Effect of Cow's Milk Exposure and Maternal Type 1 Diabetes on Cellular and Humoral Immunization to Dietary Insulin in Infants at Genetic Risk for Type 1 Diabetes

Johanna Paronen, Mikael Knip, Erkki Savilahti, Suvi M. Virtanen, Jorma Ilonen, Hans K. Åkerblom, Outi Vaarala, and the Finnish Trial to Reduce IDDM in the Genetically at Risk Study Group*

Type 1 diabetes is considered to be a T-cell–mediated autoimmune disease in which insulin-producing β-cells are destroyed. Immunity to insulin has been suggested to be one of the primary autoimmune mechanisms leading to islet cell destruction. We have previously shown that the first immunization to insulin occurs by exposure to bovine insulin (BI) in cow's milk (CM) formula. In this study, we analyzed the development of insulin-specific T-cell responses by proliferation test, emergence of insulin-binding antibodies by enzyme immunoassay, and insulin autoantibodies by radioimmunoassay in relation to CM exposure and family history of type 1 diabetes in infants with a first-degree relative with type 1 diabetes and increased genetic risk for the disease. The infants were randomized to receive either an adapted CM-based formula or a hydrolyzed casein (HC)-based formula after breast-feeding for the first 6–8 months of life. At the age of 3 months, both cellular and humoral responses to BI were higher in infants exposed to CM formula than in infants fully breast-fed (P = 0.015 and P = 0.007). IgG antibodies to BI were higher in infants who received CM formula than in infants who received HC formula at 3 months of age (P = 0.01), but no difference in T-cell responses was seen between the groups. T-cell responses to BI at 9 months of age (P = 0.05) and to human insulin at 12 (P = 0.014) and 24 months of age (P = 0.009) as well as IgG antibodies to BI at 24 months of age (P = 0.05) were lower in children with a diabetic mother than in children with a diabetic father or a sibling, suggesting possible tolerization to insulin by maternal insulin therapy. The priming of insulin-specific humoral and T-cell immunity occurs in early infancy by dietary insulin, and this phenomenon is influenced by maternal type 1 diabetes.
***Diabetes* 49:1657–1665, 2000**

Type 1 diabetes is considered to be an autoimmune disease in which T-cells destroy the insulin-producing β-cells (1). Among autoantigens implicated as playing a role in type 1 diabetes, insulin is the only known β-cell–specific antigen. Insulin-specific T-cell clones isolated from the pancreas are capable of transferring diabetes in an animal model (2). Insulin autoantibodies (IAA) are commonly found in patients with newly diagnosed type 1 diabetes (3,4), and they are predictors of the disease when combined with other islet cell antibodies (5). IAA levels have been reported to correlate with the rate of progression to type 1 diabetes (6). IAA are in particular present in affected children diagnosed at a young age (4,7), and in a birth cohort study, IAA appeared most frequently as the first antibody in offspring of diabetic parents (8). Based on these observations, immunization to insulin may be the primary event in the process leading to type 1 diabetes.

Insulin-binding antibodies measured by a solid-phase enzyme immunoassay (EIA) are frequently detected in healthy children and differ from IAA measured by the liquid phase–based radioimmunoassay (RIA) method in their affinity to insulin (9). Detection of insulin-binding antibodies by EIA can be used for detection of immunization to insulin, although they are not as closely associated with type 1 diabetes as IAA detected by RIA (10). We have previously shown that exposure to cow's milk (CM) formula elicits antibody formation to insulin in some children (10,11). Because a disturbance in oral tolerance has been implicated in patients with type 1 diabetes (12), the effect of oral insulin exposure in high-risk infants is of interest. The main emphasis of the present study was to analyze the development of T-cell immunity to insulin in the second pilot of the Trial to Reduce IDDM in the Genetically at Risk (TRIGR), in which infants with a first-degree relative with type 1 diabetes and increased genetic risk for the disease were randomized to receive either an adapted CM-based formula or an extensively hydrolyzed casein (HC)-based formula after breast-feeding until the age of 6–8 months. The emergence of cellular immunity to insulin was

From the Hospital for Children and Adolescents (J.P., E.S., H.K.Å., O.V.), University of Helsinki; the Department of Biochemistry (J.P., O.V.), National Public Health Institute, Helsinki; the Medical School (M.K.), the Department of Pediatrics (M.K., S.M.V.), Tampere University Hospital, and the School of Public Health (S.M.V.), University of Tampere, Tampere; and the Department of Virology and Turku Immunology Center (J.I.), University of Turku, Turku, Finland.

Address correspondence and reprint requests to Johanna Paronen, MD, Department of Biochemistry, National Public Health Institute, Mannerheimintie 166, 00300 Helsinki, Finland. E-mail: johanna.paronen@hus.fi.

Received for publication 15 June 1999 and accepted in revised form 6 June 2000.

*A complete listing of the TRIGR Study Group is given in the APPENDIX.

BF, breast-fed; BI, bovine insulin; BLG, β-lactoglobulin; CM, cow's milk; EAE, encephalomyelitis; EIA, enzyme immunoassay; HC, hydrolyzed casein; HI, human insulin; IAA, insulin autoantibodies; MBP, myelin basic protein; OD, optical density; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; PPD, purified protein derivative; RIA, radioimmunoassay; SI, stimulation index; TRIGR, Trial to Reduce IDDM in the Genetically at Risk; TT, tetanus toxoid.

measured by proliferation test in a group of 56 children, and the development of insulin-binding antibodies was measured by EIA and RIA in 119 children in relation to CM exposure and family history of type 1 diabetes. Antibodies to β-lactoglobulin (BLG) were analyzed by EIA to determine compliance.

RESEARCH DESIGN AND METHODS

Subjects. Infants with a first-degree relative (mother, father, or sibling) with type 1 diabetes were invited to the second pilot of TRIGR between 1995 and 1997, but only individuals at increased genetic risk (HLA-DQB1*02/*0302, *0302/x, or *02/y genotypes, where x stands for alleles other than *02, *0602, or *0603, and y stands for alleles other than *0302, *0602, or *0603) entered the study. Consecutively recruited children (n = 63) outside the Helsinki area were studied for humoral immunity to insulin only. In addition, 56 children from the Helsinki area were studied for both insulin-specific T-cell reactivity and humoral immunity. Insulin was added to the T-cell analysis only from the beginning of 1996. Infants were randomized to receive either an adapted CM-based formula (Enfamil; Mead Johnson, Evansville, IN) supplemented with 20% Nutramigen to make the two study formulas similar in taste and smell (CM group) or an extensively HC-based formula with molecular weights of <1.2 kDa (Nutramigen; Mead Johnson; HC group) after breast-feeding until the age of 6–8 months, depending on when the formula was started. According to the protocol, all infants were supposed to receive the formula for a minimum of 2 months. Breast-feeding was encouraged, and the mothers were asked to add the formula to their infant's diet at 6 months of age at the latest. During the intervention period, mothers were advised to eliminate all infant food products containing CM or beef from their infant's diet, but the diet of the lactating mothers was not modified.

