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GAD\textsubscript{65} IS RECOGNIZED BY T-CELLS, BUT NOT BY ANTIBODIES FROM NOD-MICE

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Since the 64kDa-protein glutamic acid decarboxylase (GAD) is one of the major autoantigens in T-cell mediated Type 1 diabetes, its relevance as a T-cell antigen needs to be clarified. After isolation of splenic T-cells from non-obese diabetic (NOD) mice, a useful model for human Type 1 diabetes, we found that these T-cells proliferate spontaneously when incubated with human GAD\textsubscript{65}, but only marginally after incubation with GAD\textsubscript{35}, both recombinated in the baculovirus system. No effect was observed with non-diabetic NOD mice or with T-cells from H-2 identical NON-NOD-H-2\textsuperscript{67} control mice. It has been published previously that NOD mice develop autoantibodies against a 64kDa protein detected with mouse beta cells. In immunoprecipitation experiments with sera from the same NOD mice and \textsuperscript{35}S-methionine-labelled GAD, no autoantibody binding could be detected. We conclude firstly that GAD\textsubscript{65} is an important T-cell antigen which is relevant early in the development of Type 1 diabetes and secondly that there is an antigenic epitope in the human GAD\textsubscript{65} molecule recognized by NOD T-cells, but not by NOD autoantibodies precipitating conformational epitopes. Our results therefore provide further evidence that GAD\textsubscript{65} is a T-cell antigen in NOD mice, being possibly also involved in very early processes leading to the development of human Type 1 diabetes.

KEY WORDS: Type 1 (insulin-dependent) diabetes mellitus, non obese diabetic (NOD) mouse, autoimmunity, T-cells, glutamic acid decarboxylase (GAD)

INTRODUCTION

Type 1 (insulin-dependent) Diabetes mellitus (IDDM) is an autoimmune disease leading to the destruction of insulin producing beta cells in the pancreatic islets of Langerhans [1]. T-cells are crucially involved in the process of beta cell destruction [2]. However, except from the 38kDa protein, no diabetes specific T-cell antigen has been identified [3]. A number of antibodies specific for islet cell proteins have been detected in the sera from type 1 diabetic patients and are used as markers for the evaluation of risk factors of the disease. One of the most reliable and earliest markers in Type 1 diabetes are autoantibodies directed against the 64kDa antigen, which has been identified as glutamic acid decarboxylase (GAD) [4]. Immunoprecipitating antibodies to GAD have been shown to be present in 70% of newly diagnosed type 1 diabetic patients and in 87% of their islet cell antibody (ICA)-positive first degree relatives [5] as well as in 56% of patients with Stiff Man Syndrom (SMS) [6,7]. GAD catalyzes the conversion of glutamic acid into the neurotransmitter \(\gamma\)-amino butyric acid (GABA) and is found in two isoforms, GAD\textsubscript{65} and GAD\textsubscript{67}, both in neurons and in synaptic like vesicles in rodent insulin producing beta cells [8].

Since it is not proved that autoantibodies against beta cell antigens have any deleterious effect in the pathogenesis of Type 1 diabetes, several attempts have been made to elucidate the role of GAD as a T-cell autoantigen. T-cells in the peripheral blood of diabetic patients and their first degree relatives have been shown to recognize GAD\textsubscript{65} recombinated in E. coli [9] or GAD\textsubscript{67} [10]. T-cell lines specific for Escherichia coli GAD\textsubscript{65} have been isolated from BB-rats, an animal model of Type 1 diabetes [11]. The non-obese diabetic (NOD) mouse, another well known animal model for human Type 1 diabetes mellitus, resembles the situation in humans in many clinical, immunological and immunogenetical aspects [12,13]. Antibodies in NOD mice directed against a 64kDa protein in mouse islets have been reported several years ago [14], whereas antisera to GAD\textsubscript{65}/GAD\textsubscript{67} raised in different species including sheep, rabbit and mouse could only detect small amounts of GAD\textsubscript{65}, but not GAD\textsubscript{65} in mouse islets by Western blot analysis [15].

