

# Humoral and Cellular Autoimmune Responses in Stiff Person Syndrome

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**ABSTRACT:** Stiff person syndrome (SPS) is a chronic autoimmune disease associated with humoral and cellular immune responses to glutamic acid decarboxylase (GAD) 65. Another chronic autoimmune disease, type 1 diabetes (T1D), is also associated with autoimmune responses to this antigen, but T1D patients develop SPS only extremely rarely and only a third of SPS patients develop T1D (mostly mild manifestations in adulthood). In a previous study, we described important differences between T1D and SPS in the autoimmune response to GAD 65: (1) T cells of SPS patients recognize epitopes in the middle of GAD 65 (amino residues 81–171 and 313–403), whereas patients with T1D preferentially recognize another middle (161–243) and a C-terminal region (473–555); and (2) GAD antibodies (Abs) were nearly exclusively of the Th1-associated IgG1 type in T1D, whereas SPS patients had both Th1- and Th2-associated IgG4 and IgE GAD Abs. These differences were not simply related to different HLA alleles. Fine epitope mapping revealed further distinct T cell epitopes in both diseases despite similar HLA background. Therefore, a single autoantigen can elicit different immune responses causing distinct chronic autoimmune diseases possibly related to a Th1 or Th2 bias of the disease.

**KEYWORDS:** stiff person syndrome; autoimmune disease; type 1 diabetes; glutamic acid decarboxylase 65

## INTRODUCTION

The synthesizing enzyme for the inhibitory neurotransmitter  $\gamma$ -amino butyric acid (GABA), glutamic acid decarboxylase (GAD), exists in two major isoforms, GAD 65 and 67.<sup>1,2</sup> Both proteins are encoded by two nonallelic genes and share homology that differs remarkably in different parts of the molecules. Both isoforms are expressed in human neurons, pancreatic beta cells, and other neuroendocrine tissues, but GAD 65 seems to be the major isoform expressed in human pancreatic beta cells.<sup>3</sup> Autoantibodies to GAD 65 and GAD 67 have been detected in the sera of

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patients with type 1 diabetes (T1D) and a rare neurological disorder, stiff man or stiff person syndrome (SPS).<sup>2,4</sup> SPS is diagnosed in less than one in one million of the general population and is clinically characterized by stiffness of axial muscles and painful muscular spasms.<sup>5</sup> T1D develops in about 30% of the patients with SPS,<sup>6,7</sup> mainly as mild manifestations in adulthood. GAD is implicated as an important auto-antigen in T1D.<sup>8</sup> SPS is caused by an impairment of GABA-ergic neurotransmission in the central nervous system, possibly caused directly by the anti-GAD immune attack.<sup>9,10</sup>

Islet cell antibodies (ICA) are a feature of T1D, occurring in the sera of approximately 80% of newly diagnosed cases. ICA recognize a number of different antigens including GAD, insulin, and protein tyrosine phosphatases. ICA are polyclonal and, in general, are restricted to IgG1 isotype belonging to the IgG subclass.<sup>11</sup> The isotype profile of antigen-specific antibodies has been shown to reflect T cell (Th1 or Th2) effector functions in animal models.<sup>12</sup> Interferon- $\gamma$ -dependent antibody isotypes in mice are mainly IgG2a and IgG3 (human homologues probably IgG1 and IgG3), which reflects a Th1 response, while a Th2 response is predominantly IgE or IgG1 (human homologues probably IgE and IgG4). Restriction of ICA to IgG1 in IDDM in humans may thus represent a Th1 dominant immune response. ICA are also a feature of SPS; in these patients, the antibodies are predominantly attributable to GAD antibodies. The isotype profile of antigen-specific antibodies, including GAD antibodies, in T1D and SPS has not been characterized. However, monoclonal antibodies isolated from T1D patients are usually of the IgG1 isotype.<sup>13</sup> We therefore established assays for the isotypes of GAD antibodies and estimated them in SPS and T1D patients.

