Competitive Insulin Autoantibody Assay

Prospective Evaluation of Subjects at High Risk for Development of Type I Diabetes Mellitus

PNINA VARDI, SERGIO A. DIB, MARILYN TUTTLEMAN, JEANNE E. CONNELLY, MARTA GRINBERGS, ALBERT RADIZABEH, WILLIAM J. RILEY, NOEL K. MACLAREN, GEORGE S. EISENBARTH, AND J. STUART SOELDNER

SUMMARY

A quantitative fluid-phase radioassay for autoantibodies reacting with insulin (competitive insulin autoantibody assay, CIAA) was developed. The assay's features include *1*) use of a physiologic amount of ^{125}I-labeled insulin, *2*) parallel incubations with supraphysiologic cold insulin (competitive), and *3*) an incubation time of 7 days and a single-step multiple-wash polyethylene glycol separation. Mean ± SE CIAA levels in 50 controls were 8 ± 1.4 nU/ml (range −16–33.3). In 36 cytoplasmic islet cell antibody (ICA)-positive nondiabetic first-degree relatives of type I (insulin-dependent) patients <30 yr of age, CIAA levels exceeded the normal range in 20 (55.6%) of 36 (mean 86.8 ± 17.1 nU/ml). In 26 ICA-positive relatives >30 yr of age, only 5 (19.2%) of 26 exceeded the normal range (mean 26.1 ± 9.4 nU/ml); $P < .001$ compared with younger ICA-positive relatives). Six ICA-negative HLA-identical siblings of type I diabetic patients had normal CIAA levels (mean 3.6 ± 5.8 nU/ml), and only 2 of 13 ICA-negative identical twins discordant for diabetes (mean 15.4 ± 6.6 nU/ml) exceeded the normal range. Nine (50%) of 18 ICA-positive schoolchildren exceeded the normal range (mean 105.3 ± 36.7 nU/ml). Genetically susceptible subjects negative for CIAA (with only 3 exceptions) remained negative for CIAA on multiple determinations (3 conversions observed), and CIAA levels of positive subjects were relatively stable. Linear regression of the first CIAA level versus last (interval between sampling 1 mo to 10 yr) in genetically susceptible individuals showed a highly significant correlation ($r = .95$, $P < .001$). This simple radioimmunoassay appears to have requisite specificity and sensitivity to allow screening populations at risk to develop type I diabetes. *Diabetes* 36:1286–91, 1987

From the Research Division, the Joslin Diabetes Center, Department of Medicine, Brigham and Women's Hospital, New England Deaconess Hospital, Harvard Medical School, Boston, Massachusetts, and the University of Florida, Gainesville, Florida.

Address correspondence and reprint requests to J. Stuart Soeldner, MD, Joslin Diabetes Center, One Joslin Place, Boston, MA 02215.

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Much evidence indicates that type I (insulin-dependent) diabetes of the human, the BB rat, and the NOD mouse is secondary to autoimmune β-cell destruction. Several immunologic abnormalities and progressive loss of first-phase insulin secretion to intravenous glucose are reported to precede type I diabetes (1–8). Despite this information, considerable controversy exists as to whether type I diabetes develops in a chronic progressive manner or whether the prediabetic phase is marked by major fluctuations in islet autoimmunity (9–12). We have proposed that the reported marked fluctuations in autoimmunity may be secondary to the interassay variation of several of the assays employed or the use of assays with little disease specificity (10–12). To quantify at least one aspect of the autoimmunity preceding type I diabetes, after initial studies by Palmer et al. (13), we have developed a fluid-phase competitive insulin-autoantibody radioassay (CIAA) and used this assay to study genetically susceptible individuals at multiple time points.

MATERIALS AND METHODS

We assayed coded sera stored at −20°C. Fasting samples were used. Sera was obtained from patients of the Joslin Diabetes Center, the Framingham study, and the University of Florida, Gainesville. Data on subjects are summarized in Table 1. There was no change of CIAA levels with age of normal individuals, confirming other studies.

Genetically susceptible but ICA-negative individuals were studied, including 35 ICA-negative first-degree relatives of patients with type I diabetes. Also included were six ICA-negative HLA-identical siblings of type I diabetic patients discordant for type I diabetes and 13 ICA-negative identical twins discordant for type I diabetes.