There was a difference between the two randomization groups in the age of introduction of the study formula, with a mean of 1.9 months in the CM group (n = 58) and 3.0 months in the HC group (n = 61) (P = 0.03, Mann-Whitney U test). No difference was recorded in duration of total breast-feeding between the groups (7.6 vs. 8.3 months, P = 0.53). Duration of study formula feeding was 4.8 and 3.6 months in the two groups, respectively (P = 0.01). Deviations from this protocol also occurred. In this series, 14 children (12%) were not exposed to the study formula at all (5 in the CM group and 9 in the HC group), and they were included in the breast-fed (BF) group up to 6 months of age and excluded thereafter. Two infants (one in the CM group and the other in the HC group) who dropped out early (before the 3-month visit and before the 6-month visit) and whose feeding practices were unknown were excluded from the analysis. Four children were diagnosed with CM allergy at 3.4, 4.5, 6.7, and 12 months of age; the first child was fully BF until the diagnosis, the second child received HC formula, the third child received the CM study formula after 6 months of age, and the last child received ordinary CM at 10.5 months of age and no formula at all. The two infants diagnosed with CM allergy during the dietary intervention period were excluded from the analysis after the diagnosis because of the change in diet. Infants (n = 6) of mothers with type 1 diabetes and high levels of antibodies to bovine insulin (BI) and human insulin (HI) in the cord blood (≥2 times the median optical density [OD] in the BF group at 3 months of age) were excluded from the analysis at 3 and 6 months of age because of the transplacental transfer of insulin antibodies. Three infants with high initial levels of antibodies to BLG in cord blood and decreasing levels thereafter up to 3–6 months of age were excluded from the analysis of this variable at 3 and 6 months of age. The studies were approved by the ethics committees of all participating hospitals.

Proliferation assay of peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMCs) were isolated from fresh heparinized blood by Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation. The PBMCs were suspended in RPMI-1640 containing 5% pooled human AB^+ serum (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland) and 2 mmol/l L-glutamine. They were cultured at 1×10^5 cells (200 µl) per well in quadruplicates on a U-bottomed microwell plate (Nunc, Røskilde, Denmark) with 100 µg/ml BI or HI (Sigma, St. Louis, MO), 8 µg/ml tetanus toxoid (TT) (National Public Health Institute, Helsinki, Finland), or 10 µg/ml tuberculin purified protein derivative (PPD) (Statens Serum Institut, Copenhagen, Denmark). After incubation for 5 days, 1 µCi tritiated thymidine (Amersham Life Science, Little Chalfont, Buckinghamshire, U.K.) was added, and the cultures were harvested 16 h later for thymidine incorporation measurement. Proliferation response was expressed as stimulation index (SI) = median counts per minute in the presence of antigen/median counts per minute without the antigen, and as Δ counts per minute = median counts per minute without an antigen subtracted from median counts per minute in the presence of an antigen. The samples were analyzed blindly and in sequential order, with samples of different individuals and different time points included in the same assay.

EIA for IgG antibodies to BI and HI. Polystyrene plates (Combiplate Enhanced Binding; Labsystems, Helsinki, Finland) were coated with 1 µg/well BI or HI (Sigma); 1% human serum albumin in phosphate-buffered saline (PBS) was used for residual coating, and 0.05% Tween-20 in PBS was used as a washing buffer. The samples were diluted 1:20 in PBS containing 0.2% human serum albumin and 0.05% Tween. Alkaline phosphatase-conjugated rabbit anti-human IgG antibodies (Jackson ImmunoResearch, West Grove, PA) were used as the secondary antibody, and P-nitrophenyl phosphate (Sigma) was used as a substrate. Absorbance was measured by an optical reader at 405 nm, and the results were expressed as OD units. The samples were run blindly, with each child's sequential samples on the same plate. As quality control, a pool of five known positive and negative samples was run on each plate. The mean intra-assay variation of the method was 12%, and the interassay variation was 20%. Inhibition assays were performed in serum samples taken at 6 months of age. A total of 200 µl/ml BI was incubated with the serum sample for 2 h at room temperature before analysis of the sample in the EIA for antibodies to BI. The same serum sample without the inhibitor treated in a similar manner was always run on the same plate.

The IAA radioligand assay. The IAA radioligand assay was performed with a micro-assay as previously described (13). The cutoff limit for IAA positivity was set at the 99th percentile (≥1.56 relative units) in 373 nondiabetic Finnish infants and children.

Antibodies to BLG. Antibodies to BLG were measured by EIA as previously described (14). The levels of antibodies were expressed as percentages of the standard with a very high titer of BLG antibodies.

HLA typing. HLA-DQB1 typing was performed by a technique developed for screening type 1 diabetes susceptibility based on the presence of HLA-DQB1 alleles associated with a significant risk for (HLA-DQB1*0302 and *02) or with protection against (HLA-DQB1*0301, *0602, and *0603) this disease (15).

Statistical analysis. Differences between the groups were analyzed by the Kruskal-Wallis H test or by the Mann-Whitney U test with Bonferonni correction for multiple comparisons. Differences during the follow-up within a specific group were evaluated by the Wilcoxon's signed-rank test. Correlations between different parameters were calculated by the Spearman correlation test.

RESULTS

T-cell responses to insulin and CM exposure. At 3 months of age, T-cell reactivity to BI differed among the three groups (P = 0.04, Kruskal-Wallis H test), with the SI highest in the CM group (Fig. 1*A*), whereas no difference in cellular responses to HI was detected among the groups (Table 1). The infants in the CM group had enhanced T-cell responses to BI when compared with BF infants (P = 0.015, Mann-Whitney U test; with Bonferonni correction, P = 0.045), but no difference was observed between the infants in the CM and HC groups. Reactivity to BI decreased during the intervention period from 3 to 6 months in the CM group (median SI 2.2 vs. 1.3; P = 0.006, Wilcoxon's signed-rank test), and at 6 months of age cellular responses to insulin did not differ between the groups. At 9 months of age, when all infants had been exposed to ordinary CM formula for at least 1 month, cellular responses to insulin did not differ either. When all infants had been exposed to ordinary CM formula for at least 1 month, cellular responses to insulin did not differ between the groups at 6 months of age, during the intervention period, or at 9 months of age (Fig. 1*B*, Table 1). T-cell responses to BI and HI correlated at all ages in the CM group (data not shown). T-cell responsiveness to HI above an SI of 2 was observed in the CM group in 7 of 21 infants aged 9 months, whereas earlier, the reactivity to HI was low (at 3 months of age, 0 of 14 infants had an SI >2; at 6 months of age, 1 of 19 infants had an SI >2). At 9 months of age, the same infants with cellular responses to BI also had T-cell reactivity to HI (Fig. 2). Reactivity to BI did not differ between defined HLA risk genotypes at any age (data not shown). No differences among the groups existed in the SIs to the control antigens tuberculin (PPD) or TT (Table 1).

