ORIGINAL ARTICLE

CTLA-4 gene polymorphisms predispose to autoimmune endocrinopathies but not to celiac disease

Tomáš Dallos [1], Magdalena Avbelj [2], Ľubomír Barák [3], Jiřina Zapletalová [4], Zuzana Pribilincová [1], Mária Krajčírová [1], Ľudmila Košťálová [1], Tadej Battelino [2], and László Kovács [1]

1. 2nd Department of Pediatrics, Medical Faculty, Comenius University, Limbová 1, 83340 Bratislava, Slovak Republic.
2. Department of Pediatric Endocrinology, Diabetes and Metabolism, University Children's Hospital, Vrazov trg 1, 1000 Ljubljana, Slovenia.
3. Children Diabetes Centre of the Slovak Republic, 1st Department of Pediatrics, Medical Faculty, Comenius University, Limbová 1, 83340 Bratislava, Slovak Republic.
4. Department of Pediatrics, Medical Faculty, Palacký University, Puškinova 6, 775 20 Olomouc, Czech Republic.

Correspondence to: Tomáš Dallos, 2nd Department of Pediatrics, University Children's Hospital, Limbová 1, 833 40 Bratislava, Slovak Republic.
TEL: ++421 2 5937 1109, FAX: ++421 2 5937 1850
EMAIL: dallos@dfnsp.sk

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Abstract

OBJECTIVES: The aim of the study was to determine the association of two *CTLA-4* gene polymorphisms (CT60, +49 A/G) with Hashimoto thyroiditis (HT), type 1 diabetes mellitus (T1DM) and celiac disease (CD) as well as with the occurrence of multi-organ involvement by autoimmunity in children.

METHODS: Genotyping was done by RFLP analysis in Slovak children with HT (n=63) and CD (n=120) and both Slovak and Slovene children with T1DM (n=320) and healthy controls (n=231).

RESULTS: We found a significant association of the G allele of the CT60 polymorphism with HT ($p<0.0005$) in the Slovak population and T1DM in both Slovak ($p<0.01$) and Slovene populations ($p<0.005$). The G allele of the +49A/G polymorphism was significantly, though less strongly, associated with T1DM ($p<0.05$) and HT ($p<0.05$). Distribution of genotypes of *CTLA-4* gene polymorphisms in CD patients did not differ significantly from controls. None of the polymorphisms was associated with multi-organ involvement by autoimmunity.

CONCLUSION: The G allele of both examined *CTLA-4* gene polymorphisms predisposes to HT and T1DM, but not to CD. No association with multi-organ involvement was found. The GG genotype of the CT60 polymorphism may identify CD patients at an increased risk for concomitant T1DM and HT. Further studies to assess the predictive value of *CTLA-4* polymorphisms for the co-occurrence of HT and T1DM in CD patients are needed.

Abbreviations

T1DM – type 1 diabetes mellitus;
HT – Hashimoto thyroiditis;
CD – celiac disease;
CTLA-4 – cytotoxic T lymphocyte associated antigen 4;
SNP – single nucleotide polymorphism;
HWE – Hardy-Weinberg equilibrium

INTRODUCTION

Type 1 diabetes mellitus (T1DM), Hashimoto thyroiditis (HT) and celiac disease (CD) are among the most common autoimmune diseases of childhood.

The cytotoxic T lymphocyte associated antigen 4 (CTLA-4) is a high-affinity co-stimulatory molecule expressed by activated T and B lymphocytes. Interaction of CTLA-4 with its ligands B7.1 and B7.2 leads to inhibition of the ongoing T lymphocyte activation. (Krummel *et al*., 1996) CTLA-4 action is a key immune mechanism that limits exaggerated immune reactions against self-antigens and thus an important check-point of immune tolerance. (Masteller *et al*., 2000) A fully functional native soluble form of CTLA-4 (sCTLA-4) is elevated in autoimmune diseases. (Magistrelli *et al*., 1999)

Polymorphisms of the *CTLA-4* gene region (2q33) have been associated with T1DM and HT. (Braun *et al*., 1998; Kikuoka *et al*., 2001) The exon 1 +49 A/G polymorphism has been shown to affect CTLA-4 function. Recently, polymorphisms in the 3' terminal untranslated region (possibly affecting mRNA stability and sCTLA-4 expression) were found to be significantly associated with T1DM, Graves-Basedow disease and HT. (Ueda *et al*., 2003)