Furthermore, distinct dominant epitopes of GAD have been described for GAD specific autoantibodies in T1D and SPS.<sup>6,7,14–16</sup> It was hypothesized that the different recognition of GAD by the immune system may explain the low penetration of T1D in SPS.<sup>7</sup> Our group<sup>17</sup> and others<sup>18</sup> have described dominant T cell epitopes of GAD in T1D, but nothing was known about the T cell epitopes to GAD in SPS. In this study, we investigated 8 patients with SPS (3 of them were diabetic) for their reactivity to overlapping synthetic peptides covering completely GAD 65 and 67 and compared the results to our described data on T cell reactivity to GAD peptides in T1D and healthy controls.<sup>17</sup>

## METHODS

Fourteen patients with clinical and neurological features of SPS (7 of them had T1D) were selected for this study. The diagnosis was confirmed independently by a neurologist and included typical clinical symptoms, electromyogram findings, clinical improvement by diazepam, and mostly GAD antibodies. Mean age was 45 years (range 23–67; 4 men and 10 women). All patients had no other diseases beside SPS or T1D. GAD-antibody responses of these SPS patients were compared to 17 T1D patients (mean age: 23 years; range: 3–45 years). Their duration of diabetes was similar to that of the 7 SPS patients with diabetes (3–7 years). Eight of the SPS patients were compared for T cell responses to GAD with the T1D patients (<5 years from diagnosis) and 10 healthy controls described elsewhere.<sup>17</sup> Two SPS patients

with T cell responses to region 81–171 and 1 patient for region 313–403 were studied again for responses to single peptides of these regions.

### *Antibody Assays*

*In vitro* transcribed and translated GAD labeled with  $^{35}\text{S}$ -methionine was used as the antigen, prepared as previously described.<sup>19</sup> The isotype-specific GAD antibody assay is described in detail elsewhere.<sup>20</sup>

To establish a cutoff for positivity, the results were calculated in 37 control subjects of similar age and sex ratio: (1) as the mean cpm for each antibody isotype and (2) as the mean cpm for all antibody isotypes. Positive results in patients were defined as a cpm value greater than 3 standard deviations (SD) above the mean of the control levels using both methods.

### *T Cell Assays*

All 15-mers overlapping by 11 residues covering both GAD 65 and 67 completely were synthesized by the multipin method.<sup>21</sup> Peptides were cleaved in 0.05 mol/L HEPES, pH 7.6–7.8, in 40% acetonitrile (HPLC grade) in water; the purity of peptides was confirmed by HPLC to be >80%. The peptides were used pooled (20 per pool) or as single peptides in a final concentration of 2  $\mu\text{g/mL}$  in proliferation assays. Negative control wells contained the same dilution of buffer without peptide. Both toxic and mitogenic effects of the peptide pools were excluded. Again, T cell proliferation assays are described in detail elsewhere.<sup>17</sup>

The number of positive wells for each antigen was calculated;<sup>21</sup> positive wells exceeded the mean cpm plus 3 SD (pools) or plus 2 SD (single peptides) of 32 negative control wells. Frequencies of positive responses significant at the  $p < 0.0025$  level are reported.<sup>21</sup> The pool with the most positive wells was regarded as the “dominant” pool for each individual. Fisher’s exact test was used to analyze differences between SPS, T1D, and control patients ( $p < 0.05$  was considered significant). Analysis was only done when a dominant pool was found in 2 or more SPS patients and in 3 or more T1D patients.

HLA typing of SPS patients was performed using antibodies provided by the X<sup>th</sup> HLA-Tissue Typing Workshop. Furthermore, HLA-DQ subtyping was performed by the single-step allele-specific polymerase chain reaction as described elsewhere.<sup>22</sup>

## RESULTS

Antibodies to GAD were detected in 11 of 17 T1D patients and 11 of 14 SPS patients. Only GAD antibody-positive patients are analyzed for isotype responses (11 T1D and 11 SPS patients). GAD IgG1 was the most prevalent isotype both in T1D and SPS patients. GAD isotypes other than IgG1 were detected in 0 of 11 T1D patients, but in 8 of 11 SPS patients; thus, these antibodies were more prevalent in SPS than in the T1D patients (Fisher’s exact  $p = 0.003$ ). IgG4 or IgE isotypes were found in 5 of 11 SPS compared to none of 11 T1D patients ( $p = 0.012$ ). Of the 7 SPS patients with diabetes, 5 had an antibody profile similar to that found in T1D (i.e., IgG1, but no IgG4 or IgE), but the other 2 patients had IgG4 and IgE GAD antibodies

similar to the profile in 3 of 7 patients without diabetes. GAD 65 isotypes were not related to disease duration or HLA type in SPS and T1D patients.