Antibody production to insulin in relation to CM exposure. At 3 months of age, median levels of IgG antibod-

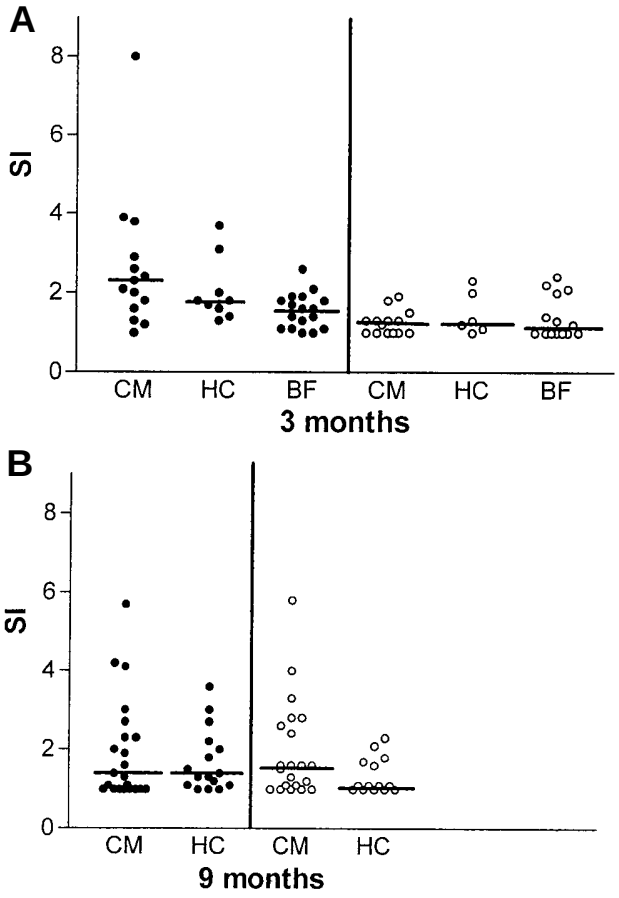

FIG. 1. T-cell responses to insulin expressed as the SI in the feeding groups during the intervention at 3 months of age (*A*) and after the intervention at 9 months of age (*B*). SI to BI (●) and SI to HI (○) are shown. Medians are represented by horizontal lines. For BI, *P* = 0.04 with the Kruskal-Wallis *H* test at 3 months of age; for HI, *P* = 0.66. *P* = 0.39 (*P* = 1.0) with the Mann-Whitney *U* test (Bonferonni correction) at 3 months for BI CM vs. HC; *P* = 0.015 (*P* = 0.045) for CM vs. BF. For BI, at 9 months of age, *P* = 0.86; for HI, *P* = 0.15.

ies to BI and HI were highest in infants exposed to CM formula when compared with infants exposed to HC or those who were fully BF (Table 2). IgG antibodies to BI and HI correlated in all age-groups ($P < 0.001$; data not shown). A trend toward an inverse correlation was detected between the age of introduction of formula and the levels of IgG antibodies to BI in the CM group at 6 months of age ($r = –0.28$, $P = 0.055$) but not in the HC group ($r = –0.18$, $P = 0.34$). The levels of insulin-binding antibodies increased from 3 to 6 months of age in children exposed to CM formula before 3 months of age (median BI-IgG 0.210 vs. 0.253, $P = 0.001$, Wilcoxon's signed-rank test) but did not increase in children who started the same formula between 3 and 6 months of age (0.183 vs. 0.199, $P = 0.36$). In the HC group, no significant changes were detected during this period (data not shown). In this series, four children converted to positivity for IAA (Fig. 3), three were in the CM group, and one did not receive the CM study formula at all but was exposed to ordinary CM formula at the age of 7 months after the intervention period (Fig. 3*B*). In inhibition experiments, soluble BI (200 μg/ml) inhibited the binding of IgG antibodies to solid-phase BI at 6 months most efficiently in the CM group. The median percentages of inhibition were 34, 19, and 19% in the CM, HC, and BF groups, respectively ($P = 0.003$, Kruskal-Wallis *H* test; CM vs. HC, $P = 0.01/P = 0.03$, Mann-Whitney *U* test/Bonferonni correction; CM vs. BF, $P = 0.003/P = 0.009$).

Relationship between cellular and humoral immune responses to insulin. T-cell responses and IgG antibodies to BI correlated in the CM group at 6 ($r = 0.47$, $P = 0.05$) and 24 ($r = 0.72$, $P = 0.02$) months of age but not at other time points (data not shown). No correlation was detected in the HC group at any age (data not shown).

The effect of maternal type 1 diabetes on immunization to dietary insulin. Cellular immune responses to BI at 9 and 24 months of age and to HI at 9, 12, and 24 months of age were lower in children with a diabetic mother than in children with a diabetic father or a sibling in the CM group (median SI to BI 1.1 vs. 2.3, $P = 0.05$, and SI to HI 1.3 vs. 2.5, $P = 0.06$ at 9 months; median SI to HI 1.2 vs. 1.7, $P = 0.014$ at 12 months; median SI to BI 1.3 vs. 1.9, $P = 0.081$, and SI to HI 1.1 vs. 1.5, $P = 0.009$ at 24 months of age, Mann Whitney *U* test) (Fig. 4). The effect of maternal type 1 diabetes on cellular immunization to dietary insulin was not observed at other time points (data not shown). Antibody responses to insulin were compared between children with a diabetic mother and children with a diabetic father or a sibling only from 9 months of age on, from which time maternal antibodies are no longer detectable in infant serum (16). IgG antibodies to BI at 24 months were lower in offspring of diabetic mothers than in children with a diabetic father or a sibling in the CM group (median 0.275 vs. 0.483; $P = 0.05$, Mann-Whitney *U* test) (Fig. 5). No significant differences were observed at other time points (data not shown). The levels of insulin-binding antibodies in children at 9, 12, 18, or 24 months of age did not correlate with maternal IAA levels in samples taken at delivery (data not shown).

Relation of BLG antibodies to CM exposure and to insulin-binding antibodies. Infants exposed to CM formula had the highest levels of BLG-IgG at 3 and 6 months of age, but elevated levels of IgG antibodies to BLG were also detected in some cases in the BF group at 6 months of age (Fig. 6). IgG antibodies to BI correlated with IgG antibodies to BLG at 6 months of age in the BF group ($r = 0.65$, $P = 0.001$) but not in the other groups. Median levels of IgG antibodies to BLG were 77.8, <10, and <10% at 3 months of age ($P = 0.0001$, Kruskal-Wallis *H* test) and 57.5, <10, and 20.4% at 6 months of age ($P = 0.0001$) in the CM, HC, and BF groups, respectively, and 68.7 vs. 38.8% at 9 months of age ($P = 0.02$, Mann-Whitney *U* test), 80.6 vs. 63.0% at 12 months of age ($P = 0.45$), 39.8 vs. 44.1% at 18 months of age ($P = 0.61$), and 58.6 vs. 58.6% at 24 months of age ($P = 0.57$) in the CM and HC groups, respectively.

