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Identification of insulin autoantibodies of IgA isotype that preferentially target non-human insulin

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Abstract Insulin autoantibodies (IAA) precede clinical type 1 diabetes in children. Immunization events leading to IAA are unknown. The aim of this study was to determine whether some IAA result from mucosal immunization. IgA-IAA and binding of IAA to non-human insulin were examined in selected high and low affinity IAA-positive samples and in first IAA-positive samples from children aged <2 years. High affinity IAA ($>10^9$ L/mol) bound strongly to human insulin and poorly to chicken insulin. In contrast, 12/13 lower affinity IAA were chicken insulin-reactive, binding equally to human and chicken insulin ($n=4$), or preferentially binding chicken insulin ($n=8$). IgA-IAA were found in association with chicken insulin-reactive IAA, and included cases where IgA-IAA predominated over IgG-IAA. Among 20 IAA-positive children aged <2 years, one had early IgA-chicken insulin-reactive IAA that were replaced by high affinity IgG-IAA. The findings suggest that some IAA can result from immunization against molecules other than human insulin at mucosal sites.

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Introduction

Type 1 diabetes is a chronic autoimmune disease caused by a selective inflammatory destruction of the insulin-producing

beta cells in the pancreatic islets of Langerhans [1]. Autoantibodies against islet beta cell antigens can appear early in life and predict the development of type 1 diabetes [1–5]. Autoantibodies against insulin (IAA) are often the first autoantibodies that are detected in childhood. Some, but not all children who have IAA also develop antibodies to other beta cell antigens, and subsequently develop type 1 diabetes [2,3,5,6]. IAA in children who progress to clinical diabetes are usually of high affinity and react with proinsulin [7].

The initiation of the IAA response at an early age has been postulated to be affected by environmental factors, including dietary constituents entering the gut [8–13]. A gut mucosal site of immunization is feasible since there is preferential trafficking of immune cells and antigens from

Abbreviations: aab, autoantibodies; cpm, counts per minute; GAD, glutamic acid decarboxylase; GADA, autoantibodies to glutamic acid decarboxylase; IA-2, insulinoma antigen 2; IA-2A, autoantibodies to insulinoma antigen 2; IAA, autoantibodies to insulin; IC50, 50% inhibitory capacity; T1D, type 1 diabetes

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the gastrointestinal tract to the pancreatic lymph nodes [14]. One hypothesis proposes dietary bovine insulin from cow's milk as the primary immunizing antigen giving rise to IAA that are reactive with human insulin [10–13]. Evidence to support this and similar hypotheses would be provided by finding IAA that preferentially bind to insulin from non-human species or to dietary proteins. A mucosal origin for the IAA would be further indicated if IAA included IgA isotype antibodies. IgA antibodies are synthesized on inner epithelial surfaces including the gut. Their primary role is to protect epithelial surfaces from infectious pathogens, including agents entering the gastrointestinal tract via ingested food.

In the course of our studies, we examined reactivity of IAA to insulin from various species [7] and have been able to identify IAA with distinct species reactivity patterns ranging from IAA that are human insulin-specific to IAA with broad species promiscuity. Some of these IAA had suboptimal affinity to human insulin and therefore could potentially target other molecules. Using these sera, we here asked whether we could find evidence for mucosal immunity to insulin. We first examined whether children had IAA that preferentially bound a non-human insulin, and second whether IAA-positive children had IgA-IAA. The findings suggest that some IAA could arise from mucosal immunization.

Materials and methods

Study cohorts, subjects and samples

The BABYDIAB Study prospectively follows children from mothers and/or fathers with type 1 diabetes. From 1989 to 2000, 1650 children were included with blood drawing and questionnaires at 9 month, 2, 5, 8, 11, 14 and 17 years of life [2,6]. The BABYDIET Study is an ongoing study started in 2000, where newborn offspring from families with type 1 diabetes are recruited and followed 3-monthly from the age of 3 months [15]. Since 1989, the Munich Family Study follows first degree relatives of patients with type 1 diabetes for the development of islet autoantibodies [16]. In all studies, autoantibodies to insulin (IAA), glutamic acid decarboxylase (GADA) and insulinoma antigen 2 (IA-2A) are measured on all serum samples obtained during follow-up, and individuals are followed for diabetes development.

