International Workshop on Lessons From Animal Models for Human Type 1 Diabetes

Identification of Insulin but Not Glutamic Acid Decarboxylase or IA-2 as Specific Autoantigens of Humoral Autoimmunity in Nonobese Diabetic Mice

Ezio Bonifacio,[1] Mark Atkinson,[2] George Eisenbarth,[3] David Serreze,[4] Thomas W.H. Kay,[5] Edwin Lee-Chan,[6] and Bhagirath Singh[6]

Several self-antigens have been reported as targets of the autoimmune response in nonobese diabetic (NOD) mice. The aim of this workshop was to identify autoantibody assays that could provide useful markers of autoimmunity in this animal model for type 1 diabetes. More than 400 serum samples from NOD (4, 8, and 12 weeks of age and at diabetes onset), BALB/c, and B6 mice were collected from six separate animal facilities, coded, and distributed to five laboratories for autoantibody measurement. Insulin autoantibodies (IAA) were measured by radiobinding assay (RBA) by four laboratories and by enzyme-linked immunosorbent assay (ELISA) in one laboratory. Using the 99th percentile of BALB/c and B6 control mice as the threshold definition of positivity, IAA by RBA were detected in NOD mice at frequencies ranging from 10 to 30% at age 4 weeks, from 26 to 56% at 8 weeks, from 42 to 56% at 12 weeks, and from 15 to 75% at diabetes onset. With ELISA, IAA signals differed significantly between control mouse strains and increased with age in both control and NOD mice, with frequencies in NOD animals being 0% at 4 weeks, 14% at 8 weeks, 19% at 12 weeks, and 42% at diabetes onset. For IAA, the ELISA results were relatively discordant with those of RBA. GAD autoantibody (GADA) and IA-2 autoantibody (IA-2A) signals obtained by RBA were low (maximum 2.5% of total) but were increased in NOD mice compared with control mice at diabetes onset (GADA 29–50%; IA-2A 36–47%). ELISA also detected GADA (42%) and IA-2A (50%) at diabetes onset, with results concordant with those of RBA. Remarkably, GADA and IA-2A frequencies varied significantly with respect to the source colony of NOD mice. Furthermore, whereas neither GADA nor IA-2A correlated with IAA, there was strong concordance between GADA and IA-2A in individual mice. Sera with increased binding to GAD and IA-2 also had increased binding to the unrelated antigen myelin oligodendrocyte glycoprotein, and binding to GAD could not be inhibited with excess unlabeled antigen, suggesting nonspecific interactions. In sum, this workshop demonstrated that IAA measured by sensitive RBA are a marker of autoimmunity in NOD mice and draw into question the true nature of GADA and IA-2A in this animal model. *Diabetes* 50:2451–2458, 2001

Autoantibodies are a hallmark of autoimmune disease (1). Their measurement in humans and in spontaneous and induced animal models of autoimmunity has been prolific and contributed substantially to our current understanding of individual autoimmune diseases. Autoantibodies have been the prime route of autoantigen identification, provide an aid in the diagnosis of disease, and can serve as indicators of the preclinical phase of disease or monitors of intervention (2).

The presence of islet autoantibody in individuals who otherwise seem healthy denotes an increased risk for later development of type 1 diabetes; hence, these individuals are suitable for participation in clinical trials aimed at disease prevention (3,4). These diagnostic and predictive abilities did not naturally present themselves, as much of the early literature regarding autoantibodies in type 1 diabetes was subject to controversies and conflicting information—factors due in large part to variances in the methods and substrates used for autoantibody detection. Considerable advances toward overcoming these difficulties came through the voluntary performance of many investigators in more than a dozen international autoantibody workshops, often organized under the auspices of the Immunology of Diabetes Workshops, an organization that later became known as the Immunology of Diabetes

From the [1]Department of Medicine, Isituto Scientifico San Raffaele, Milan, Italy, and the Diabetes Research Institute, Munich, Germany; the [2]Department of Pathology, University of Florida, Gainesville, Florida; the [3]Barbara Davis Center for Childhood Diabetes, University of Colorado Health Sciences Center, Denver, Colorado; [4]Jackson Laboratory, Bar Harbor, Maine; the [5]Autoimmunity and Transplantation Division, the Walter and Eliza Hall Institute of Medical Research, Victoria, Australia; and the [6]Department of Microbiology & Immunology, University of Western Ontario, London, Canada.

Address correspondence and reprint requests to Dr. Mark Atkinson, University of Florida, Department of Pathology, Box 100275 JHMHC, 1600 SW Archer Rd., Gainesville, FL 32610. E-mail: atkinson@ufl.edu.

Received for publication 9 May 2001 and accepted without revision 13 July 2001.

G.E. has received consulting fees from Quest Diagnostics.

BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; GAD, glutamic acid decarboxylase; GADA, GAD autoantibodies; IA-2A, IA-2 autoantibodies; IAA, insulin autoantibodies; MOG, myelin oligodendrocyte glycoprotein; OD, optical density; PBS, phosphate-buffered saline; RBA, radiobinding assays.

TABLE 1
Origin of mouse serum samples used in autoantibody workshop

Source colony	Investigator	Time point	Strain	No. of serum received
A	Singh London, Canada	4 weeks	NOD	20
			BALB/c	20
			C57/B6	20
		8 weeks	NOD	20
			BALB/c	20
			C57/B6	20
		12 weeks	NOD	20
			BALB/c	20
			C57/B6	20
		Diabetic	NOD	7
B	Eisenbarth Denver, CO	8 weeks	NOD	20
			BALB/c	19
			C57/B6	20
		12 weeks	NOD	18
			BALB/c	20
			C57/B6	20
C	Atkinson Gainesville, FL	8 weeks	NOD	10
			C57/B6	10
		12 weeks	C57/B6	10
D	Kay Melbourne, Australia	8 weeks	NOD	8
			BALB/c	8
			C57/B6	8
		12 weeks	NOD	8
			BALB/c	8
			C57/B6	8
		Diabetic	NOD	8
E	Serreze Bar Harbor, ME	8 weeks	NOD	8
		12 weeks	NOD	8
		Diabetic	NOD	5
F	Ziegler Munich, Germany	8 weeks	NOD	14

Society (5–15). Among their many findings, these workshops validated insulin, GAD, and IA-2 as autoantigens associated with type 1 diabetes in humans; identified sensitive and specific methods for autoantibody measurement; and introduced reference standards for diabetes-associated autoantibodies.

