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GAD_{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_{65}, but only marginally after incubation with GAD_{67}, 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^{g7} 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 ^{35}S-methionine-labelled GAD, no autoantibody binding could be detected. We conclude firstly that GAD_{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_{65} molecule recognized by NOD T-cells, but not by NOD autoantibodies precipitating conformational epitopes. Our results therefore provide further evidence that GAD_{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 convertion of glutamic acid into the neurotransmitter γ-amino butyric acid (GABA) and is found in two isoforms, GAD_{65} and GAD_{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_{65} recombinated in E. coli [9] or GAD_{67} [10]. T-cell lines specific for Escherichia coli GAD_{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_{65}/GAD_{67} raised in different species including sheep, rabbit and mouse could only detect small amounts of GAD_{67}, but not GAD_{65} in mouse islets by Western blot analysis [15].

In this study we examined the ability of GAD_{65} and GAD_{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^{g7} mice. Human recombinated 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 Bommice, 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^{g7} mice were obtained from L. Herberg, Diabetes Forschungsinstitut, Düsseldorf, Germany. In our experiments, 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 Eppendorf 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 vector pVL1393 (Invitrogen). After infection, Sf9 cells were cultured in SF900 medium (Gibco, Heidelberg) supplemented with 0.04% fetal calf serum. Cells were harvested 48h later and homogenized in 20 mM potassium phosphate, pH 7,0, 2 mM EDTA, 2 mM PMSF, 5 µ/ml Leupeptin, 1 mM AET, 0,2 mM PLP (buffer A). Homogenates of cells infected with the GAD recombinants, or cells infected with the vector lacking the GAD insert were used as antigens in T-cell proliferation experiment or for immunoprecipitation.

Determination of GAD enzyme activity

Enzymatic activity of GAD was determined using a modification of the method described by Miller et al. [17]. Aliqouts of the SF9 cell homogenates were diluted in 50 mM potassium phosphate buffer, pH 7.0 with 1 mM EDTA, 1 mM AET, 0.2 mM PLP to a final volume of 200 µl. L-glutamic acid was added to a final concentration of 1 mM and L-[1-^{14}C] glutamic acid was added to a final radioactivity of 500 µCi/mmol. After adding the reaction mixture into glass tubes, a filter paper soaked with 50 µl 1 M hyamin hydroxide was placed into the tubes. The tubes were closed and 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 counting and GAD enzyme activity was expressed in Units/mg protein. 1 Unit [U] was defined as the formation of 1 µmol CO_2/min under standard assay conditions.

Isolation of T-cells and proliferation assays

For each experiment 3 female NOD mice or 3 female NON.NOD-H-2^{g7} 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 homogenous 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 5×10^{-5} mol/l β-mercaptoethanol) was prepared. Mononuclear splenic cells were separated by density gradient centrifugation on Ficoll-400 cushions (Lymphoprep, Boehringer Mannheim, Mannheim FRG). The interface containing the mononuclear cells was recovered, washed and passed over 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 Fluorescence activated cell sorter analysis. Triplicate assays were carried out by culturing 3×10^4 purified T-cells in a total volume of 200 µl of complete RPMI-640 in 96-well microtitre plates in the presence of 3×10^5 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) (Amersham Buchler, Braunschweig, FRG) and harvested 20 h later. The incorporated radioactivity was determined using a Betaplate scintillation counter. Proliferation was expressed as stimulation index (SI): lymphocyte 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 x 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% Trasylol, 1% Triton x 100 for 2 h at 4°C followed by centrifugation at 33,000 g for 30 min to obtain the membrane fraction (MF) in the supernatant. 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 x 10^{-3} counts per minute (cpm) compared to background

