Significance of cow's milk protein antibodies as risk factor for childhood IDDM: interactions with dietary cow's milk intake and HLA-DQB1 genotype

T. Saukkonen¹, S.M. Virtanen², M. Karppinen³, H. Reijonen⁴, J. Ilonen⁴, L. Räsänen³, H.K. Åkerblom¹, E. Savilahti¹, and the Childhood Diabetes in Finland Study Group*

¹ Children's Hospital, University of Helsinki, Helsinki, Finland

² Tampere School of Public Health, University of Tampere, Tampere, Finland

³ Division of Nutrition, Department of Applied Chemistry and Microbiology, University of Helsinki, Helsinki, Finland

⁴ Turku Immunology Centre and Department of Virology, University of Turku, Turku, Finland

Summary Dietary factors are suspected to play an aetiological role in the development of insulin-dependent diabetes mellitus (IDDM). We analysed cow's milk formula, betalactoglobulin, and bovine serum albumin antibodies by an enzyme-linked immunoassay in unselected children with newly diagnosed IDDM and in their non-diabetic siblings and enquired about infant feeding practices by questionnaire. Among 410 diabetic sibling pairs matched for age and sex, by logistic regression analysis -- including overall duration of breast-feeding, age at introduction of dairy products, recent consumption of cow's milk and HLA-DQB1 genotype ("high/moderate" vs "low/decreased" risk of IDDM) -- bovine serum albumin IgG antibody levels (OR 2.12, 95%CI 1.25--3.57) and genetic risk (OR 3.81, 95%CI 2.43--5.17) were positively associated with IDDM; cow's milk formula IgM antibodies were in-

versely associated with the risk of IDDM (OR 0.50, 95%CI 0.29--0.87). Of the diabetic sibling pairs, 42 were identical for HLA-DQB1 alleles associated with IDDM risk or protection (DQB1*0201, *0301, *0302 and *0602/03). In these 42 pairs, children with IDDM had higher median levels of bovine serum albumin IgG, of betalactoglobulin IgG, and of cow's milk formula IgG and IgA antibodies than the non-diabetic siblings ($p < 0.05$). In conclusion, children with IDDM have higher levels of cow's milk protein antibodies than their HLA-DQB1-matched sibling controls, and these high levels of antibodies are independent risk markers for IDDM. [Diabetologia (1998) 41: 72--78]

Keywords Cow's milk protein antibodies, infant feeding, dairy products, human leucocyte antigens, IDDM, children.

Autoimmune destruction of pancreatic beta cells and development of insulin-dependent diabetes mellitus (IDDM) is controlled by genetic factors, especially those related to the HLA class II gene region. Although certain HLA class II haplotypes (e.g. DR4-DQA1*0301-DQB1*0302 and DR3-DQA1*0501-DQB1*0201) are positively associated with the risk

of IDDM, some (especially DR15-DQA1*0102-DQB1*0602) have a protective effect against IDDM, and furthermore, this protective effect seems to predominate over susceptibility. Among the HLA class II loci, HLA-DQ shows the closest association with IDDM [1]. There is strong linkage disequilibrium between DQA1 and DQB1 alleles, and the effectiveness of genotyping of the HLA-DQB1 locus alone in determining the risk of IDDM at the population level has recently been reported [2].

Association between HLA class II genotypes and beta-cell autoantibodies seems to be heterogeneous. Patients with DQA1*0301-DQB1*0302 haplotypes more often have insulin autoantibodies (IAA), while glutamic acid decarboxylase (GAD) antibodies show an association with DQA1*0501-DQB1*0201 [3]. Interestingly, despite the dominant protective effect

* see Acknowledgements

Received: 13 June 1997 and in revised form: 26 August 1997

Corresponding author: Dr T. Saukkonen, Children's Hospital, University of Helsinki, Stenbäckinkatu 11, FIN-00290 Helsinki, Finland

Abbreviations: BLG, betalactoglobulin; BSA, bovine serum albumin; GAD, glutamic acid decarboxylase; IDDM, insulin-dependent diabetes mellitus; OR, odds ratio.

against IDDM of the DQB1*0602 allele, the presence of this allele does not seem to prevent the appearance of autoantibodies [4].

Earlier studies showed increased serum levels of antibodies to cow's milk proteins, betalactoglobulin (BLG), [5--7] and bovine serum albumin (BSA) [8--10] in newly diagnosed IDDM patients. One explanation for these findings could be that breast-feeding of IDDM patients has been shorter, and their exposure to cow's milk protein has occurred earlier than in control subjects, both increasing the risk for subsequent development of IDDM [11--14].