The nonobese diabetic (NOD) mouse spontaneously develops islet autoimmunity and overt hyperglycemia and serves as a model for human type 1 diabetes (16). Similar to the human disease, insulin, GAD, and IA-2 have been implicated as target autoantigens in the NOD mouse. Also, similar to studies of humans, some of these works have been conflicting in terms of the frequency or even the mere presence of specific autoantibodies to these antigens. A recent meeting, sponsored in part by the Juvenile Diabetes Research Foundation International, was formed to review and assess the proper use of animal models (e.g., NOD mice, BB rats, and streptozotocin-induced diabetes) for type 1 diabetes in humans (17). As part of this setting, an international workshop was held to establish which, if any, of the aforementioned autoantigens commonly associated as targets of autoantibodies in type 1 diabetes in humans could also be validated as being the targets of humoral immunity in the NOD mouse. The results of this workshop form the basis for this report.

RESEARCH DESIGN AND METHODS

Workshop design. Workshop participation was open and in large part subject to investigator registration for the Lessons From Animal Models for Human Type 1 Diabetes meeting (Denver, CO, October 2000) (18) as well as past performance in using assays that monitor humoral immunity in NOD mice. The goals, priorities, and workshop design were established before the workshop by the meeting organizers. As part of this process, it was agreed that participating investigators were allowed to use methods subject to current utilization within their laboratories (shown below) rather than a common method. Serum was voluntarily collected and submitted by individuals with access to various mouse colonies, as shown in Tables 1 and 2.

Assay methods. In accordance with the workshop design, participating laboratories performed autoantibody assays as indicated below.

Laboratory 1 radiobinding assay. IAA were measured by RBA similar to that previously described for humans (18). Sera (5 μl) were incubated in duplicate with 1.159 mU of ^{125}I-labeled insulin (Hoechst) contained in 25 μl of 50 mmol/l Tris and 1% Tween 20 (pH 8.0) buffer (TBT) for 3 days on ice. Antibody was subsequently captured by adding 50 μl TBT containing 2 mg Protein A Sepharose (Pharmacia) and 6 μl Protein G (γ bind) and incubated at 4°C for 1 h with shaking. Tubes were then washed four times each with 2 ml cold TBT, and pellets were counted in a γ-counter. Results were expressed as an index calculated as follows: (cpm of test sample − cpm of negative control serum)/(cpm of positive standard sample − cpm of negative control serum) × 100.

Laboratory 2 radiobinding assay. A 96-well filtration plate micro IAA assay was performed as previously described (19). ^{125}I-insulin (Amersham) of 20,000 cpm was incubated with 5 μl serum with and without cold human

TABLE 2
Mouse samples included in the workshop

Age	NOD	BALB/c	C57/B6
4 weeks	20	20	20
8 weeks	80	47	58
12 weeks	54	48	58
Diabetic	20	—	—

insulin, respectively, at a 1:5 dilution of serum for 3 days at 4°C in buffer A (20 mmol/l Tris-HCl buffer [pH 7.4] containing 150 mmol/l NaCl, 1% bovine serum albumin [BSA], 0.15% Tween 20, and 0.1% sodium azide). Fifty microliters of 50% Protein A/8% Protein G Sepharose (Pharmacia) were added to the incubation in a MultiScreen-NOB 96-well filtration plate (Millipore), which was precoated with buffer A overnight at 24°C. The plate was then placed on a plate shaker and shaken at low speed for 45 min at 4°C, followed by two cycles of four washes each with cold buffer A containing 0.1% BSA using a Millipore vacuum-operated 96-well plate washer. After washing, 40 μl scintillation liquid (Microscint-20; Packard) was added to each well, and radioactivity was determined directly in the 96-well plate with a TopCount (96-well plate β-counter; Packard) scintillation counter. The results were calculated on the basis of the difference in counts per minute between the well without cold insulin and the well with cold insulin (Δcpm) and expressed as an index: index = (sample Δcpm − negative control Δcpm)/(positive control Δcpm − negative control Δcpm).

Laboratory 3 radiobinding assay. Autoantibodies were determined with minor modifications of those previously described (20). Mouse serum (5 μl) was incubated with 20,000 cpm of ^{125}I-insulin (Amersham) with and without 8 units/ml cold insulin (Eli Lilly) at a final 1:5 dilution of serum. Dilutions and stocks were made in assay buffer (150 mmol/l NaCl, 20 mmol/l Tris-HCl, 0.1% NaN_3, 1% BSA, and 0.15% Tween 20 [pH 7.4]). Assays were performed in low protein-binding 96-well plates. The plates were incubated for 3 days at 4°C. Microfiltration 96-well plates were blocked for 18 h with assay buffer. The buffer was removed from the filtration plates, and 25 μl of 50% Protein A Sepharose (Sigma) was pipetted into each well. Fifty microliters of serum/antigen mixture from step 1 was added to each well and incubated at 4°C with shaking for 45 min. Each well of the filtration plate was washed (Millipore plate washer) eight times under vacuum with 200 μl wash buffer (150 mmol/l NaCl, 20 mmol/l Tris-HCl, 0.1% NaN_3, 0.1% BSA, and 0.15% Tween 20 [pH 7.4]). After the plates dried, 30 μl Optisafe scintillation fluid (Fisher) was added to each well and then counted in a Wallac LKB trilux β-counter. The results were reported as an index. The Δcpm was first calculated as cpm without cold insulin − cpm with cold insulin. The index was calculated as follows: ([sample Δcpm − negative control Δcpm]/[positive control Δcpm − negative control Δcpm]) × 100.

Laboratory 4 radiobinding assay. ^{125}I-insulin (Amersham) of 20,000 cpm was incubated with 5 μl serum with and without cold human insulin, respectively, at a 1:5 dilution of serum for 7 days at 4°C in buffer A (20 mmol/l Tris-HCl buffer [pH 7.4] containing 150 mmol/l NaCl, 1% BSA, 0.15% Tween 20, and 0.1% sodium azide). Fifty microliters of 50% Protein A/8% Protein G Sepharose was added to the incubation in a MultiScreen-NOB 96-well filtration plate, which was precoated with buffer A overnight at 24°C. The plate was then placed on a plate shaker and shaken at low speed for 45 min at 4°C, followed by two cycles of four washes each with cold buffer A containing 0.1% BSA using a Millipore vacuum-operated 96-well plate washer. After washing, 40 μl scintillation liquid (Microscint-20) was added to each well and radioactivity was determined directly in the 96-well plate with a TopCount (96-well plate β-counter) scintillation counter. The results were calculated on the basis of the difference in counts per minute between the well without cold insulin and the well with cold insulin (Δcpm) and expressed as an index: index = (sample Δcpm − negative control Δcpm)/(positive control Δcpm − negative control Δcpm).

