Risk of Celiac Disease in Children With Type 1 Diabetes Is Modified by Positivity for *HLA-DQB1*02-DQA1*05* and *TNF −308A*

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OBJECTIVE — The overlap between genetic susceptibility to celiac disease (CD) and to type 1 diabetes is incomplete; therefore, some genetic polymorphisms may significantly modify the risk of CD in subjects with type 1 diabetes. This study aimed to investigate whether the susceptibility to CD in diabetic children is modified by positivity for *HLA-DQB1*02-DQA1*05* and *DQB1*0302-DQA1*03* and by alleles of single nucleotide polymorphisms within the genes encoding *CTLA4*, transforming growth factor (*TGF*)-β, tumor necrosis factor (*TNF*)-α, interferon (*IFN*)-γ, interleukin (*IL*)-1, *IL-2*, *IL-6*, and *IL-10*.

RESEARCH DESIGN AND METHODS — Genotypic data were compared between 130 case subjects (children with type 1 diabetes and CD diagnosed using endomysium antibodies) and 245 control subjects (children with type 1 diabetes only, optimally two per case, matched for center, age at type 1 diabetes onset, and type 1 diabetes duration). The subjects were recruited from 10 major European pediatric diabetes centers performing regular screening for CD. The polymorphisms were determined using PCR with sequence-specific primers, and the risk was assessed by building a step-up conditional logistic regression model using variables that were significantly associated with CD in the univariate analysis.

RESULTS — The best-fitted model showed that risk of CD is increased by presence of *HLA-DQB1*02-DQA1*05* (odds ratio 4.5 [95% CI 1.8–11], for homozygosity, and 2.0 [1.1–3.7], for a single dose) and also independently by *TNF −308A* (1.9 [1.1–3.2], for phenotypic positivity), whereas IL1-α −889T showed a weak negative association (0.6 [0.4–0.9]).

CONCLUSIONS — The results indicate that the risk of CD in children with type 1 diabetes is significantly modified both by the presence of *HLA-DQB1*02-DQA1*05* and by a variant of another gene within the major histocompatibility complex, the *TNF −308A*.

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Celiac disease (CD) is the second most prevalent autoimmune condition accompanying type 1 diabetes, after autoimmune thyroid disease. In Europe, CD is ~10 times more frequent in patients with type 1 diabetes than in the respective general populations (1). The reason for the association has not yet been fully elucidated. The environment of the ongoing diabetic autoimmunity may be a stimulant to the development of CD, a disease that possesses autoimmune features. Also, the genetic susceptibility to both conditions substantially overlaps: a certain degree of susceptibility to both diseases is associated with the HLA molecules *DQB1*0302-DQA1*03* and *DQB1*02-DQA1*05*, encoded either in *cis* or *trans*. The *CTLA4* polymorphisms have also been implicated, as well as several polymorphisms within the genes encoding for cytokines.

However, the overlap of the genetic susceptibility is incomplete. This is well demonstrated in the *HLA-DQ* region: the main determinant of CD risk is *HLA-DQB1*02-DQA1*05*, whereas for type 1 diabetes in populations of central and northern Europe, it is *HLA-DQB1*0302-DQA1*03*. There are also strong indications that certain variants of the tumor necrosis factor (*TNF*)-α gene, another gene residing within the major histocompatibility complex, are associated with CD independently of *HLA-DQ* (2–5), whereas in type 1 diabetes, the *TNF* association is secondary to that of *HLA* (6,7), although this opinion is not universally accepted (8,9). *CTLA4 +49A/G* is a marker associated with both diseases: type 1 diabetes (as well as several other autoimmune diseases) is associated with the G allele (rev. in 10), whereas for CD, the studies on the association have yielded conflicting results.