DISCUSSION

We observed that oral exposure to BI in the CM formula induced insulin-specific T-cell and antibody responses in infants at increased risk for type 1 diabetes. This finding is in accordance with our previous observations on the induction of insulin-binding antibodies by early CM feeding (10,11) and extends this phenomenon to include cellular immunity as well. The development of immune responses to dietary insulin was modified by maternal type 1 diabetes in the pres-

TABLE 1
T-cell responses to BI and HI, tuberculin PPD, and TT expressed as Δ counts per minute and SIs in the feeding groups

			Median Δ counts per minute				Median SI		
	n	Counts per minute (background)	PPD	TT	BI	HI	PPD	TT	BI	HI
3 Months										
CM	14	696	9,436	—	1,039	120	13.1	—	2.2	1.3
HC	9	554	16,366	—	352	136	24.0	—	1.8	1.3
BF	17	578	2,824	—	205	71	10.2	—	1.6	1.2
P										
All	—	0.44	0.19	—	0.008	0.66	0.39	—	0.04	0.66
CM vs. HC	—	—	—	—	0.12	—	—	—	0.39	—
CM vs. BF	—	—	—	—	0.005	—	—	—	0.015	—
6 Months										
CM	20	659	—	6,392	179	3	—	11.4	1.4	1.0
HC	11	714	—	8,564	198	93	—	11.4	1.2	1.2
BF	10	547	—	7,298	174	41	—	20.0	1.5	1.2
P										
All	—	0.22	—	0.78	0.49	0.30	—	0.54	0.96	0.73
9 Months										
CM	22	1,125	—	11,622	453	458	—	14.5	1.4	1.6
HC	16	813	—	11,079	307	36	—	12.4	1.4	1.1
P										
CM vs. HC	—	0.36	—	0.60	0.55	0.08	—	0.96	0.86	0.15
12 Months										
CM	21	446	—	7,145	286	318	—	13.3	1.6	1.3
HC	17	607	—	6,819	306	47	—	15.0	1.5	1.2
P										
CM vs. HC	—	0.21	—	0.99	0.74	0.33	—	0.52	0.47	0.33
18 Months										
CM	18	1,087	—	7,401	276	265	—	11.8	1.4	1.4
HC	15	706	—	9,810	569	470	—	10.5	1.9	1.6
P										
CM vs. HC	—	0.63	—	0.47	0.43	0.86	—	0.33	0.33	0.22
24 Months										
CM	11	573	—	11,251	407	145	—	13.7	1.6	1.2
HC	10	774	—	11,096	306	415	—	14.0	1.4	1.5
P										
CM vs. HC	—	0.62	—	0.78	0.48	0.40	—	0.27	0.23	0.84

ent study. Decreased risk of type 1 diabetes has been reported in children of mothers with type 1 diabetes when compared with children of fathers with type 1 diabetes (17,18). It has been hypothesized that this might be due to induction of immune tolerance to β-cell antigens presented in utero by exposure to maternal diabetes, but the immunologic mechanisms are unknown. We found that the immune responses to insulin were lower in children with a diabetic mother than in children with a nondiabetic mother. This phenomenon was observed after the age of 6 months, when maternal antibodies have been cleared from infant sera and, accordingly, the responses did not show correlation with maternal IAA levels. The decreased immune responses to insulin in offspring of diabetic mothers might be due to tolerization to insulin by maternal insulin therapy through transplacental transfer of insulin antibody complexes in utero (19). In experimental animals, maternal antigenic stimulation during pregnancy has been shown to decrease the antibody response to the same antigen after oral or parental challenge in offspring (20–22). In mice, maternal immunization (subcutaneously) with human γ-globulin after delivery resulted in absorption of this antigen from colostrum and in a complete antigen-specific tolerant state in the offspring (23). We suggest that exposure of offspring to maternal diabetes and insulin therapy results in tolerization to insulin and may decrease the risk for type 1 diabetes by this mechanism. Our finding supports the view that low doses of subcutaneous insulin may enhance insulin-specific tolerance (24,25). The importance of exogenous insulin as a tolerogen is further supported by the observation that the risk of type 1 diabetes in children born before maternal onset of diabetes was higher than in infants born after disease onset and who had consequently been exposed to maternal diabetes in utero and in infancy (26). It is fascinating to speculate that the exposure to insulin due to maternal diabetes may also induce susceptibility to insulin resistance and thereby may explain the increased risk of type 2 diabetes in offspring of mothers with diabetes among Pima Indians, an issue raised by Warram et al. (26) in 1991.

It is possible that the observed tolerization to insulin in infants with a diabetic mother might also have had an impact on our results. In the present study, the levels of insulin-binding antibodies in infants exposed to the CM formula were lower than those observed in our previous study in which

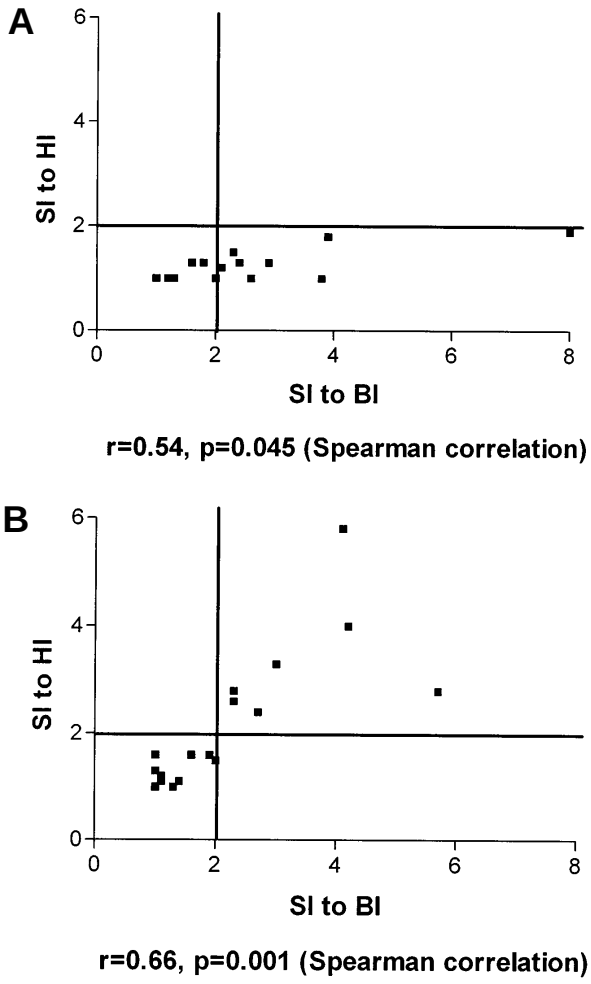

FIG. 2. Correlation of T-cell responses to BI and HI expressed as the SI at 3 (*A*) and 9 (*B*) months of age in the CM group.

none of the infants included had a mother with type 1 diabetes (10). Two other differences between the present study and our previous study may also explain the lower levels seen in this study. All infants in our previous study carried the HLA-DQB1*0302 allele, which is in linkage disequilibrium with the DR4 allele linked to high levels of IAA in diabetic patients. In the present study, infants having the DQB1*02 allele without protective alleles were also included. Because the number of children in the T-cell analyses was small, we were cautious in making any conclusions about the association between T-cell responses to insulin and HLA risk alleles. The third difference between our present and previous studies is that the majority of children in the previous study were exposed to a liquid CM formula, whereas the study formulas in the present trial were powdered. The proteins are less denaturated in liquid formulas and may thus elicit stronger immune responses.

At 3 months of age, the infants exposed to the CM formula had higher T-cell responses and levels of antibodies to BI than BF infants. The induction of insulin-specific immune responses by BI is based on the difference of three amino acids between BI and HI (amino acids 8 and 10 in the A-chain and amino acid 30 in the B-chain). When BI was used for treatment of type 1 diabetes, it induced insulin-binding antibodies (27). Interspecies differences in insulin have also been reported to be important for antigenic recognition (28) and for tolerance induction (29,30). A one–amino acid difference in the B-chain of the insulin molecule was crucial for prevention of autoimmune diabetes in NOD mice (30). T-cell responses and antibodies to BI occurred also in some infants who received HC formula. It is possible that some children in the HC group developed immunity to insulin peptides present in the hydrolysate (11,31). In this respect, prevention of early immunization to insulin is not totally avoided by the use of HC formula, although peptides can be considered less immunogenic than whole proteins. The small number of infants in the T-cell analyses may also explain the observation that no difference was seen in T-cell responses to BI between the HC and CM groups, since a difference in the levels of insulin-binding antibodies was demonstrated between the larger groups at 3 months of age. No difference was observed in cellular responses to insulin between the CM and HC groups at 6 months of age either. This result may be influenced by the decrease in T-cell responses to BI from 3 to 6 months of age in the CM group and may suggest that continuous use of the CM formula resulted in tolerization in the majority of healthy children. The occurrence of a correlation between insulin-specific T-cell and antibody responses at 6 and 24 months of age in the CM group but not in the HC group suggests that the different nature of insulin in these two formulas (whole protein vs. peptide) influences the development of insulin-specific immune responses.

