Cow's Milk Formula Feeding Induces Primary Immunization to Insulin in Infants at Genetic Risk for Type 1 Diabetes

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Insulin autoantibodies (IAAs) often appear as the first sign of islet cell autoimmunity in prediabetic children. Because cow's milk contains bovine insulin, we followed the development of insulin-binding antibodies in children fed with cow's milk formula. Bovine insulin– and human insulin–binding antibodies by enzyme immunoassay and IAA by radioimmunoassay were analyzed in 200 infants carrying HLA-DQB1*0302 but no protective alleles who participated in a Finnish population-based birth-cohort study. Based on the prospectively registered information, the first 100 infants enrolled in the study who were exposed to cow's milk formula before age 12 weeks and the first 100 infants enrolled in the study who were exclusively breast-fed for longer than their first 12 weeks of life were selected for the present study. Also, 11 children from the birth cohort who developed at least two diabetes-associated autoantibodies, 98 children with newly diagnosed type 1 diabetes, and 92 healthy children were studied. We found that the amount of IgG-antibodies binding to bovine insulin was higher at age 3 months in infants who were exposed to cow's milk formula than in infants who were exclusively breast-fed at that age (median 0.521 vs. 0.190; $P < 0.0001$). The antibodies binding to bovine insulin cross-reacted with human insulin. None of these infants tested positive for IAA. The levels of bovine insulin–binding antibodies declined in both groups at ages 12 and 18 months, whereas in the 11 children with at least two diabetes-associated autoantibodies the levels increased during the follow-up period ($P < 0.0001$). IgG antibodies correlated with IgG2 antibodies binding to bovine insulin ($r = 0.43$, $P = 0.004$) and IAA ($r = 0.27$, $P = 0.02$) in diabetic children, but not in healthy children. Cow's milk feeding is an environmental trigger of immunity to insulin in infancy that may explain the epidemiological link between the risk of type 1 diabetes and early exposure to cow's milk formulas. This immune response to insulin may later be diverted into autoaggressive immunity against β-cells in some individuals, as indicated by our findings in children with diabetes-associated autoantibodies. *Diabetes* 48:1389–1394, 1999

Immunization to insulin plays a key role in the autoimmune process leading to the loss of pancreatic β-cells and the development of type 1 diabetes. Insulin is the only known β-cell–specific autoantigen in type 1 diabetes. Insulin autoantibodies (IAAs) are commonly found in children with newly diagnosed type 1 diabetes (1–8) and predict the disease when combined with islet cell autoantibodies (ICAs) (5,8). In a prospective birth-cohort study, IAAs most frequently appeared as the first antibody in ICA-positive offspring of diabetic parents (9), suggesting that immunization to insulin may be an early event in the autoimmune process leading to type 1 diabetes.

The insulin-specific immune response is not, however, restricted to patients with type 1 diabetes (10). Low levels of IAA are often detectable in nondiabetic subjects, but without the presence of other diabetes-associated autoantibodies, these subjects are at low risk for clinical disease. Obviously healthy subjects are often immunized to insulin, implying that other features of insulin-specific immunity besides antigen-specificity are necessary for the development of type 1 diabetes. The trigger of insulin-specific immunity in humans is not known. We recently suggested that cow's milk formula may induce insulin-binding antibodies in children (11). An association between early exposure to cow's milk proteins and risk for type 1 diabetes has been observed in several epidemiological studies (12), suggesting that cow's milk may contain a factor with a diabetogenic effect.

We analyzed the development of insulin-binding antibodies by enzyme immunoassay (EIA) and liquid-phase radioimmunoassay (RIA) in relation to cow's milk exposure in 200 infants from a prospective Finnish population-based birth-cohort study, in whom the duration of exclusive breast-feeding was followed longitudinally. For comparison, 11 children from the birth-cohort study with at least two diabetes-related autoantibodies, 98 children with newly diagnosed type 1 diabetes, and 92 healthy children were also studied.

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DIPP, Diabetes Prediction and Prevention; EIA, enzyme immunoassay; GADA, glutamate decarboxylase antibody; IAA, insulin autoantibody; ICA, islet cell autoantibody; OD, optical density; PBS, phosphate-buffered saline; RIA, radioimmunoassay.

