Disease Sensitivity and Specificity of 52 Assays for Glutamic Acid Decarboxylase Antibodies

The Second International GADAb Workshop

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There is increasing interest in the use of glutamic acid decarboxylase antibodies (GADAbs) for identification of subjects at increased risk of developing insulin-dependent diabetes mellitus (IDDM). However, considerable variation exists between laboratories in the reported frequency of GADAb in various clinical situations, and disease sensitivity and specificity have not yet been compared between assays. An international workshop was held in which 101 coded freeze-dried sera, including 39 from subjects with newly diagnosed IDDM, 32 from healthy control subjects, 4 from nondiabetic subjects with Graves' disease, and 4 from islet cell antibody–positive subjects, were analyzed in 52 assays (radiobinding assay [RBA], 26; enzyme-linked immunosorbent assay [ELISA], 19; and enzymatic immunoprecipitation assay [EIP], 7). The mean sensitivity for RBAs (76.2%) was higher than for ELISAs (36.5%) and EIPs (49.9%) ($P < 0.01$). The mean specificity was similar for each assay format (RBA, 89.4%; ELISA. 89.4%; and EIP, 92.3%). The lower sensitivities of the ELISA and EIP were predominantly due to the inability of these assays to detect low levels of GADAb in IDDM. To convert results to standard units, standard curves were constructed using duplicate dilutions of the anti–glutamic acid decarboxylase monoclonal antibody MICA 3 and serum from a patient with stiff-man syndrome (SMS). Curves could be derived in 28 assays using the MICA 3 serum and in 29 using the SMS serum. The mean coefficients of variation between assays for disease and control samples were 45% when results were converted to MICA units, 77% for SMS units, and 76% for SD scores. The relatively lower scatter observed using the MICA 3 serum as a standard indicates that this serum may be useful as an international reference. Because assays for GADAb with a high level of sensitivity and specificity are now available, prospective studies are now required to establish the role of GADAb in clinical applications such as preclinical screening for IDDM. *Diabetes* 44:636–640, 1995

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CV, coefficient of variation; EIP, enzymatic immunoprecipitation assay; ELISA, enzyme-linked immunosorbent assay; GAD, glutamic acid decarboxylase; GADAb, glutamic acid decarboxylase antibody; IAA, insulin autoantibody; ICA, islet cell antibody; IDDM, insulin-dependent diabetes mellitus; RBA, radiobinding assay; rh, recombinant human; SMS, stiff-man syndrome.

Insulin-dependent diabetes mellitus (IDDM) is characterized by the presence of circulating autoantibodies, including islet cell antibodies (ICAs) (1), insulin autoantibodies (IAAs) (2), and antibodies against a 64-kDa protein (3), subsequently shown to be the enzyme glutamic acid decarboxylase (GAD) (4). Two isoforms of GAD of molecular weights 65,000 (GAD65) and 67,000 (GAD67) have been identified (5). The smaller-molecular weight form, GAD65, is the predominant form found in human islets (6) and has been shown to be the major target of antibodies in human IDDM (7). Prospective studies of first-degree relatives have shown that measurement of ICAs and IAAs allows the risk of IDDM to be quantified (8,9). The availability of purified native (10) and recombinant GAD (11) has allowed the development of assays that may be used in large-scale screening and intervention studies, and there is now considerable interest in the use of GAD antibody (GADAb) measurement for IDDM risk assessment.

A number of different assay formats have been reported for measurement of GADAb, including enzymatic immunoprecipitation assay (EIP) (4), radiobinding assay (RBA) (10–13), enzyme-linked immunosorbent assay (ELISA) (14), immunofluorescence (15,16), and Western blotting (16). The frequency of GADAb reported in patients with IDDM, using these assays, varies considerably (17). While this variation may be partly due to the age, sex, and racial composition of study groups (17–19), it is likely to reflect largely differences between the assays used. In the First International GADAb Workshop (20), 16 coded samples were examined in 34 GADAb assays. There was high between-assay concordance in the ranking of samples, but there were insufficient IDDM and control samples for meaningful comparisons of disease sensitivity and specificity of individual assays. The aims of the Second International GADAb Workshop were to compare disease sensitivity and specificity using a larger number of disease and control sera and to examine two candidate preparations for an international standard of measure.