We observed that some children who were fully BF at 6 months of age had insulin-binding antibodies but no detectable T-cell responsiveness to BI. The immunization to insulin by breast-feeding may be caused by small amounts of BI present in human milk (when the maternal diet contains CM) or alternatively by HI present in human milk. Antibodies to BLG also appeared in some fully BF children at 6 months of age, which supports the view that tiny amounts of BLG derived from the maternal CM-containing diet and present in human milk at low concentrations, i.e., 5–33 μg/l (32), may immunize some children (33). The finding that responses to BLG and BI correlated in the BF group at 6 months of age provides further support for the concept that the immune responses detected may be due to the transfer of dietary antigens to breast milk. The infant who developed IAA during the follow-up and who already had elevated levels of insulin-binding antibodies by 6 months of age during full breast-feeding (Fig. 3*B*) may be an example of immunization to low-dose antigens.

The exposure to BI in the CM formula seemed to result in immunization to insulin, which was distinguishable from that seen in the BF- and HC-fed infants at 3 months of age but not later on. However, the early exposure to the CM formula was observed in the levels of antibodies to BLG even at 9 months of age, at which time children in CM group still had higher antibody levels than children in the HC group who were exposed to ordinary CM formula only after 6 months of age. This difference in the responses to BI and BLG may be due to the modifying effect of the dose of the dietary immunogen. The insulin content in native CM varies from 1 to 10 μg/l (34), whereas the concentration of BLG is ~1,000-fold. In a study by Björksten et al. (35), small doses of mite antigen well below the suggested sensitization threshold level of 2 μg/g dust induced mite-specific T-cell responses in young children. This finding is in accordance with our present finding of T-cell responses to low doses of dietary BI.

TABLE 2
IgG antibodies to BI and HI in the feeding groups

	CM	HC	BF	*P*
3 Months				
BI	0.210 (0.022–0.541)	0.138 (0.033–0.588)	0.151 (0.028–0.736)	0.01
HI	0.221 (0.055–0.845)	0.147 (0.062–0.635)	0.150 (0.029–0.500)	0.01
n	31	21	54	
6 Months				
BI	0.246 (0.066–1.076)	0.192 (0.061–1.644)	0.261 (0.045–1.819)	0.10
HI	0.323 (0.033–0.884)	0.231 (0.050–1.694)	0.275 (0.073–1.811)	0.12
n	48	32	24	
9 Months				
BI	0.332 (0.072–0.936)	0.238 (0.072 to >3.0)	—	0.08
HI	0.311 (0.079–1.643)	0.242 (0.072 to >3.0)	—	0.12
n	49	45		
12 Months				
BI	0.303 (0.052–1.197)	0.235 (0.043–1.358)	—	0.14
HI	0.306 (0.070–1.075)	0.290 (0.062–2.668)	—	0.48
n	50	47		
18 Months				
BI	0.289 (0.065–1.555)	0.233 (0.033–1.547)	—	0.62
HI	0.303 (0.062–1.742)	0.280 (0.072–1.657)	—	0.36
n	40	40		
24 Months				
BI	0.331 (0.061–1.234)	0.267 (0.011–1.488)	—	0.57
HI	0.418 (0.069–1.285)	0.281 (0.053–1.637)	—	0.66
n	35	34	—	

Data are OD units (median and range). The *P* values of Kruskal-Wallis *H* test are shown. *P* values determined by Mann-Whitney *U* test (Bonferonni correction) areas are as follows: at 3 months of age for BI-IgG CM vs. HC, $P = 0.01$ ($P = 0.03$); CM vs. BF, $P = 0.007$ ($P = 0.02$); and for HI-IgG CM vs. HC, $P = 0.012$ ($P = 0.036$); CM vs. BF, $P = 0.009$ ($P = 0.027$). At 6 months of age for BI-IgG CM vs. HC, $P = 0.058$ ($P = 0.17$); CM vs. BF, $P = 0.74$ ($P = 1.0$); and for HI-IgG CM vs. HC, $P = 0.10$ ($P = 0.30$); and CM vs. BF, $P = 0.33$ ($P = 0.99$).

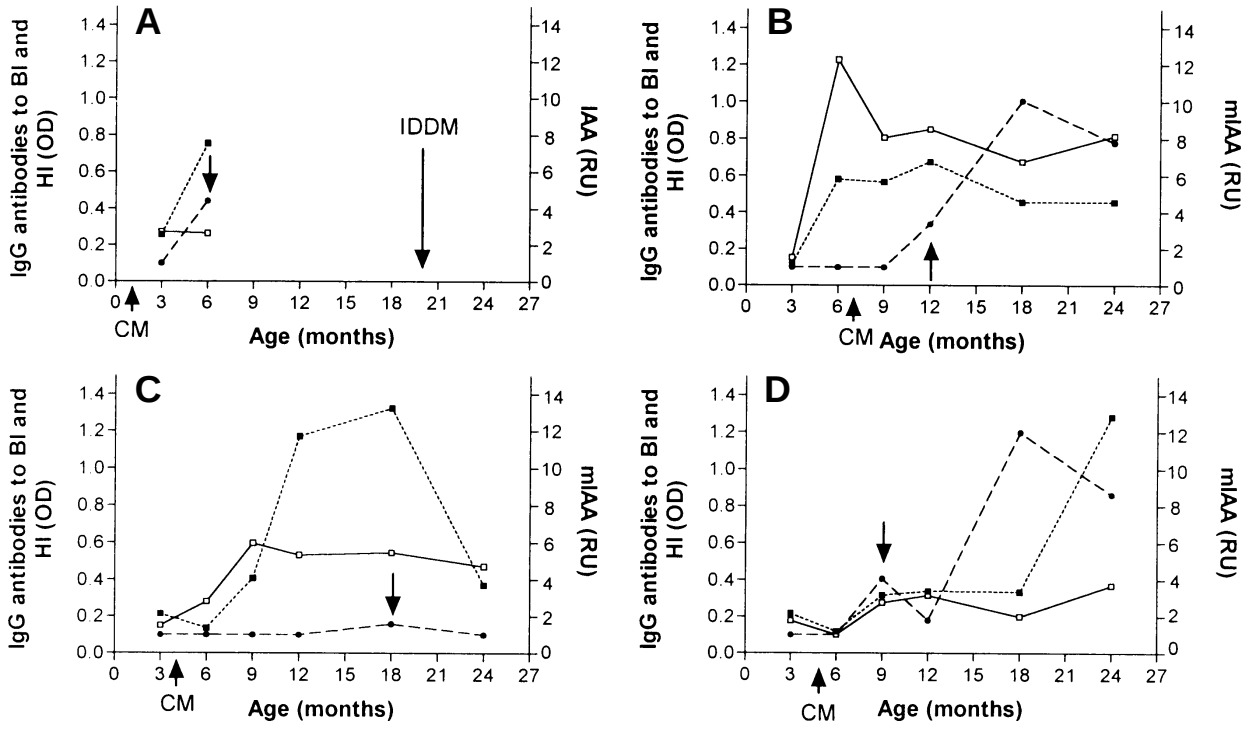

FIG. 3. Development of IgG antibodies to BI (■) and HI (□) in the four infants with IAA (●). The arrows designate the first positive IAA. The arrowheads below the *x*-axes designate the start of CM formula. RU, relative units.