Laboratory 5 ELISA. Enzyme-linked immunosorbent assay (ELISA) was performed as previously described (21). Briefly, the ELISA plate wells were coated overnight at 4°C with antigen (insulin, GAD65, and IA-2) in coating buffer (0.05 mol/l $NaHCO_3/Na_2CO_3$ buffer, pH 9.6) at a concentration of 1 μg/ml. The plates were then washed twice and saturated at 24°C for 2 h with phosphate-buffered saline (PBS)–0.05% Tween 20 (PBS-T). After washing three times with PBS-T, 100 μl diluted serum (1:80, 1:160, and 1:320) was added in each well. Samples were incubated at 24°C for 2 h. Plates were washed five times, and 100 μl alkaline phosphatase–conjugated goat anti–mouse IgG second antibody (1:1,000; BD PharMingen) was added to each well, followed by incubation at 24°C for 2 h. Finally, the plates were washed five times with PBS-T and 100 μl of phosphatase substrate (10 mg *p*-nitrophenyl phosphate disodium (Sigma) dissolved in 10 ml substrate buffer (1 mol/l diethanolamine, 3 mmol/l sodium azide, and 0.5 mmol/l magnesium chloride [pH 9.8]) added to each well. Plates were read at 405 nm at various time points. The data were expressed as OD (optical density) after subtraction of background absorbance.

Statistical analysis. Comparisons of antibody levels between control and NOD mice were performed using the Mann-Whitney *U* test. Receiver operator curves were used to compare sensitivity and specificity of individual assays. Comparisons of antibody levels at different time points in individual mice were performed using the Wilcoxon matched pairs test. Antibody levels between assays were correlated using Spearman's rank correlation.

RESULTS

Autoantibodies against insulin in NOD versus control mice. Each of the five participating laboratories observed increased IAA signals in serum samples obtained from NOD mice in comparison with control mice (Fig. 1). Using the 99th percentile of signals obtained using control mouse sera as the threshold for positivity, IAA were observed in 0–30% (4-week-old NOD mice), 12–56% (8-week-old NOD mice), 17–56% (12-week-old mice), and 15–75% of NOD mice at diabetes onset. There were substantial differences, however, in the frequency of IAA reported in each laboratory. Laboratories 1 and 2 had relatively high and comparable sensitivities. Both used an RBA but with different formats for measurement (see RESEARCH DESIGN AND METHODS). Laboratory 1 did not compete binding with excess cold insulin and reported low-level nonspecific binding in a minority of sera from control mice. The assay used by laboratory 2 included competition and observed little or no nonspecific binding in control mice. Both laboratories found increased binding in NOD mouse sera taken at each time point compared with age-matched samples from both control mouse strains. The sensitivity in laboratory 3, using an RBA with competition, was somewhat decreased when compared with laboratories 1 and 2 and observed significant differences between NOD sera and age-matched control samples in only 8- and 12-week old mice. Laboratory 4, which also used an RBA with competition, had a very low sensitivity and very low binding, as indicated by the cpm of the signals reported. Laboratory 5 measured antibodies using an ELISA. A significant difference in insulin binding was observed between control sera from BALB/c mice as compared with C57/B6 mice ($P < 0.0001$), and binding was higher in 12-week-old mice compared with 4- and 8-week-old mice in both of the control strains ($P < 0.001$). The relatively large range of binding seen in sera from control mice resulted in a low sensitivity for samples from 4- (0%), 8- (14%), and 12-week-old (19%) NOD mice. The sensitivity of the ELISA for sera from diabetic NOD mice (42%) was less than that of laboratories 1 (75%) and 2 (69%).

IAA in sequential samples from individual NOD mice. For these studies, samples were collected at 4, 8, and 12 weeks of age from 20 NOD mice. The first detection of IAA in individual mice varied between laboratories, with laboratories providing high sensitivity subjects for presentation (Fig. 2). Laboratories 1 and 3 detected IAA in mice early at age 4 weeks (note: laboratory 2 did not test 4-week-old mice). Other mice were negative at 4 weeks and became positive at 8 weeks, and some mice became IAA-positive in only their 12-week sample. Some individual mice showed large differences between IAA levels at 8 and 12 weeks of age. Only a few mice showed major decreases in IAA titer in their 12-week sample. Overall, the highest IAA levels were observed in 12-week samples ($P < 0.05$ for each laboratory). IAA at any time point were found in 60% (laboratory 1), 65% (laboratory 2), and 55% (laboratory 3) of NOD mice.

Correlation and concordance of IAA results between laboratories. IAA levels in NOD mice were highly correlated between laboratories 1 and 2 ($r = 0.77$) but much less so between laboratories 1 and 5, which used an ELISA for measurement ($r = 0.36$). For examination of concor-