We have analysed infant feeding practices, recent cow's milk consumption, serum cow's milk protein antibodies, and HLA DQB1 genotypes in a large series of unselected children with newly diagnosed IDDM and their non-diabetic siblings. We attempted to find out whether a strong humoral immune response to cow's milk proteins results from a permissive genetic background as such, or whether it is a phenomenon independently associated with the development of IDDM. HLA-matched control subjects have not previously been used to evaluate the role of putative environmental risk factors for IDDM.

Subjects and methods

Subjects. In the nationwide Finnish Childhood Diabetes in Finland (DiMe) study, from September 1986 to April 1989, 801 consecutive families including 811 children aged less than 15 years with newly diagnosed IDDM were invited to participate in a project aimed at the evaluation of the role of genetic, environmental, and immunological factors in the development of IDDM [15]. Detailed dietary, sociodemographic, and neonatal data were collected by means of structured questionnaires. A venous blood sample was drawn from each child at the time of diagnosis of IDDM (or within 3 weeks after insulin treatment was begun), and at the same time from his/her non-diabetic siblings. Serum was separated, and the samples were kept frozen at -20°C until analysed. A complete panel of cow's milk protein antibody measurements was obtained for 746 children with newly diagnosed IDDM and for 624 of their non-diabetic siblings. HLA-DQ genotyping was performed in 635 children with IDDM and in 498 non-diabetic siblings. Data of 19 siblings were omitted because they had developed IDDM during follow-up of 6 to 10 years.

We have previously compared antibody levels between children with IDDM and non-diabetic siblings in the present series using paired analysis, in which for each child with diabetes, a sibling pair of the same sex and closest in age (from a different family) was selected [7]. Among 448 such diabetic-sibling pairs, we now searched for pairs in which the diabetic proband and the sibling pair had an identical combination of DQB1*0201, 0301, 0302, 0602--03 and/or $\times$ alleles. Forty-two such pairs were identified (ages, mean $\pm$ SD: diabetic children 8.6 ± 3.3 years; non-diabetic siblings 8.7 ± 3.3 years). Dietary data were obtained from 735 children with IDDM and from 746 siblings.

The study protocols were approved by the ethics committees of the participating hospitals. Informed consent was ob-

tained from the children or their parents before enrollment in the study.

Cow's milk protein antibody assays. Antibodies to cow's milk formula, BLG and BSA were measured by solid-phase enzyme-linked immunosorbent assays (ELISA) as described in detail earlier [7, 9]. In the measurement of cow's milk formula antibodies, diluted, defatted (1:500) adapted liquid cow's milk formula (Tutteli, Valio, Finland) was used as the antigen. The levels of antibodies were expressed as arbitrary units calculated from a dilution series of a standard serum pool with a high concentration of antibodies.

HLA-DQB1 genotyping. DQB1 analysis was based on the definition of alleles known in the Finnish population to be significantly associated with either susceptibility to IDDM (DQB1*0302, DQB1*0201) or protection against IDDM (DQB1*0301, DQB1*0602--03) [16]. This technique involved solution hybridization with lanthanide-labelled oligonucleotide probes detected with time-resolved fluorometry (TRF), as described previously in detail [17].

Dietary data. We collected detailed data on infant feeding patterns and on diet later on in childhood by means of a structured questionnaire (available from the authors) [12]. The following were the dietary variables: duration of overall (total) and exclusive (no foods other than breast milk) breast-feeding (in months); age at introduction of dairy products; amount of milk and sour milk (in glasses, 1 glass = 180 ml) consumed by all children daily before entering the study, and for children with IDDM, before any symptoms of diabetes had appeared.

Statistical analysis. Statistical analyses of levels of antibodies to cow's milk, BLG, and BSA were performed by non-parametric methods, because levels were not normally distributed. The Mann-Whitney U-test was applied to compare cow's milk protein antibody levels in subjects who had vs did not have the DQB1 alleles. We also analysed differences in antibody levels between those subjects having a DQB1 genotype associated with "high/moderate" risk of IDDM (0201/0302, 0302/ $\times$ [$\times$ = 302 or undefined allele]) and those having a "low/decreased" risk of IDDM (the remaining genotypes) [2]. Differences in frequency of detectable IgA antibodies were analysed by the chi-square test. In the diabetic-sibling pairs identical for the defined HLA-DQB1 alleles, we analysed differences in the levels of cow's milk protein antibodies using the Wilcoxon matched-pairs signed-rank test.

For multivariate analyses, the levels of antibodies were dichotomized, with the 75th percentile of the siblings as the cut-off for a "positive" level of antibodies. Odds ratios (OR) served to estimate associations of dietary variables with antibody levels and to estimate associations of dietary variables and antibody levels with risk of IDDM. Adjustment for confounding variables was performed by conditional logistic regression. Statistical analyses were performed with the Statistical Analysis System, BMDP, and StatView statistical software.