T cell responses to pools of overlapping peptides of GAD 65 in SPS patients were compared to groups of T1D patients and healthy controls described previously.<sup>17</sup> The experimental design (including media or plastics used) and evaluation were absolutely identical to our previous work to get optimal comparability of the data. Six of 8 SPS patients, 12 of 17 T1D patients, and 9 of 10 controls had significant T cell responses to GAD 65 peptide pools; only these probands with T cell responses were analyzed for the comparison of epitope specificity of the T cell response. In contrast to T1D patients or healthy controls, the T cell response to GAD 65 peptides in SPS patients was mainly restricted to two regions of GAD 65, that is, 81–171 and 313–403, in the middle of the molecule. Region 81–171 was dominantly recognized in 3 of 6 SPS patients compared to 0 of 12 T1D patients ( $p < 0.025$ ) or 1 of 9 healthy controls ( $p > 0.1$ ). It should be stated that region 81–171 was also recognized in 4 of 20 long-term T1D patients (>5 years after diagnosis; unpublished data). Region 313–403 was dominantly recognized in 3 of 6 SPS patients compared to 1 of 12 T1D patients ( $p < 0.05$ ) or 1 of 9 healthy controls ( $p > 0.1$ ).

In our previous report,<sup>17</sup> we have described the region 473–555 of GAD 65 as immunodominant in T1D since the T cells of 6 of 12 T1D patients responded dominantly to this region. In our present study, none of the 6 SPS patients showed a T cell response to this region ( $p < 0.05$ ). Furthermore, the T cells of 5 of 9 healthy controls recognized region 161–243 in a dominant manner<sup>17</sup> and, again, no significant T cell reactivity to this region was observed in SPS patients ( $p < 0.05$ ).

In the same study,<sup>17</sup> 8 of 15 T1D patients and 3 of 10 healthy controls recognized dominantly region 153–243 of GAD 67, but no significant differences between healthy controls and T1D patients were found for T cell reactivity to GAD 67 pools. In this study, T cells of 6 of 8 SPS patients recognized GAD 65 and of 3 of 8 SPS patients GAD 67 peptide pools. Therefore, the T cell response in SPS can be directed to both GAD 65 and GAD 67, but also for GAD 67 the recognized epitopes in SPS patients differed from those in T1D patients and healthy controls (e.g., 73–163 or 465–555 in SPS patients and 153–243 in T1D patients and controls).

For 2 SPS patients, we were able to decode the GAD T cell response to single epitopes of the region 81–171. Remarkably, 2 peptides (101–115 and 145–159) were recognized by T cells of both patients, despite completely different HLA types. The latter epitope (145–159) overlaps a GAD 65 epitope recognized by a T cell line from a recent onset T1D patient, which was restricted by DR2.<sup>23</sup> The dominant T cell epitope in SPS patients, however, seems to be located in the middle of this region (113–127 for patient 1; 117–135 for patient 2). Peptides 113–127 and 117–135 mainly overlap a peptide (115–127) described as immunogenic in a DR401 transgenic mouse model<sup>24</sup> and, indeed, 1 patient had the DR401 haplotype. Furthermore, peptide 88–99 has been described as an immunodominant DRB1\*0101 restricted epitope in a T1D patient.<sup>25</sup> This peptide was found in our study as a subdominant epitope for 1 patient who also had the haplotype DRB1\*0101. One patient was tested again for responses to single peptides of region 313–403 and her T cells responded to an array of peptides in this region (337–351, 349–363, 369–383, 385–403), including the binding site of pyridoxal phosphate (390–402) and an epitope (339–352) described by others for SPS T cells<sup>26</sup> and showing cross-reactivity to a peptide of cytomegalovirus.<sup>27</sup>

## DISCUSSION

Our observations on GAD antibody isotypes suggest a bias in T1D patients towards a Th1-type immune response, which was not evident in the SPS patients. In contrast, SPS patients showed evidence for spreading of the immune response to IgG isotypes other than IgG1, including IgG4 and IgE, associated with a Th2 response. Such a Th2 response has been associated with at least preliminary protection from islet cell destruction,<sup>28</sup> while a bias to Th1 immunity in the cytokine network of T1D has been described already by others.<sup>29</sup> Possibly, such a Th2 bias of islet cell autoimmunity is correlated to the phase of “benign autoimmunity” in NOD mice, in contrast to the Th1-biased “malignant autoimmunity” causing islet cell destruction and full blown diabetes.<sup>30</sup> The reason for the Th1/Th2 bias in autoimmune disease may have several possible explanations. It might be due to the nature of the antigen, abnormalities in immunoregulation, or genetic factors that influence class switching.