In this study we examined the ability of GAD\textsubscript{65} and GAD\textsubscript{67} to stimulate T-cells isolated from spleens of NOD mice of different age and metabolic state of disease. As a control we used non-diabetic MHC-

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matched NON-NOD-H-2\(^{b}\) mice. Human recombi-
nated GAD\(_{65}\) and GAD\(_{67}\) were used as antigens. To
evaluate the antibody response to both antigens, we
performed immunoprecipitation experiments with the
sera from NOD mice.

MATERIAL AND METHODS

**Mice**

Female NOD mice were obtained from Bomhice,
Bomholtgard, Rye, Denmark and kept under germfree
conditions. The incidence of diabetes in this colony
was 80% by 16–18 weeks of age in females and 20–
30% in male. Control non-diabetic NON-NOD-H-2\(^{b}\)
mice were obtained from L. Herberg, Diabetes For-
schungsinstitut, Düsseldorf, Germany. In our experi-
ments, NOD and control mice were of the same age.
The metabolic status was determined by blood glucose
measurement. Blood was drawn from the portal vein
and blood glucose levels were measured in an Eppen-
dorf APC 40/50. Mice with blood glucose values
> 13 mmol/l were considered to be diabetic.

**Antigen expression and preparation**

Purified human GAD\(_{65}\) and GAD\(_{67}\) were expressed in
Spodoptera frugiperda (SF9) cells as described recently
[16]. Briefly, human GAD\(_{65}\) and GAD\(_{67}\) cDNA clones
were constructed by inserting full length human
GAD\(_{65}\) and GAD\(_{67}\) cDNAs into the baculovirus vec-
tor pVL1393 (Invitrogen). After infection, SF9 cells
was 80% by 16-18 weeks of age in females and 20-
harvested 48h later and homogenized in 20 mM potas-
sium phosphate, pH 7,0, 2 mM EDTA, 2 mM PMSF,
was added to a final radioactivity of 500 μιο/ηιηιο/. The
was prepared. Mononuclear splenic cells were sepa-
rated by density gradient centrifugation on Ficoll-400
sham Buchler, Braunschweig, FRG) and harvested
after an incubation of 1 h at 37°C, the reaction was
stopped by injecting 1 ml 5N sulphuric acid followed
by an equilibration period of 1 h to allow complete
adsorption of release \(^{14}\)CO\(_2\). The \(^{14}\)CO\(_2\) trapped to the
filter paper was measured by liquid scintillation count-
ing and GAD enzyme activity was expressed in
Units/mg protein. 1 Unit [U] was defined as the for-
mation of 1 μmol CO\(_2\)/min under standard assay con-
ditions.

**Determination of GAD enzyme activity**

Enzymatic activity of GAD was determined using a
modification of the method described by Miller et al.
[17]. Aliquots of the SF9 cell homogenates were di-
uted in 50 mM potassium phosphate buffer, pH 7.0
with 1 mM EDTA, 1 mM AET, 0,2 mM PLP (buffer
A). Homogenates of cells infected with the GAD re-
combiantors, or cells infected with the vector lacking
the GAD insert were used as antigens in T-cell prolif-
eration experiment or for immunoprecipitation.

**Isolation of T-cells and proliferation assays**

For each experiment 3 female NOD mice or 3 female
NON-NOD-H-2\(^{b}\) mice of the same age were killed.
The animals were non-fasting. Presence of insulitis
was determined by hematoxylin/eosin staining of
kryo-sections of the pancreata isolated immediately
after killing.

