

Immune reactivity to glutamic acid decarboxylase 65 in stiff-man syndrome and type 1 diabetes mellitus

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Summary

Background The immune response to an isoform of glutamic acid decarboxylase (GAD), GAD65, is associated with two clinically distinct diseases, stiff-man syndrome (SMS) and type 1 (insulin-dependent) diabetes mellitus. We sought to identify differences in the cellular and humoral immune responses to GAD in these two diseases.

Methods We compared T-cell responses in 14 SMS patients with axial disease and 17 patients with type 1 diabetes.

Findings Peripheral blood T cells of eight SMS patients recognised different immunodominant epitopes of GAD65 compared with T cells from 17 patients with type 1 diabetes. GAD regions 81–171 and 313–403 induced a dominant T-cell response in six of eight patients with SMS but in only one of 17 patients with type 1 diabetes ($p=0.001$). No SMS patients responded dominantly to GAD fragments 161–243 and 473–555 compared with ten patients with type 1 diabetes ($p=0.008$). GAD antibodies were detected in 11 of 14 SMS patients (seven with diabetes) and 11 of 17 patients with type 1 diabetes; IgG1 was dominant in both groups. SMS patients, however, were more likely than patients with diabetes to have isotypes other than IgG1 ($p=0.03$), in particular, IgG4 or IgE isotypes, which were not detected in patients with type 1 diabetes ($p=0.012$).

Interpretation Our findings indicate differences between patients with SMS and type 1 diabetes in cellular (epitope recognition) and humoral (isotype pattern) responses to GAD65. Thus the same autoantigen can elicit distinct immune responses in patients with SMS, even when associated with diabetes, compared with patients with type 1 diabetes.

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Introduction

Glutamic acid decarboxylase (GAD) isoform 65 is a major autoantigen in type 1 (insulin-dependent) diabetes mellitus and stiff-man syndrome (SMS), a rare neurological disorder that causes painful muscle spasms and rigidity. An immune response to islet-cell antigens, including GAD65, is thought to be involved in the destruction of insulin-secreting cells that causes type 1 diabetes,^{1–3} and may also cause SMS, which is associated with impairment of neurotransmission.^{4,5} Most patients with type 1 diabetes do not have SMS, but about 50% of SMS patients have diabetes.⁴ Differences between the involvement of GAD in these two disorders might explain why individuals develop diabetes but rarely develop both diabetes and SMS.⁶

The epitopes of GAD recognised by antibodies from patients with type 1 diabetes and SMS patients have been identified and the dominant epitopes are distinct.^{6–10} In type 1 diabetes these epitopes include peptides in the middle (aminoacids 245–449) and at the carboxyl terminus (amino acids 450–570) of GAD, whereas in SMS the epitope is often at the amino-terminus (first eight aminoacids) of the molecule.^{6,7} In type 1 diabetes the epitopes tend to be conformational, whereas in SMS they are linear.⁶ The distinct pattern of staining on human beta cells with GAD-positive sera in patients with SMS,¹¹ the higher titres of GAD antibodies,^{4,6} and different effects on gamma-amino butyric acid synthesis, indicate that these antibodies recognise GAD differently compared with type 1 diabetes.¹²

The GAD epitopes involved in the cellular immune response in type 1 diabetes have also been mapped to the middle and carboxyl terminus of the molecule,^{13–15} whereas T cells from controls respond to the middle of the molecule. T-cell GAD epitope mapping in SMS has not been done, although T cells in SMS patients do respond to GAD.^{16,17}

GAD antibody isotype can reflect the type of T-helper-cell response generated.¹⁸ Islet-cell antibodies, which include GAD antibody reactivity, are usually restricted to IgG1 subclass.^{19,20} The isotype profile of GAD65 antibodies has not been characterised in SMS and in type 1 diabetes, although GAD65 antibodies are dominantly IgG1 in prediabetic individuals.²¹ Moreover, monoclonal antibodies isolated from patients with type 1 diabetes are usually of the IgG1 isotype.²²

We studied differences in the cellular (epitope recognition) and humoral (isotype profile) immune response to GAD65 in patients with SMS and type 1 diabetes to characterise the possible mechanisms that lead to their distinct clinical outcome.

