Rapid Publications

Anti-Insulin Activity in Normal Newborn Cord-Blood Serum

Absence of IgG-Mediated Insulin Binding

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Insulin autoantibodies (IAAs) are present in ~60% of type I diabetes patients at onset and are used as predictors for the disease. Although the prevalence of IAAs in the general population has been reported to be <1%, preliminary data have pointed out a higher proportion of IAA positivity in newborn cord-blood serum, and some authors have suggested that they are immunoglobulin G antibodies, resulting from a hypothetical gestational insulitis. To characterize this insulin-binding activity, we analyzed cord-blood sera from 100 healthy newborns, as well as serum from 21 of their mothers at delivery, 179 new-onset type I diabetic patients, and 200 healthy control subjects. IAAs were present in 0.5% of the control subjects and 54% of new-onset type I diabetic patients. On the other hand, 96% of the newborn cord-blood sera showed anti-insulin activity, while it was detected in only 14% of their mothers. No significant differences were observed between cord sera and the general population for islet-cell or anti-GAD autoantibodies. Anti-insulin activity in cord serum was not bound by protein A or protein G, in contrast with type I diabetes–related IAA activity. We conclude that this insulin-binding activity, present in most newborn cord sera and specific to the child, is not IgG mediated. These data, together with the absence of other pancreatic autoimmunity markers in this population, suggest that it is an isolated phenomenon not related to type I diabetes or other pancreatic autoimmune processes and is due to the presence of a cross-reacting molecule in cord blood that has yet to be identified. *Diabetes* 46:713–716, 1997

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GADAb, GAD_{65} autoantibody; IAA, insulin autoantibody; ICA, islet cell antibody; IDW, Immunology of Diabetes Workshop; IgG, immunoglobulin G.

Type I diabetes is an autoimmune disease caused by the destruction of pancreatic β-cells that is present in 0.3% of the general population and in 3–4% of first-degree relatives of type I diabetic patients (1,2). Pathogenic mechanisms are not yet defined, and little is known about the triggering event that initiates the autoimmune process. To clarify this issue, screening programs in high-risk individuals (first-degree relatives) and, more recently, in the general population have been carried out. Some of these studies include initial testing and follow-up of newborns from both healthy and diabetic mothers (3,4).

Insulin autoantibodies (IAAs) and other islet-cell molecules, including the 65-kDa isoform of GAD (GAD_{65}) and the tyrosine-phosphatase–like molecule IA-2, are detected during the preclinical period preceding diabetes onset and are currently used as predictors for the disease (1–3,5–8). Although the sensitivity of different IAA detection methods may vary, the majority of the radioassays routinely used in type I diabetes prediction are very specific in the detection of anti-insulin activity. IAAs are present in ~60% of new-onset diabetic patients, whereas only 1–3% of sera samples from healthy control subjects react to insulin (4,6,7), making it an especially good marker for young children. Nevertheless, several studies have shown that anti-insulin activity may also be detected among healthy newborns, generally disappearing during the first year of life. The nature of this activity has been controversial, and it has been suggested that antibodies may be responsible for this insulin-binding activity, indicating possible gestational insulitis or natural antibodies (8,9).

In this study, we observed that >90% of newborn children present anti-insulin activity. This activity is specific to the child, but is not immunoglobulin G (IgG)-mediated, suggesting a possible cross-reaction of the assay antigen with some insulin or insulin-like binding protein still to be identified.

RESEARCH DESIGN AND METHODS

Sample data. Blood samples from 100 newborn infants born to mothers with no history of type I diabetes were collected from umbilical-cord blood vessels at the time of birth, centrifuged to separate the serum, and stored at –80°C until assayed. Serum samples from 21 of the children's mothers were also collected at the time of delivery and processed in the same manner. For comparison with the newborn group, 200 healthy control subjects with no family history of type I diabetes (mean age, 21.4 years; range, 1–64 years) and 184

TABLE 1
Antibody results in the different population groups

	n	IAA values	IAA positives	ICA positives	GADAb positives
Control subjects	200	11.6 ± 8.25	0.5	2.5	0
New-onset type I diabetic patients	179	422.6 ± 912.60	54.2*	68.4*	74.6*
Newborns	100	140.5 ± 97.19	96.0*	3.0	1.0

Data are means ± SD or %. *$P < 0.01$, compared with the control subjects (χ^2 test).

diabetes-related patients (179 new-onset type I diabetic patients, 3 first-degree relatives with IAAs, and 2 long-term type I diabetic patients with secondary antibodies to therapeutic insulin) were also analyzed.

