Developments in the Diagnostic Potential of Antibodies to Glutamic Acid Decarboxylase

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ABSTRACT. Autoantibodies to glutamic acid decarboxylase (GAD-Abs) occur in type I diabetes, polyendocrine autoimmunity (PE) and stiff-man syndrome (SMS), and are diagnostic and predictive markers. We investigated GAD-Abs in patients being assessed for SMS; comparing ELISAs employing GAD-enriched rat brain preparation with an immunoprecipitation assay using radio-labelled human GAD. An ELISA in which GAD was captured with an N-terminal-specific monoclonal antibody (mAb) showed similar sensitivity and specificity to the immunoprecipitation assay, whereas lower sensitivity and specificity were shown by an ELISA employing a capture mAb (GAD-6) specific for the C-terminal region of GAD, and by an ELISA in which the rat brain preparation was coated directly onto the plate. The direct ELISA detected additional antibodies in paraneoplastic SMS patients (probably anti-amphiphysin). We investigated autoantibodies to a subregion of GAD in PE by determining the ability of serum antibodies to inhibit the binding of GAD-6 mAb to GAD in direct ELISA. Patients were heterogeneous in the occurrence of these antibodies. There was no significant difference between PE patients with or without diabetes, although the highest levels of inhibition were given by sera from non-diabetic patients. Differential expression of subsets of GAD-Abs may be of predictive value.

Keywords: Insulin-dependent diabetes mellitus, Polyendocrine autoimmunity, Stiff-man syndrome, Glutamic acid decarboxylase.

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Introduction

Autoantibodies to glutamic acid decarboxylase (GAD) have been associated with several diseases, particularly autoimmune insulin-dependent diabetes mellitus (IDDM)\(^{[11]}\), polyendocrine autoimmunity (PE) in which organ-specific autoimmunity directed at various tissues (particularly thyroid, gastric, adrenal, and pancreatic islets) may be evident\(^{[22]}\) and the rare neurological disorder called stiff-man syndrome (SMS)\(^{[3,4]}\).

GAD is an enzyme which catalyses the synthesis of the transmitter molecule gammaaminobutyric acid (GABA). Two forms of GAD have been identified: an amphiphilic form with a molecular weight of 65kD and a hydrophilic 67D form. These two forms show 65% homology, differing mainly in their amino (N)-terminal regions. GAD-65 is the predominant form in human islet beta cells and both forms are highly expressed in GABA-secreting neurons of the central nervous system. Progression to IDDM in patients with antibodies to GAD is only about 30% for SMS and 14% for PE, respectively.

There are similarities and differences in the antibody responses to GAD in IDDM and SMS. In IDDM, serum GAD-Abs usually have a low to moderate titre, are mainly specific for GAD-65 (rather than GAD-67), and recognize mainly conformational determinants (i.e., they do not react with partially denatured GAD on western blots). In SMS, serum GAD-Abs usually have a moderate to high titre, may react with both GAD-65 and GAD-67, and recognize linear GAD epitopes as well as conformational epitopes (i.e., they will react with GAD on Western blots)\(^{[3,5]}\). In addition, a number of studies have shown differences in the antibody response to GAD in SMS compared with IDDM by examining binding to fragments of GAD generated from deletion mutants of recombinant GAD gene or as synthetic peptides or binding to recombinant molecular chimeras between GAD-65 and GAD-67\(^{[13-15]}\). These studies have shown that in both IDDM and SMS, antibodies react with conformation-dependent epitopes in the middle and carboxy (C)-terminal portions of GAD-65, whereas antibodies which bind to linear determinants and to epitopes in the first eight to sixteen amino acid residues of the amino (N)-terminal region of GAD-65 are common in SMS, but rare in primary IDDM. Less is known about the epitope specificities of GAD-Abs in PE autoimmunity.

