Progress Toward Standardization of Cytoplasmic Islet Cell–Antibody Assay

HELGA GLEICHMANN AND GIAN FRANCO BOTTAZZO

SUMMARY

Cytoplasmic islet cell autoantibodies (ICAs) of 13 coded sera were determined by 26 laboratories. ICAs were determined by 24 laboratories according to a standard protocol based on the conventional indirect-immunofluorescence technique on cryostat sections of human pancreas. In addition, these 24 laboratories had performed any of 8 modifications of the assay. Test samples were titrated to end point, and the results obtained with the different methods were compared with those obtained by the standard protocol. The remaining 2 laboratories used either exclusively pancreatic sections of monkey instead of human as substrate (lab 23) or applied immunohistochemical staining (lab 22).

By following the standard protocol, interlaboratory concordance was >90% for the presence or absence of islet cell antibodies in 7 of the 13 samples circulated. However, a wide variability of titers was recorded, ranging from negative to 128.

Of the modifications, prolonged incubation in the presence of aprotinin was performed by 10 laboratories and was found to improve the sensitivity in 57 of 70 (81%) determinations with samples that had been ICA positive by the standard protocol. Improved sensitivity was also noted by 2 laboratories with sections of monkey pancreas. Acetone-fixed sections, used by 6 laboratories, or a two-color immunofluorescence method, applied by 3 laboratories, did not change the titers in 27 of 35 (77%) and 14 of 25 (56%) determinations with samples that had been ICA positive by the standard protocol. In contrast, heat inactivation of the samples before testing, performed by 5 laboratories, resulted in a decrease in titers in 25 of 39 (64%) determinations. Four laboratories used fluorescein-labeled protein A as reagent and observed a decrease in ICA titers in 22 of 27 (81%) determinations. Complement-fixing antibodies were determined by 20 laboratories and were present in 66 of 136 (48%) determinations with ICA-positive samples, and in general, they were associated with high titers of the latter. Three laboratories reported results obtained by immunohistochemical staining with glucose oxidase, and at this stage, the data indicated the requirement of both method improvement and exchange of reference reagents for interlaboratory comparison. *Diabetes* 36:578–84, 1987

From the Diabetes-Research-Institute at the University of Düsseldorf, Düsseldorf, Federal Republic of Germany, and the Middlesex Hospital, Department of Immunology, Arthur Stanley House, London, United Kingdom.
Address correspondence and reprint requests to Dr. Helga Gleichmann, Diabetes-Research-Institute, Auf'm Hennekamp 65, D-4000 Düsseldorf 1, FRG.
Received for publication 15 April 1986 and accepted in revised form 24 November 1986.

Since the first description (1) of cytoplasmic islet cell autoantibodies (ICAs) in type I (insulin-dependent) diabetic patients, these humoral markers have been increasingly applied in clinical investigations. Thus, ICA determinations have been used to classify diabetes (2,3) as a risk factor of forthcoming insulin dependency in predisposed individuals (4–6) and as a possible correlate of impaired β-cell function (7–9), and to evaluate the therapeutic effects of immunosuppressive drugs (9,10).

Various methodic modifications have been developed (11–15) to improve the conventional indirect immunofluorescence test as originally described (1). However, until now, standardization criteria have not been attempted with any of the proposed assays. Therefore, an international workshop on the standardization of these antibodies was held recently (16). The results of the workshop demonstrate that exchange of reference sera should eventually permit interlaboratory comparison of ICA determinations (16). Here we present the complete data of ICA determinations with 13 coded samples. ICAs were determined by 24 laboratories according to a standard method and any of 8 modifications and by 2 laboratories with either immunohistochemical staining or sections of monkey pancreas only.

MATERIALS AND METHODS

Test samples. Thirteen coded plasma or serum samples were sent to 26 laboratories in 14 countries for analysis.