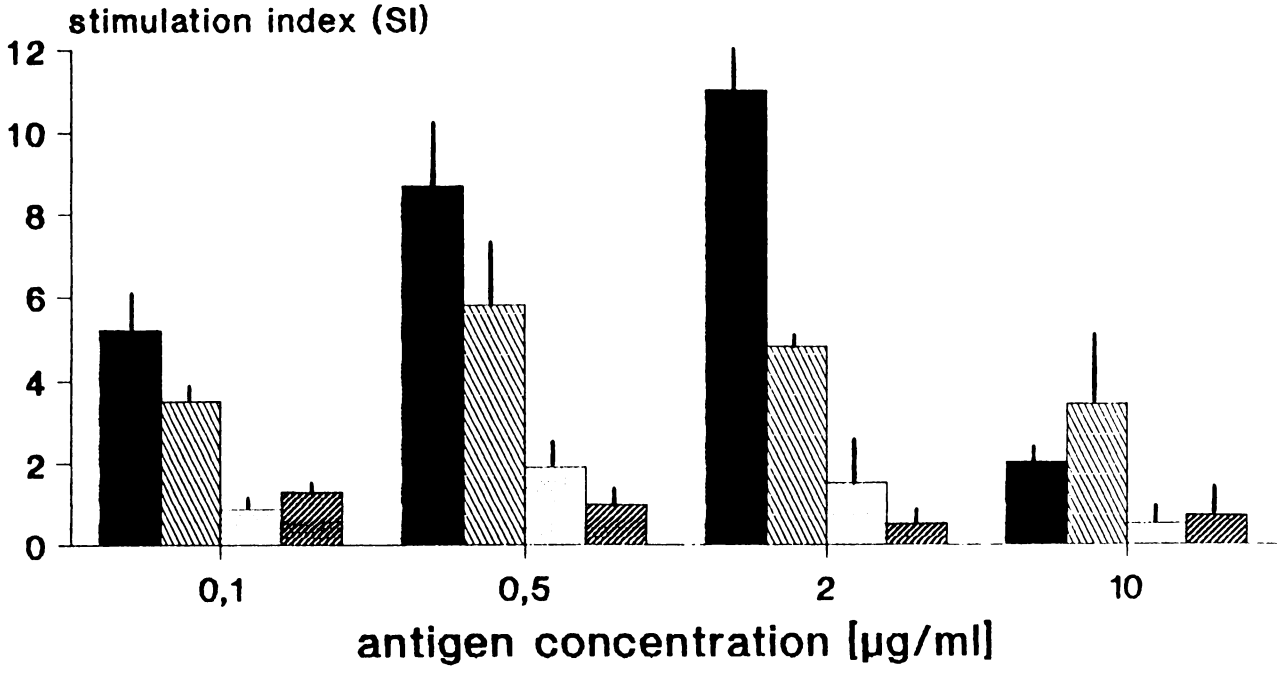

Fig. 1 Spontaneous proliferation of splenic T-cells from 20 weeks old, diabetic NOD mice after a 72 h-incubation with 0,1, 0,5, 2 or 10 µg/ml GAD_{65} (■) or GAD_{67} (▨), compared to proliferation of splenic T-cells from non-diabetic, age and H-2-matched NON-NOD control mice after incubation with GAD_{65} (▤) or GAD_{67} (▨). Proliferation is shown as stimulation index (SI) with incorporation values of 800–1500 cpm in presence and 50–100 cpm in absence of antigen. Positive control values obtained after incubation of the T-cells with Con A were between 3000 and 5000 cpm. T-cells from NOD-mice respond in a dose dependent manner both to GAD_{65} and GAD_{67}, whereas proliferation of T-cells from control mice is considerably reduced.

cpm levels of $0{,}05 - 0{,}1 \times 10^{-3}$ 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^{g7} 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 experimens, 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 ^{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 strong (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 ^{3}H-thymidine incorporation with background levels of T-cell proliferation of 50–100 counts per minute (cpm), and is expressed as stimulation index (SI) +/− 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 ('#').

antigen	age of mice [weeks]	diabetic[a] yes/no	proliferation of T-cells [SI] NOD	NON-NOD
GAD_{65}	8–10	no	10.3 ± 2.0*#	0.9 ± 0.2*
	17–20	yes	11.0 ± 0.7*#	1.9 ± 1.0*
	28–30	yes	6.7 ± 2.1	2.7 ± 1.2
GAD_{67}	8–10	no	4.6 ± 1.4**	1.2 ± 0.3**
	17–20	yes	6.1 ± 2.9	2.0 ± 0.3
	28–30	yes	1.8 ± 1.0	1.8 ± 1.3
SF9-extract	8–10	no	2.0 ± 1.4#	3.2 ± 0.8
	17–20	yes	1.7 ± 0.3#	2.5 ± 0.6
	28–30	yes	0.2	3.4 ± 0.5