HT and CD are known to occur frequently in T1DM patients, however little is known about predictors of the development of HT and CD in T1DM patients. In a number of studies, association of T1DM with *CTLA-4* SNPs was limited to patients with an associated autoimmune disorder. (Blomhoff *et al*., 2005; Djilali-Saiah *et al*., 1998; Ikegami *et al*., 2006; Vaidya *et al*., 2002) Hence, genetic factors may be responsible for the accumulation of organ-specific autoimmune disorders in some patients. Because of the central role of CTLA-4 in immune regulations and associations of its gene polymorphisms with pathophysiologically distinct autoimmune disorders we hypothesized that *CTLA-4* gene SNPs may be a risk factor for multiorgan involvement by autoimmunity.

To test this hypothesis, we examined two polymorphic markers of the *CTLA-4* gene in cohorts of pediatric T1DM patients with and without concomitant HT from two different ethnically related Slavic (Slovak and Slovene) populations. Additionally, cohorts of Slovak HT and CD patients were genotyped.

SUBJECTS AND METHODS

Patients. A total of 484 unrelated patients (T1DM n=320, HT n=63, CD n=120) and 231 healthy controls were included in the study. Patients with an associated genetic syndrome (Down, Noonan and Turner syndromes) were not included. T1DM patients and controls originated form two different, yet ethnically related Slavic populations. Some HT patients (n=24) originated from a closely related population in Moravia, Czech Republic and were included in the Slovak cohort. Anonymous healthy sex-matched blood donors that fulfilled blood donation criteria served as controls for the Slovak population (n=136). For the Slovene population, healthy, age and sex-matched children were used as controls (n=95). Characteristics of patients are summarized in table 1.

Diagnosis of T1DM was based on the finding of hyperglycemia and development of ketoacidosis. All T1DM patients were insulin-dependent at the time of inclusion into the study. In 125 T1DM patients (overall 38.1%, 37.7% of Slovak and 40.6% of Slovene T1DM patients) HT was diagnosed at a median age of 12.5 ± 5.7 years (11.3 ± 5.9 years in Slovak, 14.4 ± 5.3 years in Slovene patients). HT diagnosis was based on the finding of latent or manifest hypothyroidism, significantly elevated titers of thyroid antibodies (aTPO, aTG) and a characteristic ultrasound picture. Average levels of aTPO and aTG antibodies at the time of diagnosis of HT were 913.31 IU/ml (6–12000 IU/ml) and 544.59 IU/ml (10.3–8000 IU/ml), respectively. Every patient had a significantly elevated titer of at least one antibody at the time of diagnosis or later in the course of follow-up. All CD patients (n=120) fulfilled the revised diagnostic criteria of ESPGAN (McNeish, 1979). Total villous atrophy was found in 52% (n=63) of CD patients.

All participants or their parents gave written informed consent. The study was approved by the Ethics Committee of the University Children's Hospital in Bratislava and by the Committee for Medical Ethics in Slovenia and was carried out in accordance with the principles of the Helsinki Declaration.

***CTLA-4* gene polymorphism analysis.** Leukocytes were derived from EDTA-treated peripheral blood. Genomic DNA was extracted from leukocytes using either the phenol-chlorophorm method or a commercial DNA purification kit (Quiagen). Extracted DNA was dissolved in sterile water and stored at –18°C.

Two polymorphisms in the *CTLA-4* gene region were analyzed using standard polymerase chain reaction (PCR) amplification with purified genomic DNA as the template with 2 sets of specific primers and subsequent restriction fragment length polymorphism (RFLP) analysis.

For the +49A/G single nucleotide polymorphism (SNP) PCR, previously published primers (5'-ccacggcttcctttctcgta-3', 5'-agtctcactcacctttgcag-3') were

Table 1: Characteristics of examined subgroups of patients.