Sera from 44 IAA-positive children were selected for this study. These included selected sera from 24 children in whom IAA affinity was previously determined and ranged from $<10^6$ to $>10^{11}$ L/mol. Thirteen children were selected who had low affinity IAA ($<10^9$ L/mol). These had a median age of 5.2 years at their first IAA-positive sample. One of these 13 children developed other islet autoantibodies and none developed diabetes on follow-up. Eleven children were selected with high affinity IAA ($>10^9$ L/mol). These had a median age of 1.0 year at their first IAA-positive sample. Ten of these 11 children developed other islet autoantibodies and 4 developed diabetes on follow-up. Sera from a further 20 children who developed IAA before age 2 years were also tested in this study. All children in this study were positive for IAA on at least two occasions. As controls for the IgA-IAA measurements, samples from school children of the general population were used (median age 11.3 years; range 10.4 to 13.8 years).

IAA affinity and chicken insulin reactivity analysis

IAA affinity was measured by competitive inhibition using a protein A/G radio-binding assay as previously described [7]. Antibody binding to 7.85 fmol of human Tyr¹⁴A [¹²⁵I] insulin (Sanofi-Aventis, Frankfurt, Germany; specific activity 13.43 GBq/mg; 0.128 nmol/l) was competed with five increasing concentrations of unlabeled human insulin (Insulin Rapid, Sanofi-Aventis; 5.6×10^{-6} , 2.8×10^{-7} , 2.8×10^{-8} , 1.7×10^{-9} , 5.5×10^{-11} mol/l).

Chicken insulin was chosen for comparative competition because it contained potentially informative amino acid differences in the A chain region and because our preliminary unpublished findings suggested that some IAA bound strongly to chicken insulin. In order to determine the IAA reactivity with chicken insulin, antibody binding to [¹²⁵I] human insulin was competed with six increasing concentrations of chicken insulin (kindly provided by Dr. Louis Castano, Bilbao, Spain; 0.5, 1.8, 7.3, 29.1, 116.4, 465.5 and 1861.8 ng/ml).

Measurements were performed in a blinded fashion on coded sera and in a duplicate. Serum (5 µl per tube) was used for each concentration of competing insulin within a total volume of 55 µl. The concentration of cold insulin required for 50% inhibition of binding to human [¹²⁵I] insulin (IC₅₀) and K_d values were calculated using the GraphPad Prism3 program (GraphPad Software, San Diego, CA, USA), and IAA affinity was expressed as reciprocal K_d values (L/mol) as previously described [7]. The relative capacity of human and chicken insulins to inhibit IAA binding was expressed as the ratio IC₅₀_{human insulin}/IC₅₀_{chicken insulin}. Ratios below 1 indicated that IAA preferentially bound human insulin whereas ratios above 1 indicated that IAA preferentially bound chicken insulin.

IgA-IAA measurement

The previously described protein A/G radio-binding assay for the measurement of IgG-IAA [2,17] was used with modification. Serum (5 µl) was incubated with 7.85 fmol of human Tyr¹⁴A [¹²⁵I] insulin (Aventis, Frankfurt, Germany; specific activity 13.43 GBq/mg) in a total volume of 30 µl for 3 days. All IgG-IAA were captured by the addition of 7 µl GammaBind Plus Sepharose (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) in a total volume of 75 µl of assay buffer for 1 h at 4 °C with shaking. Following centrifugation, 60 µl supernatant was carefully removed and transferred to a new tube. Then, 10 µl Anti-human IgA-Agarose (Sigma-Aldrich, St. Louis, MO, USA) re-suspended in 40 µl assay buffer was added, and tubes were incubated for one additional hour at 4 °C with shaking. Finally, all tubes (GammaBind plus Sepharose for IgG-IAA and Anti-human IgA-Agarose for IgA-IAA) were washed 5 times in ice cold 50 mM Tris-HCl pH 8.0 1% Tween 20 to remove the unbound human [¹²⁵I] insulin and the pellets counted for 10 min (γ-counter Cobra II, Packard, Meriden, CT, USA). Results for IgG-IAA within the tubes with the GammaBind Sepharose pellet and for IgA-IAA within the tubes with the IgA-Agarose pellet were expressed as counts per minute (cpm).