RESEARCH DESIGN AND METHODS

Subjects. The present study included 200 children participating in a prospective Finnish birth-cohort survey, the Diabetes Prediction and Prevention (DIPP) Project. The DIPP study cohort comprises subjects with an increased genetic risk of type 1 diabetes—that is, they carry the HLA-DQB1*02/*0302 genotype or the *0302/x genotypes, where x stands for alleles other than *02, *0301, *0602, or *0603. The duration of exclusive breast-feeding and age at the start of formula feeding were registered sequentially at each study visit at ages 3, 6, 12, and 18 months. Based on this information, serum samples were collected from the first 100 infants enrolled in the study who were exposed to cow's milk formula before age 12 weeks (group 1; median age of exposure 3 weeks [range 0–10]) and from the first 100 infants participating in the study who were exclusively breast-fed for longer than the first 12 weeks of life (group 2; median age of exposure 17 weeks [13–26]). Serum samples were also available from 68 infants in group 1 and 79 infants in group 2 at age 12 months, and from 50 children in group 1 and 37 children in group 2 at age 18 months.

Sequential serum samples from 11 children who had developed at least two diabetes-associated autoantibodies (ICAs, IAAs, glutamate decarboxylase antibodies [GADAs], or IA2 antibodies [IA2As]) during their follow-up (range 14–31 months) in the DIPP study were also analyzed. Two of these individuals progressed to type 1 diabetes, one at age 14 months and the other at age 26 months. Of these 11 children, 9 had been exposed to cow's milk formula before age 3 months.

For comparison, insulin-binding antibodies were also studied in plasma samples from 98 children age 8.5 ± 4.0 years (mean ± SD) with newly diagnosed type 1 diabetes and 92 control children (age 8.9 ± 5.0 years) without known autoimmune disease or acute infections. The study protocol was approved by the ethical committees of the participating hospitals. Informed consent was obtained from the parents of the study subjects.

EIA for antibodies to bovine and human insulin. Polystyrene plates (Combiplate Enhanced Binding; Labsystems, Helsinki, Finland) were coated with bovine or human insulin (1 µg/well; Sigma, St. Louis, MO). We used 1% human serum albumin (HSA) in phosphate-buffered saline (PBS) for residual coating and Tween 20-PBS (0.05%) as a washing buffer. The samples were diluted 1:20 for IgG-antibodies and 1:10 for IgG1 and IgG2 antibodies in 0.2% HSA/0.05% Tween-PBS. Alkaline phosphatase-conjugated rabbit anti-human IgG or biotinylated rabbit anti-human IgG1 or IgG2 antibodies (Jackson ImmunoResearch, West Grove, PA) were used as the secondary antibody. After adding the substrate, the absorbance was measured and the results were expressed as optical density units. Two known positive and two known negative reference samples were run on each plate as controls. An equal number of samples from groups 1 and 2 taken at age 3, 12, or 18 months as well as an equal number from patients and control subjects were always run on the same plate. Intra- and interassay coefficients of variation were 12 and 15% for IgG, 15 and 16% for IgG1, and 11 and 23% for IgG2 class antibodies, respectively. For inhibition assays, 10, 100, and 1,000 µg/ml bovine insulin, human insulin, or bovine serum albumin were incubated with the serum sample for 2 h at room temperature before analyzing the sample by EIA. The same serum sample without the inhibitor, but treated otherwise in a similar manner, was always analyzed on the same plate.

The IAA radioligand assay. The IAA radioligand assay was performed as previously described (2,13). The cut-off limit for IAA positivity for serum samples from children was 68 nU/ml (mean + 3 SD in 105 nondiabetic infants) and 129 nU/ml for plasma samples (Ficoll-Paque treated) (mean + 3 SD in 92 normal children).

HLA typing. HLA-DQB1 typing was performed by a technique developed for screening for type 1 diabetes susceptibility based on the presence of HLA-DQB1 alleles associated with a significant risk for the disease (HLA-DQB1*0302,*02) or with protection against the disease (HLA-DQB1*0301,*0602,*0603) (14).

Statistical analysis. The differences between groups were analyzed by the two-tailed Mann-Whitney *U* test, and in the case of repeated measurements, by regression analysis. Correlations between different parameters were calculated by the Spearman correlation test.