Patients and methods

Patients

A consecutive series of 14 patients (mean age 45 years, range 23–67; four men, ten women) with clinical and neurological features of axial SMS, seven of whom had diabetes mellitus, were referred to us for study. Diagnosis of axial SMS was confirmed independently by a consultant neurologist and was based on typical clinical symptoms, electromyogram findings, clinical improvement on diazepam, and the presence of GAD antibodies, according to recent criteria.²³ None of the patients had diseases other than SMS or diabetes.

A consecutive series of 17 patients with type 1 diabetes mellitus (mean age 23 years, range 3–45; mean disease duration 2.0 years; eight male, nine female) who were attending a diabetes clinic were selected. Type 1 diabetes was defined according to the National Diabetes Data Group criteria.²⁴ Eligible patients were of European origin, clinically well without any other disease, had been diabetic for less than 6 years, and were without diabetic complications.

37 controls (mean age 26 years, range 9–47; 24 male, 13 female) were taken at random from a pool of over 100 normal healthy individuals without a family history of diabetes, concurrent illness, or any drug therapy, who were adults from a local insurance company, or children who were friends of young patients with diabetes. Random samples of whole-blood glucose were taken at the time of testing to exclude diabetes. Controls were compared with patients with SMS and type 1 diabetes for their T-cell responses to GAD fragments and to establish a cut-off levels for positivity for GAD isotypes.

Written informed consent was obtained from all participants, and the study was approved by the ethics committee of St Bartholomew's Hospital.

T-cell proliferation assays

We synthesised GAD65 peptides by the multipin method,²⁵ producing 15mers that overlapped each other by 11 residues to cover all regions of the GAD65 enzyme, as previously described.¹⁴ The peptides were pooled (20 per pool) in a final concentration of 2 µg/L for use in proliferation assays. Negative control wells contained the same dilution of buffer but without peptide. Pools of GAD65 peptides that did not induce a significant response were detected in all individuals tested and provided further internal negative controls. Toxic and mitogenic effects of the peptide pools were excluded. After separation of fresh peripheral blood mononuclear cells by Ficoll-Hypaque (Lymphoprep, Nycomed, Oslo, Norway) density-gradient centrifugation, cells were diluted in complete medium containing 10% autologous serum and gentamycin 50 µg/L (Gibco, Paisley, UK). Proliferation assays were done in 96-well round-bottomed plates (Nunc, Roskilde, Denmark) with 2×10^5 cells in 200 µL per well, as previously described.¹⁴

GAD antibody isotypes

In-vitro transcribed and translated GAD65 labelled with ³⁵S-methionine was used.²⁶ 50 µL GAD65 (50 000 counts per min) were incubated with 2 µL test serum overnight at 4°C in 96-well millipore filterplates. Monoclonal biotinylated antihuman antibodies (IgG1, HP6069; IgG2, HP6002; IgG3, HP6047; IgG4,

HP6046; IgM, HP6083 [Zymed, Camarillo, CA, USA]; IgE, G7-26 [Beckton Dickinson Pharmigen, Oxford, UK]) were added at concentrations of 4 µg, 4.8 µg, 2.4 µg, 1.6 µg, 2 µg, and 5 µg, respectively, and incubated at 4°C on a shaker for 1.5 h. Antigen-antibody complexes bound to biotinylated monoclonal antihuman antibodies were separated for 1.5 h at 4°C with 50 µL immobilised streptavidin agarose, which has a high affinity for the biotin moiety on the antibody molecules. Bound immune complexes were washed 12 times with Tris buffer saline–Tween on the millipore plate washer, allowed to dry at room temperature before addition of meltilex solid scintillant, and counted on the Wallac 1260 Microbeta scintillation counter (Perkin Elmer Life Sciences Wallac, Cambridge, UK). To establish a cut-off for positivity, the results were calculated in the 37 controls as the mean counts per min for each antibody isotype minus the blank counts per min, and as the mean counts per min for all antibody isotypes. Results were identical for both methods. Positive results in patients were defined as a counts per min greater than 3 SD above the mean of the control levels by both methods.