RESEARCH DESIGN AND METHODS

Samples and questionnaire. Samples used in the workshop were provided by participating laboratories. They were stored overnight at 4°C, fibrin clots were removed, and sera were stored at −20°C. They were then divided into 200-μl aliquots in glass vials and freeze-dried. A total of 101 coded freeze-dried sera were sent to each laboratory, and samples not used as standards are summarized in Table 1. There were 32 sera from healthy control subjects and 39 from subjects with IDDM of <60 months' duration (median 0 months). Serum from 21 of the IDDM

TABLE 1
Clinical and demographic characteristics of subjects from whom sera were obtained

Group	n	Age (years)	Male/Female
Control	32	33 (21–54)	21/11
IDDM	39	23 (10–71)	20/18*
Graves' disease	4	43 (30–57)	1/3
ICA^+ relative	4	33 (17–57)	1/3

Data for age are means (range). *Sex of one subject was not known.

subjects was taken at the time of commencement of insulin therapy. Four samples were from nondiabetic subjects with Graves' disease and four were from ICA^+ nondiabetic subjects, one of whom developed IDDM 9 months after the sample was drawn. The remaining samples were standards. These consisted of duplicate dilutions of the monoclonal antibody MICA 3 (21) and plasmapheresis serum from a patient with stiff-man syndrome (SMS), non-insulin-treated diabetes, and antiparietal cell antibodies, which was used in the First International GADAb Workshop (20). Standards were used undiluted and diluted in pooled normal serum from seven healthy volunteers with no family history of IDDM. The four duplicate dilutions of the MICA 3 serum at immunoglobulin concentrations of 140, 332, 719, and 1,683 ng/ml were assigned arbitrary values of 140, 332, 719, and 1,683 MICA units, and SMS serum samples undiluted and diluted at 1:50, 1:100, 1:500, and 1:1,000 were assigned values of 1,000, 20, 10, 2, and 1 SMS unit, based on a value of 1 SMS unit for the 1:1,000 dilution. Four samples of pooled normal serum, used to dilute the standards, were also included.

Samples and a questionnaire were sent to 55 laboratories. Laboratories were requested to grade samples as positive or negative, provide results using their usual unit of measure, and give details of assay methods and antigen preparation used. Participants were sent a computer disk containing the questionnaire to minimize transcription errors and to allow rapid analysis of data. Replies were received from 45 laboratories. Seven submitted results from two assays, giving a total of 52 assays. No major problems were reported with reconstitution of samples.

Data analysis. Sensitivity was defined as the percentage of IDDM sera identified as positive and specificity as the percentage of control sera reported as negative for each laboratory. To standardize the criteria for positivity of each assay, sensitivities were also calculated after adjustment of the threshold for positivity in each sample to the level of the highest healthy control serum, thus giving each assay a specificity of 100%.

Results from laboratories were interpolated to common units using three conversions: to MICA units, to SMS units, and to SD scores. To convert results to MICA and SMS units, results for IDDM, control, Graves' disease, and ICA^+ sera were interpolated using curves of best fit derived from the MICA 3 and SMS serum dilutions, respectively, using the software package Prism (GraphPad Software). Standard curves were constructed for assays in which the mean of duplicate points on the standard curve increased with increasing antibody concentration. Values above or below the highest and lowest standards were not included in this analysis. SD scores were calculated using the mean and SD of the control sera measured in each assay. To determine which unit of measure gave the least between-assay scatter, coefficients of variation (CVs) of the interpolated results for each IDDM, control, Graves' disease, and ICA^+ serum were calculated, using only samples that could be interpolated using both the MICA 3 and SMS sera.

Results are expressed as the mean and range, and the Kruskal-Wallis test was used to compare differences between groups. CVs were compared after transformation of results to MICA units, SMS units, and SD scores, using Student's paired t test.