Analytical methods

Insulin autoantibodies. IAAs were determined using a competitive fluid-phase radioimmunoassay (7) with 150 µl of serum and human A-14 monoiodinated insulin (Amersham), consisting of duplicate determinations with and without competition with unlabeled insulin, 1-week incubation at 4°C, and precipitation of immune complexes with polyethylene-glycol. The data are expressed in nanounits per milliliter. Our assay generally gives 100% sensitivity and specificity in the Immunology of Diabetes Workshop (IDW) IAA proficiency testing. The cutoff for the assay, based on the 99th percentile of the normal population, is 40 nU/ml.

GAD_{65} autoantibodies. GAD_{65} autoantibodies (GADAbs) were measured using a standard radioassay (10), consisting in overnight incubation of the samples (duplicate 2.5-µl serum samples) with in vitro translated recombinant human GAD_{65} labeled with ^{35}S-methionine and immunoprecipitation with protein A–agarose. The results are expressed as an index (index = sample cpm – negative control cpm/positive control cpm – negative control cpm). In the second IDW GADAb Proficiency Test, this assay obtained 100% sensitivity and specificity. The cutoff for this assay, based on the mean ± 3 SD of our control population (–0.04 ± 0.04), is an index of 0.08.

Islet cell antibodies. Islet-cell antibodies (ICAs) were detected using an indirect immunofluorescence assay on unfixed, snap-frozen, human blood-group O pancreas sections, using fluorescein-conjugated rabbit anti-human IgG (11). The detection threshold of our ICA assay is 10 Juvenile Diabetes Foundation units (JDF U) with a specificity ranging from 75 to 100% and a sensitivity of 75% in different ICA Proficiency Tests, performed from 1992 to 1996.

Separation of IgG. For anti-insulin activity characterization, serum samples from a subset of 12 IAA-positive newborns, 10 diabetes-related subjects (5 IAA-positive new-onset type I diabetic patients, 3 IAA-positive first-degree relatives, and 2 long-term type I diabetic patients with antibodies secondary to insulin therapy), and 1 IAA-negative healthy control subject were subjected to an IgG separation step before insulin-antibody radioassay. Briefly, samples were mixed with 1 volume of a 50% suspension of either protein A– or protein G–agarose in 0.1 mol/l Tris-HCl (pH 8.0) and incubated for 2 h at 4°C on a rocking platform. The slurry was washed with four volumes of 0.1 mol/l Tris-HCl (pH 8.0) and two volumes of 10 mmol/l Tris-HCl (pH 8.0) before elution with 1 ml of 0.1 mol/l glycine (pH 3.0). Eluates were collected in tubes with 100 µl of 0.1 mol/l Tris-HCl (pH 8.0), freeze-dried, and resuspended in sterile water to their original volume. This fraction was designated as the IgG fraction. Washes were pooled and freeze dried before being resuspended in the same way and were designated as the wash fraction.

Statistics. Data are expressed as means ± SD. Frequency differences among groups were evaluated using the χ^2 test. All tests were two-tailed, and significance was considered for $P < 0.05$.