HLA typing

HLA typing of patients was done with antibodies provided by the tenth HLA-tissue typing workshop (New York, USA, 1987). HLA-DQ subtyping was done by single-step allele-specific PCR.²⁷

Statistical analyses

The number of positive wells for each antigen was calculated by computer.²⁵ Wells were defined as positive if they exceeded the mean (about 1000) counts per min plus 3 SD of 32 negative control wells. By assuming a Poisson distribution of positive wells, we defined frequencies of positive responses as significant at $p < 0.0025$.²⁵ The pool with the most positive wells was defined as the dominant pool for each individual.

Fisher's exact test was used to analyse differences in T-cell responses and isotypes between SMS, type 1 diabetes patients, and controls at a significance level of $p < 0.05$, by use of Apple Statview version 4.01. Analysis was only done when a dominant pool was found in at least three SMS patients and in at least three patients with type 1 diabetes. To estimate the sample size we assumed 80% power, 5% significance, 40% expected positive response in type 1 diabetes (to carboxyl-terminal fragment) and 0% in SMS patients, so that a total number of at least 24 cases was needed. In case of unequal randomisation, we expected the power to be reduced by a small amount from 80% if the ratio between the two groups was kept at a ratio of about 2/1.²⁸

Results

Sera for autoantibody analysis were available from all patients. However, because of difficulty in obtaining fresh blood cells, sufficient peripheral blood mononuclear cells for HLA genotype were available from 13 of 14 SMS patients, and for T-cell studies from only eight SMS patients.

We did not detect GAD65 antibodies in three SMS patients. Of the seven SMS patients with diabetes, six were on insulin treatment and the mean duration of their diabetes was 8.4 years (range 0–22). SMS was

Patient	Age (years)	Diabetes	HLA-DQ	HLA-DR1B	GAD antibody	T-cell reactive to GAD fragments	GAD-reactive immunoglobulin isotypes
SMS							
1	45	Yes	A1*0501, B1*0301	16	Positive	313–403	G1, G3
2	43	No	A1*0103, B1*0603; A1*0201, B1*0201	13, 7	Positive	113–403, 81–171	G1, G2, G3, G4, M, E
3	67	No	A1*0101, B1*0501; A1*0102, B1*0602	1, 15	Negative	81–171	None
4	47	Yes	A1*0101, B1*0501; A1*0301, B1*0302	1, 4	Positive	81–171	IgG1
5	33	No	A1*0101, B1*0501; A1*0301, B1*0302	1, 4	Negative	81–171	None
6	23	No	A1*0101, B1*0501; A1*0102, B1*0602	1, 15	Positive	313–403	G1, G2, G3, G4
7	44	No	A1*0201, B1*0201; A1*0501, B1*0201	7, 3	Positive	None	G1, G2, G3, G4, M, E
8	29	Yes	A1*0201, B1*0201; A1*0102, B1*0502	7, 16	Positive	None	G1, G2, M
9	47	Yes	A1*0501, B1*0201	3	Positive	..	G1, G2, G3, G4, M, E
10	61	No	..	..	Positive	..	G1, G2, G3
11	60	Yes	A1*0501, B1*0201	3	Positive	..	G1
12	67	Yes	A1*0102, B1*0602	15	Positive	..	G1
13	46	Yes	A1*0102, B1*0604; A1*0501, B1*0201	13, 3	Positive	..	G1, G2, G3, G4, M, E
14	26	No	A1*0501, B1*0201	3	Negative	..	None
Type 1 diabetes							
15	10	Yes	A1*0501, B1*0201; A1*0301, B1*0302	3, 4	Positive	None	None
16	22	Yes	A1*0101, B1*0501; A1*0301, B1*0302	1, 4	Negative	473–555	None
17	24	Yes	A1*0102, B1*0604; A1*0301, B1*0302	13, 4	Negative	473–555, 153–233	None
18	19	Yes	..	..	Negative	None	None
19	30	Yes	A1*0501, B1*0201; A1*0103, B1*0603	3, 13	Negative	313–403	None
20	3	Yes	A1*0301, B1*0302	4	Negative	473–555, 223–323	None
21	11	Yes	A1*0501, B1*0201; A1*0301, B1*0302	3, 4	Negative	None	None
22	6	Yes	A1*0301, B1*0302	4	Positive	473–555	None
23	11	Yes	..	..	Positive	None	G1
24	40	Yes	A1*0102, B1*0604; A1*0301, B1*0302	13, 4	Positive	161–243	None
25	19	Yes	..	..	Positive	None	None
26	20	Yes	A1*0501, B1*0201; A1*0301, B1*0302	3, 4	Positive	161–243	G1
27	29	Yes	A1*0101, B1*0501; A1*0501, B1*0201	1, 3	Positive	161–243	None
28	40	Yes	A1*0102, B1*0604; A1*0301, B1*0302	13, 4	Positive	161–243	G1, G3
29	35	Yes	A1*0501, B1*0201	3	Positive	545–585	G1, M
30	45	Yes	A1*0501, B1*0201; A1*0301, B1*0302	3, 4	Positive	473–555	G1, G3
31	26	Yes	A1*0101, B1*0501; A1*0602, B1*0604	1, 13	Positive	473–555	None

