times with comparable results.

Discussion

To our knowledge, this is the first observation on circulating GAD 65 antigen in the peripheral blood of patients with very early IDDM. Other autoimmune disorders such as Hashimoto's disease, rheumatoid arthritis and inflammatory conditions may behave in a similar way and even overlap. Until now, GAD 65 has been considered to be strictly intracellular. Our assumption was that it has to be in the serum of patients and should be found in higher concentrations before the onset of the disease since GAD 65 has to prime the immune system as an initial step for the pathology to develop. Therefore, we set up the ultrasensitive method of FCS for detecting the antigen. In theory, it is sensitive enough to detect one single molecule in 'clean systems' such as rhodamine in distilled water [18, 19]. In our hands, the method was successful for detecting GAD 65 in some of the sera. Surprisingly, our measurements show that soluble GAD 65 occurs in reasonable amounts in the peripheral blood, leading us to the conclusion that other (less sensitive) methods such as ELISA (where the antibodies for detecting the antigen are bound on a solid carrier) will be suitable for the development of a high-throughput technique for screening patient samples.

Since GAD 65 has hitherto been considered to be exclusively intracellular, there is no record in the literature of any attempt at detecting detect GAD 65 in the peripheral blood as opposed to reactive cells [20–23] and antibodies [24–29]. The only reference we found is in relation to the 'stiff-man syndrome', and further investigations are needed to clarify its importance in the pathogenesis of diabetes mellitus and the stiff-man syndrome [22].

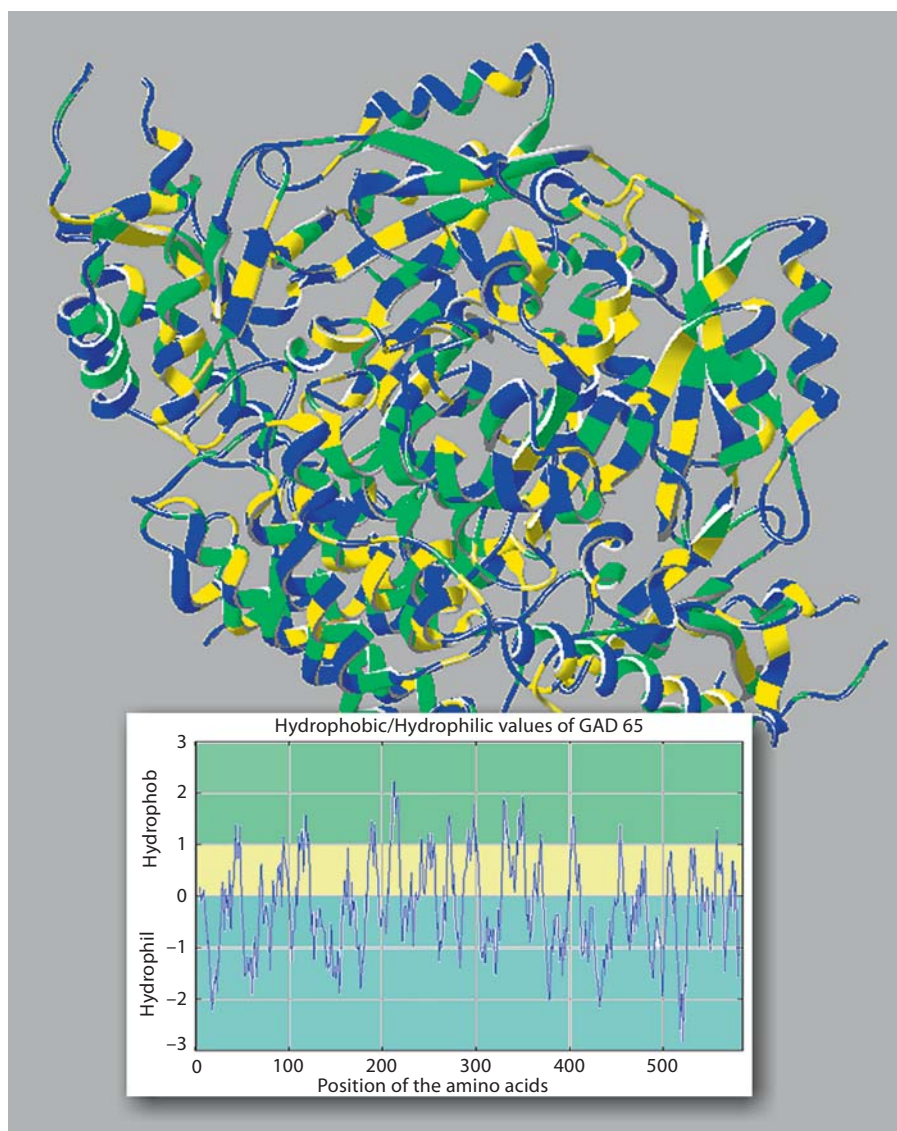
Since a primary intracellular antigen would never have access to the immune system, it is expected that the GAD 65 molecule can reversibly anchor to cell membranes. For a basic understanding of the mechanism enabling the GAD 65 molecule to interact with the hydrophobic cell membrane and to diffuse into the sera, we proposed an analysis based on a structural model. The possible interaction of GAD 65 with hydrophobic lipids of the cell membrane requires hydrophobic parts (docking sites) at the molecule surface. The structure of GAD