Thus, CD and type 1 diabetes differ in the strength or direction of genetic association with particular alleles of the same genes. Such a difference might be useful for determining the level of CD risk among patients with type 1 diabetes. This idea is appealing because genetic testing

Table 1—*Characteristics of case subjects (diabetic children with CD diagnosed using endomysial antibodies) and matched control subjects (diabetic children without CD)*

	Case subjects	Control subjects
n	130	245
The Czech Republic (Prague)	34	69
Austria (Vienna)	23	46
Slovenia (Ljubljana)	21	29
Hungary (Pecs and Budapest)	25	48
Germany (Berlin and Hannover)	18	35
Portugal (Lisbon)	5	9
Slovakia (Kosice)	4	9
M:F ratio (n)	72:58	134:111
Ethnicity (n)		
European Caucasian	128	243
Other (Asian)	2	2
Age at type 1 diabetes onset [median (interquartile range)]	4.6 (2.8–8.2)	4.6 (2.2–7.7)
Type 1 diabetes duration at study [median (interquartile range)]	7.3 (4.3–11)	6.9 (3.9–11)
Results of small bowel biopsy in case subjects		
Results available	114 (88%)	
Total or subtotal atrophy	97	
Increased intraepithelial lymphocytes	13	
Normal	4	
Results not available	16 (12%)	
Biopsy not performed–typical symptoms present	3	
Biopsy refused by parents	5	
Unsuccessful biopsy	2	
Biopsy not indicated by physician	6	

might introduce ways to better target the CD antibody screening, ultimately leading to more efficient early detection of the condition.

The hypothesis of an *HLA-DQB1*02-DQA1*05*–associated susceptibility to CD in children with type 1 diabetes has been tested in several previous single-center studies. Most of these studies suffer from a low statistical power to detect a possible effect. To overcome this difficulty, we set out to undertake a sufficiently powered international multicenter case-control study aimed to answer the question whether *HLA-DQB1*02-DQA1*05, HLA-DQB1*0302-DQA1*03, CTLA4 +49A/G* polymorphisms, and polymorphisms within selected cytokine genes modify the risk of CD among children with type 1 diabetes. The aim was to test DNA from all available children with both type 1 diabetes and CD (case subjects) and to compare their genotypes with closely matched children with type 1 diabetes but without CD (control subjects). We decided to

match for the following: center because genetic background differs among populations, age at diabetes onset because it is influenced by the *HLA-DQ* genotype, and diabetes duration because the cumulative risk of CD rises within years after the diagnosis of type 1 diabetes.

RESEARCH DESIGN AND METHODS — We performed a case-control study where the case subjects were children with both CD and type 1 diabetes and the control subjects were children with type 1 diabetes only, matched to the case subjects by center, age at type 1 diabetes onset, and duration of diabetes. Optimally, two control subjects should have been identified for each case. Ten centers from seven countries participated in the study. All participating children should have manifested with type 1 diabetes by their 15th birthday, and case subjects should have manifested with CD by their 18th birthday. All available diabetic children with CD cared for

by the center were approached and asked to participate. The nature of the study was described to the child, parents, or guardian before a written consent was obtained, in accordance with the procedures approved by the ethics committee at the particular center. Basic clinical and laboratory data were collected retrospectively from the patient's records. Two matched control subjects were identified for each case. First, the case was assigned to a 5-year band of age at type 1 diabetes onset. Then, two control children were selected at the same center from the same age-at-onset band, one being the child manifesting with type 1 diabetes just before and the other just next to the case. If the selected control subjects were not available or did not agree to participate, the selection went further to the next eligible control subject.

The characteristics of subjects are given in Table 1. The participating centers recruited 130 eligible case subjects with both type 1 diabetes and CD. The case subjects had been identified by endomysium antibody screening that was positive on two separate occasions and confirmed by small bowel biopsy in most instances. The case subjects were matched to 245 control subjects (for single case subjects, two matched counterparts could not be found). The control subjects were deemed free of CD based on the annual endomysium antibody testing. The case and control subjects were European Caucasians except for four subjects of Asian origin (one control subject from Vienna, one case subject from Berlin, and one case and one control subject from Hannover). Several exceptions were allowed from the matching criteria because the center had no better control subjects available: 25 (10%) control subjects were selected from the neighboring lower or higher band of age at type 1 diabetes onset, and seven control subjects were selected although they had manifested with type 1 diabetes after their 15th birthday, with the maximum age at onset being 17 years and 1 month.

Either genetic material was obtained from the center's DNA archives or EDTA-anticoagulated blood was drawn from the child, sent by mail to the central laboratory, and processed using the QiaAmp Blood DNA extraction kits (Qiagen, Germany). All DNA samples were quantified using a real-time assay for the human albumin gene, and 83 of 375 (22%) samples, which had an insufficient total DNA amount, were pre-amplified using the

Genomi-Phi whole-genome amplification kit (Amersham) according to the manufacturer's instructions. Because all downstream assays were based on PCR with sequence-specific primers (PCR-SSP), the quantity of the DNA was equalized among all samples by diluting, using data from real-time quantification of the human albumin gene.