Cohort	Slovak							Slovene			
Disease	**T1DM**	**T1DM+HT**	**all T1DM**	**HT**	**all HT**	**CD**	**controls**	**T1DM**	**T1DM+HT**	**all T1DM**	**controls**
N	70	56	150	63	119	120	136	101	69	170	95
M / F %	44 / 56	34 / 66	40 / 60	15 / 85	24 / 76	48 / 52	50 / 50	29 / 71	26 / 74	28 / 72	38 / 62
age of onset (SEM±SD)	7.5±3.6	7.3± 4.3	7.5± 4.0	11.3± 4.0	11.1± 4.9	11.2± 4.6	-	10.4± 4.0	9.3± 4.6	9.7± 4.3	-

Table 2: Allele and genotype frequencies of CT60 A/G and +49 A/G polymorphisms of the *CTLA-4* gene in examined groups of Slovak and Slovene patients and results of the χ^2-test for deviation from HWE (χ^2, *p*) for each subgroup.

Cohort			Slovak							Slovene			
Disease			**T1DM**	**T1DM+HT**	**all T1DM**	**HT**	**all HT**	**CD**	**controls**	**T1DM**	**T1DM+HT**	**all T1DM**	**controls**
CT60 A/G	Genotype %	AA	24.3	16.1	18.0	6.3	10.9	20.8	22.1	15.9	7.3	12.3	26.3
		AG	35.7	42.9	42.7	44.4	43.7	44.2	55.8	46.5	53.6	49.4	52.6
		GG	40.0	41.0	39.3	49.3	45.5	35.0	22.1	37.6	39.1	38.3	21.1
	Allele frequency	A	0.422	0.375	0.393	0.286	0.328	0.429	0.500	0.391	0.341	0.371	0.526
		G	0.578	0.625	0.607	0.714	0.672	0.571	0.500	0.609	0.659	0.629	0.474
	HWE deviation	χ^2	*5.10*	*0.41*	*1.68*	*0.49*	*<0.01*	*1.17*	*1.88*	*0.05*	*2.59*	*0.60*	*0.29*
		p	*0.03*	*0.52*	*0.19*	*0.48*	*0.93*	*0.28*	*0.17*	*0.82*	*0.1*	*0.44*	*0.60*
+49 A/G	Genotype %	AA	34.3	37.5	35.3	25.4	31.1	47.5	44.9	33.7	26.1	30.6	49.5
		AG	44.3	39.3	45.4	54.0	47.1	37.5	39.0	48.5	52.2	50.0	40.0
		GG	21.4	23.2	19.3	20.6	21.8	15.0	16.1	17.8	21.7	19.4	10.5
	Allele frequency	A	0.564	0.571	0.580	0.524	0.546	0.663	0.644	0.579	0.522	0.556	0.695
		G	0.436	0.429	0.420	0.476	0.454	0.337	0.356	0.421	0.478	0.444	0.305
	HWE deviation	χ^2	*0.69*	*2.19*	*0.72*	*0.42*	*0.31*	*3.13*	*3.09*	*<0.01*	*0.14*	*0.03*	*0.31*
		p	*0.41*	*0.14*	*0.39*	*0.52*	*0.58*	*0.08*	*0.08*	*0.96*	*0.71*	*0.87*	*0.58*

used. (Bittencourt *et al*., 2003) The 50 µl PCR reaction was set up to contain 5 µl 10x PCR buffer, 25 pmol of each primer, 0.02 mmol of each dNTP (Invitrogen, Brazil), 0.05 mmol $MgCl_2$ and 1.5 units of Taq polymerase (Invitrogen, Brazil). The PCR was run for 35 cycles (60 s denaturation at 94°C, 60 s annealing at 59°C, 60 s elongation at 72°C) followed by 10 minutes final extension at 72°C. The resulting 328 bp product was digested by 2 units of BbvI (NEB BioLabs, New England) for 3 hours at 37°C, which resulted in 244 bp and a 84 bp fragments in the presence of the G allele. The A allele yielded an intact 328 bp fragment.

For the amplification of a 328 bp gene fragment comprising the CT60 polymorphism oligonucleotide primers were designed (5'-atctgtggtggtcgttttcc-3' forward, 5'-aggggaggtgaagaacctgt-3' reverse) using the published human *CTLA-4* and *ICOS* gene sequence (GeneBank, NT_005403.16). Amplification was performed in a mixture of genomic DNA, 5 µl 10x PCR buffer, 25 pmol of each primer, 0.01 mmol of each dNTP (Invitrogen, Brasil), 0.05 mmol $MgCl_2$ and 1.25 units of Taq polymerase (Invitrogen, Brasil) in a final volume of 50 µl. The mixture was subjected to 35 cycles of 60 s denaturation at 94°C, 60 s annealing at 59°C, 60 s polymerization at 72°C followed by 10 minutes final extension at 72°C. The PCR product was subjected to digestion by 2.5 units of HpyCH4 IV (NEB BioLabs, New England) for 3 hours at 37°C. Digestion resulted in 252 bp and 76 bp fragments n the presence of the G allele. In the case of the A allele, no digestion occurred.