The laboratory determination of antibodies to GAD (GAD-Abs) can provide information which is clinically useful in the diagnosis and management of patients. In relation to IDDM, the occurrence of GAD-Abs in the sera of 'pre-diabetic' individuals, particularly in association with antibodies to other islet-associated autoantigens (insulin, the tyrosine phosphates-like proteins IA-2 and phosphin, and the ganglioside GM2-1), is predictive of the onset of IDDM\(^{[16]}\). This information is now being used in identifying prediabetic individuals who may benefit from immunotherapy designed to intervene in the autoimmune process and halt the progression to overt disease\(^{[17]}\). The occurrence of GAD-Abs also identifies patients with non-insulin-dependent diabetes mellitus who are likely to progress to IDDM (known as latent autoimmune diabetes
mellitus)\textsuperscript{[18]}, and women with gestational diabetes mellitus who will subsequently develop true IDDM\textsuperscript{[19]}. Not all patients with SMS possess GAD-Abs, but the presence of these antibodies can help to differentiate SMS from other neurological disorders, as well as distinguish SMS patients who are unlikely or likely to have associated neoplasms; in the latter, known as paraneoplastic SMS, GAD-Abs are usually absent but the patients frequently have serum antibodies to a 128kD brain known as amphiphysin \textsuperscript{[20-22]}. In view of the clinical utility of GAD-Abs measurements, the studies described here were undertaken to assess the relative sensitivity of different types of assays for GAD-Abs and whether the detection of subsets of antibodies directed to particular antigenic regions of GAD might enhance the clinical value of the information obtained.

A Comparison of Assays for GAD-Abs in Suspected SMA: A variety of assays have been reported for measuring GAD-Abs in serum. The results of the Second International GAD Antibody Workshop indicated that fluid-phase immunoprecipitation (IP) assays employing radiolabelled GAD were more sensitive and specific for the detection of GAD-Abs in the sera of IDDM patients than were solid-phase enzyme-linked immunosorbent assays (ELISAs) in which GAD was coated directly into plastic ELISA plates\textsuperscript{[23]}. This may be because, as discussed above, the epitopes of the GAD-Abs associated with IDDM appear to be strongly conformation dependent; these epitopes may be partially disrupted or obscured when GAD is bound to plastic. By contrast, since the serum GAD-Abs found in SMS include antibodies recognising linear as well as conformational epitopes, it is possible that ELISAs may efficiently detect GAD-Abs in SMS patients' sera although they are of lower efficacy for IDDM. A particular advantage of ELISAs is that, unlike the most sensitive IP assays, they do not require radiolabelled GAD. We have therefore compared three ELISAs with an immunoprecipitation assay for the detection of GAD-Abs in the sera of patients with suspected SMS. A direct ELISA (GAD bound directly to the plate) was compared with two capture ELISAs in which the GAD was bound via specific monoclonal antibodies (mAbs). The latter may have advantages over the direct ELISA in causing less denaturation of the GAD as well as enriching and concentrating it on the plate.

The fluid-phase IP assay employed was described by Falorni \textit{et al}\textsuperscript{[24]}. This involved the \textit{in-vitro} translation of \textsuperscript{35}S-methionine labelled recombinant human GAD-65 encoded in the plasmid vector pEX9. Serum antibodies were allowed to react with the radiolabelled GAD in the fluid phase and immune complexes were precipitated with protein A-Sepharose. The amount of radioactivity precipitated was then determined. GAD binding was calculated as a relative index of positive and negative control sera. Sera were scored positive if they gave indices greater than 10, which represented the 98th percentile of 100 normal sera.

The direct and capture ELISAs were performed as shown in Figure 1 and previously described\textsuperscript{[25]} Ninety-six well ELISA plates were coated with a GAD-enriched rat brain preparation\textsuperscript{[25]} in the direct ELISA or with one of two different GAD-specific mouse
mAbs followed by specific binding of rat brain GAD in the capture ELISAs. The rat brain preparation was produced by ultracentrifuging a rat brain homogenate, passing the supernatant through a fast protein liquid chromatography column and pooling the GAD-enriched fractions.

Fig. 1. Scheme for the direct and capture ELISAs for determining GAD-Abs.
The mABs employed in the capture ELISAs were GAD-6 (0.25 ug/ml) which is an IgG2a mABs reactive within the C-terminal region of GAD-65 between residues 475-585/529-583\(^{(6,11,26)}\) (purchased from Boehringer Mannheim U.K.), and an IgG1 mAb (GC3208/clone 11, diluted 1/4000 specific for residues 4-17 of the N-terminus of GAD-65\(^{(27)}\) (purchased from Affinity Research Products, U.K.). Patient and normal control sera were diluted 1/100 for reaction with GAD in the ELI-SAs, and bound antibodies were detected with anti-human IgG alkaline phosphatase and p-nitrophenyl phosphate enzyme substrate. Optical densities (ODs) were read at 405 nm and patient sera were considered positive for GAD-Abs if they gave ODs which exceeded the mean plus three standard deviations of the ODs given by six normal human sera.