In addition, several subjects at high risk for development of type I diabetes were evaluated for CIAA levels. We studied

TABLE 1
Demographic data of study groups

Group	*n*	Age (yr) Range	Mean ± SD	Median	Sex (F/M)
Normal	50	9–60	30.0 ± 11.8	33	23/27
Framingham study offspring controls	43	22–79	50.4 ± 13.5	56	24/19
ICA-negative first-degree relatives					
<30 yr old	29	10–22	15.4 ± 3.2	11	13/16
>30 yr old	6	30–53	37.7 ± 8.5	37	5/1
ICA-negative MZT	13	13–60	33.8 ± 15.5	35	7/6
ICA-negative HLA-identical siblings	6	0.7–35	17.5 ± 9.3	17	3/3
ICA-positive first-degree relatives					
<30 yr old	36	0.7–29.7	14 ± 6.9	15	19/17
>30 yr old	26	35–66	43.2 ± 8.5	51	17/9
ICA-positive schoolchildren	18	5.3–14.3	10.4 ± 2.7	10.1	8/10
New-onset type I diabetes	18	2–31.9	11.7 ± 7.4	14.9	10/8

ICA, islet cell antibody; MZT, monozygotic twins.

36 ICA-positive first-degree relatives of type I diabetic patients <30 yr of age and 26 >30 yr of age. The cutoff point at this age was chosen arbitrarily because initial CIAA results showed a greater tendency to be positive in younger subjects compared with older subjects. Sera from 18 newly diagnosed type I diabetic patients were studied before insulin therapy. Eighteen ICA-positive schoolchildren identified in the population-based study of Maclaren et al. (15) were also evaluated (Table 1).

Serum was tested for specific insulin binding with a highly modified method of Kurtz et al. (16) and Palmer et al. (13). Only fasting undiluted serum was assayed, because varying amounts of insulin in the sera competitively inhibit insulin binding. One hundred fifty microliters of serum with 50 μl of 0.04 M phosphate buffer (pH 7.5) containing 0.05% bovine serum albumin, 0.025% bovine γ-globulin, and 0.9% sodium chloride were incubated for at least 1 h at 4°C with another set of serum incubated with 50 μl of the same phosphate buffer containing Humulin (courtesy of Lilly, Indianapolis, IN) at a concentration of 9×10^6 nU/ml of buffer (equivalent to 3×10^6 nU/ml of serum). Two hundred microliters of gel-column–purified ^{125}I-labeled porcine insulin (sp act 140–200 μCi/μg; Cambridge Medical Diagnostic, Billerica, MA) and 7500 nU/ml of buffer (equivalent to 10,000 nU/ml of serum) diluted in the phosphosaline buffer were added, and the tubes were mixed and incubated at 4°C for 7 days. A constant amount of labeled insulin was added to each assay tube. Although ^{125}I–porcine and ^{125}I–human insulins resulted in equivalent binding in our assay (data not shown), we used ^{125}I–porcine insulin for convenience and lower expense. Each of the ^{125}I-insulins was tested repeatedly for immune integrity by binding to excess quantities of guinea pig anti-insulin sera. Specific binding at the end of the useful lifetime of multiple lots of ^{125}I-insulin ranged from 90 to 80% (60 days), and each lot was used for no longer than 42 days. After the second incubation, antibody-bound