All PCR reactions were performed on a TECHNE®-GENE TC-312 thermal cycler. In every run of PCR reactions a negative control reaction with no DNA added was included. PCR and digestion products were run for 20 min at 10V/cm on an ethidium bromide stained 3% agarose (3:1) gel in 1x TBE buffer.

Statistical analysis. All patients with T1DM, HT and CD were included in "all T1DM", "all HT" and "CD" groups, respectively. Only patients with T1DM and HT with no other concomitant autoimmune disease were included in "T1DM" and "HT" groups. To increase accuracy, only T1DM patients with no proven HT or CD during a minimum 48 month follow-up period were included in the T1DM group. Patients with T1DM and concomitant CD (*n=18*) were included in the "all T1DM" group when this was compared to controls, however were excluded when "all T1DM" and "CD" groups were compared. Patients with T1DM and both HT and CD (*n=3*) were included in both "T1DM +HT" and "CD" groups, since these were not compared (Table 1). Allele and genotype frequencies of each *CTLA-4* gene polymorphism were determined by direct counting and calculation in each group of patients and controls. Genotype frequencies were checked for deviation from Hardy-Weinberg equilibrium by the goodness of fit χ^2 test with one degree of freedom (Table 2). Allele frequencies in patients and controls were compared using Fisher's exact test. The significance of differences in genotype distributions was assessed using the χ^2 test with two degrees of freedom. For cohorts with significantly different genotype distributions, odds ratios (OR) at a 95% confidence interval (CI) were calculated using the approximation of Woolf. For all statistical tests a p value <0.05 was considered significant.

RESULTS

The distribution of CT60 genotypes in Slovak HT ("all HT") and T1DM ("all T1DM") patients was significantly different from controls ($p<0.005$ and $p<0.01$, respectively). The association of the G allele was more significant in HT patients (OR 3.1 [95% CI 1.4–7.0] for homozygosity and OR 1.7 [95% CI 0.6–2.9] for a single dose) than in T1DM patients (OR 2.2 [95% CI 1.1–4.3] for homozygosity). To eliminate bias caused by the strong association of CT60 with HT in T1DM patients, and vice versa, T1DM patients with no HT and HT patients with no T1DM were analyzed separately ("T1DM" and "HT" groups). The genotype distribution in T1DM patients remained significantly different ($p<0.05$), however a significant deviation from HWE was noted. In patients with HT only, the significance of the association remained unaffected ($p<0.005$, OR 5.8 [95% CI 1.8–18.7] for homozygosity and OR 2.4 [95% CI 0.8–7.5] for a single dose).

Genotype distribution and allele frequencies in the Slovene cohort were not different at direct comparison with the Slovak cohort. The increased frequency of the G allele and GG genotype in Slovene T1DM patients reached a similar level of statistical significance ($p<0.005$, OR 3.9 [95% CI 1.8–8.3] for homozygosity and OR 2.0 [95% CI 1.0–3.9] for a single dose), however decreased if patients with accompanying autoimmune diseases were excluded from analysis ($p<0.05$, OR 3.0 [95% CI 1.3–6.8] for homozygosity).

As to the +49 A/G polymorphism, alleles and genotypes were similarly distributed in both populations. The only significant differences were observed in the Slovene cohort of T1DM patients ($p<0.01$, OR 3.9 [95% CI 1.5–10.3] for homozygosity and OR 2.3 [95% CI 1.2–5.0] for single dose), however only in the "all T1DM" group.