Sera from 24 patients with definite or suspected SMA were screened twice in each of the four assays for GAD-Abs. The results for patients 1 to 21 given by the three ELISAs are compared with the IP assay, as given in Table 1. Patients 1-3 were unequivocally positive in all four assays. Patients 4 and 5 were clearly positive in the IP assay and the N-terminal capture ELISA, but were each positive on only one occasion in the GAD-6 capture ELISA and were both negative in the direct ELISA. Patients 6-12 were negative in the IP assay and, of these, patients 14-21 were also negative in all of the ELISAs. However, from within patients 6-13, seven (patients 6-12) were ‘false positive’ in the direct ELISA on one occasion and three in the GAD-6 capture ELISA (patients 1, 11, and 13), whereas only patient 12 gave a ‘false positive’ result in the N-terminal capture ELISA. Thus, relative to the results in the IP assay for patients 1-21, the N-terminal capture ELISA gave the highest sensitivity and specificity followed by the GAD-6 capture ELISA and then the direct ELISA.

### TABLE 1. Comparison of four assays for the determination of GAD-Abs in sera from patients with suspected SMA.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Immuno-precipitation</th>
<th>Direct ELISA</th>
<th>GAD-6 cap. ELISA</th>
<th>N-Terminal cap. ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-3</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
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<td>4, 5</td>
<td>+/-</td>
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<td>6-9</td>
<td>+/-</td>
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<td>10, 11</td>
<td>+/-</td>
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<td>12</td>
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<td>13</td>
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<td>14-21</td>
<td>+/-</td>
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</table>

+/- positive in the assay on both occasions  
-/- negative in the assay on both occasions

Sera from three other SMS patients (patients 23-25) (not shown in Table 1) were negative in the IP assay and were variably positive in the two capture ELISAs. By contrast, these three patients were consistently positive in the direct ELISA. This suggested that these patients possessed antibodies to brain component(s) other than GAD which were present in the rat brain preparation. Results consistent with this possibility were obtained in competitive binding studies in which soluble recombinant rat GAD
was used to inhibit antibody binding to the GAD-enriched rat brain preparation in the direct ELISA. At least 90% inhibition values for patients 22, 23, and 24 were only 52%, 8%, and 11%, respectively.

Patient 23 had breast cancer and patient 24 had a lymphoma, thus displaying the paraneoplastic variant of SMS [20,21]. As mentioned in the introduction, antibodies to a 128kD brain protein identified as amphiphysin have been associated with paraneoplastic SMS [20-22]. Consistent with this, antibodies of patients 23 and 24 bound components of the rat brain preparation in this molecular weight range on western blots.

The IP assay used in this study employed 35S-methionine labelled GAD [24] and has previously been shown to have high sensitivity and specificity for the detection of GAD-Abs. An IP assay employing 125I-labelled GAD has also recently been reported for the detection of GAD-Abs in SMS [28]. The direct ELISA tested in the present study appeared to have lower sensitivity and specificity for the detection of GAD-Abs. This is unlikely to be due to the use of rat GAD rather than human GAD in the ELISA since rat and human GAD-65 are highly homologous, and rat GAD was also used in the N-terminal capture ELISA which was virtually identical with the IP assay in sensitivity and specificity. A more likely possibility is that mild denaturation and masking of epitopes associated with binding the GAD to plastic decreases binding of antibodies, at least with some SMS sera. A recent study indicates that SMS sera contain GAD-Abs, recognising conformational, as well as linear, epitopes [14]. It is presumably the former whose binding is most decreased in the direct ELISA. However, one advantage of the direct ELISA with the rat brain preparation is that it detects other brain reactive antibodies which may be of diagnostic significance, as in the patients with paraneoplastic SMS.

The better performance of the capture ELISAs compared with the direct ELISA is probably because GAD, held on the plate by a mAb binding to a single epitope, maintains better antigenic conformation. However, the epitope specificity of the capture mAb does influence the sensitivity and specificity of the assay. This may be because the mAb sterically hinders the binding of some serum GAD-Abs and this will differ depending on the precise site of the mAb binding. Many SMS sera (unlike most IDDM sera) contain GAD-Abs specific for N-terminal epitopes, but the middle and C-terminal parts of GAD are also major epitopic regions for SMS antibodies [8,12,14]. This may explain why the capture ELISA with the N-terminal specific mAb performed better than the GAD-6 capture ELISA in which there would be masking of epitopes in the C-terminal region. Other possibilities are that different capture mAbs have different affects on GAD conformation, or may be differentially susceptible to competition for binding by serum GAD-Abs which might displace the mAb and thus remove GAD from the plate. In view of these considerations, further improvements in assay sensitivity may be achieved by using combinations of capture mAbs.