Results

Association of cow's milk protein antibody levels with DQB1 alleles. Study of antibody levels in siblings and children with IDDM with vs without the described DQB1 alleles revealed few differences (Tables 1 and 2). Among non-diabetic siblings, the highest levels of

Table 1. Levels of cow's milk protein antibodies in non-diabetic siblings ($n = 473$) by presence of specific HLA-DQB1 alleles

	<i>n</i>	Cow's milk formula IgA	Cow's milk formula IgG	Cow's milk formula IgM	BLG IgA	BLG IgG	BSA IgA	BSA IgG
*0201 +	133	3.4 (0.9--6.9)	3.0 (1.3--8.5)	53.2 (22.9--96.8)	0 (0--18.0)	7.8 (2.8--19.5)	0 (0--8.0)	0.7 (0--2.0)
*0201 -	340	2.8 (0.9--6.2)	2.9 (1.6--6.2)	40.9 (23.1--75.9)	0 (0--14.0)	7.1 (0--16.7)	0 (0--8.0)	0.5 (0--1.3) ^a
*0302 +	251	3.0 (0.9--7.4)	3.1 (1.5--8.1)	44.2 (23.0--80.4)	0 (0--18.0)	8.6 (2.8--18.7)	0 (0--8.4)	0.6 (0--1.3)
*0302 -	214	2.8 (0.9--6.0)	2.8 (1.5--6.3)	41.9 (22.9--80.6)	0 (0--12.0)	6.4 (0--16.2)	0 (0--7.6)	0.6 (0--1.9)
*0602 +	127	2.5 (0.8--6.1)	2.6 (1.2--6.3)	42.0 (24.7--83.8)	0 (0--12.0)	8.2 (2.5--16.4)	0 (0--9.0)	0.7 (0--1.5)
*0602 -	346	3.0 (0.9--6.7)	3.0 (1.6--7.0)	43.9 (22.8--78.6)	0 (0--16.0)	7.2 (0.6--18.0)	0 (0--7.6)	0.5 (0--1.5)
*0301 +	84	2.4 (0.7--6.1)	2.4 (1.3--4.8)	40.5 (18.9--62.5)	0 (0--11.5)	6.5 (0.3--15.1)	0 (0--7.2)	0.2 (0--1.1)
*0301 -	389	3.0 (1.0--6.5) ^b	3.0 (1.5--8.1)	44.0 (23.4--82.3)	0 (0--16.0)	7.8 (1.4--17.9)	0 (0--8.2)	0.6 (0--1.5) ^a
High risk	160 ^c	3.0 (1.0--7.5)	3.3 (1.6--8.7)	44.5 (22.9--81.7)	0 (0--20.0)	8.8 (2.9--20.2)	0 (0--8.0)	0.5 (0--1.2)
Low risk	313 ^d	2.7 (0.9--6.1)	2.8 (1.4--6.2)	41.8 (23.2--80.0)	0 (0--12.0)	6.6 (0--16.4)	0 (0--7.8)	0.6 (0--1.6)

Data are median (interquartile range). ^a $p < 0.01$, ^b $p < 0.05$ ^c "High risk" denotes DQB1 genotypes associated with high (0201/0302) or moderate (0302/x) risk for IDDM^d "Low risk" denotes DQB1 genotypes associated with low (0301/0302, 0201/0301, 0302/0602--03, 0201/x) or decreased (0602--03/x, 0301/0602--03, 0201/0602--03, 0301/x, x/x) risk for IDDM**Table 2.** Levels of cow's milk protein antibodies in children with newly diagnosed IDDM ($n = 624$) by presence of specific HLA-DQB1 alleles

