Combined Use of Autoantibodies (IA-2 Autoantibody, GAD Autoantibody, Insulin Autoantibody, Cytoplasmic Islet Cell Antibodies) in Type 1 Diabetes

Combinatorial Islet Autoantibody Workshop

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The aim of this workshop was to assess the ability of individual autoantibody (ab) assays and their use in combination to discriminate between type 1 diabetic and control sera. Coded aliquots of sera were measured in a total of 119 assays by 49 participating laboratories in 17 countries. The sera were from 51 patients with new onset type 1 diabetes and 101 healthy control subjects with no family history of diabetes. In the final analysis, data on diabetic sera were restricted to 43 subjects younger than age 30 years. The laboratories were asked to report results for these sera using their currently available anti-islet autoantibody assays. In addition, they were asked to combine information from their assays to classify sera as having high, moderate, or low probability of originating from a patient with type 1 diabetes. Actual strategies for combining assays were determined by each laboratory. There were no significant differences in sensitivity among 19 radioimmunoassays (RIAs) for IA-2 autoantibodies (cytoplasmic islet cell antibody [ICA] 512) using different constructs that included the intracellular portion of the molecule (mean sensitivity 73%). However, an enzyme-linked immunosorbent assay (ELISA) using the extracellular portion of the IA-2 molecule did not discriminate between diabetic and control sera. Among GAD autoantibody assays that achieved sensitivity >70%, 26 were RIAs and one was an ELISA. When the sera were ranked according to their autoantibody levels, the concordance for insulin autoantibodies (IAAs) in different laboratories was markedly less than for IA-2ab and GADab. Using a combination of autoantibody assays, several laboratories achieved excellent discrimination between diabetic and control sera (sensitivity up to 80% with false-positive rate of 0%). A variety of strategies for combining information from different assays were successful (e.g., those including and excluding ICA), and no one strategy emerged as clearly superior. In conclusion, IA-2/ICA512 autoantibodies are a marker of type 1 diabetes and can be measured consistently by most assays. Several different strategies for combining assays achieved high sensitivity with a low false-positive rate.
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Cytoplasmic islet cell antibodies (ICAs), measured by immunohistochemistry on sections of the pancreas, were discovered in 1974. Since that time, assays measuring antibodies directed against a series of characterized β-cell autoantigens have become available. These include autoantibodies directed against insulin, GAD, and a protein tyrosine phosphatase molecule called IA-2. Data from the screening and long-term follow-up of first-degree relatives have shown that these autoantibodies are useful in predicting the future risk of type 1 diabetes (1,2). Our aim was to evaluate the sensitivity and specificity of the autoantibody assays in an international workshop for the Immunology in Diabetes Society. Sera from subjects with and without disease were compared in blinded fashion. Previous workshops have evaluated ICA (3–7), insulin autoantibodies (IAAs) (8,9), and GADab (10,11). This was the first workshop to evaluate IA-2 autoantibodies and the use of information from multiple autoantibody assays in combination to distinguish disease from nondisease.

RESEARCH DESIGN AND METHODS

Sera. We obtained ~50 ml of serum from 51 patients with newly diagnosed type 1 diabetes (median age 13.2 years, range 5.4–44.6 years) and from 101 healthy control subjects with no family history of diabetes (median age 30.6, range 18.5–61.0 years). Sera from the diabetic patients were sampled within 7 days of the start of insulin therapy. Aliquots of each serum were lyophilized together. Coded aliquots were then distributed to 51 laboratories, 49 (from 17 countries) of which returned data (see APPENDIX for listing of participating laboratories).

Laboratories were asked to test the sera with whatever relevant assays they were currently using for the detection of prediabetes. There were 22 assays for IA-2ab, 44 for GADab, 24 for IAA, and 26 for ICA. In addition to providing results and details of the method for each assay, each laboratory was also asked to score each coded serum as having a low, moderate, or high probability of coming from a diabetic subject. This was to be done using a combination of data from the assays used in a given laboratory. For example, a laboratory measuring IA-2ab, GADab, and IAA might score sera negative for all three autoantibodies as low probability, those with a single autoantibody positive as moderate probability, and those with two or more autoantibodies positive as high probability. Laboratories were free to determine their own algorithm for combining information from multiple assays, but were asked to state the algorithm used.

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ab, autoantibody; ANOVA, one-way analysis of variance; ELISA, enzyme-linked immunosorbent assay; IAA, insulin autoantibody; ICA, cytoplasmic islet cell antibody; RIA, radioimmunoassay.

The sera from eight diabetic patients older than age 30 years (numbers 142, 18, 86, 64, 68, 114, 101, 33) were autoantibody negative in the great majority of assays and were excluded from further analysis. This was done to define a more homogeneous disease group by removing patients with possible type 2 diabetes. Of these eight sera, six were negative in all assays for IA-2ab (but all eight were negative in 50% or more of the assays), three were negative in all GADab assays (seven in 50% or more of assays), two were negative in all IAA assays (all eight in 50% or more assays), and one was negative in all ICA assays (six in 50% or more of the assays). Thus sensitivity figures in this report are for new onset diabetic patients younger than age 30 years. The median age of this group was 12.7 years (range 5.4–29.5 years).

