Rapid Publication

The IDDMK$_{1,2}$22 Retrovirus Is Not Detectable in Either mRNA or Genomic DNA From Patients With Type 1 Diabetes

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A new viral sequence (IDDMK$_{1,2}$22) similar to the human endogenous retrovirus (HERV)-K10/K18 subfamilies has recently been isolated from the culture supernatants of leukocyte-infiltrated islets from two patients who died at the onset of type 1 diabetes. It was claimed that this endogenous retrovirus is expressed in patients with type 1 diabetes but not in healthy control subjects, suggesting an important role of the retrovirus in β-cell–specific autoimmunity that results in type 1 diabetes. However, despite exhaustive attempts involving identical and expanded methods of detection, we did not observe the IDDMK$_{1,2}$22 viral sequence in genomic DNA, lymphocyte, or plasma RNA in any subject. Therefore, we believe that the viral sequence is not derived from an endogenous retrovirus and that a role for the retrovirus in the pathogenesis of type 1 diabetes must be reconsidered. *Diabetes* 48:219–222, 1999

Conrad et al. (1) reported a new human endogenous retrovirus (IDDMK$_{1,2}$22) that *1*) was isolated from the culture supernatants of leukocyte-infiltrated islets from two patients who died at the onset of type 1 diabetes; *2*) contained a Vβ7-specific superantigen sequence; *3*) was associated with reverse transcriptase (RT) activity in spleen cell cultures of both newly diagnosed and long-standing diabetic patients; *4*) was associated with RT activity in cultured islets from new-onset patients but not from those with long-standing disease; and most importantly, *5*) was specifically expressed in the plasma of diabetic patients and not in that of healthy control subjects. The IDDMK$_{1,2}$22 viral sequence was novel and had significant homology with human endogenous retroviruses of the human endogenous retrovirus (HERV)-K10/K18 subfamilies. Expression of this retrovirus was suggested as a possible precipitant of β-cell–specific autoimmunity that results in type 1 diabetes. The presence of such a retrovirus could explain the predominance of T-cells bearing a Vβ7-receptor observed in two pancreases from type 1 diabetic patients (2). In addition, if the viral genome was detectable in plasma before the onset of hyperglycemia, identification of the IDDMK$_{1,2}$22 sequence could identify subjects at increased risk for developing diabetes. Therefore, we attempted to replicate these results in peripheral blood mononuclear cells (PBMCs) and plasma from type 1 diabetic patients and healthy control subjects. Despite exhaustive attempts involving identical and expanded methods of detection, we did not observe the IDDMK$_{1,2}$22 viral sequence in any subject. Therefore, we believe that a role for the retrovirus in the pathogenesis of type 1 diabetes must be reconsidered.

RESEARCH DESIGN AND METHODS

Subjects. Blood samples were obtained from 12 patients with diabetes (seven male and five female subjects, mean age 17.5 years [range 9–23]). Six had their symptomatic onset within 2 weeks of sampling, and the remainder had been diagnosed 1–13 years before sampling. Control samples were obtained from 9 healthy subjects (five male and four female subjects, mean age 31 years [26–38]). All diabetic patients and control subjects were Caucasians living in Florida.

RNA and DNA. Plasma RNA was extracted using a Qiagen RNeasy kit as outlined by Conrad et al. (1). Total RNA and DNA were isolated from Ficoll-Hypaque–enriched PBMCs of diabetic patients and control subjects.

Polymerase chain reaction amplification. The sequences and positions of all polymerase chain reaction (PCR) primers used in this study are presented in Table 1. PCR amplifications were carried out in a final volume of 22 µl consisting of 2 µl template DNA, 4.4 pmol of each primer, 0.14 U of Taq polymerase, 50 mmol/l KCl, 10 mmol/l Tris-Cl (pH 8.3), 1.5 mmol/l $MgCl_2$, and 60 µmol/l of each dNTP. Samples were subject to 35 cycles of 30 s at 94°C for denaturing, 30 s at appropriate annealing temperature, and 30 s at 72°C for extension in a Perkin-Elmer 9600 thermal cycler. After amplification, 10 µl of product from each reaction was electrophoresed on a 2.0% agarose gel.