RESULTS

Insulin-binding antibodies at age 3 months. IgG antibodies binding to bovine insulin were higher at age 3 months in group 1 than in group 2 infants (median 0.521 [0.074–1.881] vs. 0.190 [0.049–1.481]; P < 0.0001) (Fig. 1). IgG antibodies binding to bovine and human insulin correlated positively in group 1 ($r = 0.86$; $P < 0.0001$) (Fig. 2). Bovine insulin–binding antibodies correlated inversely with the age of introduction of formula feeding in group 1 ($r = –0.31$; $P = 0.002$). IgG1 antibodies were higher in group 1 than in group 2 (median 0.185 [0.013–1.30] vs. 0.133 [0.025–1.412]; $P < 0.0001$) and correlated with the levels of total IgG antibodies binding to bovine insulin ($r = 0.59$, $P < 0.0001$). IgG2 antibodies binding to bovine insulin were low, did not differ between group 1 and group 2 (median 0.033 [0.00–0.253] vs. 0.027 [0.00–0.197]; P = 0.36), and did not correlate with total IgG antibodies binding to bovine insulin (r = 0.04; P = 0.72). None of the infants tested positive for IAA at this age.

Insulin-binding antibodies at age 12 months. No differences in IgG-antibody binding to bovine or human insulin were observed between groups 1 and 2 at age 12 months (median 0.483 vs. 0.551 [P = 0.17]; 0.415 vs. 0.423 [P = 0.73], respectively). Also by this age, bovine insulin–binding antibodies no longer correlated with age at the introduction of formula feeding ($r = –0.13$, $P = 0.12$), but did correlate with human insulin–binding antibodies ($r = 0.52$, $P < 0.0001$) and IgG1-antibody binding to bovine insulin ($r = 0.58$, $P < 0.0001$). IgG2-antibody binding to bovine insulin was low (median 0.024). All children tested negative for IAA at this age (range 0–63 nU/ml).

Insulin-binding antibodies at age 18 months. No differences were observed between groups 1 and 2 at age 18 months in IgG-antibody binding to bovine insulin (median 0.363 vs. 0.384; $P = 0.46$). Elevated IAA levels (>68 nU/ml) were found in 6 of 62 children (10%) studied. All six had been exposed to cow's milk formula, at age 3, 9, 13, 13, 15, and 17 weeks. Two of these children had also developed other diabetes-associated autoantibodies; these two children had been exposed to cow's milk formula at ages 3 and 13 weeks (Fig. 4). IAAs did not differ between groups 1 and 2 (median 42 nU/ml for both groups; $P = 0.45$).

The level of antibodies binding to bovine insulin did not differ between the infants carrying the HLA DQB1*02/*0302 genotype and those with the *0302/x genotype in either feeding group at any age studied (data not shown).

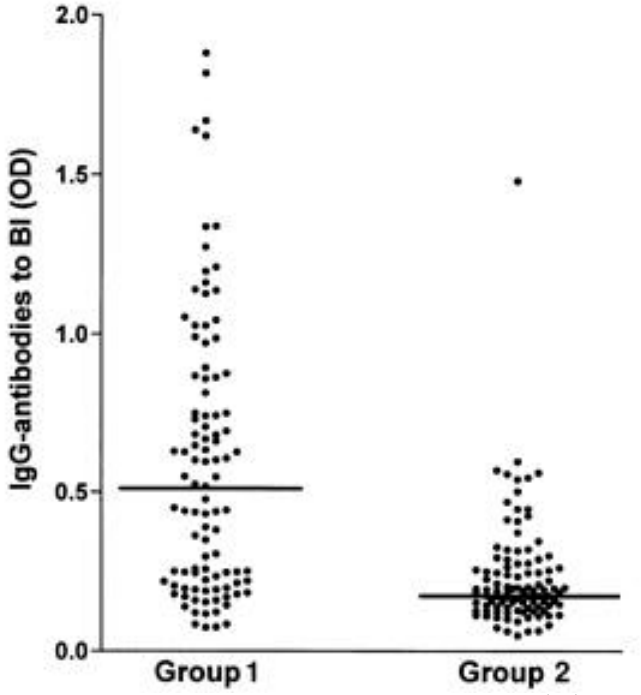

FIG. 1. The levels of IgG-antibodies to bovine insulin (BI) at age 3 months in infants who received cow's milk formula before age 12 weeks (group 1) and in infants who were exclusively breast-fed until age 12 weeks (group 2). The median is marked with a line. $P < 0.0001$, group 1 vs. group 2 (Mann-Whitney *U* test).

