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Lack of Agreement among Two Commercial Enzyme-Linked Immunosorbent Antibody Assays and a Conventional Immunofluorescence-Based Method for Detecting Islet Cell Autoantibodies

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Two commercial enzyme-linked immunosorbent assays (ELISAs) for antibodies associated with development of insulin-dependent (type 1) diabetes mellitus (IDDM) were evaluated in conjunction with a conventional indirect immunofluorescent-antibody–islet cell antibody (ICA) test and a radioimmunoprecipitation method for detection of insulin autoantibodies in sera from a selected group of patients. The anti-ICA ELISA was positive for only 1 of 17 serum samples from newly diagnosed IDDM patients but yielded false-positive results with 2 of 6 serum samples containing non-diabetes-related autoantibodies. Although the anti-glutamic acid decarboxylase ELISA did not show positive results for sera with other autoantibodies, it was positive for only 4 of 29 serum samples from recently diagnosed IDDM patients and for 49% of 37 indirect immunofluorescent-antibody–ICA test-positive sera. Until the antibodies associated with the development of diabetes are better characterized, allowing better standards for comparison, it will be difficult to evaluate commercial assays in this field.

There is a great deal of interest in the development of intervention strategies to prevent the onset of insulin-dependent diabetes mellitus (IDDM), particularly in persons thought to be at increased risk, such as first-degree relatives of IDDM patients (3, 8, 18). To this end, efforts have been directed toward identifying in vitro test results which have predictive value. Several highly regarded laboratories are working on this challenge (2, 5, 6, 11, 12, 20). Most studies have concentrated on antibody detection, since autoimmunity-mediated destruction of pancreatic islet cells is thought to be reflected by autoantibodies against islet cell components (13, 17).

The classic test, and one still thought to yield predictive results in most cases, is the indirect immunofluorescent-antibody assay (IFA) for islet cell antibodies (ICAs) (7). Unfortunately, there is no consistent, commercially available, agreed-upon standard substrate. The antibodies measured are nonspecific, and attempts to reconcile these ICAs with those detected in other test systems have been variably successful (5, 6, 22, 23), although recent studies point to antibodies against an islet cell-associated protein (ICA512) and anti-glutamic acid decarboxylase (GAD) antibodies as major contributors (15, 16). Even the use of human pancreas tissue, thought by most workers to be the most sensitive substrate, is controversial (19). To complicate matters further, most laboratories performing ICA assays produce their own substrate materials, making interlaboratory comparisons impossible. Maclaren and colleagues are attempting to remedy this problem through a voluntary proficiency testing program, but results from participating laboratories have shown some variation (7, 10). Such inconsistent results early in the standardization process are probably inevitable when the test is a subjective one, performed by laboratory scientists with various degrees of expertise using different reagents and different microscope configurations.

Advances in molecular methods have allowed the development of tests for antibodies directed against better-characterized antigens, including the 65-kDa recombinant human GAD enzyme (GAD65) and human insulin (insulin autoantibody [IAA] test) (9, 11, 21). However, most of these assays have been developed independently by different laboratories and have not been adapted for commercial use. Numerous additional tests have been utilized for predictive purposes by individual laboratories, including first-phase insulin release during intravenous glucose tolerance tests, Western blots (immunoblots) for ICA69, enzyme-linked immunosorbent assays (ELISAs) for ICA512, and others, using available groups of patients (3, 15, 16). Again, these tests are not standardized or widely available. Thus, although many studies of predictability of the various in vitro tests have been published, there is still no consensus.

The availability of a commercially available product would allow comparison of results from laboratory to laboratory and accumulation of information about much larger numbers of patients than is currently possible so the relationship between test results and patient outcome could be better evaluated. A standardized and consistent approach to in vitro testing should allow widespread access to testing for many patients and provide better predictive capabilities to clinicians.