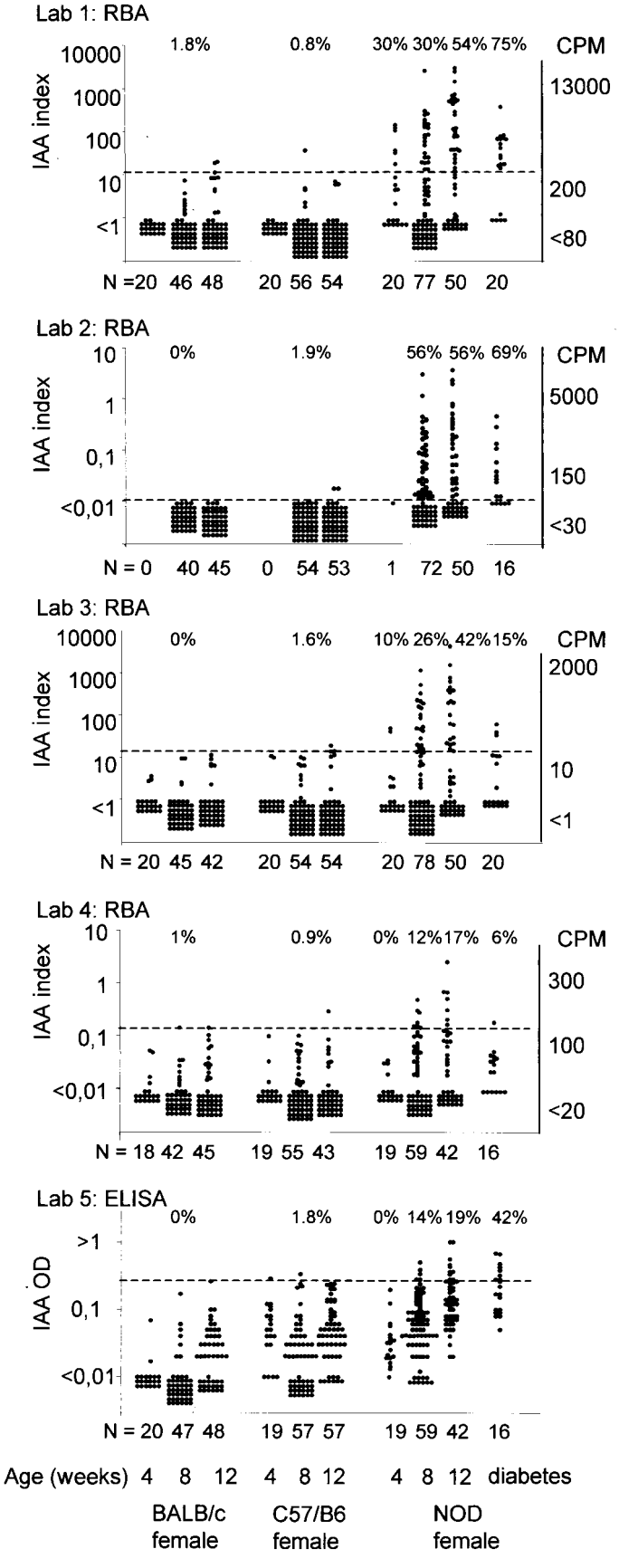

FIG. 1. IAA signals in sera included in the NOD autoantibody workshop. Antibody levels are shown for the control (BALB/c and C57/B6) mice, NOD mice at age 4, 8, and 12 weeks, and NOD mice at diabetes onset. The number of sera from each group tested is shown on the abscissa of each laboratory. Results are reported in the units reported by each of the four laboratories using RBA for measurement (Labs 1–4) and as OD for laboratory 5, which used ELISA for measurement. Approximate equivalent cpm in the RBA are shown on the right. The broken line indicates the 99th percentile of signals obtained in all control mice included in the workshop.

dance, IAA levels in NOD mice were expressed as scores of 0 (negative), 1 (doubtful), 2 (positive), and 3 (strong positive) corresponding to less than the 97th percentile of control mice samples, between the 97th and 99th percentiles of control sera, greater than the 99th percentile and less than 10 times the 99th percentile of control sera, and greater than 10 times the 99th percentile of control sera, respectively (Fig. 3). Of 151 NOD mouse sera tested in at least three laboratories, 51 (33%) were scored negative in all laboratories and 8 (5%) were scored positive or strong positive in all laboratories. The major discrepancies were due to laboratory 4, which had a very low sensitivity, and laboratory 5, which used ELISA. When only laboratories 1, 2, and 3 were examined, concordance was improved with 53 (35%) sera scored as negative and 42 (28%) scored as positive or strong positive in all laboratories. Discrepant sera among these three laboratories were often accounted for by positivity in a significant number of 8-week-old NOD mouse sera in laboratory 2 only and the decreased sensitivity in laboratory 3.

Autoantibodies to GAD and IA-2 in NOD versus control mice. Of the participating laboratories, three tested GADA and IA-2A using workshop sera (Figs. 4 and 5). Laboratories 1 and 2 used RBA with recombinant human GAD65 or IA-2, and laboratory 5 used an ELISA with recombinant mouse GAD65 and human IA-2. All laboratories observed increased signals in NOD mouse samples obtained at diabetes onset in comparison with control mouse samples, with sensitivities ranging from 29 to 50% for GADA and from 36 to 50% for IA-2A compared with 1% of control mice. Laboratories 1 and 5 also observed increased signals in 8- and 12-week-old NOD mice. Although binding for NOD mouse sera in the RBA was increased relative to control mice, it was extremely low when compared with binding levels commonly observed with human prediabetic and diabetic sera (data not shown). Signals obtained in NOD mice by laboratories 1 (RBA) and 5 (ELISA) were significantly correlated for both GADA ($r = 0.59$; $P < 0.001$) and IA-2A ($r = 0.6$; $P < 0.001$). A significant correlation between signals obtained for GADA and IA-2A was observed by laboratories 1 ($r = 0.57$; $P < 0.001$), 2 ($r = 0.57$; $P < 0.001$), and 5 ($r = 0.29$; $P < 0.01$). GADA signals did not correlate with IAA signals in all three laboratories (all $r < 0.1$). IA-2A signals were correlated with IAA signals in laboratory 5 ($r = 0.37$; $P < 0.001$) but not in laboratories 1 and 2 (both $r < 0.1$).

GADA and IA-2A signals are NOD colony dependent. As previously indicated, sera were provided by six different centers. The IAA, GADA, and IA-2A results obtained for laboratories 1 (RBA) and 5 (ELISA) relative to the center providing sera are shown in Fig. 6. For IAA, no differences in results between NOD colonies were observed. However, for both GADA and IA-2A, substantial differences were noted. Specifically, increased GADA or IA-2A signals were observed in only 2 of the 60 NOD mouse samples provided by center A, whereas sera from