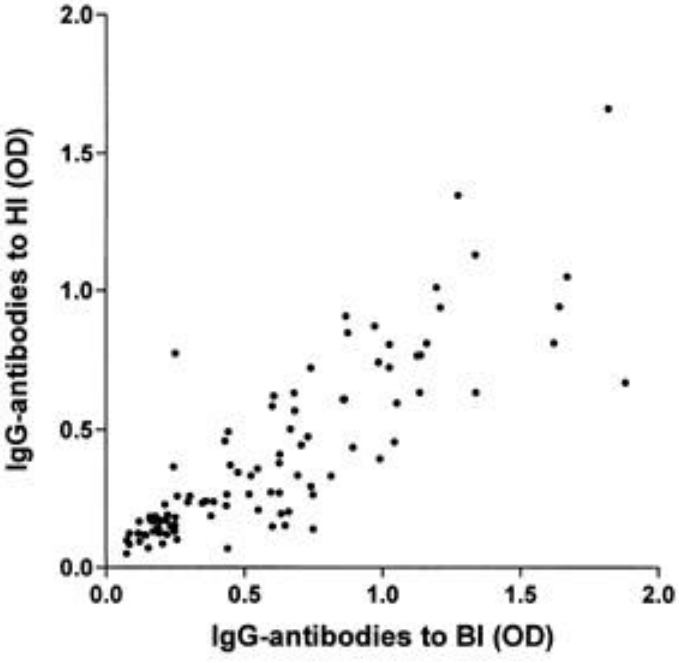

FIG. 2. Correlation between the levels of IgG antibodies to bovine insulin (BI) and human insulin (HI) at age 3 months in infants fed with cow's milk formula (r = 0.86, P < 0.0001).

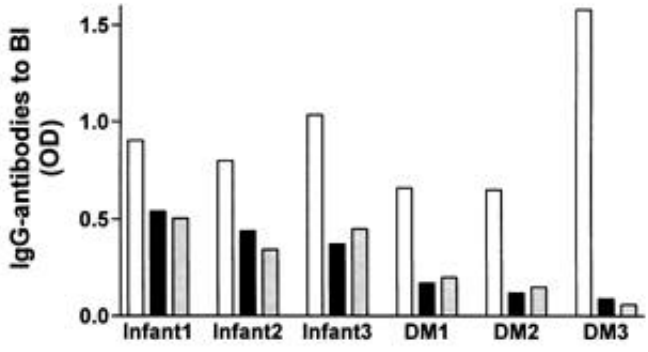

FIG. 3. Binding of IgG antibodies to solid-phase bovine insulin (BI) in the presence of no inhibitor (□), 1,000 µg/ml liquid-phase bovine (■), and human insulin (■) in sera from three infants aged 3 months who had been exposed to cow's milk formula and in sera from three patients with type 1 diabetes (DM).

Inhibition of IgG binding to solid-phase bovine insulin by bovine and human insulin. Liquid-phase bovine and human insulin inhibited the binding of IgG-antibodies to solid-phase bovine insulin in sera from infants exposed to cow's milk formulas and from children with type 1 diabetes (six examples are shown in Fig. 3). Bovine serum albumin did not inhibit the binding of IgG-antibodies to solid-phase bovine insulin in any of 20 sera studied from infants exposed to cow's milk formulas (<5% of inhibition at a concentration of 1,000 µg/ml).