The genotypes of HLA-DQB1, -DQA1 were determined using an in-house assay based on PCR-SSP. The performance of the assays is controlled by an exchange of samples with the Tissue Typing Laboratory IKEM in Prague, a laboratory accredited by the European Federation of Immunogenetics. The assay for *DQA1* and *DQB1* typing includes 24 reactions distinguishing the *DQA1*01–06* alleles and the *DQB1*02, 0301, 0302, 0303, 0304, 0401, 0402, 0501, 0502, 0503, 0601, 0602, 0603,* and *0604–9* alleles. Full-typing data were necessary to determine the dose of *DQB1*02-DQA1*05* and *DQB1*0302* in the individual. The *CTLA4 +49A/G* was determined using a previously reported PCR-SSP technique (11). The cytokine single nucleotide polymorphism (SNP) PCR-SSP typing was performed using a protocol with the sequence-specific primers developed for the cytokine component of the 13th International Histocompatibility Workshop and Conference (IHWC) (12). The laboratory was controlled within the 13th IHWC proficiency testing and achieved the accuracy of 98%. Seventeen SNPs were tested within 10 different genes for cytokines (interleukin [*IL*]*-1α −889 T/C; IL-1β −511 C/T* and *+3962 C/T; IL-2 −330 T/G* and *+160 G/T; IL-6 −174 G/C* and *+565 G/A; IL-10* haplotypes of three SNPs at −1082, −819, and −590, *ACC/ GCC/ATA; TNF −308 A/G* and *−238 G/A; TGFβ1 codon 10 C/T* and *25 G/C; IFN-γ UTR 5644 A/T*) and for IL-1–related molecules (*IL-1R 1970 C/T, IL-1RA 11100 T/C*). The design of the sequence-specific primers also allowed us to directly determine the phase of SNPs in haplotypes of *TGF-β1, TNF, IL-2, IL-6,* and *IL-10*.

The association of the variants with CD was first tested by conditional logistic regression for matched data. The outcome variable identified whether the individual was a case subject (patient with type 1 diabetes and CD, encoded as 1) or a control subject (patient with type 1 diabetes only, encoded as 0). The identifier of the matching group was used as the conditioning variable. The independent variables were the phenotype positivity

for the examined alleles or haplotypes in a univariate model. Then, the variables achieving a *P* value of the Wald statistics at <0.05 in the univariate analysis were used for step-up building of a conditional logistic regression model. The best-fitted model was determined using the likelihood ratio test statistics. All models were—apart from conditioning for the matching identifier—adjusted for the matching variables. Analyses were performed using the Stata 9.0 program (StataCorp, College Station, TX). The sample size provided an 80% power at $\alpha = 0.05$ to detect an effect size with an odds ratio (OR) of ≥1.83 for a genetic factor present in 50% of the control subjects (e.g., the *HLA-DQB1*02-DQA1*05*): this OR corresponds to a ≥65% prevalence in case subjects. Similarly, the study had an 80% power to detect an OR >1.86 for a genetic factor with a 30% prevalence among control subjects or OR >2.07 for a genetic factor with a 70% prevalence.

RESULTS — The results of the univariate analysis of the case-control status versus phenotypic positivity of the studied factors are shown in Table 2. *HLA-DQB1*02-DQA1*05* was found in 77% of the case subjects and 53% of the control subjects, being strongly associated with CD among type 1 diabetic subjects ($P < 10^{-3}$). The effect of *DQB1*02-DQA1*05* is adequately described by a codominant model taking the gene dose into account; there is a proportional rise in risk with gene dose. *HLA-DQB1*0302-DQA1*03* shows a weak negative association with CD. Among the cytokine genes, *TNF −308A* is strongly associated with CD ($P < 10^{-3}$), whereas the weak negative association of *IL1α −889* disappears after correction for multiple testing.