Some laboratories could not be sent a complete set of sera because of limited availability. In allocating sera, priority was given to those laboratories measuring IA-2ab (which was not previously evaluated in a workshop) and then to those laboratories measuring the greatest number of different autoantibody specificities. Comparing the subset of sera tested in all laboratories with the subset not tested in all laboratories, there was no significant difference in the proportion of laboratories reporting each serum as positive for the autoantibodies. For example, for IAA, 22 diabetic sera were tested by all laboratories and 21 were not (after excluding the sera from patients older than age 30 years). There was no significant difference in the percentage of laboratories finding each diabetic serum positive for IAA (median 20.8% for the 22 sera tested in all laboratories vs. 17.6% for the 21 sera not tested in all labs; $P = 0.89$, Wilcoxon test). Similarly, for IA-2ab, GADab, and ICA, the figures were 90.9, 95.5, and 96%, respectively, for the subset of sera tested in all laboratories vs. 94.7, 92.3, and 82%, respectively, for the subset not tested in all laboratories ($P = 0.12$, 0.90, and 0.96, respectively).

Sensitivity, specificity, and adjusted sensitivity. Sensitivity for each assay was calculated as the percentage of new onset diabetic sera (from patients younger than age 30 years) found positive. Specificity was calculated as the percentage of healthy control sera found negative. Sensitivity and specificity tended to have an inverse relationship, with those laboratories achieving high sensitivity tending to have lower specificity (i.e., a higher false-positive rate). This made comparison of assays difficult, as both parameters needed to be taken into account.

To better compare assays, a new workshop cutoff was calculated for each assay, based on the 99th percentile of levels measured in the control group. Thus using the workshop cutoff to derive an adjusted sensitivity figure, all assays had a 99% specificity (Tables 1–4). (For GADab assays in laboratories BK, BN, CF, CK, and CL, specificity using the workshop cutoff was 100% because all control levels were zero or because there were clusters of controls with the same antibody level.) Workshop cutoffs and adjusted sensitivity figures could not be determined for the ICA assays because the data were semiquantitative, with groupings of control sera at the various antibody levels. Workshop cutoffs could not be derived for laboratory AC-2 (GAD and IA-2 assays by immunoprecipitation and SDS-PAGE) because the results were positive/negative only.

Concordance of autoantibody levels in different assays. To compare relative levels of autoantibody from different assays, the diabetic sera were ranked from highest to lowest for each assay. The rank was expressed as a percentage rank because some sera were not tested in all assays. For each serum, this percentage rank in each assay was then plotted on the y-axis of Fig. 1, with the sera arranged in order of ascending median rank along the x-axis.

Reproducibility. Among the coded samples, 10 diabetic sera and 1 control serum were included in duplicate to allow an assessment of reproducibility. Observed agreement was determined as the percentage of duplicate pairs for which there was agreement on positive/negative status (Tables 1–4). The κ statistic was also calculated. This allowed a more valid assessment because it evaluated agreement beyond chance, on a scale from 0 (indicating no agreement beyond chance) to 1 (indicating perfect agreement). However, the κ statistic could not be calculated for some laboratories when two adjacent cells of the contingency table used for its calculation were zero. Reproducibility could not be assessed for a few laboratories that did not receive a sufficient number of duplicate sera.

RESULTS

IA-2 autoantibodies. IA-2ab were measured by 20 laboratories in a total of 22 assays (Table 1). All assays used relatively small serum volumes (1.67–16 µl). Most IA-2ab assays were radioimmunoassays (RIAs), using one of three different recombinant constructs of the human IA-2 molecule as antigen. Five assays used the full-length molecule (amino acids 1–979), seven used similar short intracellular constructs (amino acids 603–979, 605–979 or 632–979), and seven used a long intracellular construct (amino acids 256–556:630–979). The intracellular domain of the expressed molecule commences at amino acid 601. There was no significant difference comparing the adjusted sensitivity achieved by RIAs using the three different types of construct ($P = 0.46$, Kruskal-Wallis one-way analysis of variance [ANOVA]). The median adjusted sensitivity was 63% (interquartile range 61–71%) for the full-length construct, 70% (interquartile range 63–74%) for the short intracellular constructs, and 66% (interquartile range 47–72%) for the long intracellular construct. For each of the three constructs, the highest adjusted sensitivity achieved was 74% (Table 1).

Using workshop cutoffs, one of the control sera (#99) was positive in 54% of IA-2ab assays. This serum was found positive by 2% of GADab assays, 4% of IAA assays, and 44% of ICA assays.

Duplicate tests were performed on 10 diabetic sera and 1 control serum. Most laboratories reported duplicates either consistently above or consistently below the 99th percentile of controls (Table 1). Reproducibility was similar comparing RIAs using the three different constructs (median κ 1.00 for all three groups; $P = 0.63$, Kruskal-Wallis one-way ANOVA). The assay with the lowest adjusted sensitivity (laboratory AX) had poor reproducibility. Discrepant duplicates in other assays were confined to diabetic sera with relatively low-level antibodies or the control sample, suggesting difficulty in reproducing low/negative results.

Two enzyme-linked immunosorbent assays (ELISAs) came from the same laboratory. Assay CD-2 used an extracellular construct (amino acids 389–576) and could not distinguish between cases and controls. The other, CD-1, used an intracellular construct (amino acids 643–979) and achieved an adjusted sensitivity of 38%.

One laboratory (AC) measured antibodies to the 37-kD antigen (a fragment of the IA-2 molecule) by immunoprecipitation of radiolabeled native antigen and SDS gel electrophoresis. Using the in-house cutoff, sensitivity was 79% and specificity was 97%. Adjusted sensitivity could not be validly calculated for this assay as the results were positive/negative only.