Follow-up of children who developed at least two diabetes-associated autoantibodies. We studied nine infants from the DIPP study cohort who had developed at least two diabetes-associated autoantibodies (IAA, ICA, GADA, and/or IA-2A) during follow-up for IgG-antibody binding to insulin by EIA (Fig. 4). In addition, two children from groups 1 and 2 had developed at least two diabetes-associated autoantibodies by age 18 months and were included in Fig. 4. IAAs were detected as the first autoantibody alone in two cases, together with ICA in four cases, together with GADA in one case, and together with both ICA and GADA in two cases. GADA emerged as the first autoantibody in two children, one of whom was IAA negative and the other of whom developed IAA as the second autoantibody. Serum samples were not available at 3-month intervals in all children, which made it difficult to evaluate the sequential appearance of the autoantibodies. In these children, IgG-antibody binding to bovine insulin increased from ages 3 to 18 months and were higher at ages 12 and 18 months when compared with group 1 and group 2 children who did not have autoantibodies by regression analysis for repeated measurements (P = 0.007 and P < 0.0001, respectively) (Fig. 5).

Insulin-binding antibodies in children with type 1 diabetes. In patients with newly diagnosed type 1 diabetes compared with unaffected children, IgG-antibody binding to bovine insulin (0.272 [0.033–2.842] vs. 0.151 [0.030–1.451], respectively; P < 0.0001) and human insulin (0.327 [0.092–2.703] vs. 0.256 [0.034–1.701], respectively; P = 0.014) by EIA was higher. A correlation was seen between IgG-antibody binding to bovine and human insulin in patients with type 1 diabetes (r = 0.68, P < 0.001); a weaker correlation was seen in healthy children (r = 0.26, P = 0.013). Age did not correlate with the level of antibodies (data not shown). In patients with type 1 diabetes (n = 43), both IgG1 antibodies (0.323 [0.061–1.386]) and IgG2 antibodies (0.030 [0.001–0.668]) correlated with IgG-antibody binding to bovine insulin (r = 0.44, P = 0.003, and r = 0.43, P = 0.004, respectively). However, in the unaffected children (n = 62), the IgG1 antibodies (0.228 [0.03–1.451]) did correlate with IgG-antibody binding to bovine insulin (r = 0.33; P = 0.009), whereas IgG2-antibody binding to bovine insulin was rare (0.075 [0.001–1.912]) and did not show any correlation with IgG-antibody binding to bovine insulin (r = 0.13; P = 0.3). Among the patients, 21 of 69 (30%) but none of the control children (n = 59) tested positive for IAAs. IAAs in the patients did correlate with IgG-antibody binding to human and bovine insulin by EIA (r = 0.36, P = 0.003 and r = 0.27, P = 0.02, respectively). HLA-DQB1 risk alleles for type 1 diabetes were typed in 67 patients and in 60 control subjects. No significant differences in IgG-antibody binding to bovine or human insulin were detected between individuals carrying various HLA genotypes among the patients or the control subjects (data not shown). IAAs were higher in patients carrying the DQB1*0302 or *02 risk alleles when compared with control subjects matched for these risk alleles (P = 0.0003 and 0.02, respectively).

DISCUSSION

We found that oral exposure to cow's milk formulas induced bovine insulin–binding antibodies that cross-reacted with human insulin. Accordingly, dietary bovine insulin appears to be an environmental trigger of a primary immune response to a β-cell specific antigen in healthy children.

Bovine insulin has been shown to be immunogenic in humans when used in the treatment of patients with type 1 diabetes (15). A difference of three amino acids exists between bovine and human insulin (amino acids 8 and 10 in the A-chain and amino acid 30 in the B-chain). The interspecies differences have been found to be important for the antigenic recognition of insulin (16,17). Transgenic mice with