To increase the power of our study and to account for the significant deviation from HWE in the Slovak T1DM group, patients and sex- and geographically-matched controls from both populations were combined where appropriate to create larger cohorts. Deviation from HWE in all resulting groups including the T1DM group ($\chi^2= 2.69$, p=0.1 for the CT60 and $\chi^2=0.33$ $p=0.57$ for the +49A/G polymorphism) was not significant. The association of T1DM with the G allele of the CT60 polymorphism remained significant ("all T1DM" $p<0.0001$, OR 2.8 [95% CI 1.7–4.7] for CT60 G homozygosity and OR 1.3 [95% CI 0.9–2.1] for CT60 G single dose and for T1DM with no associated HT $p<0.001$, OR 2.2 [95% CI 1.2–3.9] for homozygosity). Interestingly, with the increased number of patients a significant association for the +49A/G polymorphism with T1DM could be observed ($p<0.05$, OR 1.9 [95% CI 1.1–3.4] for the GG genotype). No significant differences could be observed between T1DM patients with and without concomitant HT.

As to CD, distribution of CT60 genotypes and allele frequencies was not significantly different from healthy controls. After exclusion of all patients with accompanying autoimmune disorders (T1DM, thyroiditis, juvenile chronic arthritis, dermatitis herpetiformis Dühring, autoimmune cardiomyopathy) (n=28) from the CD group as well as of all HT patients from the T1DM group (n=56) and T1DM patients from the HT group, we analyzed a possible difference in genotype distribution and allele frequencies between both T1DM and HT patients on one side and CD patients on the other. In HT, the predisposing genotypes of the CT60 polymorphism were more frequent ($p<0.01$) with significant risk conferred by the G allele (OR 6.2 [95% CI 1.8–21.2] for homozygosity and OR 4.87 [95% CI 1.4–16.6] for a single dose). For T1DM, the difference in genotype distribution to CD patients did not reach statistical significance. We did not find any significant differences in the distribution of alleles or genotypes of the polymorphism +49 A/G in CD patients.

Genotypes were examined also in conjunction with clinical data of patients. The GG genotype of the +49 A/G polymorphism was weakly associated with an earlier onset of disease in T1DM patients (GG vs. AA 90.5 ± 45.4 months vs. 115.25 ± 50.6 months, n=171, $p<0.05$). The highest documented titre of aTPO and aTG antibodies in HT and total villous atrophy upon

histological examination at the time of diagnosis in CD patients were not associated with any genotype of the CT60 and +49 A/G polymorphisms.

DISCUSSION

We confirmed a significant association of the G allele of the *CTLA-4* CT60 polymorphism with T1DM in two related Slavic populations. Also, we found an extremely significant association of the CT60 GG genotype with HT in the Slovak cohort. These results are in line with reported data concerning the association of the CT60 G allele with HT (Ban *et al*., 2005; Ikegami *et al*., 2006; Ueda *et al*., 2003) and T1DM (Zhernakova *et al*., 2005) in other populations.

HT has been found to occur in up to 21–28% of T1DM patients (Fernandez-Castaner *et al*., 1999, Kordonouri *et al*., 2002) and thyroid function monitoring has become the standard of care for T1DM patients. Such association fulfills the clinical criteria of a type 2 autoimmune polyendocrine syndrome (at least one endocrine autoimmune disease in association with another autoimmune disease not fulfilling type 1 APS criteria) and some authors consider it a separate defect of immune tolerance. (Eisenbarth *et al*., 2004) At the same time, in some studies, associations of *CTLA-4* gene polymorphisms were limited to subgroups of patients with a concomitant autoimmune disease; T1DM and HT (Djilali-Saiah *et al*., 1998, Ikegami *et al*., 2006, Vaidya *et al*., 2002), T1DM and Graves-Basedow disease (Mochizuki *et al*., 2003), vitiligo and other autoimmune diseases (Blomhoff *et al*., 2005). Such observations may be due to either the association of the polymorphism with multi-organ involvement by autoimmunity i.e. predisposition to a type 2 APS or a strong effect of the concomitant autoimmune disorder.