	<i>n</i>	Cow's milk formula IgA	Cow's milk formula IgG	Cow's milk formula IgM	BLG IgA	BLG IgG	BSA IgA	BSA IgG
*0201 +	266	4.5 (1.8--10.2)	4.5 (2.0--19.5)	44.3 (25.0--75.0)	0 (0--28.0)	9.9 (4.2--22.8)	0 (0--14.0)	1.2 (0.5--3.2)
*0201 -	358	4.5 (1.5--9.5)	4.4 (2.1--18.3)	40.5 (23.2--63.4)	0 (0--28.0)	10.6 (3.8--22.2)	0 (0--11.6)	0.8 (0.4--2.1) ^a
*0302 +	447	4.8 (1.9--10.6)	4.5 (2.2--19.7)	40.6 (23.9--68.2)	0 (0--28.0)	10.2 (4.0--22.6)	6.4 (0--12.4)	0.8 (0.4--2.2)
*0302 -	177	3.6 (1.3--9.0) ^b	4.2 (1.4--18.0)	45.8 (25.6--75.1)	0 (0--26.0)	10.0 (4.0--22.4)	0 (0--11.9)	1.2 (0.4--3.6) ^b
*0602 +	42	5.7 (2.7--11.2)	5.7 (2.7--23.0)	45.0 (19.6--69.4)	8.0 (0--48.5)	11.7 (2.6--24.0)	0 (0--15.5)	0.8 (0.6--2.0)
*0602 -	582	4.2 (1.6--9.8)	4.3 (2.0--18.8)	41.5 (24.6--69.0)	0 (0--28.0)	10.1 (4.0--22.2)	0 (0--12.1)	0.9 (0.4--2.6)
*0301 +	90	4.9 (2.0--10.2)	6.1 (2.6--19.3)	48.3 (27.6--94.6)	0 (0--38.0)	11.7 (5.4--25.2)	6.8 (0--14.7)	1.4 (0.5--3.6)
*0301 -	534	4.2 (1.6--9.8)	4.3 (2.0--19.0)	41.0 (23.2--68.4)	0 (0--28.0)	10.0 (3.6--22.2)	0 (0--12.4)	0.8 (0.4--2.4)
High risk	381 ^c	4.7 (1.8--10.1)	4.4 (2.1--19.0)	40.6 (24.2--68.2)	0 (0--28.5)	10.6 (4.2--22.8)	6.4 (0--12.4)	0.8 (0.4--2.2)
Low risk	243 ^d	3.9 (1.4--9.7)	4.5 (1.7--19.2)	45.4 (23.8--71.6)	0 (0--26.5)	9.4 (3.6--22.0)	0 (0--13.2)	1.1 (0.4--3.4)

Data are median (interquartile range)

^a $p < 0.01$, ^b $p < 0.05$ ^c "High risk" denotes DQB1 genotypes associated with high (0201/0302) or moderate (0302/x) risk for IDDM^d "Low risk" denotes DQB1 genotypes associated with low (0301/0302, 0201/0301, 0302/0602--03, 0201/x) or decreased (0602--03/x, 0301/0602--03, 0201/0602--03, 0301/x, x/x) risk for IDDM

BSA IgG antibodies appeared in those with *0201 and lacking *0301 (Fig. 1). Levels of antibodies were similar in 0301 + 0201 + and 0301 + 0201- siblings, suggesting that the effect of 0301 is dominant over that of 0201 (Fig. 1). Levels of IgA antibodies to BSA and BLG were below the limit of detection in the majority of subjects; however, a detectable level of BLG IgA was more often observed in *0302 + siblings than in *0302- siblings (42.5 vs 33.5%, $p = 0.04$, χ^2 test). Among children with IDDM, those positive for *0201 had significantly higher levels of BSA IgG antibodies than did those negative for *0201, whereas *0302 + subjects had significantly lower BSA IgG and higher cow's milk formula IgA antibodies than did *0302 subjects (Table 2).

Median antibody levels were similar in siblings with DQB1 genotypes classified as "high/moderate risk" for IDDM (0201/0302 or 0302/x, where x denotes any other allele than 0201, 0602--03 or 0301) or having "low/decreased risk" (the remaining geno-

types) (Table 1). Nor were any differences found between IDDM children with "high/moderate risk" or having "low/decreased risk" genotypes (Table 2).

Levels of cow's milk protein antibodies in IDDM children and non-diabetic siblings matched for age, sex, and HLA-DQB1 alleles. Among our series of 448 age- and sex-matched diabetic-sibling pairs, we found 42 pairs identical for DQB1*0201, 0301, 0302, 0602--03 and/or x alleles. Of these pairs, 10 had DQB1*0201/0302, 24 had DQB1*0302/x, and 7 had DQB1*0201/x. The remaining three pairs had DQB1*0301/0302, DQB1*0302/0602--03 and x/x. Significantly higher median levels of 5 types of cow's milk protein antibodies out of the 7 tested were found in children with IDDM than in their HLA-DQB1 matched sibling pairs (Table 3).

Diet, HLA-DQB1 genotype, and the risk of IDDM. The effect of HLA-DQB1 genotype on the associa-

Fig. 1 Median levels of IgG antibodies to bovine serum albumin in non-diabetic siblings by presence of HLA-DQB1*0301 and/or *0201 allele * $p < 0.05$, ** $p < 0.001$. The number of subjects is indicated in each column

tions between infant feeding pattern and risk of IDDM was studied among diabetic sibling pairs. When either the overall duration of breast-feeding (≥ 6 months/4--5 months/2--3 months) or the age at introduction of dairy products (≥ 6 months/4--5 months/2--3 months) or recent consumption of cow's milk (≥ 3 glasses/ < 3 glasses) was included in the model simultaneously with one of the four defined DQB1 alleles or with the "high or moderate risk" DQB1 genotype, only the DQB1 genotype was significantly associated with risk of IDDM (data not shown).