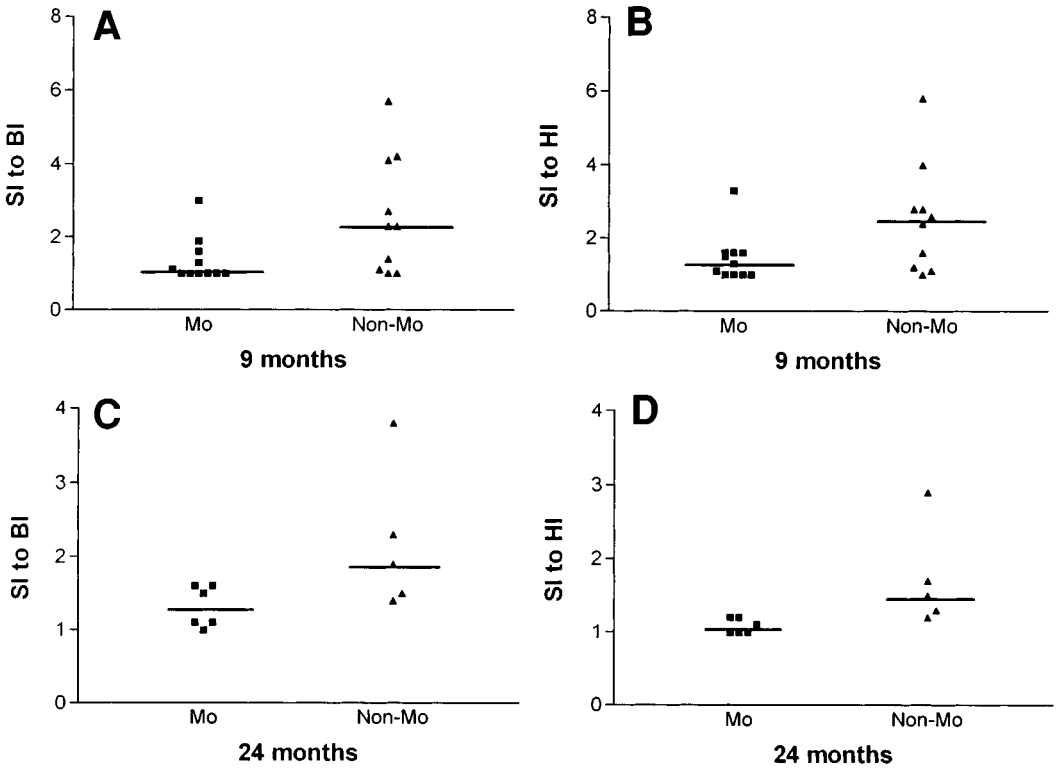

FIG. 4. T-cell responses to BI (*A* and *C*) and HI (*B* and *D*) in infants with a diabetic mother (Mo) or a nondiabetic mother (Non-Mo) at 9 (*A* and *B*) and 24 (*C* and *D*) months in the CM group. For median SI to BI 1.1 vs. 2.3, $P = 0.05$, and for SI to HI 1.3 vs. 2.5, $P = 0.06$ at 9 months. For SI to BI 1.3 vs. 1.9, $P = 0.081$, and for SI to HI 1.1 vs. 1.5, $P = 0.009$ at 24 months. Medians are represented by horizontal lines.

The inhibition of insulin-binding antibodies by liquid-phase insulin showed substantial variation. The highest affinity to liquid-phase insulin appeared in antibodies induced by the CM formula. Altogether, the affinity of these dietary insulin-induced antibodies seemed to be low. This result is expected in an early immune response, whereas a more mature response represented by autoimmunity to insulin is of higher affinity (36). Although IAA are frequently seen in patients with type 1 diabetes, only low levels of peripheral T-cell reactivity to insulin have been observed in individuals at high risk of type 1 diabetes (37) and in newly diagnosed patients before treatment with exogenous insulin (38). This observation may be explained by sequestration of insulin-specific T-cells in the pancreas, since Wegmann et al. (39) have shown that the majority of islet-infiltrating lymphocytes are insulin-reactive in NOD mice, but that peripheral T-cells do not proliferate to insulin. The observation that insulin-reactive T-cell clones isolated from NOD mice were able to transfer the disease to healthy mice suggests that insulin may play a role in the autoimmune process leading to diabetes (2). In our study, T-cell reactivity to BI had already emerged at 3 months of age in infants in the CM group; only later (by 9 months of age) was this reactivity observed to mount a cellular response to HI as well. This finding suggests that, primarily, T-cells are primed to BI and that cross-reactivity with HI develops later. Enhanced levels of antibodies to HI observed in infants in the CM group at 3 months of age are most likely due to cross-reactive BI antibodies.

Our observations raise the issue of whether oral exposure to foreign insulin plays a role in the autoimmune process leading to type 1 diabetes. In diabetes-prone animals, insulin given orally has been shown to function as a tolerogen, reducing the incidence of autoimmune diabetes (40). Some experimental studies have shown, however, that insulin may exacerbate the disease when combined with an infectious agent (41). Also, in a transgenic mouse model, oral administration of an antigen expressed in β-cells induced diabetes (42). Age of exposure also seems important because oral administration of myelin basic protein (MBP) to neonate rats primes for immune responses and enhances experimental autoimmune encephalomyelitis (EAE), whereas oral exposure in adult rats results in disease suppression (43). However, low-dose or repeated administration of MBP has been shown to exacerbate the clinical course of EAE, even in adult rats (44). All these studies emphasize the dual nature of oral antigen administration: the development of tolerance versus immunity is influenced by several modifying factors such as the age of the host, dose of the antigen, and time schedule of feeding. Only a minority of children included in the present study are likely to develop type 1 diabetes, although immunization to insulin occurs in early infancy. However, it is possible that in some genetically susceptible children, a continuous, even small-dose, early exposure to BI present in CM may lead to loss of tolerance to insulin as seen in those children who developed IAA in the present study. The initiation of insulin-specific T-cells by dietary insulin in the gut immune system may carry a risk for an autoimmune process progressing ultimately to clinical type 1 diabetes. In this process, the factors that lead to the activation of insulin-primed T-cells are unknown, but they may be associated with the regulation of the gut immune system (45).

