No Evidence for Association Between IDDMK\textsubscript{1,2,22}, a Novel Isolated Retrovirus, and IDDM

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In the past, endogenous retroviral sequences have been isolated from patients suffering from different kinds of autoimmune diseases. Recently, a full length retroviral genome, termed IDDMK\textsubscript{1,2,22}, was isolated from patients with new-onset IDDM. This genome contains a major histocompatibility complex II-dependent superantigen within its envelope gene. The viral sequence was found in ten patients with new-onset IDDM, but not in age-matched control subjects (Conrad et al. [9]). We searched for the presence of this viral genome by nested reverse transcription-polymerase chain reaction (RT-PCR) in a cohort of six patients with new-onset IDDM and six control subjects of the same age. We found all samples to be positive without any differences between patients and control subjects. The same results were obtained with supernatants of activated peripheral blood mononuclear cells. We performed isopycnic ultracentrifugation in sucrose density gradients on all samples and were unable to detect particles of the new virus in any of our samples. However, positive signals were obtained from all pellet fractions. RNase, DNase treatment and nested PCRs without reverse transcription showed that the positive signals were probably derived from intracellular RNA and DNA. In summary, no correlation between a positive nested PCR signal for IDDMK\textsubscript{1,2,22} and diabetes was found indicating that the new sequence represents just an additional member of the human endogenous retrovirus (HERV) family with lack of an exogenous counterpart. Diabetes 48:209–214, 1999
subjects, thereby having the potential ability of being transmitted as either an inherited trait or as an infectious agent.

The aim of this study was 1) to reassess the findings in our own cohort of patients, and because we were unable to do so, 2) to investigate possible explanations.

RESEARCH DESIGN AND METHODS

The blood of six IDDM patients with a disease onset 15 months (range 0.5–24) ago and of six age-matched control subjects was drawn and further processed within 10 min to 3 h. The median age at IDDM onset was 23.3 years, the median body weight was 69.3 kg, and the median insulin dose was 25 U/day.

Plasma was obtained after centrifugation at 1,200g and recentrifugation at 4,000g and stored at –80°C. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque gradient centrifugation and cultured in RPMI-1640 medium (Bio Whittaker, Verviers, Belgium), supplemented with 10% fetal bovine serum (Pansystems, Aidenbach, Germany), penicillin, and streptomycin. The cells were cultured in ~250 U/ml phytohemagglutinin. Cell culture supernatants (CCSNs) were collected three times a week and stored at –80°C after centrifugation at 1,200g and 4,000g.

RNA was extracted from 250 µl plasma, CCSNs, and sucrose density gradients with Tri Reagent BD (WAK-Chemie, Bad Homburg, Germany) in 0.1 mol/l sodium-acetate and with bichlorpropane (WAK-Chemie). RNA was precipitated with isopropanol and microcarrier and dissolved in diethylpyrocarbonate-treated water.

DNA was extracted from the same sample according to the manufacturer’s recommendations and dissolved in water.

Half the amount of RNA was reverse transcribed in the presence of 200 U of Superscript II (GibcoBRL, Eggenstein, Germany), 10 pmol of primer DM2 (5’TGGCTGAGGAGTATTAGTAAGG-3’) and ND2 (5’TGTGTTGATCATA GACAAGTAAAGG-3’). Cycle protocol was 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 45 s, and a final extension at 72°C. A total of 20 µl of the reaction was run on a 2% ethidium bromide-agarose gel.

The band at the expected size of 269 bp was excised from the gel, extracted with the Qiagen gel extraction kit (Qiagen, Hilden, Germany), and cloned into the pCR 2.1 vector (Invitrogen, Groningen, Netherlands) with a TA cloning kit. A total of 2.5 µl of the first PCR were given to the nested PCR (25 µl) with the same ingredients as in the first PCR, except for 20 pmols of each primer ND1 (5’-TGCTGAGGAGTATTAGTAAGG-3’) and ND2 (5’-GTGTTGATCATA GACAAGTAAAGG-3’). Cycle protocol was 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 45 s, and a final extension at 72°C. A total of 20 µl of the reaction was run on a 2% ethidium bromide-agarose gel.