Statistical analysis

Spearman's correlation was used to determine the correlation between relative binding of IAA to chicken insulin and

IAA affinity, and the Mann–Whitney *U* test was used to compare IAA affinities between groups. For all analyses, a two-tailed *P* value of 0.05 was considered significant. Statistical analyses were performed using the Statistical Package for Social Science (SPSS 13.0, Chicago, IL, USA).

Results

Chicken insulin reactivity of human IAA

The binding of IAA to chicken insulin relative to human insulin differed between IAA-positive samples (Fig. 1). In some samples IAA binding to human [¹²⁵I] insulin was displaced by considerably smaller amounts of cold human insulin than chicken insulin indicating low cross-reactivity to chicken insulin (Fig. 1A; displacement with chicken insulin shifted to right). In other samples, binding to human [¹²⁵I] insulin was displaced by smaller amounts of chicken insulin than human insulin indicating that chicken insulin was the preferred

ligand (Fig. 1B, displacement with chicken insulin shifted to left).

The displacement of binding to human [¹²⁵I] insulin by chicken and human insulin was expressed as an IC₅₀ (Fig. 1C), and the relative displacement by human insulin relative to chicken insulin was expressed as a ratio of IC₅₀_{human}/IC₅₀_{chicken} (Fig. 1D).

Samples from 24 selected IAA-positive children were analyzed for their reactivity to chicken insulin. The relative binding to chicken insulin as compared to human insulin varied 1750-fold in these samples. Twelve samples preferentially bound human insulin and required 3- to 70-fold less human insulin than chicken insulin for 50% inhibition of IAA binding to human [¹²⁵I] insulin (median IC₅₀_{human}/IC₅₀_{chicken} ratio, 0.07; range, 0.014–0.33; Figs. 1C and D, open circles). Four samples bound human and chicken insulin equally well, having an IC₅₀ for chicken insulin that was the same as the IC₅₀ for human insulin (Figs. 1C and D, filled squares). Remarkably, eight samples preferentially bound chicken insulin and required 4- to 25-fold more human insulin than