Characteristics of patients and findings

diagnosed after diabetes in two of seven patients with both diseases with a median duration of 1 year (range 0–13).

The T-cell reactivity profile associated with SMS was detected irrespective of whether the SMS patients had diabetes (table). SMS patients had reactivity to two dominant regions of GAD65 (81–171 and 313–403). These regions were recognised in six of eight SMS patients, but only one of 17 patients with type 1 diabetes ($p=0.001$). Type 1 diabetes patients also had reactivity to GAD65 regions, including regions previously reported to be immunodominant.^{13–15} Two dominant regions of GAD65 (161–243 and 473–555) were recognised by ten of 17 patients with type 1 diabetes but none of eight SMS patients ($p=0.008$). None of the controls had T-cell responses to GAD65.

GAD65 antibodies were detected in 11 of 14 SMS patients and 11 of 17 patients with type 1 diabetes (table). In six patients with type 1 diabetes we were unable to detect GAD immunoglobulin isotypes despite positivity for GAD antibodies, probably because of decreased sensitivity of the isotype assay. IgG1 GAD antibodies were more prevalent than other isotypes in patients with SMS or type 1 diabetes. Eight SMS patients compared with three patients with type 1 diabetes had isotypes other than IgG1 ($p=0.03$). IgG4 or IgE isotypes were detected in five of 14 SMS patients but in none of 17 patients with type 1 diabetes ($p=0.012$). Of seven SMS patients with diabetes, five had an antibody profile similar to that found in type 1 diabetes (ie, IgG1 but no IgE or IgG4), but the other two patients had IgE and IgG4 GAD antibodies, which is similar to the profile in three of seven patients without diabetes.

GAD65 antibody isotypes were not related to disease duration in SMS patients or in patients with type 1

diabetes (data not shown). In SMS patients, irrespective of their HLA genotype, particular regions of GAD65 induced T-cell proliferation and a different GAD65-antibody isotype profiles. This responsiveness in SMS patients was seen irrespective of an HLA genotype associated with type 1 diabetes (ie, HLA DQ3.2).