65 has recently been determined experimentally by X-ray diffraction [30]. Hydrophobic regions in the protein can be evaluated from the constituent residues. Specific values describing hydrophobic/hydrophilic properties are assigned to each of the amino acids [31]. Then, the hydrophobic properties are represented by plotting the course of the values along the protein sequence (fig. 2). The distribution of the hydrophobic/hydrophilic values along the peptide chains shows a certain number of hydrophobic regions [32]. The analysis was done with the protscale software (<http://au.expasy.org/tools/protscale.html>). Hydrophobic/hydrophilic properties are visualized by mapping the corresponding values onto the protein structure and by representation with color encoding (fig. 2). The resulting data and the visualization of hydrophobic properties on the structure of the molecule may explain its tendency to adhere to other structures, surfaces and membranes due to numerous hydrophobic parts of the molecular surface.

GAD 65 might become a suitable candidate for a vaccine. For analysing and planning the neutralization of GAD 65 with antibodies in the sera, the locations and shapes of epitopes are of special importance to understand the interaction with the immune receptor. This model should further enable a better understanding for the development of a vaccine. The molecular surface of the GAD 65 homodimer was calculated and the location of some epitopes projected onto the surface (fig. 3). The epitopes of GAD 65 have been identified by epitope mapping studies [33, 34]. To a large extent, the structure of GAD 65 and its physicochemical properties can be used to explain its biology. The structural visualization of the GAD 65 molecule is illustrative and may help the reader to speculate on the antigenic sites of the molecule, which might be useful for the development of active or passive immunotherapy in the near future.

Our experimental results are of special importance since they show that the sera of 4/27 pediatric and 4 adult patients with early IDDM are positive for the circulating GAD 65 antigen. The incidence of about 10% may probably be attributed to the complexed form (antigen-antibody complex) of the antigen at the time of investigation, where 'prozone' pathology has already switched to 'post-zone' phenomena, hence hiding the antigen in the antibody excess. This could make it undetectable for FCS. In the heterogeneous adult group 4/126 patients were positive for GAD 65. In this group Diabetes Mellitus Type 1 was not the prominent feature. The adult sera were initially considered as controls but retrospectively 4 cases with IDDM could be detected.

Fig. 2. The hydrophobic/hydrophilic values of the amino acids constituting the GAD 65 molecule are mapped onto the protein structure. The hydrophilic regions of the structure are encoded in blue. Low and medium hydrophobic regions are encoded in yellow whereas regions with higher hydrophobic values are encoded in green. Areas with hydrophobic values at the protein surface enable a potential interaction with hydrophobic membrane lipids, leading to a reversible anchoring of the molecule to the cell membrane.



These findings led us to the conclusion that autoimmune phenomena are more frequent than expected, can overlap, and are at least partially regulated by their respective autoantibodies [35–37]. The role of circulating autoantigen has not been studied so far and may become of special interest for further kinetic studies.

Being surprised by the high concentrations of GAD 65 present in some of the sera, we have used, in further studies, the semiquantitative surface enhanced laser desorption ionization – time of flight (SELDI-TOF) technique as well as an ELISA for the confirmation of the presence of GAD 65 in human sera (manuscript in preparation). According to our data on GAD 65, this molecule seems to behave like many other autoantigens in biologic systems.

We believe that GAD 65 may exist in trace amounts in the peripheral blood under normal conditions and that GAD 65 antibodies are operative in neutralization and regulation. Following destruction of the synthesizing organ, it is released into the blood stream where it can be found in significant amounts. It would seem that the ‘immunology dogma’, which maintains the strict intracellular localization of GAD 65, has probably discouraged intensive research on this interesting molecule, which might be a suitable candidate for diagnostic and prognostic purposes as well as for vaccination as it has been demonstrated in other immune-mediated diseases [38–43]. Another example for successful passive immunotherapy is the application of antibodies in rhesus disease [44–47].