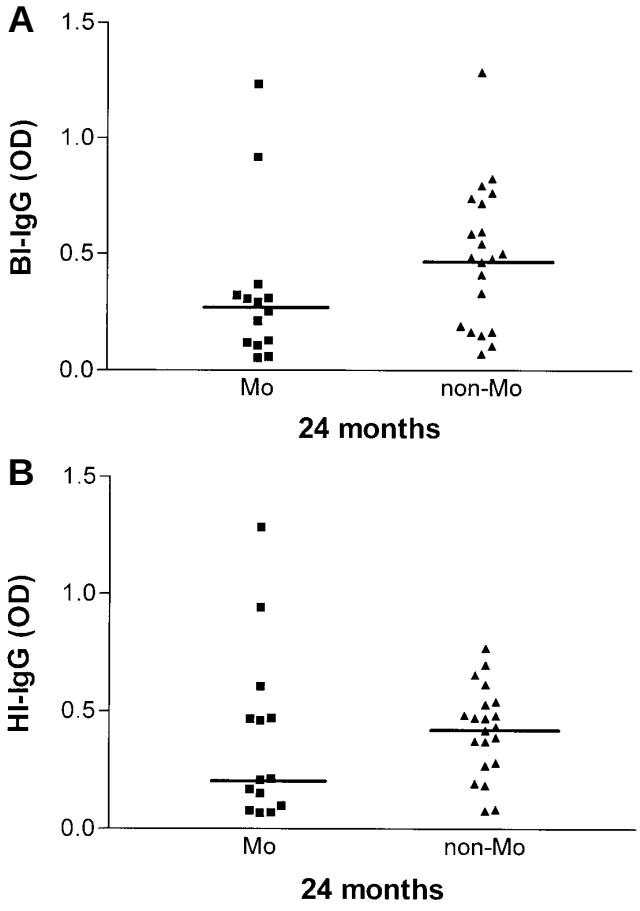

FIG. 5. IgG antibodies to BI (*A*) and HI (*B*) in children with a diabetic mother (Mo) or a nondiabetic mother (Non-Mo) at 24 months of age in the CM group. For BI-IgG 0.275 vs. 0.483, *P* = 0.05, and for HI-IgG 0.211 vs. 0.433, *P* = 0.21. Medians are represented by horizontal lines.

To summarize, we demonstrate here that primary cellular immunization to insulin may occur in infancy by oral exposure to BI present in CM. Generation of insulin-reactive T-cells by dietary insulin in early childhood may provide a pathogenic link between CM and diabetes. Our finding of lower insulin-specific T-cell reactivity in offspring of diabetic mothers than in children with an affected father or sibling may reflect tolerization to insulin by exposure to maternal diabetes and insulin therapy. We suggest that this immunologic mechanism may explain the decreased susceptibility to type 1 diabetes in the offspring of diabetic mothers compared with the offspring of diabetic fathers.

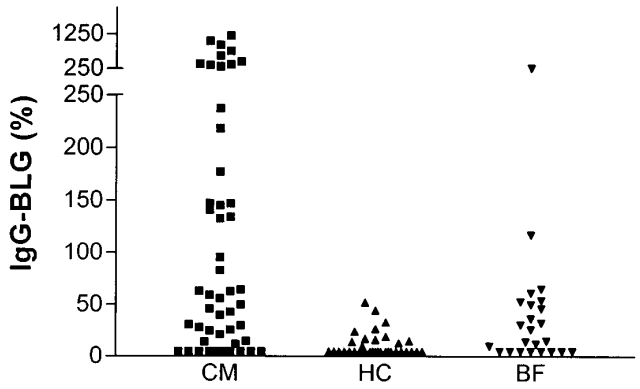

FIG. 6. Levels of IgG antibodies to BLG expressed as the percentage of the standard at 6 months of age in the feeding groups. Kruskal-Wallis *H* test, *P* = 0.0001; Mann-Whitney *U* test (Bonferonni correction), CM vs. HC, *P* = 0.0001 (*P* = 0.0003); CM vs. BF, *P* = 0.014 (*P* = 0.042).

ACKNOWLEDGMENTS

This work was supported by grants from the Foundation for Diabetes Research in Finland, the Academy of Finland, the University of Helsinki, the European Commission (contract number BMH4-CT96-0233), the Juvenile Diabetes Foundation International (Grant 195003), the Novo Nordisk Foundation, and the Sigrid Juselius Foundation.

We thank Anneli Suomela for technical assistance.

APPENDIX

The Finnish TRIGR Study Group. Principal investigator: H.K. Åkerblom. Local investigators: V. Eskola, H. Haavisto, R. Jokisalo, A.-L. Järvenpää, A.-M. Hämäläinen, U. Kaski (retired), J. Komulainen, P. Korpela, M.-L. Käär, P. Lautala, K. Niemi, A. Nuuja, M. Renlund, M. Salo, T. Talvitie, T. Uotila, and G. Wetterstrand. Special investigators: J. Ilonen, P. Klemetti, M. Knip, P.K. Kulmala, J. Paronen, A. Reunanen, T. Saukkonen, E. Savilahti, K. Savola, K. Teramo, O. Vaarala, and S.M. Virtanen.

REFERENCES

1. Eisenbarth GS: Type 1 diabetes mellitus: a chronic autoimmune disease: *N Engl J Med* 314:1360–1368, 1986
2. Daniel D, Gill RG, Schloot N, Wegmann D: Epitope specificity, cytokine production profile and diabetogenic activity of insulin-specific T-cell clones isolated from NOD mice. *Eur J Immunol* 25:1056–1062, 1995
3. Palmer JP, Asplin CM, Clemons P, Lyen K, Tatpati O, Raghu PK, Paquette TL: Insulin antibodies in insulin-dependent diabetes mellitus before insulin treatment. *Science* 222:1337–1339, 1983
4. Arslanian SA, Becker DJ, Rabin B, Atchison R, Eberhardt M, Cavender D, Dorman J, Drash AL: Correlates of insulin antibodies in newly diagnosed children with insulin-dependent diabetes before insulin therapy. *Diabetes* 34:926–930, 1985
5. Srikanta S, Ricker AT, McCulloch DK, Soeldner JS, Eisenbarth GS, Palmer JP: Autoimmunity to insulin, beta-cell dysfunction, and development of insulin-dependent diabetes mellitus. *Diabetes* 35:139–142, 1986
6. Eisenbarth GS, Jackson RA, Pugliese A: Insulin autoimmunity: the rate limiting factor in pre-type 1 diabetes. *J Autoimmun* 5 (Suppl. A):241–246, 1992
7. Karjalainen J, Knip M, Mustonen A, Ilonen J, Åkerblom HK: Relation between insulin antibody and complement-fixing antibody at clinical diagnosis of IDDM. *Diabetes* 35:620–622, 1986
8. Ziegler A-G, Hummel M, Schenker M, Bonifacio E: Autoantibody appearance and risk for development of childhood diabetes in offspring of parents with type 1 diabetes: the 2-year analysis of the German BABYDIAB study. *Diabetes* 48:460–468, 1999
9. 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
10. Vaarala O, Knip M, Paronen J, Hämäläinen A-M, Muona P, Väätäinen M, Ilonen J, Simell O, Åkerblom HK: Cow's milk formula feeding induces primary immunization to insulin in infants at genetic risk for type 1 diabetes. *Diabetes* 48:1389–1394, 1999
11. Vaarala O, Paronen J, Otonkoski T, Åkerblom HK: Cow milk feeding induces antibodies to insulin in children: a link between cow milk and insulin-dependent diabetes mellitus? *Scand J Immunol* 47:131–135, 1998
12. Vaarala O, Klemetti P, Savilahti E, Reijonen H, Ilonen J, Åkerblom HK: Cellular immune response to cow's milk β-lactoglobulin in patients with newly diagnosed IDDM. *Diabetes* 45:178–182, 1996
13. Williams AJK, Bingley PJ, Bonifacio JP, Palmer EAM: A novel micro-assay for