Cloning and sequencing. PCR products were cloned into the TA cloning vector (K2000-J10; Invitrogen, Carlsbad, CA) according to the manufacturer's specification. To verify inserts in the clones, 2 µl of bacteria culture medium was used as a template in PCR amplification. From confirmed clones, plasmid DNA was then extracted with a QIAMiniprep kit (Qiagen 27104, Valencia, CA). M13 primers were used to sequence the inserts. Each sequencing reaction (20 µl) contains 150 ng of plasmid DNA, 1 µl BigDye reaction mix (Perkin Elmer, Foster City, CA), 1 µl of primer (3.2 pmol), and 3 µl of 5X sequencing buffer (400 mmol/l Tris-HCl, 10 mmol/l $MgCl_2$, pH 9.0). After 25 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min, 1/10 vol (2 µl) of 3 mol/l NaAC (pH 5.2) and 3 vol (60 µl) of 95% ethanol were added to each sequencing reaction. After 10 min at room temperature, the samples were centrifuged for 20 min at room temperature. The pellet was washed once with 250 µl of 70% ethanol and dried in a vacuum drier, then dissolved in 20 µl of template suppression reagent (Perkin Elmer). The sequences were then read with an ABI 310 automated DNA sequencer.

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bp, base pair; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction; RT, reverse transcriptase.

TABLE 1
Nucleotide sequences for primers used in this study

Primer name	5′ → 3′ sequence	Position	Tm (°C)	Specificity
U3	AGGTATTGTCCAAGGTTTCTCC	421–442	64	Universal
F1	TGCAGTTGAGAGAAGAGGAAGA	561–582	64	K18, IDDMK$_{1,2}$22
F2	AAGACATCTGTCTCCTGCCCA	579–599	64	K18, IDDMK$_{1,2}$22
F3	GTGATAGTCTGAAATATGGCCT	447–467	62	Universal
R-polyA	TTTTTGAGTCCCCTTAGTATTTATT	910–886	64	DNA, RNA
R2-polyA	TTTTTTTTTTTTTTTTTGAGTCCC	922–898	58	RNA
R3	ATCATTCGTGGGTGTTTCTCCT	884–863	64	IDDMK$_{1,2}$22
5F1	CATCTCCCTCAGGAGAAACAC	1–21	64	IDDMK$_{1,2}$22
5F2	AAACACCCACGAATGATCAATAA	16–38	62	IDDMK$_{1,2}$22
5R1	CATCCAACAACCATGATGGAGA	201–180	64	IDDMK$_{1,2}$22
5R2	CTCGTAAGGTGCAAATGAAGAA	174–153	62	IDDMK$_{1,2}$22

The positions for the 5′ LTR primers (5F1, 5F2, 5R1, and 5R2) were based on the sequence for GenBank accession number AF012332, and the positions for all other primers were based on the sequence for AF012335. Tm, optimum annealing temperature.