When the sera were ranked according to their IA-2ab levels (as described in METHODS), the rank order of samples was similar for most assays. The RIAs, albeit using three different constructs, appeared to consistently distinguish samples as having high, moderate, or normal autoantibody levels. Most of the discordant antibody levels in Fig. 1 are accounted for by one of the full-length construct RIAs and one ELISA (643–979 construct). The rank order of samples in the ELISA using the intracellular construct was markedly different from that of RIAs for several samples, suggesting that differences in assay format may yield quantitatively different results. The ELISA using the extracellular construct (amino acids 389–576) was excluded from Fig. 1.

GAD autoantibodies. Sera were tested for GADab in 44 assays performed in 41 laboratories. The most common type of assay was the RIA with recombinant human GAD ($n = 33$, Table 2). Adjusted sensitivity for this group was 0–91% (median 81%) and 21 of 33 of these assays had 100% agreement (κ 1.00) in the assignment of duplicate samples as positive or negative. There were also three RIA assays with native porcine GAD (with adjusted sensitivity of 74–88%), two ELISAs, two assays using immunoprecipitation and ELISA, one RIA with recombinant rat GAD, one immunoradiometric assay, one immunoprecipitation and SDS gel electrophoresis,

TABLE 1
Assays for IA-2 autoantibodies

Lab	IA-2 construct (amino acids)	Serum volume (µl)	Number of sera tested: Diabetic (of 43)	Number of sera tested: Control (of 101)	In-house cutoff: Sensitivity (%)	In-house cutoff: Specificity (%)	In-house cutoff: Reproducibility Agreement (%)	In-house cutoff: Reproducibility κ	Workshop cutoff: Adjusted sensitivity (%)	Workshop cutoff: Specificity (%)	Workshop cutoff: Reproducibility Agreement (%)	Workshop cutoff: Reproducibility κ
RIA using full length IA-2 (n = 5)												
BR	1–979	2	43	101	67	99	100	1.00	74	99	100	1.00
AH-1	1–979	5	43	101	70	98	100	1.00	67	99	100	1.00
BI	1–979	16	30	101	80	72	100		63	99	100	1.00
CA	1–979	6	42	101	74	96	100	1.00	62	99	91	0.74
BN	1–979	2.5	43	101	74	93	100	1.00	60	99	91	0.74
RIA using long intracellular construct (n = 7)												
AR	256–556:630–979	5	43	101	74	100	100	1.00	74	99	100	1.00
AD	256–556:630–979	7	43	101	72	98	91	0.62	72	99	100	1.00
AQ	256–556:630–979	2	36	101	69	98	100	1.00	69	99	100	1.00
AK	256–556:630–979	5	35	100	71	95	100	1.00	66	99	100	1.00
AT	256–556:630–979	6	33	101	73	98	100	1.00	64	99	100	1.00
BE	256–556:630–979	4	43	101	63	85	100	1.00	47	99	91	0.81
AX	256–556:630–979	7	33	101	79	88	100	1.00	18	99	64	—
RIA using short intracellular construct (n = 7)												
AF	603–979	4	43	101	81	94	100	1.00	74	99	100	1.00
BV	603–979	5	43	101	84	96	100	1.00	74	99	100	1.00
AL	603–979	5	43	100	81	97	100	1.00	72	99	100	1.00
AI	603–979	2	43	101	79	95	91		70	99	91	0.62
AH-2	605–979	5	43	101	70	99	100	1.00	70	99	100	1.00
AY	603–979	5	30	99	70	92	91	0.62	63	99	100	1.00
AV	632–979	8	43	101	65	98	100	1.00	60	99	91	0.62
ELISA (n = 2)												
CD-1	643–979 (intracellular)	1.67	37	101	54	96	100	1.00	38	99	100	1.00
CD-2	389–576 (extracellular)	1.67	37	101	5	95	100	1.00	5	99	100	1.00
IP and SDS-PAGE (n = 1)												
AC	Native human 37-kD antigen	5	43	100	79	97	100	1.00	—	—	—	—

n = 22. For each autoantibody, the results are grouped according to assay type and/or the specific antigen used, with assays of each type ordered according to the adjusted sensitivity achieved. Adjusted sensitivity was calculated using a workshop cutoff determined from the 99th percentile of levels in the control sera. Diabetic sera from subjects older than age 30 years were excluded from analysis. Reproducibility was assessed on 11 sera included in duplicate, using observed agreement (positive/negative) and the κ statistic (as described in METHODS). Note the intracellular region commences at amino acid 601. IP, immunoprecipitation.

and one assay using enzymatic immunoprecipitation with native fetal pig GAD (Table 2). Of note, the ELISA using recombinant human GAD (laboratory BU) did well in comparison with the RIAs, achieving adjusted sensitivity of 77% and agreement of 100% for the assignment of duplicates as positive or negative (κ 1.00). Figure 1 shows the concordance of GADab levels in the diabetic sera measured in the different laboratories, revealing good concordance compared with the IA-2ab and IAA assays. GADab assays using miscellaneous methods (laboratories CE, CF, CK, BK, CH-2, AC-2, and AN) were excluded from Fig. 1 for clarity.

Insulin autoantibodies (IAA). Data for IAA were returned by 24 laboratories. The majority of laboratories used RIAs with unlabelled insulin competition and polyethylene glycol precipitation, either with or without a preceding charcoal extraction step to remove endogenous insulin from the sample. There was no significant difference in adjusted sensitivity (P = 0.87, Wilcoxon test) or reproducibility assessed with the κ statistic (P = 0.11) between the charcoal versus no charcoal groups. Two assays used other techniques, one an RIA with precipitation by protein A, the other an ELISA.