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TABLE 1
Islet cell–antibody (ICA) end-point titer in field trial with 13 samples assayed by standard protocol

Laboratory code number	Sample 1	2	3	4	5	6	7	8	9	10	11	12	13
1	8	–	4	2	8	2	16	2	–	4	32	32	4
2	2	–	4	–	–	–	–	–	–	–	–	8	–
3													
Substrate 1	16	–	16	–	NT	1	–	2	1	1	16	32	8
Substrate 2	32	–	16	–	NT	2	–	2	2	4	16	64	8
4	8	–	4	–	–	–	–	–	–	2	8	32	4
5	128	–	32	2	–	4	–	4	–	4	32	128	32
6	8	–	8	–	–	–	–	–	–	2	2	8	–
7	8	–	8	1	–	8	TF	4	2	4	8	16	16
8	16	–	8	–	?	–	?	–	–	2	4	16	2
9	1	–	4	–	–	–	8	–	–	–	–	4	4
10	8	–	8	–	64	2	–	–	–	2	8	16	4
11	2	–	4	–	–	1	–	–	–	–	–	–	2
12	16	–	8	–	–	1	–	–	–	1	8	32	4
13	+	–	4	–	–	–	–	1	–	–	2	4	2
14													
Substrate 1	16	–	8	–	–	4	–	–	–	4	8	8	8
Substrate 2	8	–	4	–	–	4	–	–	–	2	8	8	4
15	32	–	16	–	–	2	–	–	–	4	64	128	32
16	4	–	8	–	1	–	TF	–	–	2	4	8	2
17	16	–	8	–	–	2	–	–	–	8	16	32	16
18	32	–	16	–	–	–	–	–	1	1	16	256	16
19	64	–	32	–	16	–	–	–	–	–	16	64	8
20	8	–	8	–	–	–	–	–	–	1	8	64	2
21	8	–	16	–	4	–	–	4	4	>16	>16	>16	>16
24	4	–	16	–	–	–	–	–	–	–	16	64	16
25	128	8	256	8	256	32	–	64	32	32	128	256	64
26	16	–	16	2	–	8	–	4	–	16	128	128	16

–, Negative; ?, difficulties in defining the antibody; NT, not tested; TF, technical failure.

Plasma samples 1–4, 6, 8–11, and 13 were collected from newly diagnosed diabetic children undergoing plasmapheresis treatment. Samples 1 and 2 were disclosed to be positive and negative controls and were used to define the sensitivity of the pancreatic sections in each laboratory. Serum samples 5, 7, and 12 (supplied by G.F.B.) were pooled sera containing autoantibodies known to react with determinants of the exocrine and endocrine pancreatic tissue. Sample 5 was prepared from sera of ICA-negative nondiabetic patients with primary biliary cirrhosis and was positive for mitochondrial antibodies (AMA), sample 7 was from ICA-negative nondiabetic patients and was positive for nuclear antibodies (ANA), and sample 12 was derived from diabetic patients positive for ICA and from nondiabetic patients positive for ANA but negative for ICA.

Pancreas. The glands were collected and selected individually by laboratories.

Antisera. FITC-labeled anti-human IgG reagents were purchased individually by laboratories. Antisera were produced in goats, rabbits, or sheep.

Assays for ICA determination. The 13 test samples were analyzed for ICA by 24 of the 26 participating laboratories with the indirect immunofluorescence assay according to a standard protocol as previously described (16). The remaining 2 laboratories used either exclusively pancreatic sections of monkey instead of human as substrate (lab 23)

TABLE 2
Analysis of islet cell–antibody (ICA) determination in field trial with 13 samples assayed by standard protocol

	Sample 1	2	3	4	5	6	7	8	9	10	11	12	13
Relative frequencies of ICA (%)	100.0	4.2	100.0	25.0	28.6	47.8	9.5	33.3	20.8	75.0	91.7	100.0	91.7
Number of laboratories	24	24	24	24	21	23	21	24	24	24	22	24	22
ICA end-point titer													
Median	16	–	8	–	–	–	–	–	–	2	8	32	8
Minimum	1	–	4	–	–	–	–	–	–	–	–	4	0
Maximum	128	8	256	8	128	32	16	64	32	32	128	256	64
Number of laboratories	22	24	23	23	21	23	21	24	24	23	22	22	22

–, Negative.