The differences in epitope recognition between SPS patients and T1D patients or controls may simply reflect differences in HLA types rather than disease specificity. However, 2 SPS patients had the T1D-related HLA haplotype DR4/DQ0302 highly correlated with T cell responses to GAD 473–555 in T1D in our previous study,<sup>17</sup> but the T cells of these SPS patients still responded preferentially to GAD 81–171. In this region 81–171, we found responses to peptides of GAD 65 described as immunogenic in the HLA transgenic mice, supporting the feasibility of our approach for epitope decoding. Some epitopes detected in region 81–171 had been described as epitopes of T cell lines of T1D patients of the same HLA haplotype, but were rather subdominant epitopes in our SPS patients. However, we cannot completely exclude HLA dependence of T cell epitope specificity because it is impossible to match SPS and T1D patients for HLA since the HLA linkage differs between these diseases.<sup>31</sup>

Region 313–403 of GAD 65 was recognized by 3 of 8 of our SPS patients and contains 2 linear epitopes of GAD antibodies (residues 354–368 and 390–402) described by others.<sup>7</sup> Moreover, region 390–402 contains the binding site of the GAD cofactor, pyridoxal 5'-phosphate, and an immune attack against this region has been proposed as a possible pathogenetic mechanism in SPS.<sup>6</sup> Remarkably, the 3 SPS patients with dominant T cell responses to region 313–403 elicited very high GAD antibody titers and a broad antibody response with isotypes other than IgG1. Larger studies about T cell responses to GAD epitopes are necessary to define a possible link between T and B cell epitopes in SMS since the existence of linear GAD antibody epitopes in SPS,<sup>6,7</sup> in contrast to the preferentially conformational GAD antibody epitopes in T1D,<sup>14</sup> may offer such a direct link.

The comparison of immune responses to GAD between SPS and T1D patients or healthy controls is hampered by the small number of SPS patients available (SPS is diagnosed in  $<1/10^6$  of the general population).<sup>7</sup> Nevertheless, regions 81–171 and 313–403 of GAD 65 seem to be recognized by T cells of a remarkable fraction of SPS patients, while the region 473–555 of GAD 65 was never recognized in SPS patients in contrast to T1D patients, although 3 of 8 SPS patients had developed T1D. There may be different explanations for the absence of T1D in most of the SPS patients: (1) T1D is progressing very slowly in these patients such that the future development of T1D in these individuals cannot be excluded; (2) differences in HLA types as proposed by Pugliese<sup>31</sup> are responsible for T1D development; or (3) a Th2-like immune response protects SPS patients from development of T1D. Supporting

**TABLE 1. T cell autoimmunity against a single autoantigen, GAD65, in two distinct diseases, SPS and T1D**

|                 | Type 1 diabetes (T1D)              | Stiff person syndrome (SPS) |
|-----------------|------------------------------------|-----------------------------|
| Dominant T cell | 477–555                            | 88–171                      |
| Epitopes        | 161–243                            | 313–403                     |
| GAD-Ab isotypes | IgG1 (IgG3)                        | IgG1 + IgG4 + IgE           |
| Th1/Th2 bias    | pure Th1                           | mixed Th1/Th2               |
| Islet outcome   | “Malignant” islet cell destruction | “Benign” islet inflammation |

the second possibility, some SPS patients not developing T1D had HLA DQ6 subtypes that were proposed as protective against T1D in SPS patients.<sup>31</sup> In support of the third outcome, all 3 patients with GAD antibody isotypes suggestive for a Th2-type immune response were free of diabetes. This is in line with numerous reports on a defective Th2 immune response in T1D.<sup>29,32–34</sup>

In summary, the isotype profile of the GAD antibody response and the dominant T cell epitopes of GAD 65 and GAD 67 differed between SPS and T1D. Therefore, the immune response to a single autoantigen may be qualitatively and/or quantitatively different, resulting in distinct or only partially overlapping autoimmune disease entities (TABLE 1). These differences may explain the low penetration of T1D in SPS and, more importantly, the Th1- or Th2-like preference of the immune attack in both diseases.<sup>35</sup> Since the switch of a Th1- to a Th2-like immune response was proposed as a therapeutic approach in T1D,<sup>36</sup> the differences in epitope recognition between SPS and T1D may give clues about the conditions that prime the different immune reactions to GAD 65 and may possibly be exploited in therapeutic interventions. On the other hand, the development of SPS has to be considered as a possible hazard in such interventions.

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