RESULTS

Assay methods. Descriptions of the assays used are summarized in Table 2. Native GAD was used as the antigen in 19 assays (pig brain, $n = 8$; rat brain, $n = 6$; fetal pig brain, $n = 2$; human brain, $n = 1$; rat pancreas, $n = 1$; and human insulinoma, $n = 1$) and recombinant GAD was used in 33 (human GAD65, $n = 30$; rat GAD65, $n = 2$; and rat GAD67, $n = 1$).

Three assay formats were used: RBA ($n = 26$), ELISA ($n = 19$), and EIP ($n = 7$). In the RBAs, the antigen was labeled with [^{35}S]methionine ($n = 17$) or with ^{125}I ($n = 9$). Serum was incubated with antigen and precipitated with a solid-phase precipitation reagent, or serum immunoglobulin was prebound to the solid phase and then incubated with the antigen. Bound antigen was detected by counting in a beta ^{35}S or gamma ^{125}I counter or by fluorography after separation on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. In the ELISAs, antigen was immobilized directly onto a microtiter plate or onto pins (assays 29, 30, 32, and 36) or captured onto a microtiter plate using GAD-1 monoclonal antibody (assay 42). After incubation with test serum, bound antibody was detected using an enzyme-labeled second antibody and color development. One immunodot assay using rat brain GAD (assay 41) was included under the ELISA category. In EIPs, antigen was immunoprecipitated with test serum. Bound GAD was then detected by enzymatic assay, by incubation with [^{14}C]glutamate and detection of liberated $^{14}CO_2$ by beta scintillation counting.

Assay sensitivity and specificity. Disease sensitivity and specificity are summarized in Table 2. The mean sensitivity of RBAs (76.2%) was significantly higher than that of EIPs (49.9%) and ELISAs (36.5%, $P < 0.01$). Six RBAs and one ELISA had a sensitivity >80% and specificity >90%. Of these RBAs with high sensitivity and specificity, four used ^{35}S-labeled recombinant human (rh) GAD65, one used ^{125}I-labeled rhGAD65, and one used ^{125}I-labeled immunoaffinity-purified pig brain GAD. The ELISA with high sensitivity (number 28) used biotinylated rhGAD65 and streptavidin-coated plates. The details of this method will be published elsewhere (H. Mehta, personal communication). Mean specificity was 89.4% for RBAs, 83.3% for ELISAs, and 92.3% for EIPs (NS). The assay using recombinant GAD67 (number 26) detected GADAb in 48.6% of the IDDM samples and in none of the control subjects. In the four sera from subjects with Graves' disease in the absence of IDDM, positivity was more frequently reported in ELISAs than in RBAs and EIPs (means 23.7, 3.8, and 7.1%, respectively; $P < 0.01$). This difference was accounted for by ELISAs in which nonrecombinant sources of GAD were used as antigen (mean positivity for nonrecombinant GAD 43.8% vs. 9.1% for recombinant GAD, $P < 0.005$). All four sera from ICA^+ subjects were positive in 25 of 26 RBAs, 14 of 19 ELISAs, and 4 of 7 EIPs.

Sensitivities calculated after adjustment of the threshold to give a 100% specificity are shown in Table 2. In nine of the assays, sensitivity was reduced by >50%. Sensitivity increased in 12 assays, and in one of these the increase was >50%. The mean adjusted sensitivity of RBAs was 66.7%, of ELISAs was 24.7%, and of EIPs was 40.6%.

Among the RBAs, the adjusted sensitivity was similar in those assays using recombinant protein (mean 67.0%, range 5.1–87.2) and those using native protein (mean 65.1%, range 30.8–84.6, NS). The adjusted sensitivities of RBAs using ^{35}S-labeled GAD (mean 63.6%, range 5.1–82.1) were not significantly different from those using ^{125}I-labeled GAD (mean 72.1%, range 30.8–87.2, NS).