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TABLE 3
Differentiation between islet cell (ICA), mitochondrial (AMA), and nuclear (ANA) antibodies by laboratories using standard protocol

Laboratory code number	Sample 5		Sample 7		Sample 12	
	ICA	AMA	ICA	ANA	ICA	ANA
1	+	+	+	+	+	NI
2	−	+	−	+	+	+
3	NI	NI	−	+	+	+
4	−	NI	−	NI	+	NI
6	−	?	−	NI	+	NI
7	−	+	TF	+	+	+
8	?	+	?	+	+	+
9	−	NI	+	NI	+	NI
10	+	NI	−	NI	+	NI
11	?	NI	−	+	+	NI
12	−	+	−	+	+	+
13	−	+	−	+	+	NI
14	−	+	−	+	+	+
15	−	+	−	+	+	+
16	+	NI	TF	TF	+	+
17	−	NI	−	+	+	+
18	−	+	−	+	+	+
19	+	+	−	+	+	NI
20	?	+	−	NI	+	NI
21	+	NI	−	+	+	NI
24	−	+	−	+	+	+
25	+	NI	−	+	+	NI
26	−	+	−	+	+	+

+, Present; −, absent; ?, difficulties in defining the antibody; NI, not indicated; TF, technical failure.

or performed immunohistochemical staining only (lab 22). In addition, any of the following 8 modifications were performed: *1*) acetone fixation of cryostat sections for up to 3 min (n = 6); *2*) heat inactivation of samples at 50°C (n = 1) and 56°C (n = 5) for 30 min before testing; *3*) dilution of the test samples in aprotinin followed by 24-h incubation on cryostat sections (lab 13; n = 10); *4*) use of FITC protein A as second-step reagent instead of conventional conjugates (lab 14; n = 4); *5*) application of two-color immunofluorescence assays with FITC-labeled anti-human IgG and monoclonal antibodies binding either to human proinsulin (lab 15; n = 2) or to other human islet determinants (n = 1), both detectable with Texas red–labeled antisera; *6*) immunohistochemical staining with glucose oxidase (lab 12; n = 3); *7*) use of sections of monkey pancreas (n = 2); and *8*) determination of complement-fixing ICA (CF-ICA) (lab 11; n = 20).

Comparison of the results. End-point ICA titers obtained with the modifications were compared with those determined by the standard method. Samples 5 and 7 were excluded from comparisons because AMA and ANA in these ICA-negative probes were not readily differentiated from ICA by some participants. Thus, a comparison obtained with a modified test would not exclusively reflect the effect on the end-point ICA titers. Sample 12, in contrast, was included because all participants readily recognized the presence of ICA. In the analysis of the data, each laboratory is represented by a code number.

RESULTS

STANDARD PROTOCOL

ICA. The ICA results of samples 1 and 2 were reported as originally disclosed, positive and negative, by all laboratories except one (Table 1).

Excluding samples 1 and 2, the qualitative analysis of the results obtained with the 11 samples tested on a blind basis showed that the interlaboratory concordance was >90% for 5 of the samples (samples 3, 7, and 11–13; Table 2). These samples were of high titers, except number 7; for the other lower-titered samples the degree of concordance varied between 52 and 79%. Quantitative analysis, however, revealed a broad range of titers. A positive median of titers was obtained for 5 of the 11 samples tested (Table 2). The use of different FITC-labeled anti-human IgG reagents in the field

TABLE 4
Analysis of results in field trial for samples containing mitochondrial (AMA) or nuclear (ANA) autoantibodies

	Sample 5				Sample 7				Sample 12			
	ICA+	ICA−	AMA+	AMA NI	ICA+	ICA−	ANA+	ANA NI	ICA+	ICA−	ANA+	ANA NI
Relative frequencies (%)	31.6	68.4	59.1	40.9	10.0	90.0	77.3	22.7	100.0	0.0	52.2	47.8
Number of laboratories	6	13	13	9	2	18	17	5	23	0	12	11

+, Present; −, absent; NI, not indicated.