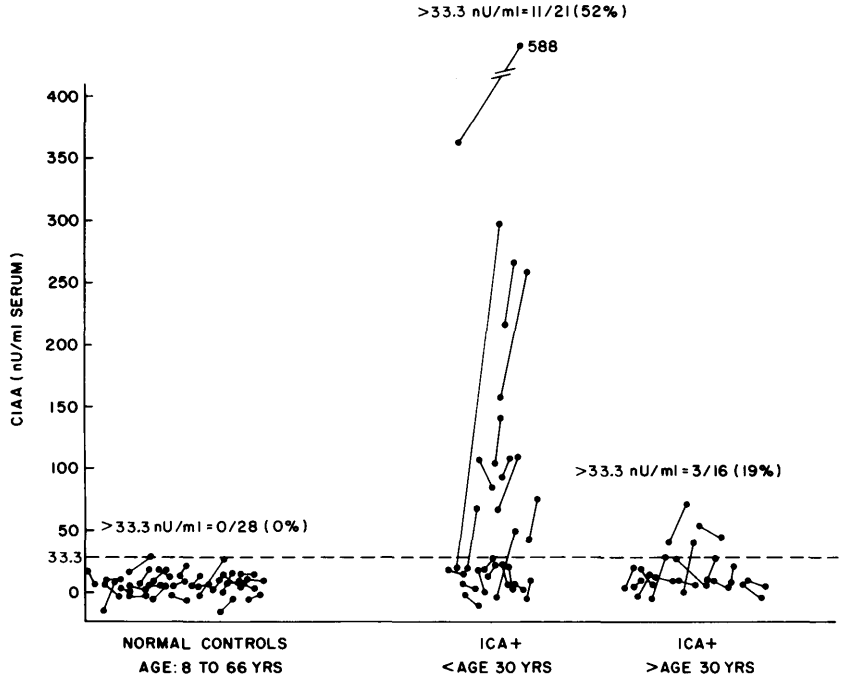

FIG. 1. Comparison of competitive insulin autoantibody radioassay (CIAA) levels (nU/ml) in normal and high-risk subjects for development of type I diabetes obtained with 3- and 7-day incubation of tracer with sera.

insulin was precipitated by adding 1.5 ml of ice-cold 14.3% polyethylene glycol prepared in 0.05 M Veronal buffer (Fisher Scientific, Boston, MA; pH 8.6) with 0.1% Tween 20 (Fisher Scientific), vortexing and centrifuging at 1200 relative centrifugal force for 30 min at 4°C. The supernatant was decanted into a clean tube for counting. The pellet was then washed twice with 1.5 ml 11.0% polyethylene glycol in 0.05 M Veronal buffer (pH 8.6) with 0.1% Tween 20, vortexed, and centrifuged. The second supernatant was decanted into the same tube as the first; the final supernatant was discarded.

The pellet and supernatants were counted for 9 min each. The background counts were subtracted, and the following equation was used to calculate the total insulin binding (TIB)

$$\frac{\text{cpm pellet}}{\text{cpm pellet} + \text{cpm supernatant (without unlabeled insulin)}} - \frac{\text{cpm pellet}}{\text{cpm pellet} + \text{cpm supernatant (with unlabeled insulin)}} \times \text{nU tracer/ml serum} = \text{TIB}$$

The difference in percent binding was translated to the absolute amount of labeled hormone displaced from the binding sites by the competition with the cold ligand. The amount of labeled hormone was left constant; thus the expression of the results as the absolute amount of the radioactive ligand displaced by the competition was not significantly different from expression of the results as specific percent binding. Negative CIAA levels were statistical computational products where the counts with cold displacement were slightly greater than counts in the absence of unlabeled insulin.

Statistical analysis. The insulin-binding capacities of all the groups were compared with those of the controls by χ^2-analysis and Fisher's exact test. The variation of CIAA levels in the sera of 28 ICA-positive subjects with more than one time point was calculated with regression analysis.

RESULTS

With a 36-h incubation, 8 of 21 cytoplasmic ICA-positive relatives of type I diabetic subjects <30 yr of age and 2 of 16 >30 yr of age were positive for CIAA, but 11 of 21 and 3 of 16 were found to be positive when a longer incubation time of 7 days was used. Twenty-eight normal control sera did not differ in their CIAA levels and remained negative with a 36-h or 7-day incubation period (Fig. 1). Based on these data, all subsequent studies used a 7-day assay.