Results of a comparison of positivity (adjusted to a specificity of 100%) of each IDDM serum are shown in Fig. 1. RBAs detected GADAb more frequently than ELISAs in all but one sample (number 4) and more frequently than EIPs in all but four samples (numbers 27, 28, 32, and 39) (Fig. 1). While the lower sensitivity of ELISAs and EIPs appears to be

TABLE 2
Assay methods, specificity, sensitivity, percentage of Graves' disease sera positive, and adjusted sensitivity

Laboratory	GAD substrate	Detection method	Specificity (%)	Sensitivity (%)	Graves' disease positive (%)	Adjusted sensitivity (%)
RBA						
1	Pig brain	^{125}I	65.6	56.4	25	30.8
2	Pig brain	^{125}I	93.7	82.1	0	74.4
3	Pig brain	^{125}I	100.0	79.5	0	84.6
4	Pig brain	^{125}I	100.0	79.5	0	79.5
5	Pig brain	^{125}I	87.5	69.2	0	56.4
6	rhGAD65	^{125}I	100.0	79.5	0	82.1
7	rhGAD65	^{125}I	84.4	87.2	0	79.5
8	rhGAD65	^{125}I	96.9	74.4	0	74.4
9	rhGAD65	^{125}I	100.0	82.1	0	87.2
10	rhGAD65	^{35}S	100.0	79.5	0	82.1
11	rhGAD65	^{35}S	75.0	76.9	0	46.2
12	rhGAD65	^{35}S	78.1	79.5	0	51.3
13	rhGAD65	^{35}S	100.0	69.2	0	76.9
14	rhGAD65	^{35}S	96.8	71.8	0	71.8
15	rhGAD65	^{35}S	81.2	79.5	0	76.9
16	rhGAD65	^{35}S	90.6	86.8	0	82.1
17	rhGAD65	^{35}S	81.2	82.1	0	69.2
18	rhGAD65	^{35}S	87.5	74.4	0	71.8
19	rhGAD65	^{35}S	84.4	84.6	25	79.5
20	rhGAD65	^{35}S	96.77	87.2	0	82.1
21	rhGAD65	^{35}S	96.9	46.2	0	—
22	rhGAD65	^{35}S	43.7	92.3	50	5.1
23	rhGAD65	^{35}S	93.7	69.2	0	10.2
24	rhGAD65	^{35}S	93.7	82.1	0	82.1
25	rratGAD65	^{35}S	96.9	82.1	0	82.1
26	rratGAD67	^{35}S	100.0	48.6	0	48.6
ELISA						
27	rratGAD65	—	62.5	48.7	25	2.6
28	rhGAD65	—	93.7	82.0	0	79.5
29	rhGAD65	—	100.0	15.4	0	30.8
30	rhGAD65	—	100.0	26.3	25	31.6
31	rhGAD65	—	84.4	20.5	25	2.6
32	rhGAD65	—	100.0	18.0	0	20.5
33	rhGAD65	—	100.0	18.4	0	23.1
34	rhGAD65	—	96.0	15.6	0	15.6
35	rhGAD65	—	96.9	23.1	0	20.5
36	rhGAD65	—	93.7	35.9	25	25.6
37	rhGAD65	—	100.0	23.1	0	28.2
38	Human insulinoma	—	43.7	53.9	50	18.0
39	Human brain	—	62.5	25.6	50	5.1
40	Rat pancreas	—	50.0	28.2	25	2.6
41	Rat brain	—	21.9	84.6	100	—
42	Rat brain	—	90.6	38.5	50	25.6
43	Pig brain	—	93.7	41.0	25	30.8
44	Pig brain	—	96.9	30.8	25	30.8
45	Pig brain	—	96.9	64.1	25	51.3
EIP						
46	rhGAD65	—	90.6	51.3	25	15.4
47	Rat brain	—	100.0	43.6	0	53.9
48	Rat brain	—	100.0	38.5	0	38.5
49	Rat brain	—	96.9	65.8	0	65.8
50	Rat brain	—	64.6	60.5	25	15.4
51	Fetal pig brain	—	100.0	33.3	0	46.2
52	Fetal pig brain	—	96.9	56.4	0	48.7