RESULTS

Antibody results are shown in Table 1. Among the healthy control subjects, 1/200 (0.5%) was positive for IAAs (Fig. 1), none were GADAb positive, and 5/200 (2.5%) were ICA positive. In the new-onset type I diabetic group, 54.2, 68.4, and 74.6% of the samples were positive for IAAs, ICAs, and GADAbs, respectively. When the type I diabetic population was subdivided into age-groups, differences of IAA distribution could be observed, being most significant for children <10 years of age (90% positive) and adults >30 years of age (37.5%) (Table 2), and no significant age distributions were observed for ICAs or GADAbs. Surprisingly, 96/100 cord sera from healthy newborns (96%) were positive for IAAs (Fig. 1), but only 3 and 1% were positive for ICAs and GADAbs, respectively. When considered as an independent population, insulin-binding activity values for newborns were 140.5 ± 97.19 nU/ml, and only one sample (1%) exceeded the mean ± 3 SD. Only 3 of the 21 mothers showed anti-insulin activity without GADAbs or ICAs, being concordant with their respective child.

Comparison among groups showed significant differences between the control subjects and new-onset type I diabetes patients for all antibodies, but only for IAAs when cord-blood sera from newborns and control subjects were compared (Table 1).

IAAs in serum fractions. Newborn cord-blood serum samples with high insulin-binding activity, including the sample over the mean ± 3 SD of the group, were selected for characterization. All of the IgG fractions from these sera (n = 12)

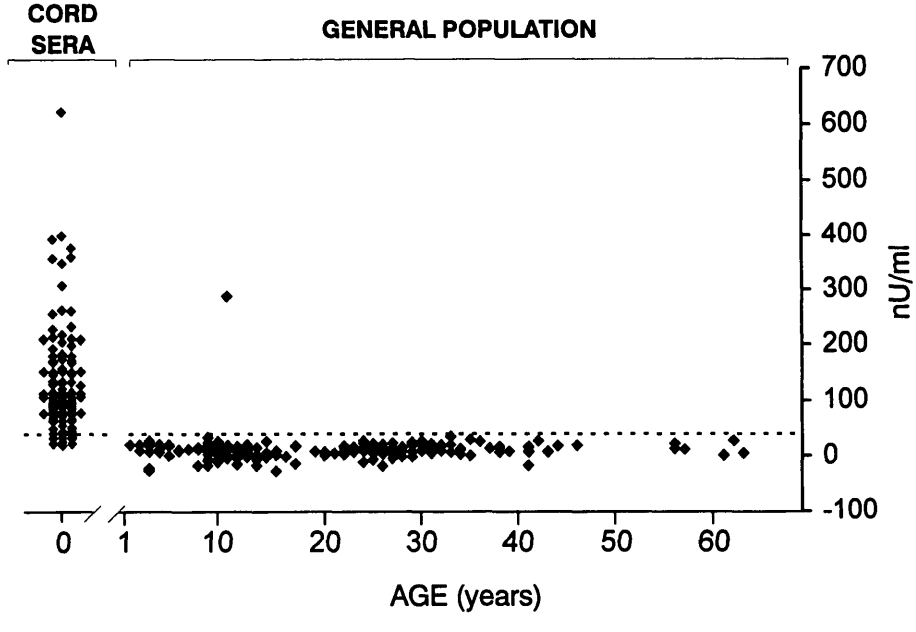

FIG. 1. Age distribution of anti-insulin activity among healthy individuals. Dashed line represents the cutoff value (40 nU/ml).