The best-fitted regression model is shown in Table 3. Although *HLA-DQB1*0302-DQA1*03* completely lost its effect after being adjusted for *HLA-DQB1*02-DQA1*05*, both cytokine SNP alleles retained the association observed in the univariate analysis. Homozygosity for *HLA-DQB1*02-DQA1*05* increased the risk of CD roughly fourfold, whereas the presence of a single dose of *DQB1*02-DQA1*05* doubled the risk relative to individuals carrying no *HLA-DQB1*02-DQA1*05*. In the final model, both *HLA-DQB1*02-DQA1*05* and *TNF −308A* retained the statistical significance, indicating an independent effect: the risk was roughly doubled also by the presence of the *−308A* variant of the *TNF*. While the

association of *HLA-DQB1*02-DQA1*05* was dose dependent, such an effect could neither be confirmed nor refuted for the *TNF −308A* dose. *IL-1α −889T* was associated with a marginal decrease in CD risk. No interaction terms between the variables could further improve the fit.

The results did not change after exclusion of four subjects having other than European Caucasian descent (e.g., the OR for a double dose of *DQB1*02-DQA1*05* was 4.45 [95% CI 1.75–11.3], in the whole dataset vs. 4.27 [1.67–10.9], after exclusion of the subjects of non-European descent). Including sex into the analysis as one of the predictors neither improved the fit nor changed the results at the first two decimal positions of the risk estimate.

CONCLUSIONS — The present study clearly demonstrates that factors inside HLA significantly modify the risk of CD in children with type 1 diabetes. The *HLA-DQB1*02-DQA1*05* increases the risk in a dose-dependent manner, with a significant contribution of *TNF −308A*.

The observed effect of *HLA-DQB1*02-DQA1*05* may have been expected in populations where *DQB1*02-DQA1*05* confers a lower risk of type 1 diabetes than CD but has seldom been documented. The first study to indicate that *DQB1*02* is overrepresented among diabetic children with CD was published 10 years ago by Saukkonen et al. (13), based on a longitudinal observation of 775 diabetic children that identified 18 case subjects of CD diagnosed after type 1 diabetes onset. A later Czech study suggested that *HLA-DQB1*0201-DQA1*05* is associated with a fourfold increased risk of CD in children with type 1 diabetes (14), based on data from 15 children with CD identified from a cohort of 345 diabetic children, of whom 186 were genotyped. However, the CI of the risk estimate was wide. The association was not replicated in an Italian study of 25 children with CD and type 1 diabetes compared with 79 children with type 1 diabetes only (15): the *DQB1*0201-DQA1*05* frequency was 68% in the former group and 62% in the latter group. The authors concluded that the previously seen association may have been population specific, which is likely particularly for the Italian population where *DQB1*0201-DQA1*05* is frequent among diabetic subjects. Another study comes from Australia (16): an increased frequency of *HLA-DQB1*0201-DQA1*05*

Table 2—*Univariate analysis of genetic factors tested for association with CD in children with diabetes*