At least two commercially available test kits that yield either quantitative or qualitative results have been marketed for research use. The Elias Synelisa GAD II Antibodies (elias usa, inc., Osceola, Wis.) used a baculovirus vector-produced recombinant human GAD II (GAD65) antigen in a unique ELISA format in which the antigen is presented on plastic pins (14). The Isletest-ICA (Biomerica, Newport Beach, Calif.) uses a more traditional ELISA format to detect the presence or ab-

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TABLE 1. Results obtained by four methods with 22 serum samples from patients with type 1 diabetes

Test(s) showing positive results	No. (%) of patients	
	Newly diagnosed, no previous insulin[a]	Previously diagnosed, receiving insulin[b]
IAA	3 (18)	1 (20)
IAA, ICA-IFA	5 (29)	1 (20)
IAA, ICA-IFA, Synelisa	6 (35)	1 (20)
IAA, ICA-IFA, Synelisa, Isletest	1 (6)	0 (0)
IAA, Synelisa	1 (6)	1 (20)
IAA, Isletest-ICA	0 (0)	1 (20)
None	1 (6)	0 (0)
Total no. IAA positive	16 (94)	5 (100)

[a] $n = 17$.
[b] $n = 5$.

sence of antibodies against pancreatic cytoplasmic antigens (proprietary preparation method) (4).

We sought to determine if either of these test systems can yield results comparable to those derived by traditional methods of in vitro testing for ICAs and if the ELISA results correlate with clinical parameters. We tested well-characterized sera, including sera with positive IAA test results, antinuclear antibodies of various specificities, and rheumatoid factor; sera positive for ICAs by IFA; and sera from clinically characterized early-onset IDDM patients. Unfortunately, there was almost no correlation among the results generated by the different methods.

MATERIALS AND METHODS

Sera. Most of the sera evaluated had been sent to Endocrine Sciences for diagnosis and monitoring of patients with type 1 and 2 diabetes, including new-onset diabetes. Several sera from newly diagnosed IDDM patients were kindly submitted to us by their diabetologists for this study only. Of the 22 serum samples from patients with IDDM that were tested by all four methods, 11 were from females (age range, 5 to 15 years; average age, 8.8 years) and 11 were from males (age range, 4 to 41 years; average age, 12.4 years). Five patients had been diagnosed 2 months to 20 years before the current serum samples were obtained, and all were receiving insulin; the remaining 17 patients were newly diagnosed and either had not received insulin prior to procurement of the serum samples tested or had received the first dose within hours of sample collection (Table 1). Six serum samples with antinuclear antibodies (homogeneous, antispindle, and nonspecific cytoplasmic speckled by conventional fluorescent-antinuclear-antibody tests) and two serum samples with rheumatoid factor were graciously donated (with patient identification marks removed) by local laboratories. Sixteen normal control serum samples were drawn from employees, and a normal sibling of a diabetic patient provided another normal control. An additional 77 serum samples, tested in one or the other ELISA system, were obtained from patients with previously diagnosed IDDM who were currently taking insulin. The same serum was not tested in both ELISA systems for these additional tests because of volume restrictions.

All tests for autoantibodies were repeated in house with commercial components (Sanofi Diagnostics Pasteur, Chaska, Minn.). Again because of low volumes of some sera, not all sera yielding negative results by both of the two ELISAs were tested for all other parameters. In total, 126 serum samples were tested in at least one ELISA system and other tests were performed as necessary and as sample quantity allowed.

IAAs. IAAs were determined with a highly sensitive radioimmunoprecipitation assay, similar to that of Greenbaum and coworkers (9). The key features of this assay are that endogenous insulin is removed by dextran-charcoal treatment, antigen-antibody complexes are precipitated with polyethylene glycol and centrifugation, and radioactivity in the pellet is compared with a standard curve based on antibodies with quantified binding capacity. The sensitivity of this assay is 2 μU of insulin-binding capacity per ml. For this evaluation, we used 5 μU of insulin-binding capacity per ml as a negative cutoff value.

ICAs. The IFA for ICAs was performed with a human pancreatic tissue substrate harvested from heart-beating organ donors. Tissue was flash-frozen, acetone fixed, and then cut into 5 μm-thick sections (MarDx, Carlsbad, Calif.). Such treatment has been shown to yield results comparable to those obtained with non-acetone-fixed tissue (7). Fluorochrome-conjugated $F(ab')_2$ (Kallestad Diagnostics, Austin, Tex.) or fluorochrome-conjugated anti-human immunoglobulin G (Fab specific; Sigma Immunochemicals, St. Louis, Mo.) was used as the antiserum. An internal standard calibrated with respect to the Juvenile Diabetes Foundation standard serum (10) was included with each assay.