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TABLE 5
Comparison of islet cell–antibody (ICA) end-point titers determined on monkey and human pancreatic sections by standard protocol

Laboratory number	Origin of section	Sample										
		1	2	3	4	6	8	9	10	11	12	13
7	Human	8	–	8	1	8	4	2	4	8	16	16
7	Monkey	128	–	128	8	4	4	4	8	128	>16	>128
23	Monkey	32	–	64	–	4	–	64	8	128	256	16

–, Negative.

trial appeared not to influence the results despite variations in the choice of donor animal species and the use of widely differing FITC-protein ratios and dilutions of the antisera (range 1:10 to 1:80) (results not shown).

Differentiation of interfering autoantibodies. Although there was no strict request to comment on the presence of AMA and ANA, most of the participants submitted specific information (Table 3). The majority identified AMA and ANA. ICAs were more readily differentiated from ANA than AMA (Table 4).

MODIFICATIONS

Acetone fixation of sections. In most determinations this modification did not change the end-point titers of ICA as determined by the standard method (Fig. 1). In 27 of 35 (77%) determinations the same ICA titers were obtained by both methods, and on acetone-fixed sections only a twofold increase or decrease of ICA titers was found in 6 and 2 determinations, respectively. Five of 19 (26%) ICA-negative determinations by the standard method became positive on acetone-fixed sections; 4 of these 5 changes, however, were observed in a single laboratory.

Heat inactivation of samples. Heat inactivation of samples before ICA determination resulted in a two- to fourfold decrease in titers in 25 of 39 (64%) determinations with samples that had been ICA positive by the standard method (Fig. 2). In 9 (23%) determinations the titers remained unchanged; in 5 (12%) a twofold increase was observed and 1 determination became negative because the heat-inactivated sample was used.

Prolonged incubation in aprotinin. The sensitivity for ICA determination was improved in the majority of determinations by prolonged incubation in aprotinin (Fig. 3). Fifty-seven of 70 (81%) determinations with samples that had been ICA positive by the standard method had a 2- to 16-fold increase in ICA titers, and 15 of 36 (42%) determinations with samples that had been ICA negative by the standard method became positive, with ICA titers ranging from 2 to 16.

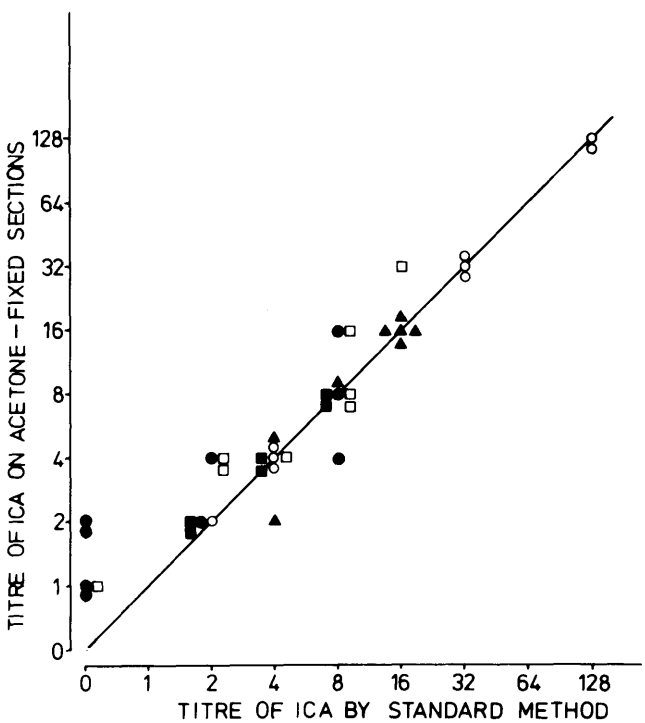

FIG. 1. Comparison of ICA end-point titers obtained by standard method and by use of acetone-fixed sections; laboratories 5 (○), 6 (●), 10 (□), 16 (■), 21 (▲).