Previous studies have indicated that 30-60% of SMS patients are negative for GAD-Abs [4,20]. This may partly explain the high proportion of patients in which GAD-Abs
were not detected, even in the IP assay, plus SMS being eliminated as the diagnosis for patients. Indeed, sera from a further 19 patients not included in Table 1 were negative in all four assays. Overall, this study indicates that a fluid phase IP assay with radiolabelled GAD is the most reliable option for the detection of GAD-Abs in SMS, as in IDDM. However, in situations where this is not practical (e.g., where use of radioactivity is discouraged), a carefully designed capture ELISA can yield results with high sensitivity and specificity.

Assays for other antibodies may then be helpful in the further investigation of individual patients. Thus, particularly patients who are negative for GAD-Abs should be assessed for antibodies to amphiphysin, which may be indicative of having an associated tumour. On the other hand, patients with GAD-Ab who are found to have other islet-reactive antibodies (e.g., anti-IA-2, anti-insulin) are most at risk of developing IDDM (see Fig. 2).

**Fig. 2.** A possible decision tree for the assessment of autoantibodies in the laboratory investigation of suspected SMS.

**The Detection of a Subset of GAD-Ab to a Particular Antigenic Region of GAD:** Progression to overt IDDM in patients with antibodies to GAD is only about 30% for SMS and 14% for PE. This suggests that, compared with 'primary' IDDM, the anti-islet reactivity in SMS and PE is less damaging to beta-cells and therefore is less likely to precipitate dependence on exogenous insulin. As discussed above, the epitopic spec-
Specificities of GAD-Abs differ in IDDM and SMS. This suggests that the antibody response to GAD may differ in different individuals in ways which would reflect the degree to which the autoimmune process is damaging to beta-cells and therefore whether or not it is likely to lead to clinical IDDM. In other words, GAD-Abs with different specificities may differ in their prognostic significance with being more closely associated than others with the type of anti-islet autoimmunity which is most likely to precipitate overt IDDM. The diagnostic value of measuring subsets of antibodies to defined regions of GAD has been highlighted by Falorni et al.\textsuperscript{115} who found, using GAD-65/GAD-67 chimeras, that antibodies to the C-terminal region (but not to the middle region) of GAD were significantly higher in diabetic children than in non-diabetic children with anti-GAD antibodies.

As an approach to determining the occurrence of a defined subset of anti-GAD antibodies, we investigated whether serum antibodies from PE patients (with or without diabetes) could complete for binding to native GAD with the mouse monoclonal antibody GAD-6 which has been widely used in antigenic studies of GAD. The epitope of GAD-6 has previously been mapped to amino acid residues 475-585/529-585\textsuperscript{[6,11]} within the C-terminal region of GAD-6, which is a major epitopic region for anti-GAD antibodies in IDDM and SMS\textsuperscript{[14]}. In this way we defined a subset of GAD antibodies which bound to GAD within the region of the epitope for GAD-6.

Serum samples were obtained from nineteen PE patients who possessed GAD-Abs who were non-diabetic, and from twelve diabetic PE patients with GAD-Abs. The ability of these sera to inhibit GAD-6 binding to rat brain GAD was determined in a competitive direct ELISA as indicated in Fig. 3. Semi-purified rat brain GAD was coated onto wells of ELISA plates and from PE patients were applied to the wells for 2 hours. Plates were washed and GAD-6 (0.25\mu g/ml) was added to all wells for 2 hours. Following further washes, anti-mouse IgG alkaline phosphatase conjugate was added to all wells for 1 hour: this anti-mouse IgG conjugate showed negligible cross-reactivity with human antibodies. Plates were washed and p-nitrophenyl phosphate substrate was added. Optical density (OD) was read at 405 nm. The mean OD of the GAD-coated wells was corrected by subtracting the mean OD of equivalent BSA blocked in wells incubated with patient’s serum relative to GAD-6 binding alone.

This was calculated using the formula:

$$\frac{\text{mean corrected OD in wells incubated with patient serum}}{\text{mean corrected OD in wells incubated with GAD-6 alone}} \times 100$$

In order to use inhibition of GAD-6 binding as a measure of differences in the epitope specificities of the patients’ GAD-Abs, equipotent dilutions of sera showing equivalent levels of antibody binding to GAD were employed. As shown in Fig. 4, the individual PE patients’ sera varied widely in their ability to inhibit GAD-6 binding to GAD, ranging from no inhibition to almost 80% inhibition of GAD-6 binding. This indicates that PE patients differ in the proportion of their GAD-Abs which are directed to
Fig. 3. Scheme for the determination of patients' serum antibodies which competitively inhibit GAD-6 binding to GAD in a direct ELISA.

the region of the GAD-6 epitope of GAD.