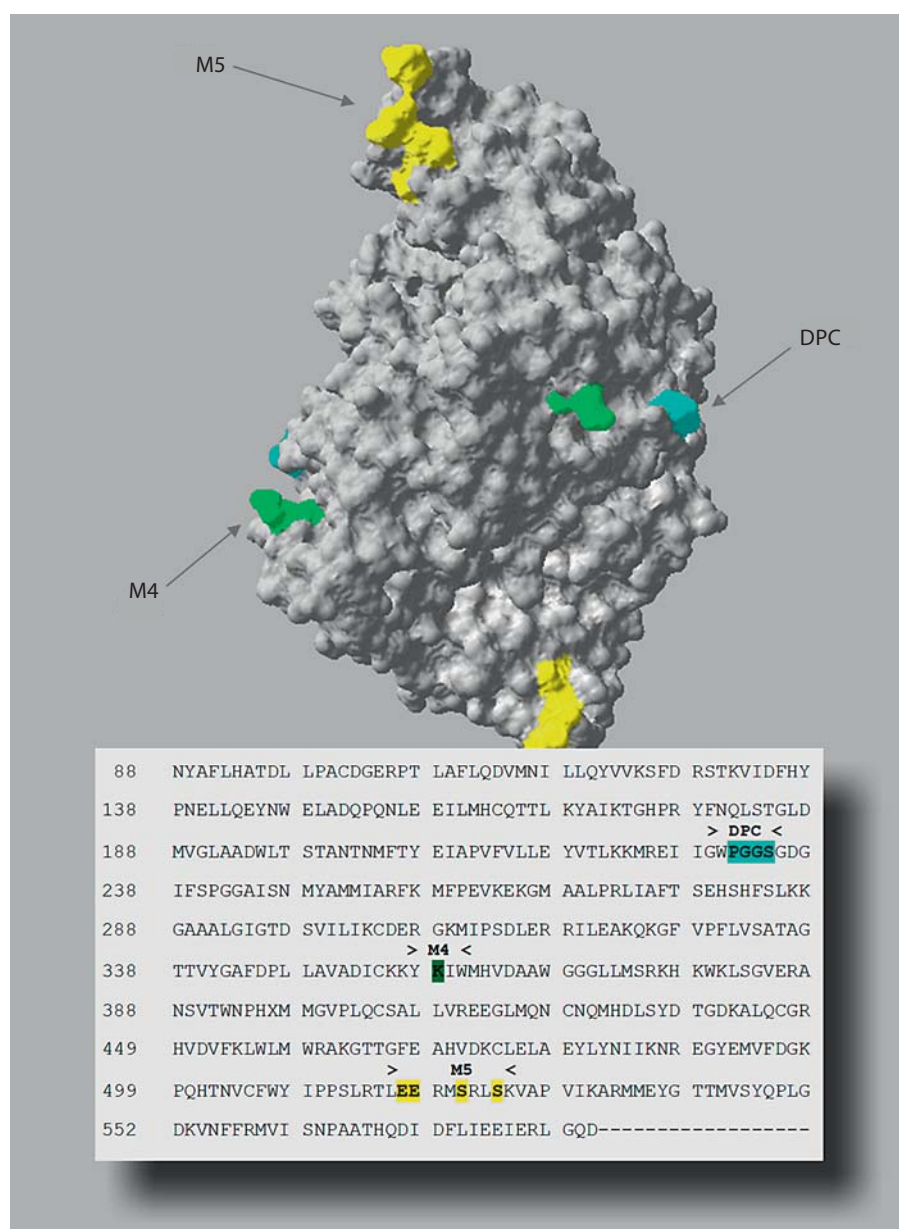


Fig. 3. Visualization of the locations of some epitopes at the GAD 65 molecule. The protein is represented by its molecular surface and the locations of the epitopes are mapped onto the surface and color encoded. Visualization of the epitopes at the molecule surface gives insights into mechanisms of antigenicity by showing complementary surfaces to the corresponding antibody. The following epitopes of GAD 65 have been selected for visualization: M5 (E517, E520, E521, S524, S527), DPC (P231, G232, G233, S234) and M4 (K358).

Conclusion

In conclusion, the results show that GAD 65 occurs in sufficiently high amounts in the peripheral blood and that the molecule is sufficiently stable. This is encouraging for further investigations on the nature and role of this interesting molecule in diseases such as IDDM, stiff-man syndrome and other autoimmune diseases.

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