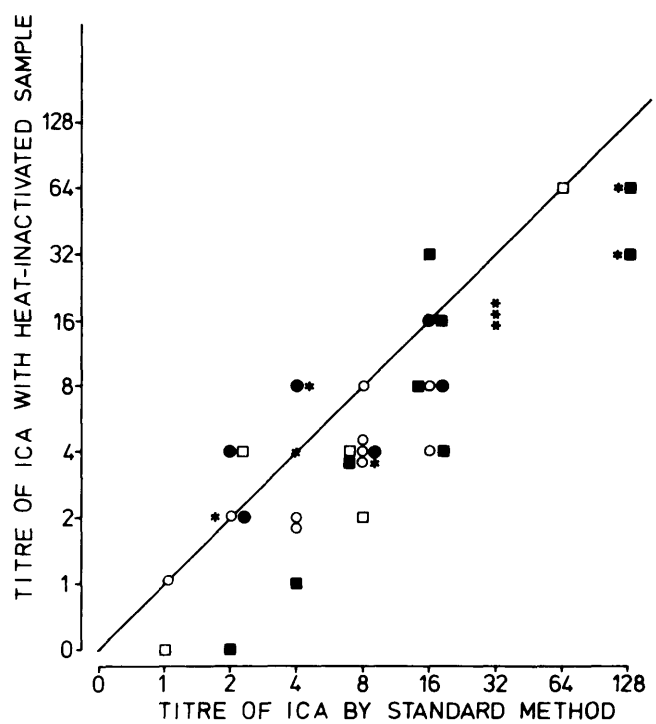

FIG. 2. Comparison of ICA end-point titers obtained by standard method and after heat inactivation of sample; laboratories 5 (*), 7 (○), 8 (●), 20 (□), 26 (■).

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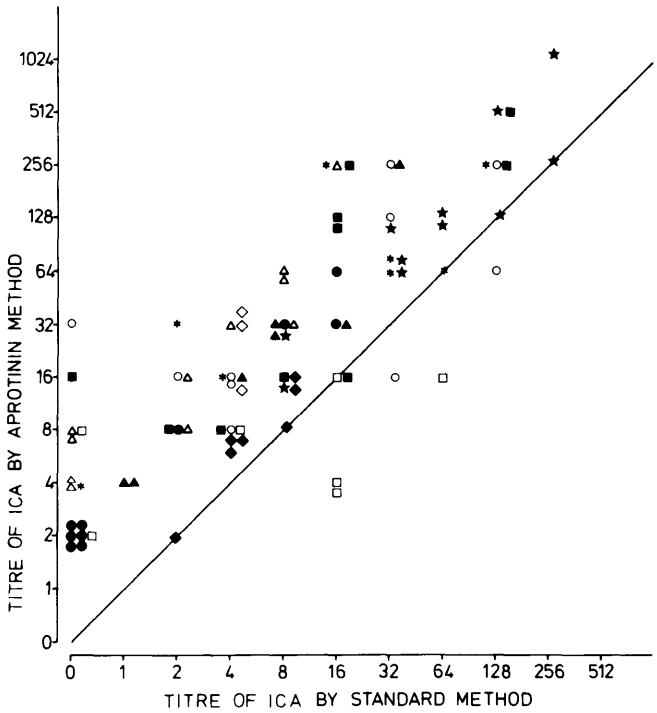

FIG. 3. Comparison of ICA end-point titers obtained by standard method and after prolonged incubation in aprotinin; laboratories 5 (○), 8 (●), 9 (◇), 10 (△), 12 (▲), 14 (♦), 15 (*), 24 (□), 25 (★), 26 (■).