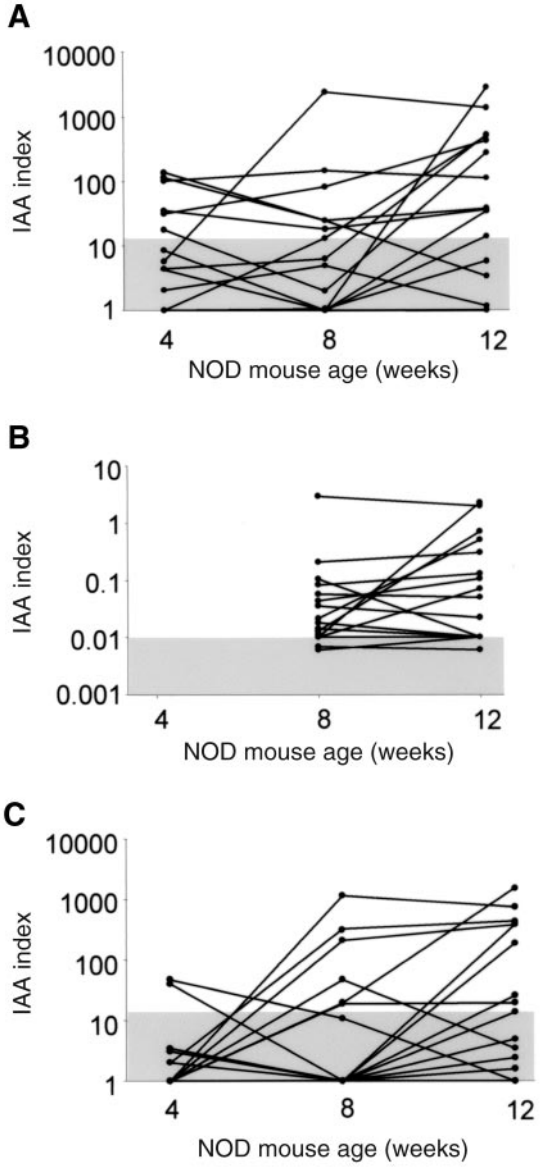

FIG. 2. IAA signals in sequential sera obtained at age 4, 8, and 12 weeks from individual NOD mice. Results are shown for laboratories 1 (*A*), 2 (*B*), and 3 (*C*), which represent those with the highest sensitivity. The shaded area represents the 99th percentile of signals obtained in all control mice included in the workshop.

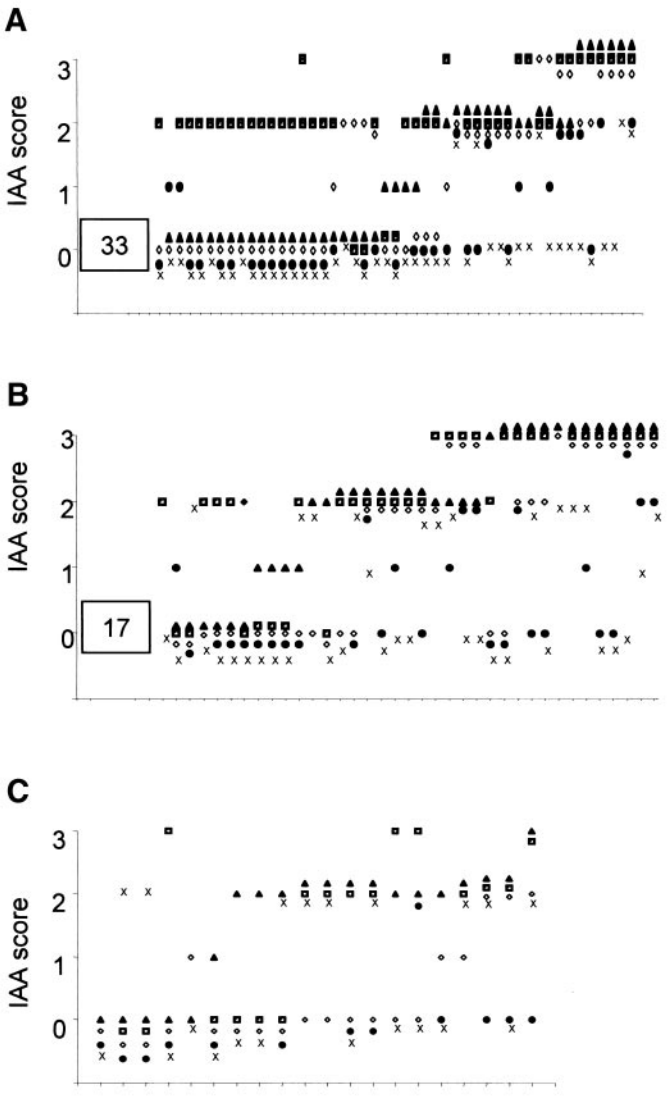

FIG. 3. Concordance between IAA measurements obtained in the five laboratories. IAA signals in 8-week-old (*A*), 12-week-old (*B*), and diabetic (*C*) NOD mice were scored as 0 (<97.5th percentile of signals obtained in control mice), 1 (>97.5th–99th percentile of signals obtained in control mice), 2 (>99th percentile to 10-fold of the 99th percentile of signals obtained in control mice), and 3 (>10-fold of the 99th percentile of signals obtained in control mice) for each laboratory. The score obtained for each laboratory is shown on the ordinate axis for individual NOD mouse sera shown along the abscissa. ▲, scores for laboratory 1; □, scores for laboratory 2; ◇, scores for laboratory 3; ●, scores for laboratory 4; X, scores for laboratory 5, which used ELISA. For 8- and 12-week-old mice, the number of sera with scores of 0 in all laboratories is shown in the boxes in the left-hand bottom corner.

diabetic NOD mice from centers D and E were often positive for both GAD and IA-2 antibodies.

GADA and IA-2A are not specific for diabetes. The observations that GADA and IA-2A signals were weak, that they correlated between RBA and ELISA, that they were colony-dependent, and that positive GADA and IA-2A signals were often observed in the same samples raised the question of whether the signals were due to specific autoantigen binding. As a first step, antibodies to the protein myelin oligodendrocyte glycoprotein (MOG), unrelated to type 1 diabetes, were determined in selected positive and negative NOD mouse sera using the same assay system used by laboratory 1. Antibody binding to MOG did not correlate with IAA ($r = -0.15$) but remarkably did correlate with both GADA ($r = 0.63$; $P < 0.01$) and IA-2A ($r = 0.77$; $P < 0.01$) signals measured by that laboratory. Next, the GADA assay was performed with and without competition with an excess of cold GAD65 (Fig. 7). Competition of binding in a serum from a mouse immunized with GAD65 was complete, whereas the binding from the unimmunized NOD mice was unaffected by competition with GAD65, suggesting that the binding was not antigen-specific. Binding in the IAA RBA was completely inhibited by competition with excess insulin.