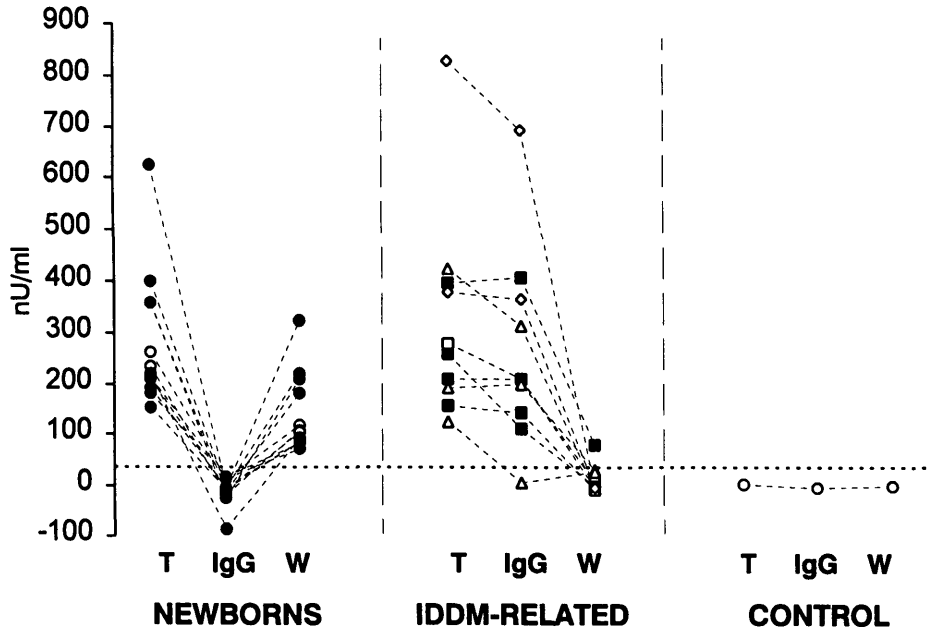

FIG. 2. Insulin-binding activity in serum fractions detected by IAA assay. Dashed line represents the cutoff value (40 nU/ml). Open and filled symbols represent purifications with protein A– and protein G–agarose, respectively. □, type I diabetic patients at onset; △, first-degree relatives of individuals with type I diabetes; ◇, long-term type I diabetic patients. T, whole serum; IgG, immunoglobulin G fraction; W, wash fraction.

were negative for IAAs, and the activity remained in the wash fraction (Fig. 2). No differences were observed in relation to original IAA titers. No activity was observed in the normal control subjects, and for the diabetes-related subjects (n = 10), the insulin-binding activity was concentrated in the IgG fraction. In one of the samples, some binding still remained in the wash fraction, probably due to incomplete washing of the resin. Activity was completely lost in the sample that had the lowest initial IAA titer, possibly due to the prolonged acid treatment of the eluate (Fig. 2). No differences were observed when the purifications were carried out with either protein-A or protein-G (Fig. 2). Serum from the healthy control subject who showed significant insulin-binding activity in the initial screening (Fig. 1) was also subjected to the purification procedure, and all activity remained in the IgG fraction.

DISCUSSION

Confirming previous reports, our data show that IAAs are present in the majority of type I diabetic patients at diagnosis, being a very specific marker for type I diabetes at a young age, with the exception of newborn children. In this large group, we observed a high proportion of IAA positivity (96%) in cord-blood sera. These results agree with previous reports that suggest the presence of anti-insulin activity in this population (8,9). Differences in assay sensitivity, especially for the detection of low-medium positive values, could account for the higher proportion of positive samples in our study. Additionally, the activity is specific to the child and is rarely found among their mothers. The nature of insulin binding in newborn cord serum has been very controversial, and we have made an attempt to characterize it. The purification of IgG from diabetic and newborn sera shows molecular differences between these two insulin-binding activities, which is mostly immunoglobulin among diabetes-related patients but clearly non-IgG in the newborns, in contrast with the preliminary data reported by other groups (9). On the other hand, no other diabetes-related autoantibody was detected in significant proportion, indicating that this anti-insulin activity is an isolated event.

Our data suggest that this activity is probably due to cross-reacting molecules that bind insulin and are present at elevated concentrations in cord blood, based on the fact of non-IgG implication, and the absence of other diabetes-related autoantibodies, thus probably not being due to a real immune response to pancreatic antigens (related to type I diabetes or insulitis). Further investigations are needed to purify and characterize this non-IgG insulin-binding molecule, which could distort IAA evaluation in general population follow-up studies that begin at birth.

TABLE 2
Age distribution of IAA positivity among new-onset type I diabetic subjects

Age-group	n	IAA positives
<10 years	31	90.0*
10–20 years	58	58.6*
20–30 years	42	40.5*
>30 years	48	37.5*

Data are n or %. *$P < 0.01$ (χ^2, 3 df).

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