[a]for NOD-mice
* = $p < 0.001$
** = $p < 0.01$
# = $p < 0.001$

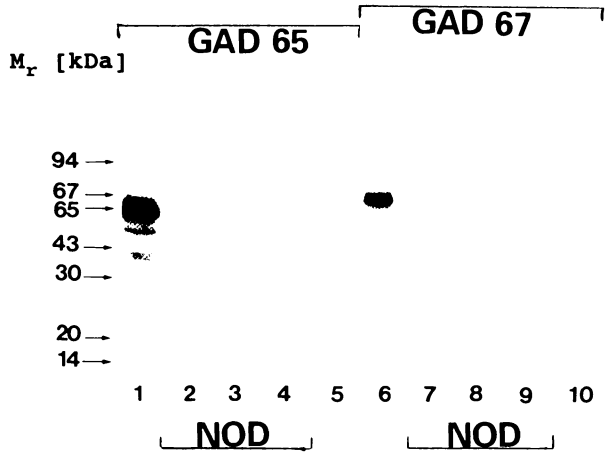

Fig. 2 Autoradiography after immunoprecipitation of human recombinated ^{35}S-methionine-labelled GAD_{65} (lane 1–5) and GAD_{67} (lane 6–10). As positive controls, the mouse monoclonal antibody GAD 1 precipitating GAD_{65} (lane 1) and the rabbit polyclonal antibody K2 precipitating GAD_{67} (lane 6) were used. The molecular weights (M_w) are indicated. With undiluted sera from NOD mice aged 8–10 weeks (lanes 2 and 7), 17–20 weeks (lanes 3 and 8) or 28–30 weeks (lanes 4 and 9) or with undiluted NON-NOD mouse sera (lanes 5 and 10) no binding signal was obvious.

In our study, non-diabetic, H-2 identical control mice which did not show any signs of insulitis, did not respond to either form of GAD. This could implicate an MHC-independent mechanism of GAD_{65} recognition by the T-cells from NOD mice, but further blocking experiments with anti-H2 antibodies will be necessary to prove the influence of the NOD-mouse specific H-2 complex in the recognition of diabetes specific antigens. In a previous study we reported the presence of T-cell autoantigens in very young NOD mice [18]. 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 we have already been able to show a positive binding signal with ICA^+ sera from human type 1 diabetic patients and GAD_{65} recombinated in Baculovirus by using this immunoprecipitation assay [5] and in this study the murine monoclonal antibody GAD1 was able to precipitate GAD_{65}, which implicates that the missing GAD_{65} or GAD_{67} binding signal by using NOD mouse sera is not due to technical problems.

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 existance 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

1. Eisenbarth GS. Type 1 Diabetes Mellitus: A chronic autoimmune disease. *N Engl J Med* 1986: **314**: 1360–1368.
2. Roep BO, DeVries RRP. T lymphocytes and the pathogenesis of type 1 (insulin-dependent) diabetes mellitus. *J. Clin. Invest.* 1992: **22**: 1–15.
3. Roep BO, Kallan AA, Hazenbos WL, Bruining GJ, Baileys EM, Arden SD *et al.* T-cell reactivity to 38kD insulin secretory granule protein in patients with recent-onset type 1 diabetes. *Lancet* 1991: **337**: 1439–1441.
4. Baekkeskov S, Aanstoot AJ, Christgau S, Reetz A, Solimena M, Cascalho F *et al.* Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthezising enzyme glutamic acid decarboxylase. *Nature* 1990: **347**: 151–156.
5. Seissler J, Amann J, Mauch L, Haubruck H, Wolfahrt S, Bieg S *et al.* Prevalence of autoantibodies to the 65- and

67-kDa isoforms of glutamate decarboxylase in insulin-dependent diabetes mellitus. *J Clin Invest.* 1993: **92**: 1394–1399.