FITC–protein A as conjugate. Except for laboratory 11, a multifold decrease in ICA titers was observed with FITC–protein A as a conjugate compared with the results obtained by the standard method (Fig. 4). Decreased titers were reported in 14 (52%) and absence of ICA in 8 (30%) of the 27 determinations performed. Higher or unchanged titers occurred in 3 (11%) and 2 (7%) determinations, respectively, and 3 of 15 (20%) ICA-negative determinations became positive.

Interestingly, a high concordance for ICA titers was found between 2 laboratories using different methods: laboratory 11 applied this modification and laboratory 12 used the standard method. Discordance was observed in one sample only (results not shown).

Two-color immunofluorescence method. ICA titers obtained by the standard method correlated well with those observed by simultaneous counterstaining with monoclonal antibodies directed toward proinsulin or other islet cell determinants (Fig. 5). Of 23 determinations with samples that had been ICA positive by the standard method, 14 (61%) had the same, 8 showed a twofold increase or decrease, and 1 showed a fourfold decrease in ICA titers. The results obtained with monoclonal antibodies directed toward either proinsulin or other islet cell determinants were comparable; the ICA titers remained unchanged in 7 of 12 and 7 of 11 determinations, respectively (Fig. 5). None of 5 determinations with samples that had been ICA negative by the standard method became positive with this modification.

Thus, simultaneous counterstaining of islet cells is a suitable modification facilitating the recognition of islets, especially when ICA-negative samples are tested.

Immunohistochemical staining with glucose oxidase. Three laboratories (labs 10, 22, and 24) used immunohistochemical staining with glucose oxidase; two of them (labs 10 and 24) also submitted the results obtained by the standard method. The limited data were comparable, but it was difficult to distinguish AMA and ANA from ICA. At this stage, this modification appeared to require careful selection of reagents because high background staining was reported by several other investigators. Although immunohistochemical staining in general is a well-established method for histological examinations, it remains to be studied why it could not be readily applied for ICA determinations.

Use of monkey pancreas. The data of two laboratories (labs 7 and 23), although limited, suggested that the use of monkey pancreas may improve the sensitivity of ICA determination (Table 5). Laboratory 7 observed a two- to eightfold increase in titers compared with the results obtained on human sections. Similarly, the titers determined by laboratory 23 were mostly above those obtained by most of the investigators applying the same method on human sections (Table 1). Because samples 4 and 8 were found to be positive on sections of monkey pancreas only by laboratory 7 and not by laboratory 23, the ICA-binding cytoplasmic determinants appear to vary in different monkey pancreases.

CF-ICA. Sixty-six of 136 (49%) determinations with samples that had been ICA positive by the standard method were CF-ICA positive (Fig. 6). A strong association was found between the titers of ICA and presence of CF-ICA. None of 78 determinations that had been ICA negative was positive