Table 3 shows the results for individual assays grouped according to assay type. Adjusted sensitivity was 0–50%. There was an association between adjusted sensitivity and the volume of serum used (r = 0.63, P < 0.001, Spearman rank cor-

TABLE 2
Assays for GAD autoantibodies

Lab	Serum volume (μl)	Number of sera tested: Diabetic (of 43)	Number of sera tested: Control (of 101)	In-house cutoff: Sensitivity (%)	In-house cutoff: Specificity (%)	In-house cutoff: Reproducibility Agreement (%)	In-house cutoff: Reproducibility κ	Workshop cutoff: Adjusted sensitivity (%)	Workshop cutoff: Specificity (%)	Workshop cutoff: Reproducibility Agreement (%)	Workshop cutoff: Reproducibility κ
RIA with recombinant human GAD (n = 33)											
AF	4	43	101	86	99	100	1.00	91	99	91	0.62
AX	7	33	101	73	100	91	0.79	88	99	100	1.00
AZ	2	31	101	87	98	100	1.00	87	99	100	1.00
BX	7	31	101	87	99	100	1.00	87	99	100	1.00
BI	16	30	101	87	98	100	1.00	87	99	100	1.00
AH	5	43	101	88	98	100	1.00	86	99	100	1.00
CH-1	2.5	43	101	86	99	100	1.00	86	99	100	1.00
AI	2	43	101	79	100	91	0.79	86	99	91	0.79
AT	6	33	101	85	100	100	1.00	85	99	100	1.00
AU	7	33	101	91	92	91	0.74	85	99	100	1.00
CN	20	39	101	87	98	100	1.00	85	99	100	1.00
AD	7	43	101	86	96	100	1.00	84	99	100	1.00
AL	5	43	100	88	97	100	1.00	84	99	100	1.00
AR	5	43	101	84	99	100	1.00	84	99	100	1.00
AB	6	36	101	64	100	100	1.00	83	99	91	0.74
BN	2.5	43	101	81	100	100	1.00	81	100	100	1.00
BV	5	43	101	79	100	100	1.00	81	99	100	1.00
AK	5	35	101	80	100	100	1.00	80	99	100	1.00
AY	5	30	101	87	89	91	0.74	80	99	100	1.00
CL	5	27	99	78	100	100	1.00	78	100	100	1.00
BD	?	31	100	81	95	90	0.74	77	99	90	0.78
AC-1	5	43	100	86	96	91	0.74	77	99	91	0.79
BB	8	33	101	79	97	100	1.00	76	99	100	1.00
CA	6	42	101	76	96	91	0.79	74	99	100	1.00
BF	2	35	101	86	87	100	1.00	66	99	100	1.00
BH-1	20	34	101	68	98	100	1.00	62	99	91	0.81
BE	4	43	101	65	97	100	1.00	58	99	91	0.81
CM	?	28	100	46	99	100	1.00	54	99	86	0.59
AP	6	35	101	63	93	73	0.42	43	99	82	0.63
BQ	6	31	101	81	81	100	1.00	42	99	100	1.00
AQ	2	36	101	78	92	82	0.42	42	99	64	0.35
AV	8	43	101	77	96	100	1.00	37	99	91	0.81
CB	7	33	97	61	95	82	0.62	0	99	100	—
RIA with native porcine GAD (n = 3)											
BR	25	43	101	86	100	100	1.00	88	99	100	1.00
BC	50	38	101	82	94	100	1.00	74	99	100	1.00
BH-2	20	34	101	65	100	100	1.00	74	99	100	1.00
ELISA (n = 2)											
BU (recombinant human)	50	43	101	70	100	100	1.00	77	99	100	1.00
CE (unspecified)	10	30	101	73	98	100	1.00	47	99	90	0.78
IP and ELISA with recombinant human GAD (n = 2)											
CF	45	29	100	66	100	100	1.00	66	100	100	1.00
CK	30	26	32	77	78	—	—	42	100	—	—
Miscellaneous methods (with recombinant human GAD unless specified otherwise; n = 4)											
BK (RIA; recombinant rat)	60	30	101	73	100	100	1.00	73	100	100	1.00
CH-2 (IRMA)	5	43	101	67	100	91	0.79	67	99	91	0.79
AC-2 (IP and SDS-PAGE)	5	43	100	88	98	91	0.74	—	—	—	—
AN (enzymatic IP; native fetal pig)	30	43	100	28	100	91	0.79	37	99	73	0.48

n = 44. For each autoantibody, the results are grouped according to assay type and/or the specific antigen used, with assays of each type ordered according to the adjusted sensitivity achieved. Adjusted sensitivity was calculated using a workshop cutoff determined from the 99th percentile of levels in the control sera. Diabetic sera from subjects older than age 30 years were excluded from analysis. Reproducibility was assessed on 11 sera included in duplicate, using observed agreement (positive/negative) and the κ statistic (as described in METHODS). IP, immunoprecipitation; IRMA, immunoradiometric assay.