Diet, HLA-DQB1 genotype, and the levels of cow's milk protein antibodies. To analyse the effect of dietary variables and HLA-DQB1 genotype on the levels of cow's milk protein antibodies in children with IDDM, we included age of subject, overall duration

of breast-feeding (≥ 4 months vs < 4 months), age at introduction of dairy products (≥ 4 months vs < 4 months), recent milk consumption (≥ 3 glasses vs < 3 glasses daily), and DQB1 genotype ("high/moderate" vs "low/decreased" risk) in a logistic regression model explaining one cow's milk protein antibody level at one time. We found the overall duration of breast-feeding to be inversely associated with BLG IgG antibodies (OR 0.53, 95% CI 0.30--0.93), age of introduction of dairy products to be inversely associated with cow's milk formula IgA (OR 0.57, 95% CI 0.33--0.96) and IgG (OR 0.56, 95% CI 0.33--0.95) antibody levels, and recent milk consumption to be positively associated with BSA IgA (OR 1.71, 95% CI 1.11--2.64) and IgG antibodies (OR 1.77, 95% CI 1.15--2.74). Age was inversely associated with cow's milk formula IgG (OR 0.89, 95% CI 0.85--0.95), BLG IgG (OR 0.92, 95% CI 0.87--0.97), and BSA IgG (OR 0.89, 95% CI 0.84--0.94) antibodies. DQB1 genotype (high/moderate risk vs low/decreased risk) was not significantly associated with any of the cow's milk protein antibody levels. However, when specific DQB1 alleles were studied in the same models instead of high or moderate risk genotype, the DQB1*0201 allele was associated with increased BSA IgG antibody levels (OR 1.68, 95% CI 1.15--2.46).

Diet, HLA-DQB1 genotype, cow's milk protein antibodies, and risk of IDDM. All IgA and IgG class cow's milk antibody levels were positively associated, with risk of IDDM independently of DQB1 genotype (high/moderate vs low/decreased risk) (Table 4). When overall duration of breast-feeding, age at introduction of dairy products, recent cow's milk consumption, genetic risk of IDDM and all cow's milk protein antibodies were included in the logistic model at the same time, BSA IgG antibodies and genetic

Table 3. Levels of antibodies to cow's milk proteins in diabetic-sibling pairs matched for age, sex, and HLA-DQB1 alleles (42 pairs)

	Children with IDDM	Non-diabetic siblings	<i>p</i> value
Cow's milk formula IgA	5.0 (2.3--11.4)	3.0 (1.4--5.8)	< 0.05
Cow's milk formula IgG	5.0 (2.5--19.6)	3.4 (1.6--9.2)	< 0.05
Cow's milk formula IgM	40.9 (19.9--72.7)	50.2 (23.4--87.9)	0.1
BLG IgA ^a	0 (0--39.0)	0 (0--5.0)	< 0.05
BLG IgG	14.4 (7.1--22.4)	6.9 (1.5--17.0)	< 0.05
BSA IgA	7.2 (0--12.5)	0 (0--11.6)	0.3
BSA IgG	0.8 (0.5--3.5)	0.6 (0--1.2)	< 0.05

Data are median (interquartile range). ^a BLG IgA was detectable in 45% of children with IDDM and in 31% of control children

Table 4. ORs and 95 % CIs for risk of IDDM by dietary variables, levels of cow's milk protein antibodies, and DQB1 genotype among 410 diabetic-sibling pairs