Consistent with this interpretation, further studies with the non-diabetic PE patients' sera confirmed the level of GAD-6 inhibition was not simple related to either the level of serum antibody binding to GAD or the function affinities of the GAD-Abs.

There was no significant difference in the occurrence of antibodies to the region of the GAD-6 epitope between the groups of PE patients with or without diabetes. However, a greater proportion of non-diabetic PE patients had antibodies which show a high percentage inhibition of GAD-6 binding compared to PE patients with diabetes (Fig. 4). Some of the non-diabetic polyendocrine patients may still progress to diabetes and it is possible that there is differential expression of subsets of anti-GAD antibodies in progressive versus slow or non-progressive anti-islet autoimmune responses.

In summary, autoimmunity to GAD is associated with a variety of clinical conditions, and the appropriate detection of GAD-Abs can have diagnostic and/or prognostic value.
Fig. 4. Comparison of the inhibition of GAD-6 binding to GAD by PE sera from patients with or without diabetes.

Acknowledgments

This work was funded by the British Diabetic Association, the Trent Regional Health Authority Research Committee, and the Special Trustees for Nottingham University Hospitals. ML was supported by "L’Associazione per l’aiuto al giovane diabetico" (Milan, Italy) and The Autoimmune Disease Charitable Trust.

References


التطورات في كيفية تشخيص الأجسام المضادة لنازعة الكاربوكسيليز حامض الجلوتاميك

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مانويليتا لاسأًا،* وامان بوناتيز**

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المستخلص: تتكون الأجسام المضادة الذاتية ضد نازعة الكاربوكسيليز حامض الجلوتاميك في النوع الأول من مرض السكر ونزعة الذاتية للغدد الصماء، وتزامنا مع ظهور الرجل المتيس. وهي تشكل جزءًا من مجموعات الأجسام المضادة الذاتية. فقد استخدمنا الأجسام المضادة الذاتية تازعة الكاربوكسيليز للمسح الجلوتاميك (GAD) لمجربي متلازمة الرجل المتيس (س م) بمقارنة (GAD) من تجارب بين الجرذان بطرق مختلفة. ونتيجة لهذه التجارب، نستنتج أن نازعة السكر والتوترات من النظام العصبي المزمن يمكن استخدام نتائج متشابهة من (GAD) من الإنسان. إن تقنية جرذان GAD مركزية لتشخيص التورم النسيجي - بينما كانت هناك خاصية خصوصية أقل بالنسبة للإيلوزا التي أمسك فيها GAD بواسطة جسم مسكيني نازعة وحيد النسيجية. وقد أظهرت هذه الدراسة المعاشرة مع تقنية GAD، GAD، (MAB) في جرذان (GAD) نتائج متشابهة مع تقنية اصطناعية (GAD) في جرذان (GAD) في إدGirl. نزعة السكر والتوترات من النظام العصبي المزمن يمكن استخدام نتائج متشابهة من (GAD) من الإنسان. إن تقنية جرذان GAD مركزية لتشخيص التورم النسيجي - بينما كانت هناك خاصية خصوصية أقل بالنسبة للإيلوزا التي أمسك فيها GAD بواسطة جسم مسكيني نازعة وحيد النسيجية. وقد أظهرت هذه الدراسة المعاشرة مع تقنية GAD، GAD، (MAB) في جرذان (GAD) نتائج متشابهة مع تقنية اصطناعية (GAD) في جرذان (GAD) في إدGirl. ينتشر هذا التطور في نازعة السكر والتوترات من النظام العصبي المزمن يمكن استخدام نتائج متشابهة من (GAD) من الإنسان. إن تقنية جرذان GAD مركزية لتشخيص التورم النسيجي - بينما كانت هناك خاصية خصوصية أقل بالنسبة للإيلوزا التي أمسك فيها GAD بواسطة جسم مسكيني نازعة وحيد النسيجية. وقد أظهرت هذه الدراسة المعاشرة مع تقنية GAD، GAD، (MAB) في جرذان (GAD) نتائج متشابهة مع تقنية اصطناعية (GAD) في جرذان (GAD) في إدGirl.