	Case subjects (%)	Control subjects (%)	OR (95%CI)	P
n	130	245		
HLA-DQB1*02-DQA1*05				
Dominant model*	77	53	2.97 (1.78–4.97)	<0.001
Codominant model†				
Two doses of *DQB1*02-DQA1*05*	17	7	5.94 (2.54–14)	<0.001
Single dose of *DQB1*02-DQA1*05*	60	46	2.53 (1.48–4.34)	0.001
No *DQB1*02-DQA1*05*	23	47	1.0: reference	
HLA-DQB1*0302-DQA1*03	52	65	0.58 (0.36–0.93)	0.024
Cytokines and related molecules				
IL-1α −889 T/C				
T	38	49	0.64 (0.42–0.98)	0.041
C	94	90	1.69 (0.72–3.93)	0.23
IL-1β				
−511 C	88	91	0.84 (0.42–1.69)	0.64
−511 T	59	56	1.12 (0.72–1.74)	0.61
+3962 C	98	97	1.84 (0.47–7.2)	0.38
+3962 T	41	45	0.82 (0.54–1.26)	0.37
IL-1R 1970 C/T				
C	88	87	1.15 (0.58–2.30)	0.69
T	59	55	1.18 (0.76–1.87)	0.46
IL-1RA 11100 T/C				
T	89	93	0.59 (0.27–1.31)	0.20
C	53	59	0.81 (0.52–1.26)	0.36
IFN-γ UTR 5644 A/T				
A	84	82	1.19 (0.68–2.10)	0.54
T	72	72	1.00 (0.61–1.63)	1.0
TGF-β1				
Codon 10 C	68	60	1.35 (0.87–2.11)	0.12
Codon 10 T	81	84	0.77 (0.43–1.37)	0.37
Codon 25 G	100	99		0.99
Codon 25 C	12	15	0.75 (0.38–1.46)	0.40
TNF-α				
−308 G	90	93	0.59 (0.26–1.36)	0.22
−308 A	68	45	2.7 (1.7–4.31)	<0.001
−238 G	100	100		0.99
−238 A	5	10	0.44 (0.18–1.1)	0.080
IL-2				
−330 T	95	95	1.01 (0.34–2.7)	0.98
−330 G	52	47	1.22 (0.78–1.91)	0.38
+160 G	85	83	1.36 (0.74–2.48)	0.32
+160 T	54	63	0.67 (0.43–1.03)	0.068
IL-6				
−174 G	79	82	0.84 (0.5–1.44)	0.54
−174 C	71	70	1.2 (0.73–1.99)	0.46
nt565 G	82	86	0.76 (0.44–1.33)	0.34
nt565 A	67	68	1.05 (0.65–1.71)	0.83
IL-10 haplotype				
ACC	59	50	1.52 (0.98–2.34)	0.064
ATA	47	51	0.77 (0.49–1.19)	0.24
GCC	61	64	0.98 (0.68–1.52)	0.94
CTLA4				
+49A	86	80	1.44 (0.81–2.57)	0.21
+49G	59	64	0.9 (0.59–1.38)	0.64

The ORs come from conditional logistic regression models where the outcome variable was the presence of CD in the diabetic child (i.e., whether the individual is a case = 1 versus a control = 0). The conditioning variable was the identifier of the matched set (matching stratum). The model was adjusted for the matching variables of age at diabetes onset and duration of diabetes. The presented *P* values are not corrected for multiple testing. *In the dominant model, the predictor variable was 1 when any *DQB1*02-DQA1*05* was present (irrespective of dose) and 0 when no *DQB1*02-DQA1*05* was present. †In the codominant model, two indicator variables compare the levels of risk associated with the double dose and the single dose of *HLA-DQB1*02-DQA1*05*, relative to its absence.

Table 3—*The best-fit multiple regression model for risk of CD in children with type 1 diabetes*

Genetic factor	OR (95%CI)	Wald *P*
*HLA-DQB1*02-DQA1*05*		
Double dose*	4.45 (1.75–11.3)	0.002
Single dose*	2.04 (1.11–3.76)	0.022
TNF-α −308A	1.87 (1.10–3.20)	0.021
IL-1α −889T	0.58 (0.37–0.93)	0.023

The variables associated with CD in the univariate model were used to build a step-up multiple model. The predictor variables included two indicator variables showing a double and single dose of *HLA-DQB1*02-DQA1*05* and dichotomous variables showing the phenotypic positivity for a given allele of a cytokine SNP. The adequacy of inclusion of predictor variables into the model was tested using the log-likelihood ratio test. No interaction terms improved the model. The effect of *HLA-DQB1*0302-DQA1*03* observed in the univariate analysis was lost after adjusting for *HLA-DQB1*02-DQA1*05*. *Two indicator variables compare the levels of risk relative to an absence of *HLA-DQB1*02-DQA1*05*.

was seen in diabetic subjects with CD (10/13, 77%) compared with subjects with type 1 diabetes but not CD (70/118, 59%), but this difference was not statistically significant. A clear limitation to these studies is the low number of identified case subjects (diabetic children also having CD): a single-center study therefore has a very substantial risk of type II error, i.e., not refuting the false null hypothesis due to low statistical power. Negative results reported by some of the studies should therefore be interpreted with caution. Two articles were published by a group from Denver, Colorado, who used positivity for the transglutaminase antibody as an end point, thus increasing the number of case subjects: the studies indicate a significant contribution of genetic factors to CD risk in diabetic individuals, since an increased prevalence of transglutaminase antibodies was reported in diabetic children carrying *DQB1*02-DQA1*05* or being *DQB1*02-DQA1*05* homozygous (17,18). Possible weak points of these two reports are that the antibodies were measured only one time and that analysis on ethnicity is not reported in a population that is likely to contain ethnic minorities.