	Risk of IDDM		
	Unadjusted	Adjusted for genetic risk	Adjusted for all other variables in model
	OR 95 % CI	OR 95 % CI	OR 95 % CI
Overall breast-feeding (≥ 4 months)	0.81 (0.58--1.11)	0.83 (0.55--1.25)	1.13 (0.57--2.21)
Supplementary milk feeding (< 4 months)	1.27 (0.92--1.76)	1.23 (0.83--1.83)	1.14 (0.59--2.22)
Current milk consumption (≥ 3 glasses)	1.13 (0.82--1.55)	1.48 (0.99--2.23)	1.44 (0.89--2.32)
Cow's milk formula IgA (4 th quartile)	1.78 (1.32--2.42) ^c	2.02 (1.35--3.02) ^c	1.38 (0.83--2.32)
Cow's milk formula IgG (4 th quartile)	1.48 (1.08--2.03) ^a	1.56 (1.03--2.36) ^a	0.82 (0.48--1.42)
Cow's milk formula IgM (4 th quartile)	0.73 (0.52--1.04)	0.66 (0.42--1.05)	0.50 (0.29--0.87) ^b
BLG IgA (4 th quartile)	1.62 (1.20--2.19) ^b	1.53 (1.04--2.25) ^a	1.38 (0.86--2.23)
BLG IgG (4 th quartile)	1.47 (1.07--2.01) ^b	1.57 (1.06--2.34) ^a	1.26 (0.76--2.09)
BSA IgA (4 th quartile)	1.90 (1.40--2.59) ^c	2.27 (1.51--3.43) ^c	1.54 (0.96--2.48)
BSA IgG (4 th quartile)	1.65 (1.21--2.26) ^c	2.82 (1.82--4.38) ^c	2.12 (1.25--3.57) ^b
DQB1*0201/0302 or *0302/x ^d	3.16 (2.18--4.58) ^c		3.81 (2.43--5.97) ^c

^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$ for significant ORs and their 95 % CIs

^d x denotes any allele other than 0201, 0301 or 0602--03

risk were positively associated with IDDM, whereas cow's milk formula IgM antibodies were inversely associated with risk of IDDM among the diabetic sibling pairs (Table 4). BSA IgG antibody level was also analysed in the same model with dietary variables and the DQB1*0201 allele, since a significant association between DQB1*0201 and BSA IgG was detected in the previous analysis. In this model also, BSA IgG was associated with risk of IDDM (OR 1.71, 95 % CI 1.13--2.58).

Discussion

The present analysis of a large series of children with newly diagnosed IDDM and their non-diabetic sibling controls showed a significant association between serum levels of several cow's milk protein antibodies and the risk of IDDM, independently of the disease-associated HLA-DQB1 alleles. Children with IDDM had higher levels of cow's milk protein antibodies than their age-, sex-, and HLA-DQB1 matched non-diabetic sibling controls. Furthermore, in multivariate analysis including data on infant feeding and dietary milk consumption as well as cow's milk protein antibodies and HLA-DQB1 genotype, BSA IgG antibodies were positively associated with the risk of IDDM, and cow's milk formula IgM antibodies were inversely associated with the risk of IDDM.

A positive association between the DQB1*0201 allele and the levels of IgG antibodies to BSA was seen both in children with IDDM and in their non-diabetic siblings, suggesting that the presence of this allele results in a stronger humoral response to BSA irrespective of diabetes status. In multivariate analysis adjusted for age, the DQB1*0201 allele was positively associated with the level of BSA IgG antibodies in

children with IDDM independently of infant feeding and recent cow's milk consumption. An association between DQB1*0201 and GAD antibodies has been reported previously [3]; therefore, it is possible that subjects with this genotype are more susceptible to autoimmunity in general.

In studying factors that influence susceptibility to IDDM, controlling for the effect of HLA genotype is considered extremely important [18]. The major difficulty with this kind of approach, however, is in obtaining sufficient numbers of control subjects with HLA genotypes associated with high risk of IDDM [19]. Our solution to this problem was studying siblings of IDDM subjects, siblings who during a period of follow-up had not contracted the disease.

We found no difference in cow's milk protein antibody levels in subjects having or not having the major "IDDM-protective" allele DQB1*0602--03. Those siblings positive for DQB1*0301, an allele associated with a weaker protective effect for IDDM [2], had significantly lower levels of BSA and cow's milk formula IgG antibodies. One may speculate that the IDDM susceptibility effect associated with absence of the DQB1*0301 allele is somehow related to the production of IDDM associated cow's milk protein antibodies. The lack of influence of DQB1*0602--03 emphasizes the possibility that the pathogenesis of IDDM is a multistep process in which the HLA alleles associated with disease risk and protection also have heterogeneous effects.

The finding of an independent association of BSA IgG antibody levels with IDDM differs from our earlier results from Finnish and Swedish populations [20, 21]. In those studies, the HLA genotype was not included in our multivariate analyses. Immune response to BSA has received much attention since the work of Karjalainen et al. [8] which showed elevated BSA IgG antibodies in all newly diagnosed diabetic

patients studied. Although such marked differences in antibody levels between IDDM patients and control subjects have not been seen by other investigators, a number of well-controlled studies from several countries have shown moderately increased humoral BSA responses in newly diagnosed patients with IDDM compared with unrelated control subjects [9, 10, 22, 23]. Interestingly, Krokowski et al. [10] found higher BSA antibodies in DR3- and/or DR4-positive recently diagnosed diabetic adults compared with values for DR3- and/or DR4-positive controls, a result reminiscent of that in our paired analysis of HLA-DQB1-matched diabetic-sibling pairs. Clearly, further research on HLA-matched subjects is needed before conclusions can be drawn as to the role of immunity in relation to BSA in the pathogenesis of IDDM.