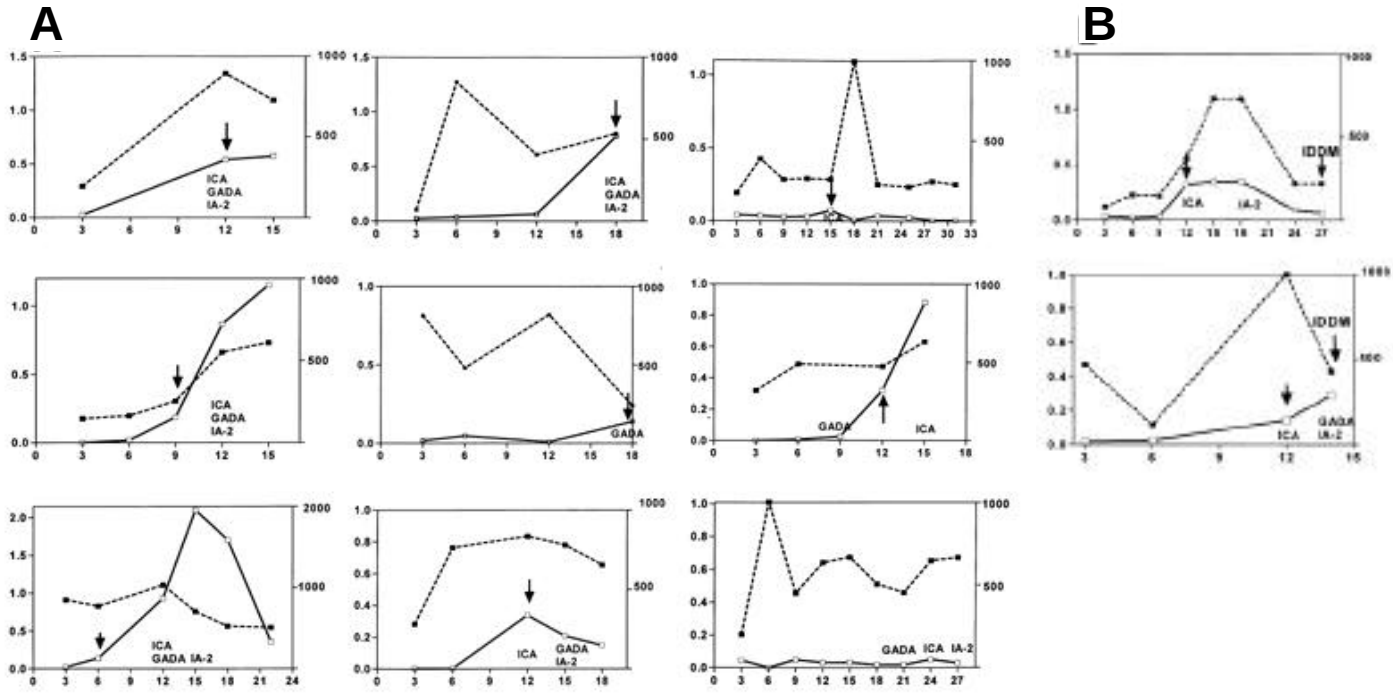

FIG. 4. Follow-up of insulin-binding antibodies in nine children who developed islet-cell autoimmunity (*A*) and two children who developed type 1 diabetes (*B*) during follow-up. The *x*-axis shows the age of the child in months. The left *y*-axis indicates the levels of IgG-antibody binding to bovine insulin by EIA (- - - ■ - - -) and the right *y*-axis indicates the levels of IAAs (—- □ —-). The arrow marks the age when IAAs for the first time exceeded the cutoff limit for positivity. The age when ICAs, GADAs, and IA2As became positive is marked. The first two cases on the first row in *A* were exposed to cow's milk formula between ages 3 and 6 months, whereas the others were exposed before age 3 months. In B, arrows labeled with IDDM indicate onset of type 1 diabetes.

expression of the human insulin gene in their β-cells produced insulin-specific antibodies when injected with bovine insulin, whereas they were tolerant to human insulin (18). Low levels of antibodies binding to insulin in exclusively breast-fed children indicates that human insulin in breast milk is less immunogenic than bovine insulin. All children in the DIPP study carry the HLA DQB1*0302 allele, which is in linkage-equilibrium with the HLA DR4 allele. This may have had some impact on our results, since children with the HLA DR4 allele may be more susceptible to developing an insulin-specific immune response than children without this risk allele (6). In the patients and control subjects, the levels of bovine insulin– or human insulin–binding antibodies did not differ between individuals carrying various HLA genotypes, suggesting that immunization to insulin is not HLA restricted.