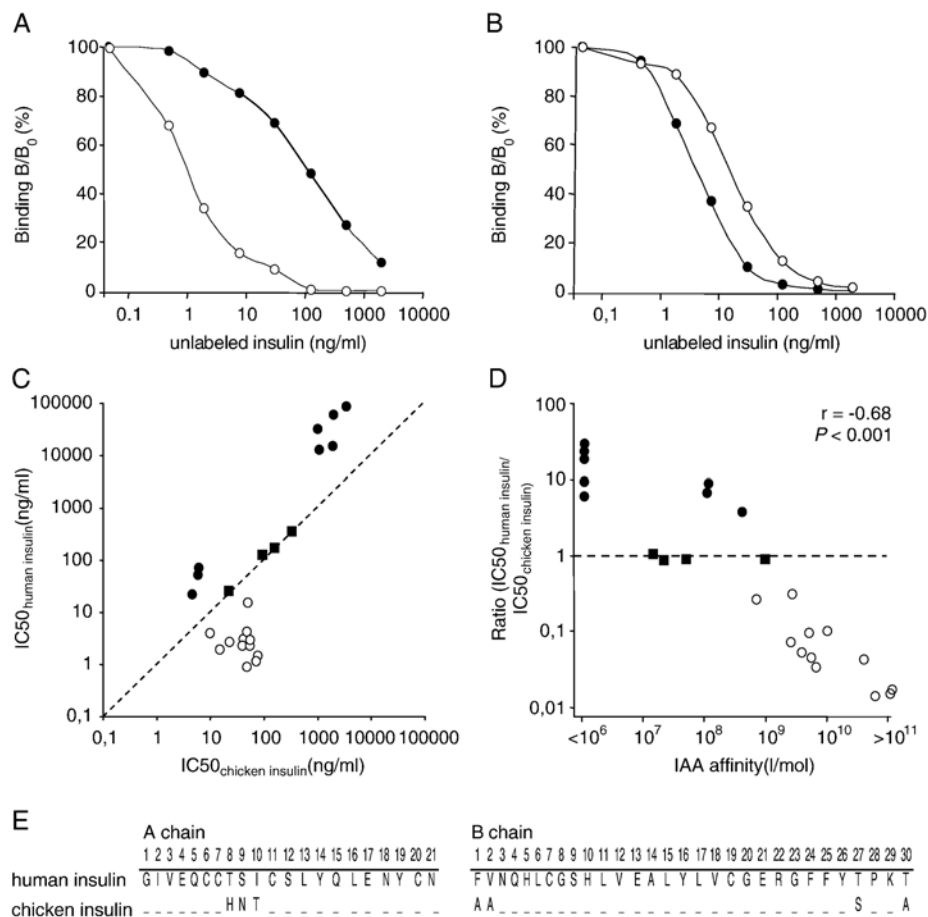


Figure 1 Representative examples of competition curves of (A) IAA with high affinity that preferentially bind to human insulin (open circles) than to chicken insulin (filled circles) and (B) IAA that preferentially bind to chicken insulin (filled circles) than to human insulin (open circles). (C) Relative displacement of IAA from human [¹²⁵I] insulin by chicken (x axis) and human (y axis) insulin. The capacity to displace the radiolabeled human insulin is expressed as the IC₅₀ (concentration of competitor required to reduce binding by 50%). From 24 selected samples, 12 samples preferentially bound human insulin (open circles), 4 samples bound human and chicken insulin equally well (filled squares) and 8 samples preferentially bound chicken insulin (filled circles). (D) Negative correlation between the relative IAA binding to human and chicken insulin (ratio of IC₅₀_{human insulin} to IC₅₀_{chicken insulin}) and IAA affinity to human [¹²⁵I] insulin ($r = -0.68$, $P < 0.001$). (E) The amino acid sequences of human and chicken insulin differ at seven residues.

chicken insulin for 50% inhibition of IAA binding to human [^{125} I] insulin (median $IC_{50_{human}}/IC_{50_{chicken}}$ ratio, 11; range, 4–25; Figs. 1C and D, filled circles).

The $IC_{50_{human}}/IC_{50_{chicken}}$ ratio was correlated with IAA affinity ($r=-0.68$, $P<0.001$; Fig. 1D). All samples with IAA affinity $>10^9$ L/mol preferentially bound human insulin, whereas all but one sample with an IAA affinity $<10^9$ L/mol did not preferentially bind human insulin ($P<0.0001$).

IgA-IAA and relationship to chicken insulin reactivity

Samples from healthy control subjects did not have IgA binding to human [^{125} I] insulin (Fig. 2). The background cpm for IgA-IAA in controls was 31 cpm (range 20 to 36 cpm). IgA binding to human [^{125} I] insulin above background was observed for 5 of the 24 IAA-positive children in whom the chicken insulin reactivity was determined. Increased IgA binding to human [^{125} I] insulin was found in 4 of 8 children with IAA that preferentially bound chicken insulin. In two children, the IgA component bound more human [^{125} I] insulin than the IgG component (IgG-IAA 198 cpm vs. IgA-IAA 507 cpm and IgG-IAA 182 cpm vs. IgA-IAA 481 cpm; Fig. 2). A third child had IAA with moderately high binding to human [^{125} I] insulin for both IgG (191 cpm) and IgA (95 cpm). These three children had IAA with very low affinity to human [^{125} I] insulin ($<10^6$ L/mol). A fourth child from this group and one of the 4 children with IAA binding equally well to human and chicken insulin had weak binding of IgA together with very strong IgG-IAA. None of the 12 samples with high affinity IAA and low chicken insulin reactivity had IgA-IAA.