DISCUSSION

A significant portion of our knowledge regarding the pathogenesis of type 1 diabetes has been derived from studies of animal models of the disease and in particular, the NOD mouse. However, one of the more disappointing features of studies involving NOD mice has been a relative

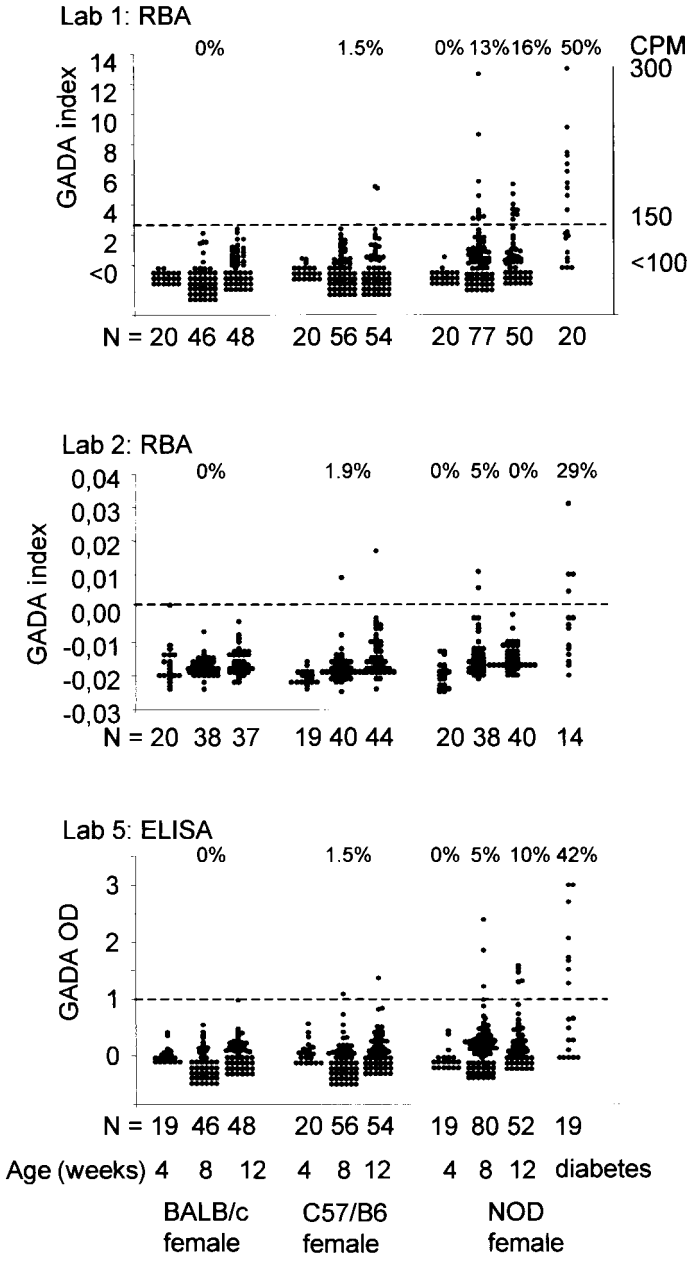

FIG. 4. GADA signals in sera included in the NOD autoantibody workshop. Antibody levels are shown for the control BALB/c and C57/B6 mice and for the NOD mice at age 4, 8, and 12 weeks and for NOD mice at diabetes onset. The number of sera from each group tested is shown on the abscissa of each laboratory. Results are reported in the units reported by laboratories 1 and 2, which used RBA for measurement, and as OD for laboratory 5, which used ELISA for measurement. Approximate equivalent cpm in the RBA assay is shown on the right for laboratory 1. The broken line indicates the 99th percentile of signals obtained in all control mice included in the workshop.

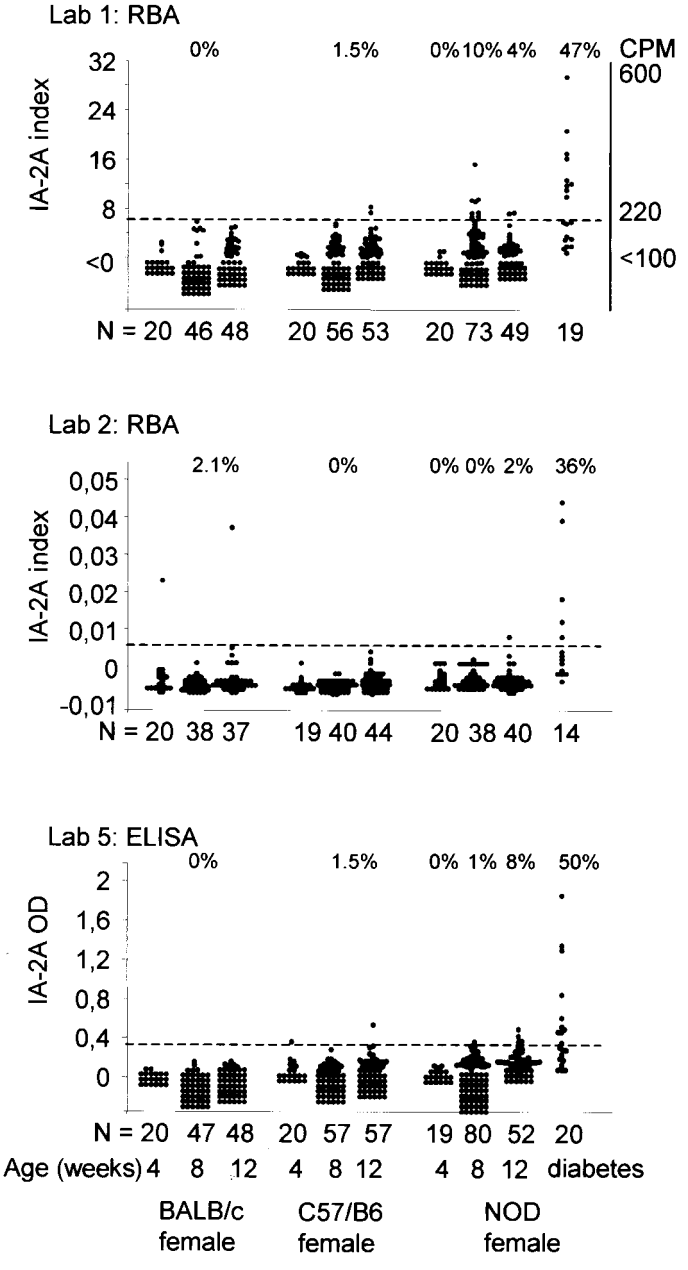

FIG. 5. IA-2A signals in sera included in the NOD autoantibody workshop. Antibody levels are shown for the control BALB/c and C57/B6 mice and for the NOD mice at age 4, 8, and 12 weeks and for NOD mice at diabetes onset. The number of sera from each group tested is shown on the abscissa of each laboratory. Results are reported in the units reported by laboratories 1 and 2, which used RBA for measurement, and as OD for laboratory 5, which used ELISA for measurement. Approximate equivalent cpm in the RBA is shown on the right for laboratory 1. The broken line indicates the 99th percentile of signals obtained in all control mice included in the workshop.

lack of consensus in terms of investigator agreement of target antigens of β-cell autoimmunity. Indeed, although much enthusiasm has been generated toward the potential pathogenic significance of immunities to the autoantigens insulin, GAD, and heat shock protein in NOD mice, significant controversies remain. The outcomes of this workshop should, in part, bring partial resolution to these questions and again, in part, work to resolve existing controversies.