TABLE 3
Assays for insulin autoantibodies

Lab	Serum volume (µl)	Number of sera tested: Diabetic (of 43)	Number of sera tested: Control (of 101)	In-house cut off: Sensitivity (%)	In-house cut off: Specificity (%)	In-house cut off: Reproducibility: Agreement (%)	In-house cut off: Reproducibility: κ	Workshop cut off: Adjusted sensitivity (%)	Workshop cut off: Specificity (%)	Workshop cut off: Reproducibility: Agreement (%)	Workshop cut off: Reproducibility: κ
RIAs without charcoal extraction step (n = 14)											
AK	600	32	101	44	100	82	0.63	50	99	82	0.61
AQ	600	34	100	44	100	82	0.63	50	99	82	0.61
AR	600	43	101	58	98	73	0.44	49	99	82	0.63
AX	600	27	100	59	91	100	1.00	37	99	100	1.00
AZ	100	31	101	36	93	82	0.63	32	99	91	0.81
AT	300	33	101	30	99	91	0.79	30	99	91	0.79
CA	450	40	99	30	98	91	0.79	30	99	91	0.79
AS	300	35	100	49	97	100	1.00	29	99	100	1.00
BH	200	34	101	38	96	91	0.81	24	99	91	0.79
AN	600	37	100	30	93	91	0.74	22	99	100	1.00
CN	100	39	101	23	98	91	0.74	21	99	100	1.00
AE	300	30	101	30	93	100	1.00	17	99	100	1.00
AP	300	35	101	17	89	82	0.42	6	99	100	—
AB	300	36	98	33	86	91	0.79	0	99	100	—
RIAs with charcoal extraction step (n = 8)											
AU	200	33	101	36	98	91	0.81	36	99	91	0.81
CK	200	27	100	15	100	100	—	26	99	71	0.30
AV	160	43	101	37	98	91	0.79	21	99	100	1.00
BF	100	35	100	17	99	100	1.00	20	99	91	0.74
BB	80	33	101	52	78	82	0.63	18	99	100	1.00
CB	100	30	100	17	99	—	—	17	99	—	—
CM	80	28	100	7	94	100	—	7	99	100	—
BY	20	30	101	7	95	90	—	7	99	100	—
Miscellaneous assays (n = 2)											
AH (protein A)	10	43	101	23	96	82	0.56	14	99	100	1.00
CG (ELISA)	10	28	101	36	60	57	0.16	4	99	100	—

n = 24. Except for the two miscellaneous assays, all RIAs used unlabeled insulin competition, plus precipitation with polyethylene glycol. For each autoantibody, the results are grouped according to assay type and/or the specific antigen used, with assays of each type ordered according to the adjusted sensitivity achieved. Adjusted sensitivity was calculated using a workshop cutoff determined from the 99th percentile of levels in the control sera. Diabetic sera from subjects older than age 30 years were excluded from analysis. Reproducibility was assessed on 11 sera included in duplicate, using observed agreement (positive/negative) and the κ statistic (as described in METHODS).

relation; graph not shown). The three assays with highest sensitivity were laboratories AK, AQ, and AR, all of which were similar RIAs using a large volume of serum (600 µl) and no charcoal extraction step. There was no significant association between reproducibility (assessed with the κ statistic) and serum volume (r = 0.32, P = 0.20).

When the diabetic sera were ranked according to IAA level in each assay (see METHODS), there was wide variability in the levels obtained in different assays. Even allowing for the fact that more of the diabetic sera were negative for IAA (Fig. 1*C*, left half) than for other autoantibodies, the IAA assays showed markedly less concordance than the IA-2ab and GADab assays.

One of the control sera (#105) from a subject age 44.5 years was found to be positive for IAA by 78.3% of laboratories, including four of five assays with the highest adjusted sensitivity (laboratories AR, AX, AU, AZ, all with polyethylene glycol precipitation) and by the assay with protein A precipitation (AH), in which levels were 2–10 times the assay cutoff. This serum was negative in all GADab and IA-2ab assays and positive in only 4% of ICA assays.

ICAs. A total of 26 ICA assays were involved in the workshop. The results for one laboratory (BC) could not be evaluated because ICAs were measured only on samples positive for GADab as part of the combined antibody strategy in that laboratory.

Among the 101 control sera, 28 were reported as positive by more than 10% of the laboratories. Four control sera were reported as positive by >33% of laboratories (#19 by 48%, #134 by 44%, #95 by 44%, and #93 by 79%, with the latter reported to show a mitochondrial staining pattern by laboratory AH).

In all, 21 assays used immunofluorescence on human pancreas, 2 used immunoperoxidase on human pancreas (laboratories BH and AD), 1 used immunofluorescence on monkey pancreas (laboratory CG), and 1 did not specify the method used (laboratory CM). The assay using monkey pancreas had the second lowest specificity. The in-house cutoffs used varied from 1.25 to 12 Juvenile Diabetes Foundation (JDF) U. Using in-house cutoffs, the median sensitivity was 81% (range 44–100%). The median specificity was 96% (range 64–100%). Some laboratories with high sensitivity achieved this only at the