Isolation of T-cells was performed as described [18].
Briefly, spleens were removed aseptically and a ho-
mogenous cell suspension comprising spleen cells from
all three animals in complete RPMI-640 medium
(RPMI-1640, supplemented with 10% fetal calf serum,
10 units/ml penicillin/streptomycin, 200 mmol/l
L-glutamine and 5 x 10\(^{-5}\) mol/l β-mercaptoethanol)
was prepared. Mononuclear splenic cells were sepa-
rated by nylon wool columns to obtain purified T-cells. Purity
of the T-cell preparations was > 90% as analyzed by
anti-CD3-antibody labelling and subsequent Fluores-
cence activated cell sorter analysis. Triplicate assays
were carried out by culturing 3 x 10\(^{6}\) purified T-cells in
a total volume of 200 μl of complete RPMI-640 in
96-well microtitre plates in the presence of 3 x 10\(^{5}\)
irradiated (3000 rad) syngeneic spleen cells as antigen
presenting cells (APC). For proliferation assays, the
antigenic fractions were used in concentrations of 10,
2, 0,5 and 0,1 μg/ml to determine their respective
optimal concentration for stimulation of the T-cells. In
each case concanavalin A (1 μg/ml) was used as a
positive control for T-cell proliferation. The basal
stimulatory activity was ascertained in the presence of
complete cell culture medium without antigen. The
cultures were incubated for 72–86 h, then pulsed with
1 μCi per well \(^{3}\)H-methyl-thymidine (\(^{3}\)H-TdR) (Amer-
sham Buchler, Braunschweig, FRG) and harvested
20 h later. The incorporated radioactivity was deter-
mained using a Betaplate scintillation counter. Prolifer-
ation was expressed as stimulation index (SI): lympho-
cyte DNA synthesis/\(^{3}\)H-TdR incorporation/counts per
min (cpm) in the presence of antigen over cpm in the
absence of antigen.
Immunoprecipitation assay

Immunoprecipitation was performed with sera from NOD and control mice. As a positive control we used the murine monoclonal antibody GAD 1 recognizing GAD$_{65}$ and the polyclonal rabbit antibody K2 shown to recognize both GAD$_{65}$ and GAD$_{67}$ [8]. The assays were performed following a modification of the method published by Seissler et al [19].

Infected SF9 cells were cultured in Grace’s medium (Gibco). After 36 h, $^{35}$S- methionine (200 μCi/5 × 10$^6$ cells) were added. 6 h later the cells were harvested and homogenized in buffer A. Homogenates were centrifugated at 33,000 g for 30 min to separate the soluble cytosolic fraction (CF). The cell pellets were resuspended in 20 mM potassium phosphate, 50 mM NaCl pH 7.2, 2 mM EDTA, 2 mM PMSF, 1% Tranysol, 1% Triton × 100 for 2 h at 4°C followed by centrifugation at 33,000 g for 30 min to obtain the membrane fraction (MF). MF was incubated with a pool of normal human serum (25 μl/100 μl lysate) for 6 h and then preabsorbed with protein A sepharose (Pharmacia, Freiburg). 100 μl of the precleared extracts were precipitated with 25 μl Type 1 diabetes or NOD mouse serum, followed by adsorption of the immunocomplexes by a mixture of protein A and protein G sepharose. After extensive washing the bound proteins were eluted with 65 mM Tris/HCl pH 6.8, 2% SDS, 5% β-mercaptoethanol and analysed by fluorography.

Statistical analysis

Data of T-cell assays are shown as means + / − standard deviation (SD) from at least three identically performed experiments. The results were assessed by one-way analysis of variance. Differences with p-values less than 0.01 were considered to be significant.