RESULTS AND DISCUSSION

We first attempted to detect the expression of the IDDMK$_{1,2}$22 retrovirus in the PBMCs of diabetic patients and control subjects. Using the published U3/R-polyA primer pair that was claimed to be specific for cDNA of IDDMK$_{1,2}$22, we detected a single fragment of the expected size (468 base pair [bp]) in all PCR reactions, whether or not the RNA had been treated with RT (Fig. 1). These results suggest that the primer pair can amplify sequences from residual gDNA in RNA preparations. This outcome was expected because the R-polyA primer is comprised at its 3′ end of 20 bases of the viral genomic sequence, with only five thymidines at its 5′ end (Table 1). We therefore designed the R2-polyA primer, which contains only seven bases specific for the viral genomic sequence at its 3′ end and 17 thymidines at its 5′ end. When the R2-polyA primer was used together with U3 in amplification of PBMC RNA (64°C for annealing and with 5% DMSO), a single product of expected size was once again detected in PCR reactions with RNA samples before RT reaction in both diabetic patients and normal control subjects (Table 2). These results were unexpected because the R2-polyA primer should not be able to initiate amplification from gDNA. We therefore cloned the PCR products into the TA cloning vector and sequenced 20 clones from two diabetic patients and two normal control subjects. Surprisingly, both the 5′ and 3′ ends of our clones contained the U3 primer sequence. A review of the sequence of IDDMK$_{1,2}$22 and other members of the K10 and K18 families revealed that eight bases at the 3′ end of the U3 primer match exactly the genomic sequence near the R-polyA primer. The U3 primer alone can be used as a forward and reverse primer to amplify the U3/R-polyA fragment (Fig. 2). Interestingly, none of the 20 clones exactly matched the IDDMK$_{1,2}$22 sequence, although they were all identical or very similar to those reported for the HERV-K18 family. Because we have demonstrated that the published U3 primer is not specific for IDDMK$_{1,2}$22 and that the R-polyA primer is not specific for viral RNA, serious doubt is raised about the reported results of Conrad et al. (1).

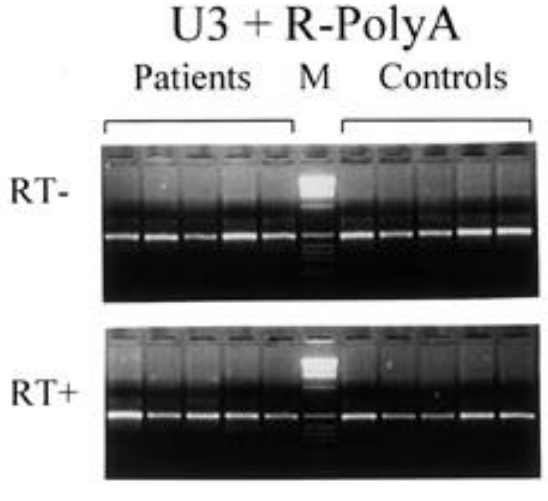

FIG. 1. Nonspecific amplification with primers U3 and R-polyA. PCR products amplified with the U3 and R-PolyA primers from total PBMC RNA before (RT–) and after (RT+) reverse transcription were electrophoresed on a 2.5% agarose gel. Results from five type 1 diabetic patients and five healthy control subjects are shown.

We then designed the F1 and F2 forward primers (Table 1), both of which distinguish the 3′ LTR sequences of IDDMK$_{1,2}$ 22 and HERV-K18 viruses from other similar retroviruses by their unique 3′ terminal base. Neither the F1/R2-polyA nor the F2/R2-polyA primer pairs yielded the expected amplicon from gDNA or cDNA of PBMCs (Table 2). The ability of F1 and F2 to initiate PCR amplification was proven with amplification of the expected sequence from gDNA, using R-polyA as a reverse primer (data not shown). Subsequently, we designed the forward primer F3, which is universal for a large number of endogenous retroviruses. This primer, together with the RNA-specific R2-polyA primer, amplified the expected fragment from RT-treated RNA, but not from RT-untreated RNA or gDNA (Table 2 and Fig. 3). However, sequencing of the PCR products did not reveal any IDDMK$_{1,2}$22 sequence. These results provide convincing evidence that IDDMK$_{1,2}$22 is not present in PBMC RNA.