TABLE 4
Assays for ICAs

Lab	Serum volume (μl)	Threshold for positivity (JDF U)	Number of sera tested		In-house cutoff			
			Diabetic (of 43)	Control (of 101)	Sensitivity (%)	Specificity (%)	Reproducibility Agreement (%)	κ
Immunofluorescence on human pancreas (n = 21)								
BU	50	≥10	43	101	74	100	91	0.79
BF	?	≥4	34	100	50	100	91	0.81
BQ	40	≥5	32	101	44	99	90	0.80
AI	25	≥5	43	101	88	98	91	0.62
AH	20	≥5	43	100	86	98	100	1.00
AB	25	≥5	35	101	83	98	100	1.00
AU	20	≥12	33	101	73	98	91	0.74
AV	200	≥10	43	101	63	98	91	0.74
AS	30	≥10	35	101	91	97	91	0.62
AP	20	≥10	35	101	66	97	91	0.74
CN	10	≥5	39	101	85	96	91	0.62
AN	?	≥5	42	101	81	96	82	0.56
CA	25	≥5	42	101	86	95	73	0.38
BX	25	≥2.5	31	101	81	94	91	0.62
AK	60	≥10	35	101	77	90	82	0.39
AE	50	≥5	30	101	70	88	80	0.52
BD	50	≥6	31	100	100	88	80	—
AF	40	≥5	43	100	81	86	82	—
CB	30	≥5	31	97	52	78	40	0.00
CK	100	≥1.25	27	100	100	73	86	—
BB	60	≥2.5	33	101	91	64	100	—
Immunoperoxidase on human pancreas (n = 2)								
BH	300	≥5	31	101	74	99	73	0.42
AD	4	≥5	43	101	65	98	100	1.00
Immunofluorescence on monkey pancreas (n = 1)								
CG	50	≥5	28	101	89	72	100	—
Method not stated (n = 1)								
CM	?	≥2	28	100	54	95	100	1.00

n = 25. Adjusted sensitivity figures could not be calculated for ICA (see text). JDF, Juvenile Diabetes Foundation. For each autoantibody, the results are grouped according to assay type, with assays of each type ordered according to the specificity achieved. Diabetic sera from subjects older than age 30 years were excluded from analysis. Reproducibility was assessed on 11 sera included in duplicate, using observed agreement (positive/negative) and the κ statistic (as described in METHODS).

expense of low specificity, with many controls reported with high levels of ICA. Adjusted sensitivity figures could not be determined for ICA because the results were semiquantitative, reported on a discontinuous scale in most laboratories, often with no final end point titer determined. Reproducibility appeared to be a problem with borderline positive sera. The median κ value was 0.68 (range 0–1.00) (Table 4). This compares with median κ values of 1.00 (range 0.62–1.00) for IA-2ab assays, 1.00 (range 0.35–1.00) for GADab assays, and 0.81 (range 0.30–1.00) for IAA assays.

Combinations of assays. Laboratories were asked to score each serum as having low, moderate, or high probability of having originated from a new onset diabetic patient. This can be translated into a high-sensitivity strategy (low probability = negative, moderate, or high probability = positive) and a high-specificity strategy (low or moderate probability = negative, high probability = positive).

A total of 37 laboratories participated in the combined antibody component of the workshop, using 72 strategies based on analysis of one (5 strategies), two (26 strategies), three (31 strategies), or four (4 strategies) antibody markers. The antibody combination used by three laboratories was not specified. All strategies were necessarily applied using the in-house cutoffs for individual assays.

Several laboratories achieved specificity of 100% while maintaining useful sensitivity. For example, laboratory AI measured ICA, GADab, and IA-2ab. Sera with one or no antibody positive were considered low risk, whereas those with two or more antibodies positive were considered high risk. With the sera included in the workshop, this strategy achieved a sensitivity of 80% and a specificity of 100%. Laboratory AR measured GADab, IA-2ab, and IAA; sera with two or more antibodies positive were scored as high risk. This strategy achieved a sensitivity of 65% and a specificity of 100%.

Neither sensitivity nor specificity were significantly related to the number of autoantibodies included in the strategy. Combining high-sensitivity and high-specificity strategies for analysis, the proportion of strategies achieving an arbitrary target of