The data presented gave unanimous support to the concept of the autoantigenic role for insulin molecule as a target of humoral autoimmunity in NOD mice. The ability of every laboratory to detect IAA lends support to this notion as do the facets of relative disease specificity (i.e., presence in NOD vs. non-NOD strains), good concordance in sample identity (again, despite significant differences in assay formats), and the observations of frequencies of IAA following those predicted for the natural history of diabetes. RBA for IAA in NOD mice seemed superior to ELISA

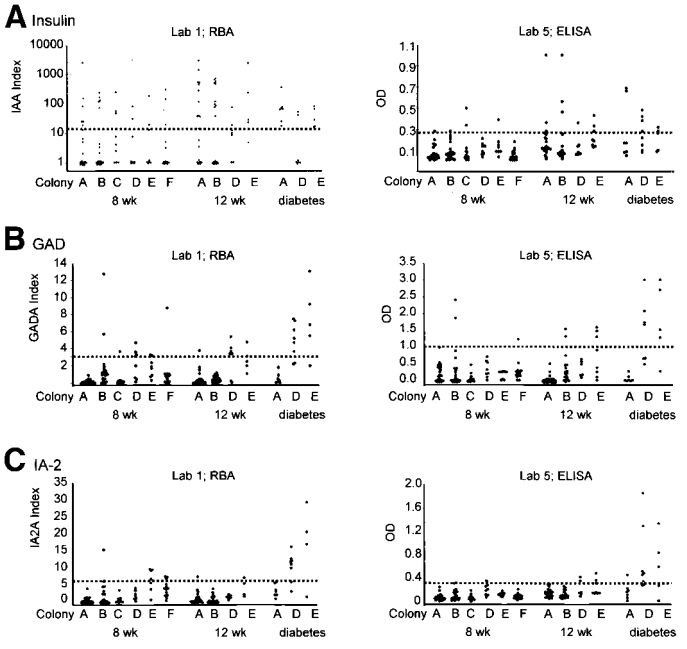

FIG. 6. GADA and IA-2A signals vary with respect to NOD colony. IAA (*A*), GADA (*B*), and IA-2A (*C*) signals in NOD mice are shown for laboratory 1 (RBA) and laboratory 5 (ELISA) according to where the NOD mice sera were obtained (colonies A–F). The 99th percentile of control mice sera is indicated by the broken line. No significant differences were seen between colonies for IAA, but for both GADA and IA-2A, sera obtained from colonies D and E had higher signals than those obtained from colony A.

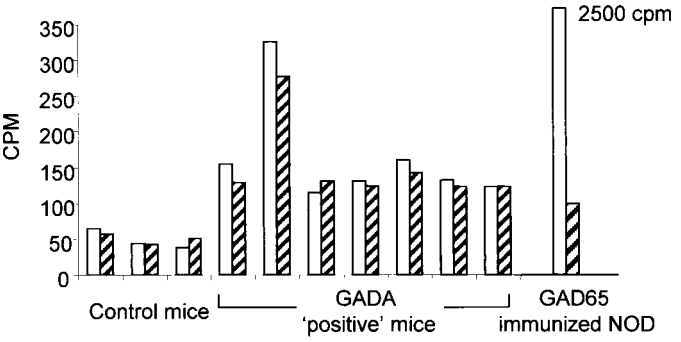

FIG. 7. Competition of GADA signals. Binding (cpm) to GAD in the RBA in laboratory 1 is shown in the absence (□, uninhibited) and presence (▨, inhibited) of 10 μg of GAD65. Binding in control BALB/c mice was ~50 cpm with no reduction by preincubation with GAD65. The binding obtained in sera from NOD mice with signals above the 99th percentile of control mice (GADA positive mice) could not be inhibited by preincubation with GAD65, indicating that GAD65 binding was not specific. As a positive control, binding in a serum from a GAD65-immunized NOD mouse was shown to be markedly inhibited by preincubation with GAD65.

when viewed as a function of sensitivity. Furthermore, unlike the RBA, the ELISA format provided results that were both age- and control strain–dependent and that were relatively discordant with those obtained by RBA. These findings comparing assay format for detecting IAA are consistent with those for detection involving human sera, where IAA by RBA represent a clearly superior choice over that of ELISA. Indeed, the ability of investigators even to detect these autoantibodies using NOD sera has become possible in large part only as a result of efforts involving the development of a microassay (22). Despite these opinions, the workshop studies clearly would have been aided by the inclusion of additional laboratories using ELISA formats for IAA detection, a factor that would have allowed for improved comparisons as were performed with the RBA.

As to the two other markers of anti-islet autoimmunity tested, GADA and IA-2A, the results of this workshop call into question their role as a target of humoral autoimmunity. Such a conclusion came only after a series of initial observations that gave some credence and potentially interesting values. Approximately half of diabetic NOD mice were found to have increased GADA and IA-2A signals compared with control mice by both ELISA and RBA. Although increased signals were also detected before diabetes onset, they were infrequent, suggesting that they were secondary to the appearance of IAA. However, a series of observations led us to question whether the increased GADA and IA-2A binding were specific for autoantigen in the first place. First, unlike the results obtained with IAA and unlike results in sera from humans with type 1 diabetes, binding in NOD mouse sera was extremely low (<2.5% of total antigen). Second, whereas IAA were consistently detected in all NOD mouse colonies, GADA and IA-2A elevations were determined to be colony-variant (e.g., observed in sera obtained from colonies D and E). Third, neither GADA nor IA-2A signals correlated with IAA signals but were significantly correlated with each other, and sera with elevated GADA almost always had elevated IA-2A. Testing sera against a fourth unrelated antigen (mouse MOG) showed elevated binding in the same sera with GADA and IA-2A, further suggesting nonspecific interactions with labeled antigen. Finally, attempts to compete binding to GAD autoantigen with excess cold GAD using a protocol that effectively competes 100% of the binding of sera from humans with type 1 diabetes and from GAD-immunized mice was ineffective in the NOD mouse sera, strongly suggesting that the weak elevations seen in the GADA and IA-2A were not specific binding of autoantibody. These observations call for a more critical assessment of data in which small quantitative differences between disease and control groups are found.