Specificity was calculated by number of control sera negative divided by number of control sera. Sensitivity was calculated by number of IDDM sera positive divided by number of IDDM sera. Adjusted sensitivity is sensitivity after adjustment of specificity to 100%; it could not be calculated for assays 21 and 41, as no quantitative results were given.

due to a relative inability of these assays to distinguish low levels of GADAb in IDDM from levels in control sera, results for occasional sera indicate additional discrepancies between assay formats. Serum 15, for example, was scored positive in 42% of ELISAs but in none of the EIPs, while serum 13 was positive in only 10.5% of ELISAs and in 57% of EIPs. Both of these sera were scored positive in 73% of RBAs.

Interpolation of results from standard curves. Standard curves were constructed using the dilutions of MICA 3 and SMS serum in assays in which the mean of duplicate points on the standard curve increased with increasing antibody concentration. Using this criterion, standard curves were able to be constructed in 28 of 52 assays with the MICA 3 serum (ELISA, 12 of 19; RBA, 13 of 26; and EIP, 3 of 7) and 29 of 52 assays with the SMS serum (ELISA, 10 of 19; RBA, 17 of 26; and EIP, 2 of 7). A prozone-like standard curve, in which there was a falloff in readout at high antibody concentration, was seen with the MICA 3 serum in 10 of 52 assays

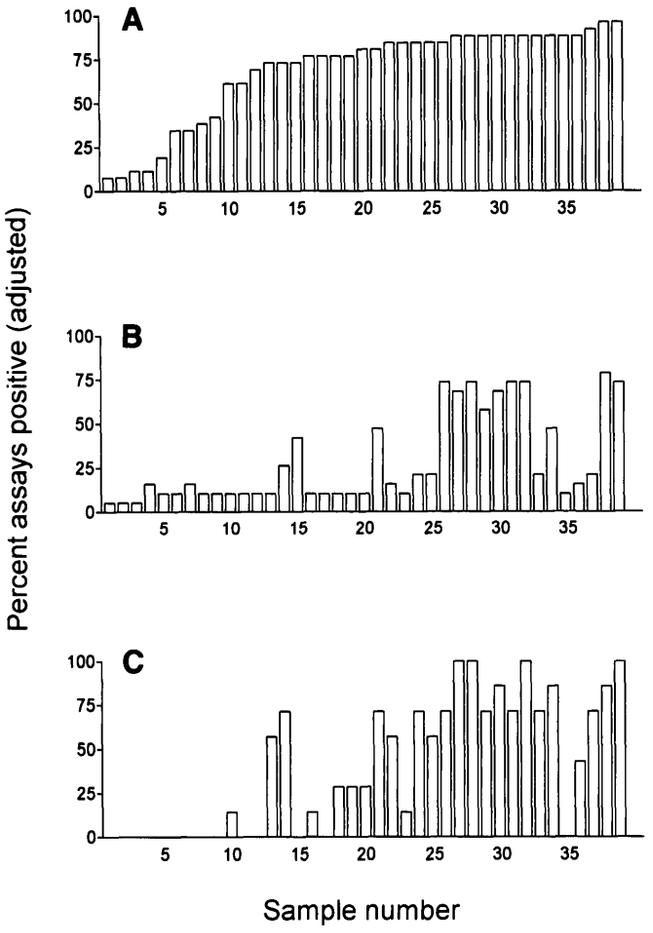

FIG. 1. Adjusted positivity of each IDDM sample (defined as GADAb level greater than highest control serum), according to assay format. Samples are ranked according to positivity in RBAs. *A:* RBA. *B:* ELISA. *C:* EIP.

(ELISA, 1 of 19; RBA, 6 of 26; and EIP, 3 of 7) and with the SMS serum in 5 of 52 assays (ELISA, 1 of 19; RBA, 1 of 26; and EIP, 3 of 7).