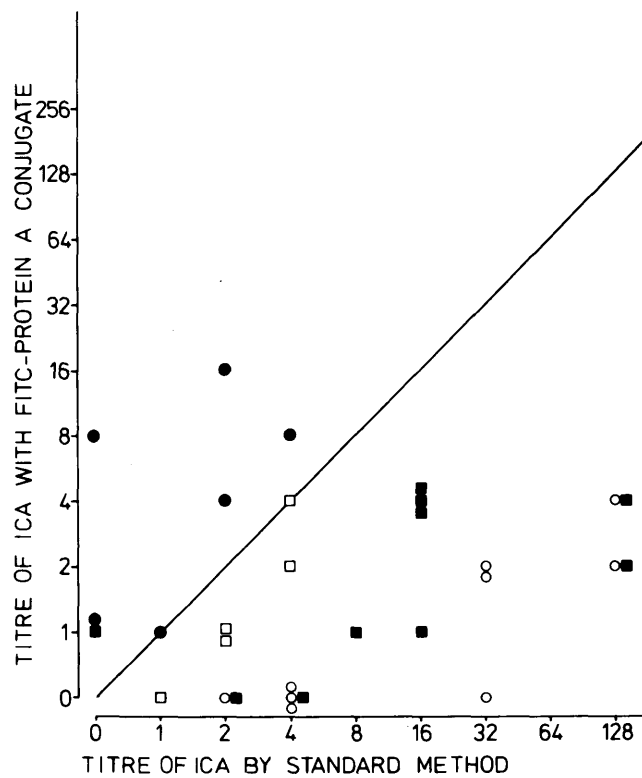

FIG. 4. Comparison of ICA end-point titers obtained by standard method and by use of FITC–protein A reagent; laboratories 5 (○), 11 (●), 13 (□), 26 (■).

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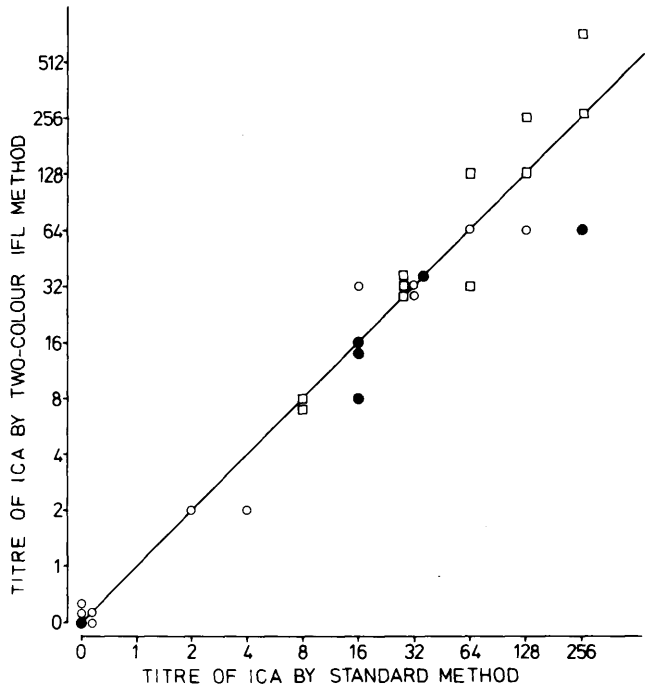

FIG. 5. Comparison of ICA end-point titers obtained by standard and by 2-color immunofluorescence methods; laboratories 15 (○) and 18 (●) used monoclonal antibodies directed toward proinsulin, 25 (□) toward other islet cell determinants.

for CF-ICA (results not shown). The frequency of CF-ICA correlated positively with increased titers of ICA. Fifty-four of 83 (65%) determinations with ICA titers ≥8 and 29 of 32 (90%) determinations with ICA titers ≥32 had CF-ICA. No correlation was found between ICA and CF-ICA titers, although the latter generally were lower.

DISCUSSION

Several methodic modifications have been developed (11–15) to improve the determination of cytoplasmic ICA by the originally applied indirect immunofluorescence method (1). By comparison of end-point titers of ICA as determined by a standard method with those obtained with various modifications, the following conclusions were drawn.

1. Improved sensitivity, which resulted in increased ICA titers in most samples, was observed with prolonged incubation of the test sample in the presence of aprotinin (Fig. 3). Preliminary data indicate that an increased sensitivity may be also achieved by the use of sections prepared from monkey pancreas (Table 5).

2. No major effect on ICA titers of ICA was exerted by either acetone fixation of the sections (Fig. 1) or use of the two-color immunofluorescence methods (Fig. 5).

3. A decrease in sensitivity, which resulted in diminished ICA titers, was found after heat inactivation of the test samples (Fig. 2), by use of FITC-labeled protein A as reagent (Fig. 4), and by testing for CF-ICA (Fig. 6).