This workshop validated insulin as a major target of autoimmunity also in the NOD mouse model of autoimmune diabetes, identified sensitive RBA as the method of choice for IAA measurement in mice, and brought into question GAD and IA-2 as targets of humoral autoimmunity in the NOD mouse. By doing so, the workshop demonstrated similarities and differences between disease characteristics of the NOD mouse and human, in which insulin is also a major early target of humoral autoimmunity but high levels of GADA and IA-2A are detected in the majority of patients before and at diabetes onset. This and previous workshops in humans using large numbers of sera from control and disease-related cohorts tested in a blinded manner have now proved to be invaluable in validating antigens and assays in type 1 diabetes. The findings of these workshops should also provide important lessons for other autoimmune diseases. As to the question of future workshops, such efforts seem necessary and should focus on determination of IAA isotypes in NOD mice, the inclusion of more laboratories that use ELISA formats, and analysis of autoantibodies in BB rats.

ACKNOWLEDGMENTS

These studies were performed, in part, with funding provided by the Juvenile Diabetes Research Foundation International. The authors thank LiPing Yu, Clive Wasserfall, Tamir Ellis, Kerstin Koczwara, Heike Naserke, Vito Lampasona, Chantal Coehlho, and Shane Gellert for technical and scientific assistance in performing these assays.

REFERENCES

1. Dighiero G, Rose NR: Critical self-epitopes are key to the understanding of self-tolerance and autoimmunity. *Immunol Today* 20:423–428, 1999
2. Gottlieb PA, Eisenbarth GS: Diagnosis and treatment of pre-insulin dependent diabetes. *Annu Rev Med* 49:391–405, 1998
3. Rabinovich A, Skyler JS: Prevention of type 1 diabetes. *Med Clin North Am* 82:739–755, 1998
4. Pietropaolo M, Becker DJ: Type 1 diabetes intervention trials. *Pediatr Diabetes* 2:2–11, 2001
5. Gleichmann H, Bottazzo GF: Progress toward standardization of cytoplasmic islet cell-antibody assay. *Diabetes* 36:578–584, 1987
6. Bonifacio E, Lernmark Å, Dawkins RL: Serum exchange and use of dilutions have improved precision of measurement of islet cell antibodies. *J Immunol Methods* 106:83–88, 1988
7. Wilkin T, Schoenfeld SL, Diaz J-L, Kruse V, Bonifacio E, Palmer J: Systemic variation accounts and differences in insulin autoantibody measurements. *Diabetes* 38:172–181, 1989
8. Palmer JP, Wilkin TJ, Kurtz AB, Bonifacio E: The Third International Workshop on the Standardisation of Insulin Autoantibody Measurement. *Diabetologia* 33:60–61, 1990
9. Bonifacio E, Boitard C, Gleichmann H, Shattock MA, Bottazzo GF: Assessment of precision, concordance, specificity and sensitivity of islet cell antibody measurement in 41 assays. *Diabetologia* 33:731–736, 1990
10. Greenbaum CJ, Palmer JP, Kuglin B, Kolb H: Insulin autoantibodies measured by radioimmunoassay methodology are more related to insulin-dependent diabetes mellitus than those measured by enzyme-linked immunosorbent assay: results of the Fourth International Workshop on the Standardization of Insulin Autoantibody Measurement. *J Clin Endocrinol Metab* 74:1040–1044, 1992
11. Greenbaum CJ, Wilkin TJ, Palmer JP: Fifth International Serum Exchange Workshop for Insulin Autoantibody (IAA) Standardization: the immunology and diabetes workshops and participating laboratories. *Diabetologia* 35:798–800, 1992
12. Greenbaum CJ, Palmer JP, Nagataki S, Yamaguchi Y, Molenaar JL, Van Beers WA, MacLaren NK, Lernmark A: Improved specificity of ICA assays in the Fourth International Immunology of Diabetes Serum Exchange Workshop. *Diabetes* 41:1570–1574, 1992
13. Schmidli RS, Colman PG, Bonifacio E, Bottazzo GF, Harrison LC: High level of concordance between assays for glutamic acid decarboxylase antibodies: the First International Glutamic Acid Decarboxylase Antibody Workshop. *Diabetes* 43:1005–1009, 1994
14. Schmidli R, Colman PG, Bonifacio E: Disease sensitivity and specificity of fifty-two assays for glutamic acid decarboxylase antibodies: the Second International Glutamic Acid Decarboxylase Antibody Workshop. *Diabetes* 44:636–640, 1995
15. Verge CF, Stenger D, Bonifacio E, Colman PG, Pilcher C, Bingley PJ, Eisenbarth GS: Combined use of autoantibodies (IA-2 autoantibody, GAD autoantibody, insulin autoantibody, cytoplasmic islet cell antibodies) in type 1 diabetes: Combinatorial Islet Autoantibody Workshop. *Diabetes* 47:1857–1866, 1998
16. Atkinson MA, Leiter EH: The NOD mouse model of type 1 diabetes mellitus: too much of a good thing? *Nat Med* 5:601–604, 1999
17. Haskins K, Atkinson M: Predicting disease in animal models of type 1 diabetes: lessons for studies of humans? *J Autoimmun* 16:15–19, 2001
18. Naserke H, Dozio N, Ziegler AG, Bonifacio E: Comparison of a novel micro-assay for insulin autoantibodies with the conventional radiobinding assay. *Diabetologia* 41:681–683, 1998
19. Yu L, Robles DT, Abiru N, Kaur P, Rewers M, Kelemen K, Eisenbarth GS: Early expression of antiinsulin autoantibodies of humans and the NOD mouse: evidence for early determination of subsequent diabetes. *Proc Natl Acad Sci U S A* 97:1701–1706, 2000
20. Wasserfall C, Atkinson M, Jodoin E, Schatz D, She JX, Ellis TM: Glutamic acid decarboxylase and IA-2 autoantibodies in type 1 diabetes: comparing sample substrates for autoantibody determination. *Pediatr Diabetes* 1:10–16, 2000
21. Qin HY, Elliott JF, Lakey JRT, Rajotte RV, Singh B: Endogenous immune response to glutamic acid decarboxylase (GAD67) in NOD mice is modulated by adjuvant immunotherapy. *J Autoimmun* 11:591–602, 1998
22. Williams AJK, Bingley PJ, Bonifacio E, Palmer JP, Gale EAM: A novel microassay for insulin autoantibodies. *J Autoimmun* 10:473–478, 1997