We explored a possible effect of the *CTLA-4* gene polymorphism on the occurrence of HT in T1DM by comparing subgroups of T1DM patients with and without concomitant HT. Association of the CT60 G allele with T1DM and HT was significant in both groups when compared to controls and direct comparison of T1DM and T1DM+HT groups did not yield significant results. This is in line with a similar study of Asian populations. (Djilali-Saiah *et al*., 1998) This result speaks for the association of the CT60 polymorphism with both T1DM and HT and against the possibility that the association with T1DM is due to the effect of concomitant occurrence of latent HT. (Ikegami *et al*., 2006) Nevertheless, we have to critically state, that the exclusion of concomitant HT in T1DM patients may not have been reliable in all cases. In up to 46% of our T1DM patients, HT was diagnosed later than 48 months after T1DM manifestation. Elevated aTPO and aTG antibodies are considered to indicate thyroid autoimmunity in euthyroid subjects (Hollowell *et al*., 2002) and may precede HT manifestation by years (Kordonouri *et al*., 2002). We may not have recognized all cases of thyroid autoimmunity in our cohort of T1DM patients, since thyroid autoantibody testing had not been carried out in all patients. Euthyroid T1DM patients with unrecognized elevated thyroid antibody titers may have affected our analysis, since aTPO and aTG antibody levels are associated with the genotype of *CTLA-4* gene polymorphisms. (Howson *et al*., 2007)

Based on our data, we propose, that the G allele of the CT60 polymorphism predisposes to both T1DM and HT, though more strongly to HT. This is in line with the central role of CTLA-4 in immune responses and the published associations of polymorphisms of its gene with a wide range of autoimmune diseases. Thus *CTLA-4* gene polymorphisms may act as one of a number of universal predisposition loci for autoimmune diseases.

Studies on the association of *CTLA-4* gene polymorphisms with CD published so far have yielded controversial results; the G allele of the CT60 polymorphism showed borderline (van Belzen *et al*., 2004) or no association with CD (Rueda *et al*., 2005) and was part of predisposing (Hunt *et al*., 2005) as well as protective haplotypes (Amundsen *et al*., 2004). We did not find an association of the CT60 polymorphism with CD in our cohort. Hence, CTLA-4 may not play a crucial role in CD pathogenesis. Nonetheless, an up to 10-fold increase in the occurrence of autoimmune thyroiditis and T1DM has been reported in CD patients. (Cuoco *et al*., 1999; Lorini *et al*., 1996; Talal *et al*., 1997) The increased prevalence of autoimmune diseases in CD patients is often explained by the sharing of common genetic susceptibility factors. In the case of T1DM and HT, this may not be true with respect to the examined *CTLA-4* gene polymorphisms. An alternative explanation may be that celiac disease may start off a sequence of immunopathologic events that result in the initiation of organ-specific autoimmune phenomena in genetically predisposed individuals. This is supported by the fact that autoimmune complications of celiac disease are in relation to the duration of gluten-exposure and dietary intervention can prevent the development of autoimmunity in CD patients (Cuoco *et al*., 1999; Ventura *et al*., 1999) as well as decrease levels of organ-specific autoantibodies (Sategna-Guidetti *et al*., 2001)

As a novel observation, we suggest that the GG genotype of the CT60 *CTLA-4* gene polymorphism (present in 34.7% of our CD patients) may modify the risk of T1DM and HT in CD patients and may have the potential to identify CD patients at an increased risk for the development of concomitant T1DM and HT. Identified early, these patients might benefit from regular selective sonographic and autoantibody screening. Reduction of hidden morbidity from undiagnosed disease and individualization of the strict-

ness of gluten-free diet might be based on the patient's genotype. This hypothesis should be confirmed in studies comparing a representative cohort of CD patients with and without concomitant T1DM and HT. A similar approach was applied in a study of T1DM patients with and without CD, in which no association of the +49 A/G *CTLA-4* gene polymorphism with the occurrence of CD in T1DM was found. (Šumník *et al*., 2006) However, polymorphisms of the 3´ terminal region of the *CTLA-4* gene were not examined and comparison of CD patients with and without T1DM was not done in that study. The small number of T1DM patients with CD in our study (n=19) did not allow us to analyze such associations. Interestingly, however, none of the CD patients with concomitant T1DM carried the protective AA genotype, and 9 patients (47.4%) carried the predisposing GG genotype.

In conclusion, we confirmed that the CT60 polymorphism predisposes to the development of T1DM and HT in children of Slavic origin. This association is stronger than that of the exon 1 polymorphisms in position +49. No role of any of the examined polymorphisms in predisposition to HT in T1DM or in celiac disease could be demonstrated. Genotyping of CD patients for the CT60 polymorphism may be a useful clinical tool to identify patients at an increased risk for the development of concomitant HT and T1DM.

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