Interassay and intra-assay variation was performed on sera showing high-positive, moderately positive, and negative (normal control) CIAA levels. For interassay coefficient of variation (C.V.) high-positive C.V. was 7.1% (mean 635 ± 57.9 nU/ml, n = 8, range 557.4–711.1); low-positive C.V. was 10.3% (mean 78.7 ± 8.1 nU/ml, n = 7, range 67.3–90.5); samples deemed negative had a C.V. of 23.3% (mean 5.8 ± 1.3 nU/ml, n = 7, range 3.8–7.3). For intra-assay variation the high-positive C.V. was 5.4% (mean 546.8 ± 29.6 nU/ml, n = 10, range 493.8–599); low-positive C.V. was 10.0% (mean 42.52 ± 4.3 nU/ml, n = 8, range 35.8–50.4). Values of CIAA in a negative normal control ranged from −6.3 to 4.6 nU/ml (mean ± SD, 1.45 ± 3.3).

The mean ± SE of CIAA levels of 50 controls was 8 ± 1.4 nU/m (range −16–33.3). None of the 43 subjects from the Framingham offspring study of nondiabetic individuals exceeded the normal range. They showed a mean of 7.3 ± 1.5 nU/ml (range −11.2–26.6). Of 29 first-degree relatives

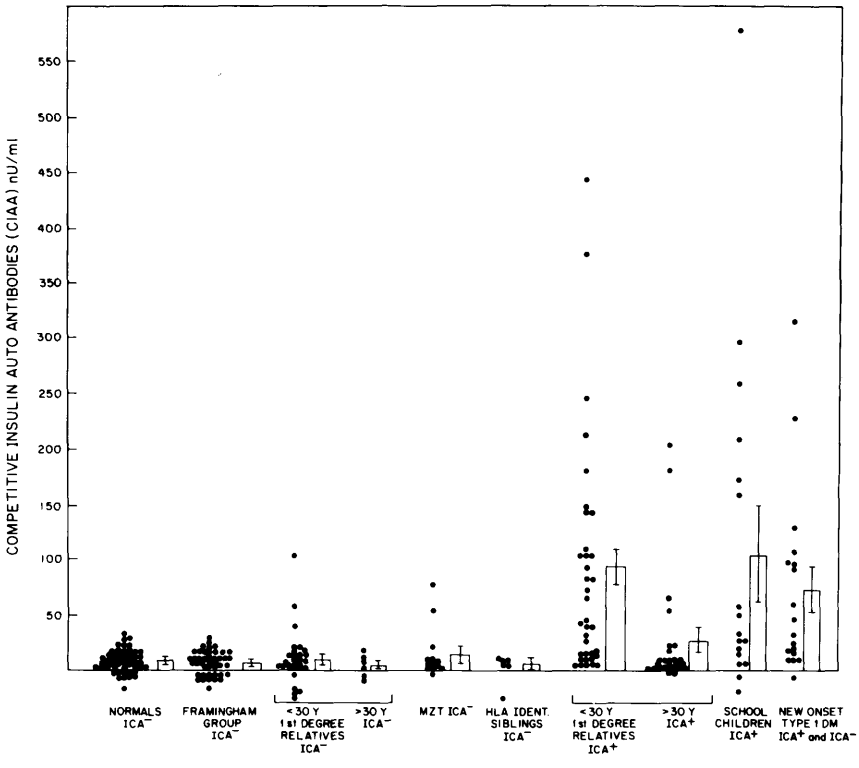

FIG. 2. CIAA levels (nU/ml) in different groups studied. *Bars* represent means ± SE.

TABLE 2
Competitive insulin autoantibodies in various groups studied

Group	*n*	Range (nU/ml)	Mean ± SE (nU/ml)	Percent positive (>33 nU/ml)
Normal subjects	50	−16.0–33.3	8.0 ± 1.4	0.0
Framingham controls	43	−11.2–26.6	7.3 ± 1.5	0.0
ICA-negative first-degree relatives				
<30 yr old	29	−23.0–110.0	10.9 ± 4.6	10.3
>30 yr old	6	−6.1–17.3	5.0 ± 3.9	0
ICA-negative MZT	13	−1.9–79.3	15.4 ± 6.6	15.3
ICA-negative HLA-identical siblings	6	−25.2–14.2	3.6 ± 5.8	0.0
ICA-positive first-degree relatives				
<30 yr old	36	3.5–444.0	86.8 ± 17.1	55 6*
>30 yr old	26	−2.3–204.0	26.8 ± 10.1	19.2*
ICA-positive schoolchildren	18	−34.0–582.0	105.3 ± 36.7	50.0†
New-onset type I diabetes	18	−4.2–228.0	73.3 ± 20.0	50.0†