6. Solimena M, De Camilli P. Autoimmunity to glutamic acid decarboxylase (GAD) in Stiff Man Syndrome and insulin dependent diabetes mellitus. *Trends in Neurosciences* 1991: **14**(10): 452–457.
7. Solimena M, Folli F, Aparisis R, Pozza G, DeCamilli P. Autoantibodies to GABA-ergic neurons and pancreatic beta cells in Stiff Man Syndrome. *Engl J Med* 1991: **322(22)**: 1555–1560.
8. Christgau S, Aanstoot HJ, Schierbeck H, Begley K, Tullin S, Hejnaes K *et al.* Membrane anchoring of the autoantigen GAD_{65} to microvesicles in pancreatic β-cells by palmitoylation in the NH2-terminal domain. *J Cell Biol* 1992: **118**: 309–320.
9. Atkinson M A, Kaufman DL, Campbell L, Gibbs KA, Shah SC Bu D *et al.* Response of peripheral-blood mononuclear cells to glutamate decarboxylase in insulin-dependent diabetes. *Lancet* 1992: **339**: 458–459.
10. Honeyman MC, Cram DS, Harrison LC. Glutamic acid decarboxylase 67-reactive T-cells: a marker of insulin-dependent diabetes. *J Exp Med* 1993: **77**: 535–540.
11. Diaz JL, Ways J, Hammonds P. T-lymphocyte lines specific for glutamic acid decarboxylase (GAD), the 65K β-cell antigen of IDDM. *Diabetes* 1992: **41**: 118–121.
12. Ohneda A, Kobayashi T, Nihei J, Tochino Y, Kanaya H, Makino S. Insulin and glucagon in spontaneously diabetic non-obese mice. *Diabetologia* 1984: **27**: 460–463.
13. Leiter EH, Prochazka M, Coleman DL. Animal model of human disease — the non-obese diabetic mouse. *Am J Path* 1987: **28**: 380–383.
14. Atkinson MA, Maclaren NK. Autoantibodies in non obese diabetic mice immunoprecipitate 64,000-Mr islet antigen. *Diabetes* 1988: **37**: 1587–1590.
15. Velloso LA, Kämpe O, Eizirik DL, Hallberg A, Andersson A, Karlsson FA. Human autoantibodies react with glutamic acid decarboxylase antigen in human and rat but not in mouse pancreatic islets. *Diabetologia* 1993: **36**: 39–46.
16. Mauch L, Seissler J, Haubruck H, Cook NJ, Abney, CC, Berthold H *et al.* Baculovirus mediated expression of human 65 kDa and 67 kDa glutamic acid decarboxylase in SF9 cells and their relevance in diagnosis of insulin-dependent diabetes mellitus. *J Biochem* 1993: 113: 699–704.
17. Miller LP, Martin DL, Mazumder A, Walters JR. Studies on the regulation of GABA synthesis: substrate promoted dissociation of pyridoxial-5′-phosphate from GAD. *J. Neurochem.* 1978: **30**: 361–369.
18. Bieg S, Bailyes EM, Yassin N, Amann J, Herberg L, McGregor AM *et al.* A multiplicity of protein antigens in subcellular fractions of rat insulinoma tissue are able to stimulate T-cells obtained from non-obese diabetic mice. *Diabetologia* 1993: **36**: 385–390.
19. Seissler J, Hering B, Richter W, Glück M, Yassin N, Bretzel RG *et al.* Antibodies to the MR 64.000 (64K) protein in islet cell antibody positive non-diabetic individuals indicate high risk for impaired beta-cell function. *Diabetologia* 1992: **35**: 550–554.
20. Christie MR, Brown TJ, Cassidy D. 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. *Diabetologia* 1992: **35**: 380–384.
21. Peters PJ, Neefjes JJ, Oorschot V, Ploegh HL, Geuze HJ. Segregation of MHC class II from molecules HC class I molecules in the Golgi complex for transport to lysosomal compartments. *Nature* 1992: **349**: 669–676.
22. Lie W, Myers NB, Gorka J, Rubocki RJ, Connolly JM, Hansen TH. Peptide ligand-induced conformation and surface expression of the Ld class I MHC molecule. *Nature* 1990: **344**: 439–441.
23. Hackett CJ. Later for the rendezvous: antigen presentation *Nature* 1991: **349**: 655–656.
24. Alzari PM, Lascombe MB, Poljak RJ. Three dimensional structure of antibodies. *Ann Rev Immunol.* 1988: **6**: 555–580.
25. Jennings PA, Boyle DB, Andrew ME. Class I MHC restricted cytotoxic T-cells efficiently recognize haemagglutinin that is defective in protein folding and cell surface expression. *Molecular Immunology* 1988: **25(2)**: 1371–1376.