4. At this stage, immunohistochemical staining with glucose oxidase and application on pancreatic sections needs to be further studied to eliminate the observed interfering background staining.

5. CF-ICAs were present in ~50% of ICA-positive samples and were more prevalent in ICAs of higher titers (Fig. 6).

Presence of ICA in the negative control sample was reported by one laboratory. Because this sample was obtained from a diabetic child, differing specificities or increased sensitivity of cytoplasmic antigenic determinants in individual pancreases could account for the observed discrepancy.

Prolonged incubation in the presence of aprotinin apparently improved the sensitivity without loss of substrate specificity. Experience of prolonged incubation without dilution of the test sample in aprotinin also resulted in improved sensitivity as reported by several participants. A reduced quality of the morphology, however, often ensued. Thus, it remains to be clarified if the proteinase inhibitor is required for stabilization of the pancreatic substrate.

Unless the ICA-binding cytoplasmic determinants are isolated and characterized on a molecular basis to establish standard curves for ICA determinations, the various immunofluorescence and immunohistochemical assays as presented here remain the method of choice to evaluate a possible ongoing anti–islet cell autoimmunity. To overcome the numerous variabilities of these different assays, a current proposal for standardization of ICA was presented by Roger Dawkins and Ezio Bonifacio at The First International Workshop on the Standardisation of Cytoplasmic Islet Cell Antibodies (16). They calibrated standard curves for the individual laboratories to enable expression of ICA results in arbitrary units. Such an approach could promote the ultimate goal of reliable interlaboratory comparisons. Ongoing investigations in a second-stage workshop are being conducted to test whether this approach is feasible. If ICA determinations are expressed in arbitrary units, it is assumed that future studies can contribute to define the ICA concentration predicting forthcoming insulin dependency. Moreover, general

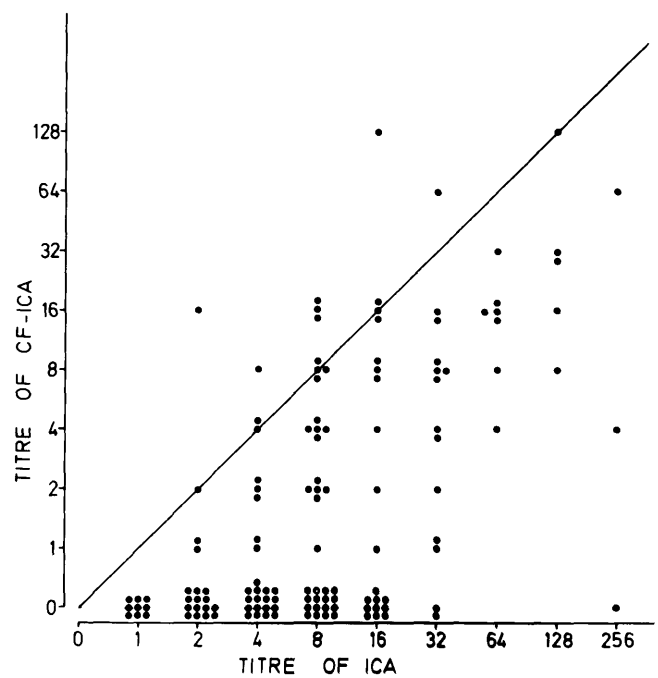

FIG. 6. Comparison of ICA and CF-ICA end-point titers determined according to standard protocol by 20 laboratories.

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use of standard curves could enable studies providing data to calculate the probability at what time overt insulin deficiency might be expected.

ACKNOWLEDGMENTS

We thank Drs. B. M. Dean, G. S. Eisenbarth, and Å. Lernmark for critical reading of the manuscript and D. Wellems for fine secretarial help.

This is an analysis of data presented at the First International Workshop on the Standardisation of Cytoplasmic Islet Cell Antibodies.

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