ICA, islet cell antibody; MZT, monozygotic twins discordant for type I diabetes.
*$P < .001$, <30- vs. >30-yr-old subjects ($\chi^2 = 15.1$).
†$P < .0001$ compared with normal group, Fisher's exact test.

of patients with type I diabetes negative for ICA under age 30 yr, only 3 (10.3%) exceeded the normal range (mean 10.9 ± 4.6 nU/ml, range −23–110). None of 6 ICA-negative first-degree relatives >30 yr old exceeded the normal range (mean 5.0 ± 3.9 nU/ml, range −6.1–17.3). In 13 ICA-negative monozygotic twins and 6 ICA-negative siblings HLA identical to a proband with type I diabetes, CIAA levels exceeded the normal range in 2 (15.3%) of 13 and 0 of 6, respectively (Fig. 2). Although these individuals are genetically susceptible, most are unlikely to develop type I diabetes because they are ICA negative, discordant for diabetes >5 yr (twins), and have a normal first-phase insulin secretion on intravenous glucose tolerance test (IVGTT) (8). The 2 ICA-negative subjects with elevated levels of CIAA are from a set of identical triplets, and their first-phase insulin release has decreased from the 10th to the 1st percentile over only 10 mo of follow-up (data not shown).

In contrast to immunologically normal relatives, 25 (40.3%) of 62 ICA-positive first-degree relatives had elevated CIAA levels. When this group is divided into two subgroups, 36 subjects younger and 26 older than age 30 yr, CIAA levels averaged 86.8 ± 17.1 nU/ml (range 3.5–444 in the younger group) and 26.8 ± 10.1 nU/ml (range −2.3–204 in the older group). Twenty (55.6%) of 36 young subjects exceeded the normal range ($P < .001$), whereas only 5 (19.2%) of 26 relatives >30 yr of age were positive (Fig. 2). There was a significant difference between the results of CIAA levels in these age groups ($P < .001$).

Among 18 schoolchildren found to be ICA positive [detected in the population-based study of Maclaren et al. (15)], 9 (50%) showed high levels of CIAA ($P < .0001$ compared with controls) with a mean of 105.3 ± 36.7 and a range of −34–582 nU/ml (Fig. 2). Nine (50%) of 18 newly diagnosed type I diabetic patients before insulin therapy had CIAA levels that exceeded the normal range ($P < .0001$, mean 73.3 ± 20.0, range −4.2–228.0 nU/ml). Five (45%) of 11 of the ICA-positive newly diagnosed diabetic children were also positive for CIAA, whereas 4 (57%) of 7 ICA-negative newly diagnosed diabetic subjects had CIAA levels exceeding the normal range, and therefore insulin autoantibodies did not differ in newly diagnosed diabetic children relative to ICA status (Table 2; Fig. 2).

Fourteen monozygotic twins negative for ICA were tested for CIAA levels at two or more time points during follow-up (Fig. 3). Twelve (86%) of 14 were negative for CIAA at the first examination and remained so at follow-up. Two previously ICA-negative "twins" presented elevated levels of CIAA at the first examination, and at follow-up their level of CIAA remained slightly above the normal range (Fig. 3).

CIAA levels of 16 ICA-positive relatives of type I diabetic patients were evaluated serially. Ten subjects (63%) were positive for CIAA on initial examination (Fig. 4). CIAA levels were within the normal range in 6 subjects. One subject became highly and continuously CIAA positive after 7 yr of follow-up, coinciding with the time of detection of cytoplasmic ICAs in his sera (17). Five years later he developed overt type I diabetes. Two other subjects presented initially negative CIAA levels that exceeded the normal range on further examination. One of the subjects developed type I diabetes, whereas the other did not, but her first-phase insulin release was under the 1st percentile.