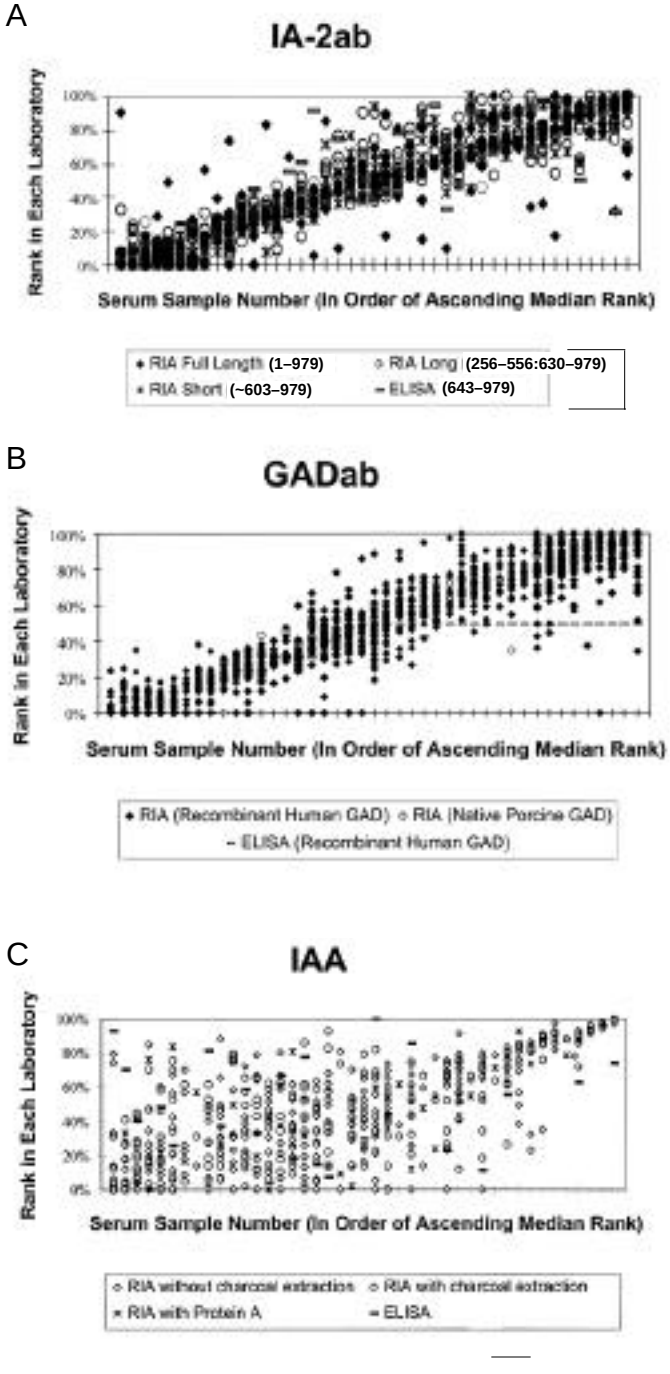

FIG. 1. The concordance of autoantibody levels in diabetic sera in different laboratories: IA-2ab (*A*), GADab (*B*), and IAA (*C*). For each diabetic serum, the rank order of the antibody level in each laboratory is plotted on the *y*-axis (expressed as a percentage rank). The sera are ordered along the *x*-axis from lowest to highest autoantibody level (determined using the median of the ranks in the assays for each serum). For IA-2ab, assay AC was excluded as the levels were semiquantitative; assay CD-2 (extracellular portion of the molecule) was also excluded as it did not discriminate between diabetic and control sera. GADab assays using miscellaneous methods (CE, CF, CK, BK, CH-2, AC-2, and AN) were excluded for clarity.

≥60% sensitivity with ≥95% specificity was 50% using four markers, 39% using three markers, 38% using two markers, and 40% using only one marker ($P = 0.98$). Sensitivity ≥70% sensitivity and specificity ≥98% was achieved by 19% of strategies using two markers and 13% of strategies using three markers, and none of the strategies using one or four markers ($P = 0.55$).

Table 5 shows an analysis of the use of all possible combinations of IA-2ab, GADab, IAA, and ICA. For each of IA-2ab, GADab, and ICA, data from the assay with the highest adjusted sensitivity was used (laboratories AR, AF, and BU, respectively). For IAA, laboratories AK, AQ, and AR had adjusted sensitivity figures of 50, 50, and 49%, respectively. Laboratory AR was selected because neither AK nor AQ had tested all diabetic sera. As shown in the table, many combinations achieved useful results.

DISCUSSION

This is the first workshop to evaluate IA-2ab assays and the first to evaluate the use of assays in combination. Workshop participants were blinded to the case and control status of the coded sera. Laboratories used their previously established in-house cutoffs to score each serum as positive or negative. Low cutoffs yield high sensitivity (i.e., high proportion of individuals with disease found positive) at the expense of low specificity (high false-positive rate). Increasing the cutoff for a given assay lowers sensitivity but at the same time improves specificity. This reciprocal relationship between sensitivity and specificity makes the comparison of assays difficult. We therefore calculated new workshop cutoffs for each assay, based on the 99th percentile of levels in the controls, and applied these to determine adjusted sensitivity for each assay, as in a previous GADab workshop (10). This approach allowed us to compare the sensitivity of different assays with specificity held constant at 99%. Using their in-house cutoffs, many laboratories achieved specificities considerably less than 99%. Because the prevalence of type 1 diabetes is ~0.3%, it is likely that many laboratories detect a relatively large number of false positives using their in-house cutoffs, too many to allow screening of the general population for at-risk individuals.

The results of the workshop confirmed that IA-2 autoantibodies are a marker of type 1 diabetes and can be measured consistently by most laboratories. No significant differences in the ability to discriminate between disease and nondisease were found between RIAs using the full-length molecule and partial constructs, including the COOH-terminal intracellular portion of the molecule. However, one assay using only the extracellular portion of the molecule could not distinguish diabetic cases from controls. This is in agreement with previous reports showing that diabetes-associated IA-2 antibodies are directed against the intracellular region of the protein (12). Samples were ranked consistently by many assays, although the ELISAs and the immunoprecipitation assay on native 37-kD antigen gave quantitatively different results for some samples. The similarity of results from the RIAs suggests that the introduction of a reference standard is likely to improve the comparability of results from assays using similar formats.

GADab was the most frequently measured antibody in the workshop, being measured in 44 assays. The majority of assays performed well, and the most common format was a RIA similar to that for IA-2ab. When GADab levels for the sera were ranked in each laboratory, the concordance of GADab levels was greater than for IA-2ab and markedly greater than for IAA.