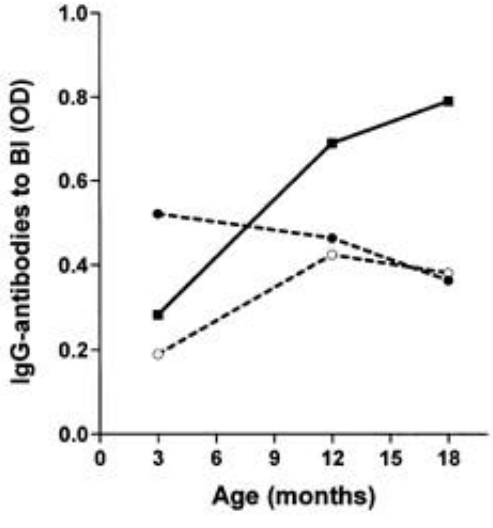

FIG. 5. Median levels of IgG-antibodies to bovine insulin at ages 3, 12, and 18 months in children who received cow's milk formula before age 3 months (●), children who were exclusively breast-fed until age 3 months (○), and children who developed at least two diabetes-associated autoantibodies (■).

The frequent detection of insulin-binding antibodies in healthy children has limited their use as a risk marker for type 1 diabetes (10). IAAs detected by liquid-phase RIA have been more closely associated with type 1 diabetes than insulin-binding antibodies detected by EIA. In the present study, because we wanted to study the immunological sensitization to bovine insulin in nondiabetic infants, we used EIA. The specificity of the antibodies against insulin was confirmed by inhibition studies. IAAs were not observed in any infant age 3 months among infants fed cow's milk formula. This is likely due to differences in the affinity and avidity of the antibodies detected by these two assays, as suggested previously (19). RIA detects IAAs that are insulin-binding antibodies that have high affinity to insulin and are associated with β-cell autoimmunity, whereas EIA detects a heterogeneous group of insulin-binding antibodies showing variation in their affinity. Supporting the high affinity of insulin-binding antibodies in type 1 diabetes, bovine insulin–binding antibodies were more effectively inhibited by liquid-phase insulin in patients than in nondiabetic children. Furthermore, bovine insulin–binding antibodies correlated with IAAs in patients, but not

in nondiabetic children. Also, the presence of IgG2 antibodies to insulin in patients with type 1 diabetes but not in healthy children suggests that the insulin-specific immune response is more mature in those children who develop clinical diabetes.

Despite the occurrence of IgG-antibody binding to insulin in type 1 diabetes, only low reactivity to insulin has been detected by peripheral T-cells from patients with type 1 diabetes before treatment with exogenous insulin (20,21). Wegmann et al. (22) showed that in NOD mice the majority of the islet-infiltrating lymphocytes recognize insulin, although peripheral T-cells do not proliferate to insulin (22). Insulin-reactive T-cells were also able to transfer the disease to healthy mice, suggesting that insulin may play a key role in the pathogenesis of autoimmune diabetes (23).

Our observations raise the question of whether the priming and/or later exposure to bovine insulin in cow's milk products may be involved in the development of β-cell destructive autoimmunity. The levels of antibodies binding to bovine insulin declined during the follow-up period in both feeding groups, which would support the development of tolerance to insulin with age, as has been reported for other cow's milk antigens (24). The generation of an immune response to oral insulin during infancy may be protective for autoimmune diabetes in a majority of immunized infants, as has been suggested in animal studies using insulin as a tolerogen (25,26). In contrast, in the children who developed at least two diabetes-associated autoantibodies, the insulin-binding antibodies increased steadily toward age 18 months, and there was no indication of the development of tolerance; 9 of these 11 infants were exposed to cow's milk formula before age 3 months. Enhanced immune responses to several cow's milk proteins have been reported in type 1 diabetes (27–29) and could be considered as markers of disturbed oral tolerance (28,30). Among these associated cow's milk proteins, dietary bovine insulin may, however, be the most likely trigger of autoimmune diabetes, as immunity to insulin appears to be an early sign of β-cell autoimmunity in humans. In some experimental studies, feeding autoantigens has been shown to induce or enhance the development of autoimmune-mediated diabetes (31).

We demonstrated here that primary immunization to insulin is induced in infancy by oral exposure to cow's milk insulin, indicating sensitization to insulin in nondiabetic children. Our data further suggest that this early insulin-specific immune response is not normally regulated in those children who will develop islet cell–related autoimmunity. The possibility that insulin-specific lymphocytes induced by cow's milk feeding may be later activated in some children needs to be considered as a possible mechanism leading to autoimmune destruction of β-cells and subsequent progression to clinical type 1 diabetes.

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