We next sought the IDDMK$_{1,2}$22 sequence in the gDNA of our subjects. The reverse primer R3, specific for IDDMK$_{1,2}$22 based on sequences in GenBank, was used together with F1 in PCR amplification from gDNA. A single fragment of 325 bp was amplified and cloned into the TA cloning vector, and 15 clones from three American subjects (two diabetic patients

TABLE 2
Results of PCR amplification

		Diabetic patients				Control subjects			
			PBMC	Plasma RNA			PBMC	Plasma RNA	
Primer pairs	RT	gDNA	RNA	PCR1	PCR2	gDNA	RNA	PCR1	PCR2
U3 + R-polyA	–	+	+	–	±	+	+	–	±
	+	ND	+	–	±	ND	+	–	±
U3 + R2-polyA	–	+	+	–	ND	+	+	–	ND
	+	ND	+	–	ND	ND	+	–	ND
F1 + R-polyA	–	+	+	–	±	+	+	–	±
	+	ND	+	–	±	ND	+	–	±
F1 + R2-polyA	–	–	–	–	–	–	–	–	–
	+	ND	–	–	–	ND	–	–	–
F2 + R2-polyA	–	–	–	–	ND	–	–	–	ND
	+	ND	–	–	ND	ND	–	–	ND
F3 + R2-polyA*	–	–	–	–	ND	–	–	–	ND
	+	ND	+	–	ND	ND	+	–	ND
F1 + R3*	–	+	+	ND	ND	+	+	ND	ND
5F1 + 5R1	–	–	–	–	–	–	–	–	–
5F2 + 5R2	–	–	–	–	–	–	–	–	–

*All amplified sequences are similar or identical to viruses in the K18 family, but are different from IDDMK$_{1,2}$22. ND, not determined; PCR1, one round of amplification; PCR2, nested PCR using primers described in the text.

and one control subject) were sequenced. While all of the cloned sequences were very similar to the 3′ LTR of HERV-K18 retroviruses, none matched the IDDMK$_{1,2}$22 sequence. Because the study subjects in the original publication were Italians, we also sequenced the 325-bp fragment amplified from gDNA of Italian subjects. In three pairs of affected siblings and their nondiabetic parents, no IDDMK$_{1,2}$22 sequence was detected. Finally, using four primer pairs (combinations of 5F1, 5F2, 5R1, and 5R2) specific for the 5′ LTR of IDDMK$_{1,2}$22, a region that is very divergent from other retroviruses, no PCR products were detectable. These results together suggest that IDDMK$_{1,2}$22 is not an endogenous retrovirus. Conrad and associates were misled in their studies because they did not sequence their PCR products amplified by the U3/R-PolyA, a primer pair that is specific for neither IDDMK$_{1,2}$22 nor RNA templates.

The plasma RT-PCR results of Conrad and associates could still be explained if IDDMK$_{1,2}$22 was an infectious virus whose RNA was detectable only in plasma. Therefore, we attempted to detect mRNA of IDDMK$_{1,2}$22 from plasma of diabetic patients and control subjects. Using primer pairs that are specific for RNA sequences of both IDDMK$_{1,2}$22 and K18 viruses (F1/R2-polyA and F2/R2-polyA), we were unable to

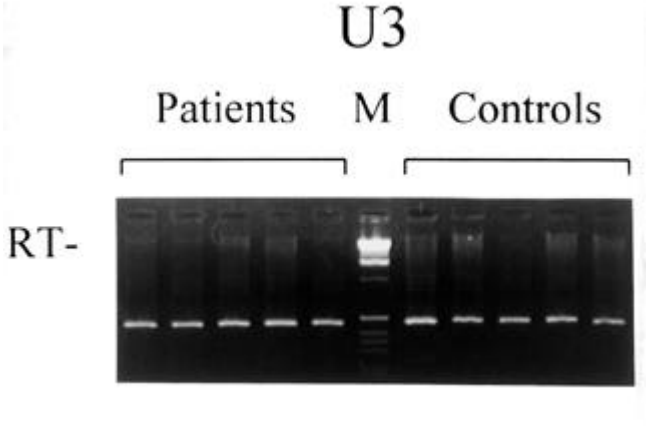

FIG. 2. Amplification of retroviral gDNA initiated by primer U3 alone. PCR products amplified with the U3 primer alone from total PBMC RNA before reverse transcription were electrophoresed on a 2.5% agarose gel. Results from five type 1 diabetic patients and five healthy control subjects are shown.