In order to determine whether IgA-IAA was a common feature of early insulin immunization, the first available IAA-positive sample from 20 additional children was subsequently tested for IgA-IAA (Fig. 2). One of these children had IgA-IAA. This sample is from a 9-month-old girl who also had GAD and IA-2 antibodies and who developed type 1 diabetes at age 2.8 years. The sample is remarkable because it has both high (4.8×10^9 L/mol) and low affinity (1.2×10^6 L/mol) IAA. Follow-up samples showed a gradual loss of the low affinity IAA component and a rise in the high affinity IAA. The low affinity IAA component in this sample preferentially bound chicken insulin ($IC_{50_{human}}/IC_{50_{chicken}}$ ratio=1.6) and contained the IgA binding, whereas the high affinity IAA component reacted poorly with chicken insulin ($IC_{50_{human}}/IC_{50_{chicken}}$ ratio=0.06) and was IgA-IAA negative (data not shown). Follow-up samples had decreasing IgA-IAA and increasing IgG-IAA.

Discussion

Immune responses to dietary constituents that are associated with autoimmunity would provide evidence for causal factors in the pathogenesis of autoimmune disease. Here, we show that a minority of children with persistent autoantibodies to insulin preferentially bound non-human insulin and therefore could target molecules other than insulin. We further showed that some of these non-human insulin preferred IAA had an IgA component, suggesting that they may have arisen from mucosal exposure to the immunizing antigen. Together these data suggest that autoantibodies that bind to human insulin can arise from exposure to exogenous antigen found at mucosal surfaces.