RESULTS

Prior to performance of the proliferation experiments, enzymatic activity of the cell lysates was determined. For GAD$_{65}$ it was 55 mU/mg and for GAD$_{67}$ 69 mU/mg. Splenic T-cells from NOD mice of 8–10, 17–20 and 28 weeks of age were incubated with different concentrations of GAD$_{65}$ or GAD$_{67}$. As shown in Figure 1, both GAD$_{65}$ and GAD$_{67}$ were recognized by the NOD mouse T cells in a dose dependent manner with an incorporated radioactivity of 0.8 – 1.5 × 10$^{-3}$ counts per minute (cpm) compared to background.
cpm levels of 0.05 - 0.1 \times 10^{-3} \text{ cpm}. Table 1 shows that the proliferation of the T-cells obtained from NOD mice at every age class and metabolic state studied were increased after stimulation with GAD_{65}. Proliferative responses towards GAD_{67} could also be observed, but were considerably reduced when the corresponding SI-values in each of the age groups were compared. This effect was also observed with T-cells from 8–10 weeks old NOD mice, which had not yet developed overt diabetes, but had mononuclear cells infiltrating the islets. T-cells from these mice showed marginal responses after incubation with GAD_{67}. When we used T-cells from non-diabetic, MHC-matched NON-NOD-H-2\kappa^7 control mice, only a small effect could be seen either after incubation with GAD_{67} or with GAD_{65} (Table 1). By using homogenates from SF9 cells not expressing GAD as an antigen to test unspecific T-cell response to SF9-cellular proteins, no significant proliferation of the T-cells both from NOD and NON-NOD mice was detected (Table 1). T-cells from non diabetic NOD mice which did not show any histological signs of islet infiltration did not respond to GAD_{65} or GAD_{67} (data not shown).

In immunoprecipitation experiments, sera from the same mice which have been used for T-cell isolation in the above described experiments were tested. Immunoprecipitation studies were performed with extracts from GAD_{65} expressing SF9 cells which had been metabolically labelled by \textsuperscript{35}S-methionine incorporation. As a control for the suitability of the immunoprecipitation method for mouse sera, we used the monoclonal mouse antibody GAD 1 which recognizes GAD_{65} and the polyclonal rabbit antibody K2 recognizing mainly GAD_{67}. As shown in Figure 2, the monoclonal antibody GAD 1 and the polyclonal antibody K2 could precipitate GAD_{65} or GAD_{67} from the SF9 cell extract. None of the NOD mouse or NON-NOD mouse sera gave a positive binding signal.

**DISCUSSION**

With this study evidence is provided that human GAD_{65} contains an antigenic epitope or antigenic epitopes for NOD T-cells, different from the epitope(s) recognized by NOD autoantibodies. In a previous study, reactivity of peripheral blood T-cells isolated from human type 1 diabetic patients towards GAD expressed in E.coli was reported [9]. When recombinated GAD_{65} from E.coli or E.coli extract without GAD_{65} was used, T-cells isolated from NOD mice and from non-diabetogenic control mice proliferated equally strongly (data not shown). This might be due to E.coli contaminating proteins which stimulated the T-cells in an unspecific manner. When we used human GAD_{65} and GAD_{67} recombinated in the baculovirus system and expressed in SF9 cells, the T-cell proliferation signals in response to GAD_{65} were increased compared to GAD_{67}; background levels of proliferation to SF9 cell extract were significantly lower. These results are remarkable since in a recent report only GAD_{67} was immunologically detectable on mouse islet cells [15]. It might yet be possible that the amount of GAD_{65} molecules on mouse islets is too small to be detected in immunoassays, but sufficient to prime specific T-cells in vivo. The human form of GAD_{65} apparently provides an epitope which is immunologically effective for stimulation of NOD T-cells.

**Table 1** Proliferation of T-cells from NOD- and NON-NOD-mice after in vitro stimulation with human recombinated GAD_{65} or GAD_{67}. Extract of SF9 cells not expressing GAD was used as a negative tissue control. Proliferation was measured after \textsuperscript{3}H-thymidine incorporation with background levels of T-cell proliferation of 50–100 counts per minute (cpm), and is expressed as proliferation stimulation index (SI) \pm standard deviation (SD) from at least three identically performed experiments. The table shows significantly higher proliferation of NOD-mouse T-cells after stimulation with GAD_{65} compared to the respective results obtained with T-cells from non-diabetic, H-2 compatible NON-NOD-control mice of the same age group (** for GAD_{65} and *** for GAD_{67}) and compared to the respective T-cell proliferation after incubation with SF9 cell homogenate (#).