In the present report, we show a significant effect of *HLA-DQB1*02-DQA1*05* positivity that is probably a compound of the effects of *DQB1*02-DQA1*05* itself and other genetic factors carried on the *DQB1*02-DQA1*05* haplotypes, namely the *TNF −308A*. Whereas the pathogenic effect of *DQB1*02-DQA1*05* in CD is well established and the association is primary, the association of the *TNF −308A* may be due either to its functional significance as a primary risk modifier or to a linkage disequilibrium to another variant on the haplotype. The former alternative is plausible for TNF-α,

a potent proinflammatory cytokine involved in T-cell immunity. However, the data on the functional significance of its polymorphisms are conflicting (rev. in 19), and the *TNF −308A* may be just a passive marker traveling on a proinflammatory haplotype. The *TNF-α* gene lies between HLA class II and class I in what is termed the class III region. *TNF −308A* is known to be associated with the proinflammatory 8.1 ancestral haplotype, so other components of the haplotype may significantly contribute as well. There are many genes with known inflammatory roles in the close vicinity of TNF-α: lymphotoxin α, the heat-shock protein-70 complex, the complement components 4A and 4B, and others.

Although the *TNF −308A* remains associated when adjusted for *DQB1*02-DQA1*05* positivity, this fact does not allow us to deem the genetic association truly independent. Adjusting or matching only for *DQB1*02-DQA1*05* phenotypic positivity is not enough, because we do not control the other chromosome in heterozygotes carrying one dose of *DQB1*02-DQA1*05* (20). An alternative approach to dissecting the association may be to analyze only subjects positive for a fixed *HLA-DQ* genotype, e.g., the frequent *HLA-DQB1*02-DQA1*05/DQB1*0302-DQA1*03*. However, the power of this type of analysis of our dataset is low, because there are only 23 matching groups where a case and at least one control are positive for this genotype. In this small subset, we observed indications that the *TNF −308A* is associated with an increased risk of CD (OR 2.4 [95% CI 0.63–9.1], *P* = 0.20).

An optimal design for a future study verifying the independence of the *TNF −308A* effect in determining the CD risk among diabetic individuals would involve matching for the primarily associ-

ated *DQB1*02-DQA1*05* on both chromosomes. As seen in Table 2, there are not many *DQB1*02-DQA1*05* homozygotes, only 17% among the case subjects and 7% among the control subjects. Thus, for such a relatively rare combination of diseases, it would be especially difficult to recruit a sufficient number of *HLA-DQB1*02-DQA1*05* homozygous diabetic individuals with CD and to match them adequately with control subjects with type 1 diabetes but without CD.

The present study was the first to use matching for age at diabetes onset and duration of diabetes. This is because the age at onset is known to be influenced by the child's HLA genotype and because the cumulative incidence of CD rises with duration of exposure to type 1 diabetes. This careful matching, together with matching for study center, should eliminate possible bias due to the recent shift toward a younger age at type 1 diabetes onset observed in some populations. Another important reason for matching is the heterogeneity in the CD prevalence in diabetic children: the prevalences previously reported from populations in this study range from 3 to 12%, moreover with significant variations over time (14,21–25). Consequently, reliable estimations of absolute risks attributable to particular genotypes are impossible, although knowledge of them might be of interest for the clinician.

An important source of technical error was eliminated by equalizing the DNA content across the samples using real-time quantification. All the genotyping reactions we used were based on PCR with sequence-specific primers, a technique known to be sensitive to uneven DNA content. The adequacy of this approach is well documented by the high success rate in IHWC proficiency testing.

In conclusion, at least in some European populations, the risk of CD in children with type 1 diabetes is substantially modified by the child's genotype, with a contribution from both *HLA-DQB1*02-DQA1*05* and the *TNF −308A* variant. Although there had been previous indications that children with diabetes differ in their risk of CD based on their HLA genotype, our study has conferred the strongest evidence so far, having collected the largest adequately matched case-control dataset. Although an immediate transition of the knowledge into clinical use is unlikely, in the future, genetic testing may be useful for stratifying the frequency of

testing diabetic children for antibodies indicative of CD.

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