To assess the variation of CIAA levels at more than one time point, multiple sera samples were tested from 28 sub-

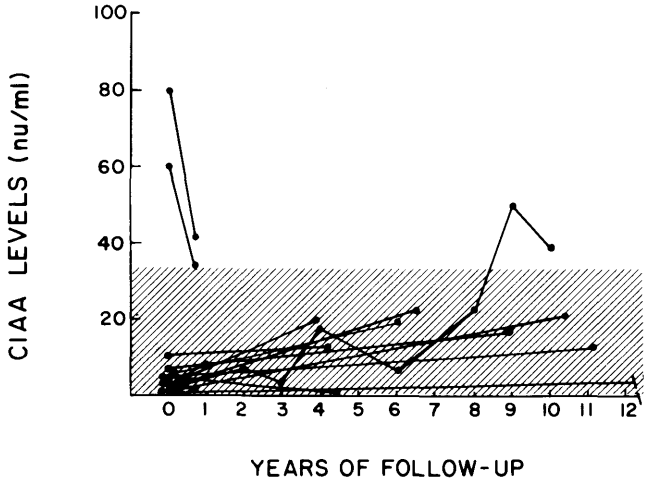

FIG. 3. CIAA levels tested at several time points during follow-up of ICA-negative monozygotic twins of patients with type I diabetes. Normal range is represented by *shaded area*.

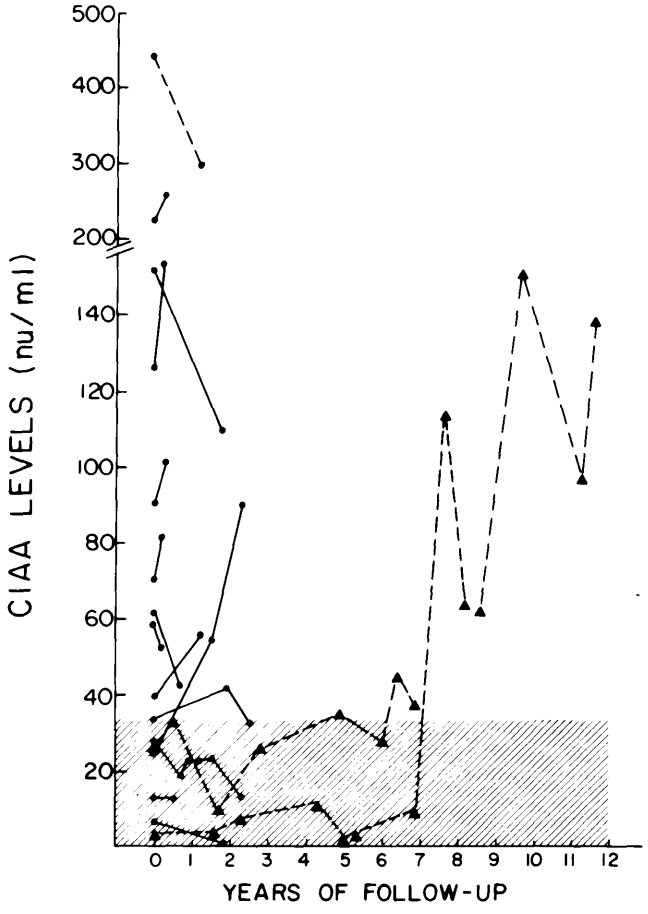

FIG. 4. CIAA levels (nU/ml) evaluated at >1 time point in ICA-positive first-degree relatives and identical twins of patients with type I diabetes. *Broken line* represents subjects that developed type I diabetes. *Shaded area* represents normal range.

jects genetically at risk of developing type I diabetes. The range of time between the first and last samples was 1 mo to 10 yr, with a mean follow-up between samples of 46.1 ± 8 mo. The correlation coefficient between first and last samples was .95 ($P < .001$; Fig. 5). The correlation was also significant when only patients shown in Fig. 4 with at least one positive value were evaluated ($n = 10$, $r = .97$, $P < .001$).

DISCUSSION

Prior studies have reported the occurrence of insulin autoantibodies in individuals with newly diagnosed diabetes before insulin therapy and in some nondiabetic identical twins with a type I diabetic twinmate (9–13,17–22). To date, fluid-phase anti-insulin antibody assays have detected as positive 50% (23) of ICA-positive individuals and ELISA assays are positive in as many as 8% of normal individuals (22) and 50% of ICA-negative long-term discordant monozygotic twins (9).