<table>
<thead>
<tr>
<th>antigen</th>
<th>age of mice [weeks]</th>
<th>diabetic*</th>
<th>proliferation of T-cells [SI]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>yes/no</td>
<td>NOD</td>
</tr>
<tr>
<td>GAD_{65}</td>
<td>8–10</td>
<td>no</td>
<td>10.3 ± 2.0*#</td>
</tr>
<tr>
<td></td>
<td>17–20</td>
<td>yes</td>
<td>11.0 ± 0.7*#</td>
</tr>
<tr>
<td></td>
<td>28–30</td>
<td>yes</td>
<td>6.7 ± 2.1</td>
</tr>
<tr>
<td>GAD_{67}</td>
<td>8–10</td>
<td>no</td>
<td>4.6 ± 1.4**</td>
</tr>
<tr>
<td></td>
<td>17–20</td>
<td>yes</td>
<td>6.1 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>28–30</td>
<td>yes</td>
<td>1.8 ± 1.0</td>
</tr>
<tr>
<td>SF9-</td>
<td>8–10</td>
<td>no</td>
<td>2.0 ± 1.4#</td>
</tr>
<tr>
<td>extract</td>
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<td>yes</td>
<td>1.7 ± 0.3#</td>
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<tr>
<td></td>
<td>28–30</td>
<td>yes</td>
<td>0.2</td>
</tr>
</tbody>
</table>

\*\text{for NOD-mice}

* \( p < 0.001 \)

** \( p < 0.01 \)

*** \( p < 0.001 \)
NOD mouse sera is not due to technical problems.

We have already been able to show a positive binding signal with ICA + sera was obtained in contrast to positive binding by undiluted NOD mouse sera (lanes 5 and 10) no binding signal was observed. Here we show that GAD 65 is one of the autoantigens eliciting T-cell response towards the pancreatic islets, which is relevant in the early development of insulitis and diabetes.

When immunoprecipitation experiments with metabolically labelled GAD 65 and GAD 67 were performed, no binding signal with undiluted NOD-mouse sera was obtained in contrast to positive binding by the control antibodies GAD 1 and K2. Nevertheless, the assay itself is suitable for detection of both human and mouse antibodies: In one of our previous studies the assay itself is suitable for detection of both human and mouse antibodies: In one of our previous studies we reported the presence of autoantibodies towards a 64kDa protein in the serum of NOD mice was reported several years ago by Atkinson et al. [14], although evidence is still lacking that this 65kDa-protein equals GAD 65. immunoprecipitation studies performed with tryptic islet fragments indicate the existence of another 64kDa-protein in islet beta cells [20]. Another group recently reported enzymatic GAD activity in mouse islets, but also noted species specific differences in the expression of immunologically relevant forms of the antigen [15].

T-cells recognize proteogenic peptides comprising a small number of amino acids processed by antigen presenting cells and presented in conjunction with molecules of the major histocompatibility complex (MHC) [21,22,23]. In contrast, antibodies bind three dimensional structures of protein molecules [24]. In epitope studies with influenza virus haemagglutinin (HA) it has been demonstrated that amino acid changes in the molecule affect the conformational structure and the antibody binding, whereas cell surface localization and response of cytotoxic T-cells was not impaired [25]. It is therefore possible that amino acid sequences within a protein molecule serve as a T-cell antigen, but are cryptic to B-cell recognition by conformational structures. This might be the case with GAD 65, since it is shown here that in NOD mice, T-cells are able to recognize GAD as an antigen whereas no antibody response was detected.

Our results contribute to the examination of the role of autoreactive T-cells in the pathogenesis of Type 1 diabetes and demonstrate the difference in immunological recognition of GAD as a beta cell antigen. We would suggest that the presentation of the T-cell epitope reacting with GAD-specific T-cells is one of the factors triggering beta cell destruction. The B cell response seems to be secondary to the T-cell response to GAD and independent from this process. Further investigations will be necessary to affirmate the triggering effect of GAD reactive T-cells in the NOD mouse or in human Type 1 diabetes.

References

![Fig. 2](image-url)


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