IAA assays using high volumes of serum (600 µl) were more likely to have high sensitivity. However, five of the assays used 600 µl of serum with the same assay protocol, and it is possible that some other aspect of this protocol may be

TABLE 5
Combination of assays: the sensitivity and specificity achieved using different combinations of IA-2ab, GADab, IAA, and ICA

	High-sensitivity strategy (≥1 antibody positive)		High-specificity strategy (≥2 antibody positive)	
	Sensitivity	Specificity	Sensitivity	Specificity
Single assay				
IA-2 (lab AR)	74	99	—	—
GAD (lab AF)	91	99	—	—
IAA (lab AR)	49	99	—	—
ICA (lab BU)	74	100	—	—
Two assays				
IA-2, GAD	98	99	67	100
IA-2, IAA	77	99	47	100
IA-2, ICA	88	99	60	100
GAD, IAA	93	98	47	100
GAD, ICA	98	99	67	100
IAA, ICA	79	99	44	100
Three assays				
IA-2, GAD, IAA	98	98	72	100
IA-2, GAD, ICA	98	99	88	100
IA-2, IAA, ICA	88	99	67	100
GAD, IAA, ICA	98	98	74	100
Four assays				
IA-2, GAD, IAA, ICA	98	98	88	100

Data are %. For each autoantibody, one laboratory that performed well and tested all diabetic and control sera was selected. For each combination of assays, both high sensitivity (one or more autoantibody positive) and high specificity (two or more autoantibodies positive) strategies were evaluated. Sensitivity and specificity for individual assays are shown for comparison. Workshop cutoffs (derived from the 99th percentile of levels in control sera; see METHODS) were used.

responsible for the higher sensitivity apart from serum volume. A high-sensitivity method that requires only 30 µl of serum and uses protein A rather than polyethylene glycol precipitation was recently reported by Williams et al. (13). This assay was developed after the workshop was held, but testing the workshop sera for comparison with assays participating in the workshop gave an adjusted sensitivity of 60% (specificity 99%). Most of the assays in the workshop used polyethylene glycol precipitation, and there was no apparent difference between assays with or without a charcoal extraction step. The control serum that was positive in the majority of IAA assays may have been a true positive, given that it was also positive in the protein A assay (laboratory AH) and in the Williams et al. protein A assay (13). This is consistent with the expectation that a small proportion of the general population will be positive for autoantibodies. There was a wide variability in the relative ranking of IAA levels for individual sera in different laboratories. This was less marked for sera with very high levels of IAA, emphasizing the need to include sera with low levels of IAA in future workshops. Differences in the literature on the frequency of IAA may relate to differing assay sensitivity.

Although further analysis with larger numbers of subjects is needed, the combined antibody results revealed that a variety of strategies can result in useful discrimination between diabetic and control sera. No one strategy emerged as superior. Combining data from one assay of each type (IA-2ab, GADab, IAA, and ICA), as shown in Table 5, suggests several strategies for economical, rapid screening of large numbers of samples.

The results of the workshop need to be interpreted with some caution as there are several potential sources of bias. First, the analysis was restricted to diabetic patients under age 30 years to achieve a more homogeneous disease group. Sera from patients over age 30 years were negative in the majority of assays, suggesting either that the clinical diagnosis of type 1 diabetes was incorrect or that the frequency of autoantibodies is much lower in older patients. The clinical differentiation of type 1 (autoimmune) and type 2 diabetes in adults may be difficult, and at least one of these patients is known to have had the initial clinical diagnosis revised to type 2 diabetes by the treating physician before antibody results were known. Second, the control subjects were older than the cases. Third, although the diabetic subjects were randomly selected, young children may have been underrepresented because large sample volumes are less likely to be available. In particular, this may have affected the results for IAAs, which are more frequent in young children with type 1 diabetes than in older patients (14). Fourth, comparison of individual assays was limited by the relatively small number of sera tested (although larger than in most previous workshops). Sensitivity and specificity estimates based on 43 diabetic and 101 control sera have relatively large 95% confidence intervals (CIs). For example, the highest adjusted sensitivity achieved for IA-2ab in this data set, namely 74%, had a 95% CI of 61–88%, and the highest adjusted sensitivity achieved for GADab, 91%, had a 95% CI of 82–99%. Similarly, the data on reproducibility must be interpreted with caution as only a small number of samples could be tested in duplicate. The analysis therefore may have low power to determine differences among performances unless they are markedly different. Finally, the workshop could determine sensitivity (the probability of a positive test given the presence of disease), but it could not directly estab-

lish positive predictive value (the probability of disease given a positive test) for the autoantibody markers and their combinations. The latter requires the long-term follow-up of autoantibody positive individuals to determine their risk for developing diabetes (1,2). Large volumes (50 ml) of sera collected on individuals who were subsequently followed for the development of diabetes were not available for inclusion in the workshop.

Nevertheless the results obtained are encouraging for diabetes prediction and for future recruitment into intervention trials. Intervention trials are currently underway in at-risk relatives, and large-scale screening of the general population may become a priority in the near future if an effective intervention is found.

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APPENDIX

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Author Queries (please see Q in margin and underlined text)

Q1: In text and tables, you refer to the 49 labs with a 2-letter abbreviation. However, there is no way to know which abbreviation goes with which lab. Can you please mark in the Appendix which abbreviations go with which lab? If abbreviations do not correspond with that in the Appendix, provide a separate listing explaining the abbreviations. Thanks.

Q2: Correct that you mean radioimmunoassays throughout the text? OK to proceed and change radioassay to radioimmunoassay (RIA)? Please advise.
Q3: Please spell out IA.
Q4: Compare 26 ICA assays here with 25 listed in Table 4.
Q5: Table 4: Second paragraph of METHODS (MS p. 4) states there were 26 assays for ICA. Please confirm which number is correct.
Q6: Table 5: Under column heading "High-sensitivity strategy," should "≥ 1 antibody positive" be "≤ 1 antibody positive"?
Q7: At end of sentence beginning "Third," addition of "for children" as meant?
Q8: Ref. 3: Is this an abstract, letter/editorial, or one-page article? If not, please give full page range.
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