TABLE 2. Characteristics of 100 serum samples tested[a]

Group	Total no. of serum samples tested	No. positive/no. tested (% positive)	
		Synelisa	Isletest-ICA
Recent clinical diagnosis of IDDM	29	4/29 (14)	1/17 (6)
IAA positive	27	3/27 (11)	2/19 (11)
ICA positive	37	18/37 (49)	1/18 (6)
Autoantibody positive	6	0/6 (0)	2/6 (33)
Rheumatoid factor positive	2	0/2 (0)	0/2 (0)
Normal controls	17	0/17 (0)	0/15 (0)
IAA and ICA positive	13	3/13 (23)	1/13 (8)

[a] Not all sera were tested by both ELISA methods.

ELISAs. The Synelisa GAD II test includes six standards and positive and negative controls. The horseradish peroxidase enzyme used tetramethyl benzidine as the substrate. Standards were tested in duplicate, and a standard curve was generated; 1,500 mU of GAD activity per ml was considered the positive cutoff point, and results were reported in milliunits of GAD activity per milliliter. The Isletest-ICA included only positive and negative controls. Results had been standardized, presumably against a pool of normal (negative) sera. An arbitrary positive cutoff point was determined by multiplying the average of duplicate optical density readings for the negative control by 2.5. Since results were qualitative, reported as the presence or absence of ICAs only, no units were suggested. The Isletest-ICA used an alkaline phosphatase–*para*-nitrophenyl phosphate enzyme-substrate combination. For comparison of the 22 serum samples tested by all four methods, both ELISA systems were tested with the same serum samples on the same day. Results were read on a plate reader spectrophotometer (Ceres 900; Biotek Instruments, Inc., Winooski, Vt.) at the wavelengths specified for each kit. A four-parameter logistic curve-fitting routine was used for evaluation of results. Sera were diluted into the appropriate buffers (supplied in the kits), reagents specific to each system were used, and the manufacturer's recommendations were followed for each kit. Results for controls were within acceptable limits.

RESULTS

No single test identified all patients with type 1 diabetes (Table 1). Two serum samples were positive in the Synelisa and negative by the ICA test but positive by the IAA test. The IAA test results were positive for 21 of 22 serum samples from patients with diabetes tested by all four methods, making it the most sensitive among the methods evaluated here, with a sensitivity of 95%. In one patient identified as having new-onset IDDM, no antibodies were identified by any of our tests. An additional serum sample from a patient newly diagnosed with IDDM was positive in the ICA test and neither ELISA, but because the IAA test was not performed on that serum sample because of low sample volume, it was not included in Table 1. The normal control sera did not react in any of these tests (data not shown). The Isletest-ICA yielded false-positive results for two of the six autoantibody-positive serum samples tested (Table 2). It is probable that the producers did not include such sera in the control pool from which normal values were developed. Rheumatoid factor did not seem to cross-react in any test system. Although the Synelisa did not react with these autoantibody-positive sera, only half of the sera positive by the traditional ICA test were positive with this assay and only 14% of the sera from newly diagnosed IDDM patients were positive (Table 2).

Correlation among the test methods was not observed (Table 2). Only one serum sample, from a 41-year-old male with newly diagnosed IDDM and positive by both the IAA test (5.2 μU/ml) and the ICA test (1:4), was positive by both ELISAs.

DISCUSSION

The two ELISAs evaluated here purport to measure antibodies thought to be important in the development or detection of diabetes. Although none of the assays measure the same product, it would be expected that the majority of patients with newly diagnosed diabetes and those positive for ICAs or IAAs by other methods might also yield positive results for ICAs (measured by an IFA) or GAD65 antibodies. This was not the case. The Synelisa has been compared with an immunoprecipitation method for detection of anti-GAD65 antibodies, and results were found to be equivalent (14). We sought to determine whether either of these two ELISA systems could substitute for the more labor-intensive and subjective ICA IFA. Although the recombinant or mammalian GAD65 used for other ELISAs has been considered to be similar to or a component of the islet cell cytoplasmic antigen detected by IFA, this is probably not true for this assay (1, 14). After preparation of this report, the Synelisa was withdrawn from the market. The company now produces a radioimmunoassay for anti-GAD65, which we are currently evaluating.

The results of this study highlight the difficulty in evaluating immunologic tests for predicting development of diabetes by using sera from newly diagnosed diabetics, the only ready patient base. Only long-term studies of large numbers of persons at risk, such as siblings of patients with IDDM, can adequately address the utility of such tests. Until the immunologic characteristics of this disease are more definitively ascertained, combinations of tests must be used and some degree of uncertainty is likely to prevail.

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