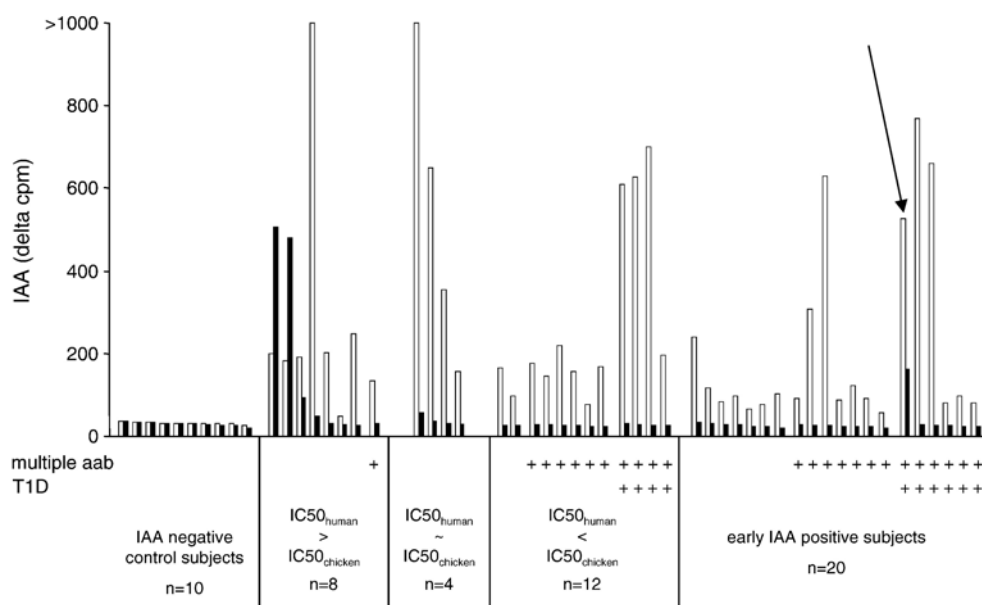


Figure 2 Total IgG-IAA and IgA-IAA for each analyzed sample. The samples are grouped into IAA-negative control children ($n=10$), samples from IAA-positive children who had IAA preferentially binding human insulin, children who had IAA that bound human and chicken insulin equally well, children who had IAA that preferentially bound chicken insulin and the first IAA-positive sample from 20 children who developed IAA before age 2 years. IgG-IAA are represented as open bars and IgA-IAA are represented as filled bars and are expressed as bound radioactivity in counts per minute (cpm). The sample marked by an arrow represents a child who has IgA-IAA of low affinity concomitantly with high affinity IgG-IAA, multiple autoantibodies and who developed type 1 diabetes.

The study used a panel of IAA-positive sera selected as informative on the basis of their affinity to human insulin. The panel included sera with relatively low affinity to human insulin since we considered that human insulin may not be the primary target antigen of the IAA in these cases. In searching for supportive evidence, we took advantage of natural differences found in insulin from non-human species. Of the insulin molecules available, chicken insulin was selected as potentially informative. Chicken insulin differs from human insulin at positions 8, 9 and 10 of the A chain and positions 1, 2, 27 and 30 of the B chain. As expected from previous results using other insulin species, the high affinity IAA were poorly reactive with chicken insulin. Novel was the finding that all IAA of very low affinity and around half of the moderate affinity IAA preferentially bound chicken insulin rather than human insulin. Thus, human insulin was not the preferred ligand of the low affinity IAA. Although these IAA have a higher affinity to chicken insulin, the amount of chicken insulin required to inhibit binding to insulin is still relatively high, suggesting that chicken insulin is not the high affinity ligand and therefore unlikely to be the immunizing antigen. Of relevance to previous hypotheses [10–13], bovine insulin is also unlikely to be the primary target of these antibodies since no preferential binding to bovine insulin over human insulin was observed for any of the sera tested (data not shown).

Regardless of the true identity of the immunizing antigen for the low affinity IAA, the findings suggest that humoral immunity to insulin can arise from immune responses to other antigens. We further show that the site of immunization for these responses is likely to include mucosal surfaces. Using a novel assay for measuring antibodies of minor isotype classes, we found insulin-binding antibodies of IgA isotype in sera that had chicken insulin reactive low-affinity IAA. The assay includes an IgG-pre-clearing step that removes all IgG, including the insulin-binding IgG and therefore eliminates false-positive IgA-IAA due to excess IgG-IAA. Moreover, the two-step assay identified a minority of sera with relatively high titer antibodies to insulin in which the IgA component was dominant, suggesting that mucosal exposure to antigen may be driving the IAA response in some cases.

The relevance of the findings to the pathogenesis of type 1 diabetes is unclear. The high affinity IAA that are characteristic of pre-type 1 diabetes bound poorly to chicken insulin and did not have an IgA component suggesting that the mechanism of mucosal immunity indicated here is not the cause of type 1 diabetes. There is, however, one exception in which IgA-chicken insulin reactive IAA were found concomitant with the typical high affinity IgG-IAA in a 9-month-old child who later developed diabetes. The natural history of the IAA response in this child showed an early IgA response that was rapidly replaced with the typical high affinity IgG-IAA. This is consistent with an early mucosal immunization to a molecule other than human insulin and a rapid conversion to an islet antigen-driven response. It is possible that such a rapid conversion was missed in other children and that IgA responses of this kind are not always detected in the circulation. Hence, early IgA-IAA responses may not be a rare event in the pathogenesis of type 1 diabetes.

In conclusion, we show a mechanism of humoral insulin immunity that involves molecules other than human insulin

and which is consistent with mucosal immunization. Depending upon whether this mechanism is causal or protective for type 1 diabetes, identification of the target antigens of this mucosal immunization may identify causal factors of insulin autoimmunity or novel ways to induce protection.

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