Results for all control, IDDM, Graves' disease, and ICA^+ samples (n = 4,108) were converted to MICA and SMS units using the respective standard sera. In assays in which standard curve readouts rose in a dose-dependent manner, converted results were within the range of the standards in 408 of 4,108 (10%) samples using the MICA 3 standards and in 1,028 of 4,108 (25%) samples using the SMS standards. CVs for each sample were calculated, and the means of the CVs were derived using the samples that fell within the range of both the MICA 3 and SMS standards (n = 311). The mean CVs for samples standardized using the MICA 3 serum, SMS serum, and SD score were 45, 77, and 76%, respectively, the mean CV for the MICA 3 serum being significantly lower than the SD score ($P < 0.01$).

DISCUSSION

In this study, a large number of disease and control sera have been used to compare disease sensitivity and specificity of GADAb assays in a blinded serum exchange. The results demonstrate that current RBAs have higher sensitivities than ELISAs (except for one assay) and EIPs and that the lower sensitivity of ELISAs and EIPs is predominantly due to an inability of most of these assays to detect low levels of GADAb in IDDM sera. Many RBAs had sensitivities >80%, which is similar to the rate of positivity in several published series in which the RBA format was used (7,10). However, sensitivities of RBAs varied considerably, and several detected GADAb in less than half of the IDDM sera. Differences in performance between RBAs could not be related to the use of native or recombinant antigen or to the mode of antigen labeling (^{125}I or ^{35}S). All but one ELISA had low sensitivity. As opposed to RBAs and EIPs, most ELISAs scored at least one of the four Graves' sera as positive. This was true for all ELISAs using nonrecombinant GAD as the antigen source. However, RBAs using nonrecombinant GAD rarely detected GADAb in these sera, suggesting that nonrecombinant GAD is more likely to give nonspecific positivity in ELISAs. GADAbs have been demonstrated in some subjects with nondiabetic autoimmune disease, including thyroid disease (17, 22).

In addition to the assay format used, the threshold for positivity had a considerable effect on the sensitivity and specificity of assays. The low incidence of IDDM in the general population and in first-degree relatives necessitates a high assay specificity to have a suitably high positive predictive value. In this workshop, however, IDDM specificity was <90% in 17 assays, 10 of which had a specificity <80%. The low specificity in these assays is unlikely to be due to sample selection or preparation because 15 assays did not report GADAb in any of the control subjects and a further 10 assays reported GADAb in only one control subject. A more likely explanation for this low specificity is an inappropriate selection of positive threshold or a change in assay performance since the assay's threshold was determined.

An important goal is the establishment and evaluation of a standard serum for international calibration of results. In this workshop, two candidate preparations were evaluated. The first was a human monoclonal GAD antibody MICA 3 and the second was serum with high levels of GADAb from a patient with SMS and diabetes which was used in the first workshop. Standard curves could be constructed for over half of the assays using dilutions of either serum, with the remainder of assays giving curves in which the readout did not increase with increasing antibody concentration. Some assays gave a prozone-like standard curve in which there was a falloff in the reading at very high antibody concentrations; quantitative measurement of GADAb in these assays requires the use of sample dilutions. This type of curve was most common in EIPs and occurred with both standard preparations. The use of the MICA 3 serum to standardize results appeared to give better concordance than use of the SMS serum. However, as the range of antibody concentrations covered by the dilutions of the two preparations differed considerably, it was not possible to directly compare their performance. Although the range of antibody concentrations covered by the standard dilutions was small and did not include low antibody levels, the mean CVs for units interpolated from MICA 3 standard curves were significantly lower than those of SD scores. Additionally, the MICA 3 serum can be produced in indefinite quantities, can be standardized for antibody concentration, and has an epitope specificity that appears to be shared by the majority of sera from patients with IDDM of recent onset (21).

In conclusion, we have shown, using a large number of IDDM and control sera, that the RBA format is more sensitive than ELISA or EIP for measurement of GADAb. Use of a monoclonal antibody as a standard serum resulted in better

between-assay concordance than conversion of results to SD scores. The availability of highly sensitive and specific assays and an international reference of measure will greatly assist large multicenter trials in establishing the role of GADAb in such applications as preclinical IDDM screening.

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APPENDIX

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