The level of IgM antibodies to cow's milk formula was inversely correlated with risk of IDDM in multivariate analysis, and accordingly, the levels tended to be lower in children with IDDM than in non-diabetic siblings in the paired analysis, thus being similar to earlier findings on IgM antibodies to BSA [8]. Levels of IgM antibodies typically decrease, and levels of IgG and IgA antibodies increase during maturation of an antigen-driven immune response, due to a phenomenon known as isotype switching. Therefore, our finding of an association between increased IgG antibodies to BSA and decreased IgM antibodies to cow's milk formula with the risk of IDDM suggests that the possible encounter with cow's milk proteins, leading to the activation of the immune system, in children with IDDM does not take place during or just before their development of overt symptoms of diabetes, but at an earlier age, perhaps as early as during their first year(s) of life.

Dietary intervention trials have been planned for infants with an increased genetic risk of IDDM in order to explore the effect of avoidance of dietary cow's milk in preventing IDDM [24]. In our comparison of age-, sex-, and HLA-DQB1-matched children with IDDM and siblings, higher levels of antibodies to cow's milk protein antigens were found in the former. A majority of the pairs (34/42) had a combination of DQB1 alleles associated with a high risk of IDDM (0201/0302 or 0302/non-protective allele). It thus seems that an enhanced immune response to cow's milk proteins is specifically associated with IDDM in a population with a high genetic risk of the disease.

Acknowledgements. This work was financially supported by the Foundation for Pediatric Research, Finland, the Sigrid Juselius Foundation, the Finnish Academy of Sciences, and the Finnish Diabetes Research Foundation. The "Childhood Diabetes in Finland" (DiMe) project was also supported by the National Institutes of Health, the Association of Finnish Life Insurance Companies, the University of Helsinki, Nordisk

Insulinfond, and the Dorothea Olivia, Karl Walter, and Jarl Walter Perkle Foundation.

The Childhood Diabetes in Finland Study Group:

Principal Investigators: H. K. Akerblom, J. Tuomilehto

Co-ordinators: R. Lounamaa, L. Toivanen, E. A. Kaprio

Data managers: E. Virtala, J. Pitkaniemi

Local investigators: A. Fagerlund, M. Flittner, B. Gustafsson, C. Häggqvist, A. Hakulinen, L. Herva, P. Hiltunen, T. Huhtamäki, N-P. Huttunen, T. Huupponen, M. Hyttinen, T. Joki, R. Jokisalo, M.-L. Käär, S. Kallio, E. A. Kaprio, U. Kaski, M. Knip, L. Laine, J. Lappalainen, J. Mäenpää, A.-L. Mäkelä, K. Niemi, A. Niiranen, P. Ojajarvi, T. Otonkoski, K. Pihlajamäki, S. Pöntynen, J. Rajantie, J. Sankala, J. Schumacher, M. Sillanpää, M.-R. Stahlberg, C.-H. Strahmann, T. Uotila, M. Väre, P. Varimo.

Special investigators: A. Aro, M. Hiltunen, H. Hurme, H. Hyöty, J. Ilonen, J. Karjalainen, M. Knip, P. Leinikki, A. Miettinen, T. Petäys, H. Reijonen, L. Räsänen, A. Reunanen, T. Saukkonen, E. Savilahti, E. Tuomilehto-Wolf, P. Vähäsalo, S. M. Virtanen.