Discussion

We found that the GAD antibody response in type 1 diabetes and SMS commonly includes IgG1. In type 1 diabetes, GAD65 autoantibodies were highly restricted, comprising only IgG1 and IgG3, which are thought to be associated with a cell-mediated T-helper type 1 response in human beings. By contrast, there were no IgE or IgG4 GAD isotype antibodies, which are thought to be associated with a humoral T-helper type 2 response, so there was no evidence of a humoral response in type 1 diabetes. The main isotype of islet-cell antibodies is probably IgG1.^{19,20} Previous reports have shown a bias towards cell-mediated immunity in the peripheral T-helper 1/T-helper 2 balance, as indicated in the cytokine network, in patients with type 1 diabetes and non-obese diabetic mice.^{30,31} Our results are consistent with that finding in that a dominant GAD65-specific cell-mediated response is seen in type 1 diabetes.

The variations in GAD65 antibody isotypes in SMS patients are consistent with a broad-based autoantibody response to GAD65, reflecting humoral differences from type 1 diabetes. Our results are in line with the assumption that SMS patients have a notable humoral response as well as a cell-mediated response to GAD65.²⁹ Indeed, IgE GAD antibodies were detected in only one of 124 patients with type 1 diabetes (M Hawa, R D G Leslie, unpublished data), but in this study the IgE isotype was detected in two of seven SMS patients

with diabetes. If confirmed, then IgE isotypes may be a feature of SMS, irrespective of whether diabetes is present; alternatively, the form of diabetes associated with SMS may be immunologically distinct from type 1 diabetes.

The implications of a broad-based humoral immune response to GAD in SMS, but not in type 1 diabetes, remains unclear, particularly since SMS patients can develop diabetes. Subclass restriction, identified here in type 1 diabetes patients, has been shown in several autoimmune diseases that are thought to be driven by T-helper type 1 cells, and could reflect the nature of the antigen, mode of presentation, abnormalities in immunoregulation, or genetic factors (in particular different HLA), which influence class switching.²⁹

We extended our analysis of the GAD65 reactivity to the cellular immune response, and found that dominant GAD65 peptides that induce T-cell proliferation were also different between SMS and type 1 diabetes. Interestingly, these differing patterns of reactivity were maintained despite the fact that a few SMS patients had genotypes associated with susceptibility to type 1 diabetes.³² One SMS patient who was studied with T-cell lines raised against GAD65 was recently shown to recognise GAD peptide 331–360, which is contained within one of the regions (313–403) we assessed.¹⁷ The number of SMS patients that we studied was small, which was inevitable given the rarity of the disorder, and it was therefore not possible to study T-cell reactivity to GAD65 in all SMS patients. Nevertheless, our results show a clear difference between the two diseases despite recognition of the same autoantigen.²⁹

SMS patients also offer a unique approach towards understanding the potential role of HLA in the development of diabetes. Indeed, some SMS patients express HLA DQ6, which may protect against diabetes.³³ The numbers of SMS patients we studied is too small to come to a clear conclusion; however, three SMS patients with diabetes had HLA susceptibility alleles for type 1 diabetes, whereas three patients without diabetes had HLA diabetes-protective alleles.³² Nevertheless, the T-cell epitope profile in SMS patients did not reflect the presence or absence of HLA diabetes-associated alleles. Thus the T-cell epitope response to GAD65 in SMS is determined by factors other than HLA. There was no difference in the seven SMS patients with diabetes compared with those without diabetes in terms of their T-cell epitope profile or antibody isotype profile. However, SMS patients with diabetes showed GAD65 reactivity (T-cell proliferation and isotypes) not found in type 1 diabetes patients. Although the nature of factors determining the immune distinctions between SMS and type 1 diabetes remains unclear, understanding mechanisms for differential epitope display and isotype profiles will be important if we are to understand the reasons behind the development of either disease.²⁹

Contributors

Tobias Lohmann, Mohammed Hawa, David Leslie, and Marco Londei designed the study, co-ordinated the project, and wrote the report. Russell Lane advised on clinical diagnoses, and Jean Picard did the HLA typing. Tobias Lohmann and Mohammed Hawa contributed equally.

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