Our assay was designed to maximize sensitivity but remain a fluid-phase assay to maximize specificity. With this competitive method, we detected as positive for CIAA <1% of our normal controls and ~50% of newly diagnosed diabetic children and immunologically high-risk individuals. With our assay, most ICA-negative first-degree relatives and monozygotic twins, who are very unlikely to develop diabetes in contrast to ICA-positive relatives, were negative for CIAA. Approximately 55% of first-degree relatives with cytoplasmic ICAs and ICA-positive schoolchildren showed high CIAA levels, whereas only 19% of similarly ICA-positive adults exceeded the normal range. The finding of an inverse age relation to CIAA levels in our series is consistent with several reports that indicated that the highest levels of insulin autoantibodies are found in the youngest children (18–21).

Results of testing multiple sera samples during follow-up of high-risk populations indicate that negative subjects usually, but not always, remain negative for CIAA. ICA-positive first-degree relatives show limited fluctuation of CIAA levels during follow-up, and, as in the former group, most of the observed fluctuations are in the range of our inter- and intra-assay variation. Three subjects found to be initially negative became positive for CIAA. Two of these subjects developed overt diabetes, and another is not diabetic, but her first-phase insulin release on IVGTT is under the 1st percentile. Note that individuals positive for CIAA do not differ consistently over time, as indicated by the correlation coefficient of .95 comparing initial with final sera sample and by the data in Figs. 3 and 4, although some fluctuations in CIAA levels were found.

The constancy of CIAA levels we have observed contrasts with a report of insulin autoantibodies determined with a solid-phase ELISA assay (9). Regarding the different reading between radioimmunoassay and ELISA, it is apparent from initial exchange of sera in an international workshop that there is little correlation between what was detected by ELISA assays and radioimmunoassays. Further standardization and study are required to determine the utility of each assay format, but we believe that a major problem with ELISA assays for predicting development of diabetes relates to the high levels of insulin antibodies (indistinguishable from most

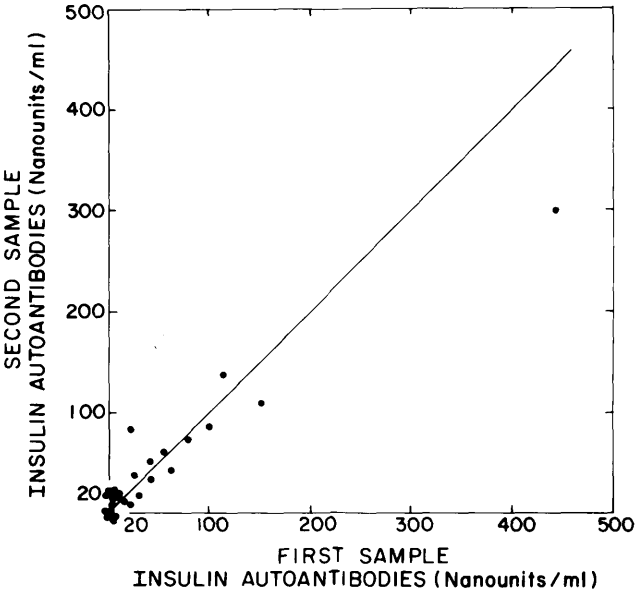

FIG. 5. Linear regression of CIAA levels in sera of 28 subjects genetically and/or immunologically at risk to develop type I diabetes. For each individual, first and final CIAA levels during prospective evaluation are plotted. $r = .95$. $P < .001$.

prediabetics) detected in as many as 8% (22) of control populations and 50% of ICA-negative long-term discordant twins (9).

We use a cytoplasmic ICA assay and our CIAA assay in screening first-degree relatives of patients with type I diabetes. Among children, each assay is positive in ~60% of newly diagnosed diabetic subjects. By combining both markers, 90% of the children are positive with one, the other, or both abnormalities. The consistency of anti-insulin autoantibodies over time, their prevalence in high-risk groups and low prevalence in normal controls, and their presence for years before diabetes onset all indicate that measurement of insulin autoantibodies should aid in identifying individuals at high risk to develop type I diabetes.

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