References

1. Thorsby E, Ronningen KS (1993) Particular HLA-DQ molecules play a dominant role in determining susceptibility or resistance to type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* 36: 371-377
2. Ilonen J, Reijonen H, Herva E, et al. (1996) Rapid HLA-DQB1 genotyping for four alleles in the assessment of risk for IDDM in the Finnish population. *Diabetes Care* 19: 795-800
3. Hagopian WA, Sanjeevi CB, Kockum I, et al. (1995) Glutamate decarboxylase-, insulin-, and islet cell-antibodies and HLA typing detect diabetes in a general population-based study of Swedish children. *J Clin Invest* 95: 1505-1511
4. Huang W, She JX, Muir A, et al. (1994) High risk HLA-DR/DQ genotypes for IDDM confer susceptibility to autoantibodies but DQB1*0602 does not prevent them. *J Autoimmun* 7: 889-897
5. Savilahti E, Akerblom HK, Tainio V-M, Koskimies S (1988) Children with newly diagnosed insulin dependent diabetes mellitus have increased levels of cow's milk antibodies. *Diabetes Res* 7: 137-140
6. Dahlquist G, Savilahti E, Landin-Olsson M (1992) An increased level of antibodies to β -lactoglobulin is a risk determinant for early-onset type 1 (insulin-dependent) diabetes mellitus independent of islet cell antibodies and early introduction of cow's milk. *Diabetologia* 35: 980-984
7. Savilahti E, Saukkonen TT, Virtala ET, Tuomilehto J, Akerblom HK, Childhood Diabetes in Finland Study Group (1993) Increased levels of cow's milk and β -lactoglobulin antibodies in young children with newly diagnosed IDDM. *Diabetes Care* 16: 984-989
8. Karjalainen J, Martin JM, Knip M, et al. (1992) A bovine albumin peptide as a possible trigger of insulin-dependent diabetes mellitus. *N Engl J Med* 327: 302-307
9. Saukkonen T, Savilahti E, Vaarala O, et al. (1994) Children with newly diagnosed insulin-dependent diabetes mellitus have increased levels of antibodies to bovine serum albumin but not to ovalbumin. *Diabetes Care* 17: 970-976
10. Krokowski M, Caillat-Zucman S, Timsit J, et al. (1995) Anti-bovine serum albumin antibodies: genetic heterogeneity and clinical relevance in adult-onset IDDM. *Diabetes Care* 18: 170-173

11. Borch-Johnsen K, Mandrup-Poulsen T, Zachau-Christian B, et al. (1984) Relation between breast-feeding and incidence rates of insulin-dependent diabetes mellitus. *Lancet* ii:1083--1086
12. Virtanen SM, Räsänen L, Aro A, et al. (1991) Infant feeding in Finnish children < 7 yr of age with newly diagnosed IDDM. *Diabetes Care* 14: 415--417
13. Virtanen SM, Räsänen L, Ylönen K, et al. (1993) Early introduction of dairy products associated with increased risk for insulin-dependent diabetes mellitus in Finnish children. *Diabetes* 42: 1786--1789
14. Gerstein HC (1994) Cow's milk exposure and type I diabetes mellitus -- a critical overview of the clinical literature. *Diabetes Care* 17: 13--19
15. Tuomilehto J, Lounamaa R, Tuomilehto-Wolf E, et al. (1992) Epidemiology of childhood diabetes mellitus in Finland -- background of a nationwide study of type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* 35: 70--76
16. Reijonen H, Ilonen J, Knip M, Akerblom HK (1991) HLA-DQB1 alleles and absence of Asp-57 as susceptibility factors of IDDM in Finland. *Diabetes* 40: 1640--1644
17. Sjöroos M, Iitiä A, Ilonen J, Reijonen H, Lövgren T (1995) Triple-label hybridization assay for type-1 diabetes-related HLA alleles. *Biotechniques* 18: 870--877
18. Pozzilli P, Buzzetti R, Bottazzo GF, Tosi R (1993) The selection of control subjects for case/control analysis of susceptibility to type 1 (insulin-dependent) diabetes mellitus. *Diabetologia* 36: 1208--1209
19. Petrovsky N, Harrison LC (1995) HLA-matched control subjects are essential in studies of susceptibility to IDDM. *Diabetologia* 38: 125--126
20. Baisch JM, Weeks T, Giles R, Hoover M, Statsny P, Capra JD (1990) Analysis of HLA-DQ genotypes and susceptibility in insulin-dependent diabetes mellitus. *N Engl J Med* 322: 1836--1841
21. Virtanen SM, Saukkonen T, Savilahti E, et al. (1994) Diet, cow's milk protein antibodies and the risk of IDDM in Finnish children. *Diabetologia* 37: 381--387
22. Saukkonen T, Savilahti E, Landin-Olsson M, Dahlquist G (1995) IgA bovine serum albumin antibodies are increased in newly diagnosed patients with insulin-dependent diabetes mellitus, but the increase is not an independent risk factor for diabetes. *Acta Paediatr* 84: 1258--1261
23. Lühder F, Schlosser M, Michaelis D, Ziegler B, Kohnert K-D, Ziegler M (1994) No association between anti-bovine serum albumin antibodies and islet cell reactive antibodies in newly diagnosed type 1 diabetic patients. *Diabetes Res Clin Pract* 26: 35--41
24. Pardini VC, Vieira JGH, Miranda W, Ferreira SRC, Velho G, Russo EMK (1996) Antibodies to bovine serum albumin in Brazilian children and young adults with IDDM. *Diabetes Care* 19: 126--129
25. Akerblom HK, Savilahti E, Saukkonen TT, et al. (1993) The case for elimination of cow's milk in early infancy in the prevention of type 1 diabetes -- the Finnish experience. *Diabetes Metab Rev* 9: 269--278