insulin autoantibodies. *J Autoimmun* 10:473–478, 1997
14. Savilahti E, Saukkonen T, Virtala ET, Tuomilehto J, Åkerblom HK: Increased levels of cow's milk and β-lactoglobulin antibodies in young children with newly diagnosed IDDM: The Childhood Diabetes in Finland Study Group. *Diabetes Care* 16:984–989, 1993
15. Sjöroos M, Iitiä A, Ilonen J, Reijonen H, Lövgren T: Triple-label hybridization assay for type-1 diabetes-related HLA alleles. *Biotechniques* 18:870–877, 1995
16. Martikainen A, Saukkonen T, Kulmala PK, Reijonen H, Ilonen J, Teramo K, Koskela P, Knip M, Åkerblom HK: Disease-associated antibodies in offspring of mothers with IDDM. *Diabetes* 45:1706–1710, 1996
17. Warram JH, Krolewski AS, Gottlieb MS, Kahn CR: Differences in risk of insulin-dependent diabetes in offspring of diabetic mothers and diabetic fathers. *N Engl J Med* 311:149–152, 1984
18. Tuomilehto J, Lounamaa R, Tuomilehto-Wolf E, Reunanen A, Virtala E, Kaprio EA, Åkerblom HK: Epidemiology of childhood diabetes mellitus in Finland: background of a nationwide study of type 1 (insulin-dependent) diabetes mellitus: The Childhood Diabetes in Finland (DiMe) Study Group. *Diabetologia* 35:70–76, 1992
19. Gergely J, Sarmay G: FcγRII-mediated regulation of human B cells. *Scand J Immunol* 44:1–10, 1996
20. Peri BA, Rothberg RM: Specific suppression of antibody production in young rabbit kits after maternal ingestion of bovine serum albumin. *J Immunol* 127:2520–2255, 1981
21. Davis BK, Gill TJ: Decreased antibody response in the offspring of immunized high responder rats. *J Immunol* 115:1166–1168, 1975
22. Yamaguchi N, Shimizu S, Hara A, Saito T: The effect of maternal antigenic stimulation upon active immune responsiveness of their offspring. *Immunology* 50:229–238, 1983
23. Halsey JF, Benjamin DC: Induction of immunologic tolerance in nursing neonates by absorption of tolerogen from colostrum. *J Immunol* 116: 1204–1207, 1976
24. Keller RJ, Eisenbarth GS, Jackson RA: Insulin prophylaxis in individuals at high risk of type 1 diabetes. *Lancet* 341:927–928, 1993
25. Naik RG, Palmer JP: Preservation of β-cell function in type 1 diabetes. *Diabetes Rev* 7:154–182, 1999
26. Warram JH, Martin BC, Krolewski AS: Risk of IDDM in children of diabetic mothers decreases with increasing maternal age at pregnancy. *Diabetes* 40:1679–1683, 1991
27. Kurtz AB, Matthews JA, Mustaffa BE, Daggett PR, Nabarro JDN: Decrease of antibodies to insulin, proinsulin and contaminating hormones after changing treatment from conventional beef to purified pork insulin. *Diabetologia* 18: 147–150, 1980
28. Diaz JL, Wilkin T: Differences in epitope restriction of autoantibodies to native human insulin (IAA) and to heterologous insulin (IA). *Diabetes* 36:66–72, 1987
29. Ottensen JL, Nilson P, Jami J, Weiguny D, Duhrkop M, Bucchini D, Havelund S, Fogh JM: The potential immunogenicity of human insulin and insulin analogues evaluated in a transgenic mouse model. *Diabetologia* 37:1178–1185, 1994
30. Homann D, Dyrberg T, Petersen J, Oldstone MBA, von Herrath MG: Insulin in oral immune "tolerance": a one-amino acid change in the B chain makes the difference. *J Immunol* 163:1833–1838, 1999
31. Vaarala O, Saukkonen T, Savilahti E, Klemola T, Åkerblom HK: Development of immune response to cow milk proteins in infants receiving cow milk formula or hydrolyzed formula. *J Allergy Clin Immunol* 96:917–923, 1995
32. Jakobsson I, Lindberg T, Benediktsson B, Hansson B-G: Dietary bovine β-lactoglobulin is transferred to human milk. *Acta Paediatr Scand* 74:342–345, 1985
33. Jakobsson I, Lindberg T: A prospective study of cow's milk protein intolerance in Swedish infants. *Acta Paediatr Scand* 68:853–859, 1979
34. Aranda P, Sanchez L, Perez MD, Ena JM, Calvo M: Insulin in bovine colostrum and milk: evolution throughout lactation and binding to caseins. *J Dairy Sci* 74:4320–4325, 1991
35. Björksten B, Holt BJ, Baron-Hay MJ, Munir AK, Holt PG: Low-level exposure to house dust mites stimulates T-cell responses during early childhood independent of atopy. *Clin Exp Allergy* 26:775–779, 1996
36. Tikhomirov OY, Thomas JW: Preference for IgG mAb binding insulin in solution or on surfaces is related to immunoglobulin variable region structures. *J Autoimmun* 10:541–549, 1997
37. Keller RJ: Cellular immunity to human insulin in individuals at high risk for the development of type 1 diabetes mellitus. *J Autoimmun* 3:321–327, 1990
38. McCuish AC, Jordan J, Campbell CJ, Duncan LJP, Irvine WJ: Cell-mediated immunity in diabetes mellitus: lymphocyte transformation by insulin and insulin fragments in insulin-treated and newly-diagnosed diabetics. *Diabetes* 24:36–43, 1975
39. Wegmann DR, Norbury-Glaser M, Daniel D: Insulin-specific T-cells are a predominant component of islet infiltrates in pre-diabetic NOD mice. *Eur J Immunol* 24:1853–1857, 1994
40. Zhang JZ, Davidson L, Eisenbarth G, Weiner HL: Suppression of diabetes in nonobese mice by oral administration of porcine insulin. *Proc Natl Acad Sci U S A* 88:1052–1056, 1991
41. Bellmann K, Kolb H, Rastegar S, Jee P, Scott FW: Potential risk of oral insulin with adjuvant for the prevention of type 1 diabetes: a protocol effective in NOD mice may exacerbate disease in BB rats. *Diabetes* 41:844–847, 1998
42. Blanas E, Carbone FR, Allison J, Miller JFAP, Heath WR: Induction of autoimmune diabetes by oral administration of autoantigen. *Science* 274:1707–1709, 1996
43. Miller A, Lider O, Abramsky O, Weiner HL: Orally administered myelin basic protein in neonates primes for immune responses and enhances experimental autoimmune ecephalomyelitis in adult animals. *Eur J Immunol* 24: 1026–1032, 1994
44. Meyer AL, Benson JM, Gienapp IE, Cox KL, Whitacre CC: Suppression of murine chronic relapsing experimental autoimmune encephalomyelitis by the oral administration of myelin basic protein. *J Immunol* 157:4230–4238, 1996
45. Vaarala O: Gut and induction of immune tolerance in type 1 diabetes. *Diabetes Metab Res Rev* 15:353–361, 1999