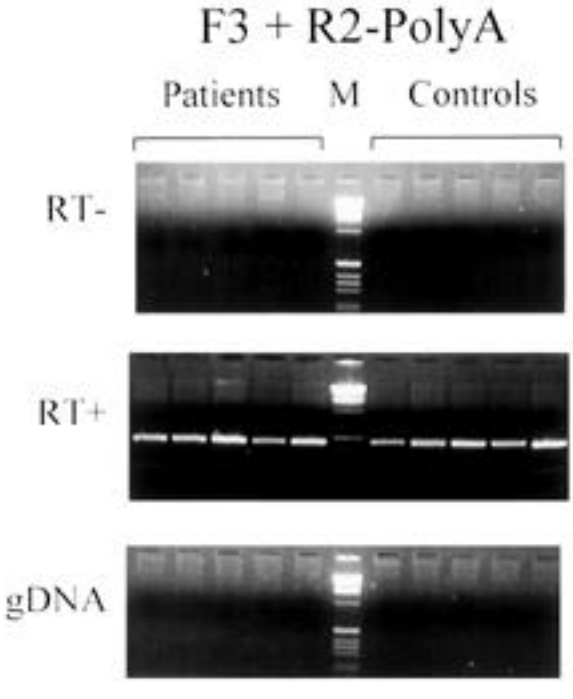

FIG. 3. Specific amplification of retroviral RNA with primers F3 and R2-polyA. PCR products amplified with the F3 and R2-PolyA primers from gDNA and total PBMC RNA before (RT–) and after (RT+) reverse transcription were electrophoresed on a 2.5% agarose gel. Results from five type 1 diabetic patients and five healthy control subjects are shown.

detect any product from the plasma of six diabetic patients and two control subjects. Nested PCR (with F2/R3) from PCR products with outside primers (F1/R2-polyA) also did not yield visible PCR bands in either diabetic patients or control subjects. The negative results were not likely due to inefficient RNA extraction because the same procedure successfully amplified Hepatitis C virus sequence in the plasma of infected patients. Since nested PCR is capable of detecting low levels of template, our results suggest that the IDDMK$_{1,2}$22 virus is unlikely to be present in plasma of diabetic patients or control subjects.

Finally, we conducted several experiments to determine whether the reported results can be explained by residual gDNA in plasma RNA preparations. Although no bands were visible in PCR reactions with the U3/R-PolyA or F1/R-polyA primer pairs, which can amplify from gDNA, nested PCR using F2/R-polyA after the first round of amplification yielded PCR products in some, but not all, diabetic patients and control subjects. The nested PCR amplification was not consistent and did not correlate with disease status of the donors. The results confirm, as expected, that a very small amount of gDNA is present in plasma RNA preparations. In the reported studies, Southern blot analysis using an internal probe was performed to detect the amplified PCR products from plasma RNA (1). The sensitivity of their detection method is similar to that by nested PCR. Our experiments suggest that the detected PCR bands with U3/R-polyA may be from gDNA contamination and that different levels of gDNA contamination between diabetic and control groups studied by Conrad and associates may provide a possible explanation for the reported difference between the two groups. The only other explanation for their results, although very unlikely, is viral infection that occurred only in their patients.

We therefore conclude that IDDMK$_{1,2}$22 is not present in the human genome and is not expressed in the PBMCs or plasma of either the diabetic patients or the healthy control subjects of our study population. It is unlikely that this virus, if it truly exists, contributes to the pathogenesis of type 1 diabetes.

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REFERENCES

1. Conrad B, Weissmahr RN, Boni J, Arcari R, Schupbach J, Mach B: A human endogeneous retroviral superantigen as candidate autoimmune gene in type 1 diabetes. *Cell* 90:303–313, 1997
2. Conrad B, Weldmann E, Trucco G, Rudert WA, Behboo R, Ricordi C, Rodriquez-Rilo H, Finegold D, Trucco M: Evidence for superantigen involvement in insulin-dependent diabetes mellitus aetiology. *Nature* 371:351–355, 1994