The band at the expected size of 269 bp was excised from the gel, extracted with the Qiagen gel extraction kit (Qiagen, Hilden, Germany), and cloned into the pCR 2.1 vector (Invitrogen, Groningen, Netherlands) with a TA cloning kit. Large-scale preparations of the vector, obtained by the Qiagen Plasmid Maxi Kit, were used for cycle sequencing with the Sequi Therm Excell Sequencing Kit (Epicycle Technologies, Madison, WI). Sequencing products were run on a MWG 4000 sequencer (MWG-Biotech, Ebersberg, Germany).

Southern blots. Gels were soaked in 0.4 mol/l NaOH for 30 min, and DNA was transferred to a Hybond-N+ (Amersham, U.K.) membrane by capillary transfer in 0.4 mol/l NaOH. Membranes were neutralized in 0.4 mol/l Tris (pH 7.2) and DNA was immobilized by ultraviolet crosslinking (Stratagene, La Jolla, CA). Membranes were prehybridized in 6× SSPE, 0.1% SDS, 0.5% bovine lacto-transfer-technique optimizer BLOTTO (0.5% nonfat dried milk powder, 0.02% sodium azide) at 65°C. Hybridization was carried out with 500,000 dpm/ml of the cDNA, labeled to a specific activity of >1×10⁶ dpm/µg with 32P. The 269-bp cDNA fragment lying between the ND1 and the ND2 primer of the U3 region was used as the probe. The cDNA probe was cut out of the vector by EcoRI digestion. The most stringent washing condition was 0.1× SSPE, 0.1% SDS at 65°C. Membranes were exposed to BIOMAX-MR-films (Kodak, Rochester, NY) for 2 h to 6 days.

DNA was digested with 2 U of RNase-free Amp-Garde DNase (GibcoBRL) or with 15 U of RNase-free DNase (Pharmacia, Freiburg, Germany) in 10 x buffer before RT reaction. Reaction was stopped with 1 µl 25 mmol/l EDTA (pH 8) and heat inactivation at 65°C for 10 min. EDTA addition did not influence subsequent enzymatic steps (data not shown).

DNA was digested with 15 U of DNase-free RNase (Boehringer Mannheim, Mannheim, Germany) on 10 x buffer after RT reaction. Reaction was stopped with 1 µl 25 mmol/l EDTA (pH 8) and heat inactivation at 65°C for 10 min. EDTA addition did not influence subsequent enzymatic steps (data not shown).

RNase digestion was performed with 15 U of DNase-free RNase (Boehringer Mannheim, Mannheim, Germany) before reverse transcription. Ultracentrifugation. A total of 2 ml of plasma was mixed with 10 ml phosphate-buffered solution (PBS) (pH 7.2) and pelleted at 70,000g in sterile tubes. The pellet was dissolved in PBS and laid on a linear 20–63% sucrose density gradient in PBS. Ultracentrifugation was carried out at 200,000g for 20 h in a sw 41.14 rotor (Kontron, Germany) at 5°C. A total of 20 fractions of each gradient were obtained by puncturing the side wall of the tube, thereby avoiding churning up the pellet. Fractions were either directly taken for RNA and DNA extraction, or

FIG. 1. Results of first and nested PCR after RNA extraction of plasma (A) or cell culture supernatant (B) of activated PBMCs. Experiments were performed in the presence (+RT) or absence of reverse transcriptase (–RT) reaction in order to detect DNA contamination. H₂O controls were performed to exclude false-positive results.
fractions were pooled, diluted with PBS, and pelleted at 70,000g in sterile tubes. Pellets were dissolved in PBS, and nucleic acids were extracted.

RESULTS
In contrast to the study by Conrad et al. (9), we found all samples of patients and control subjects to be positive for IDDMK1,22 sequences by using the same PCR primers for the first PCR and the same cycle profile as used in the original study, but a more sensitive secondary detection method (Fig. 1A). Similar results were obtained with plasma samples and supernatants of activated PBMCs (Fig. 1B). All signals had the expected size of ~269 bp (data not shown), and cloning and sequencing revealed the expected sequence of IDDMK1,22 with a sequence homology of 93.7% (GeneBank accession numbers AFO 12327–12337) (data not shown). No difference could be seen between control subjects and IDDM patients. The same results were obtained with a more stringent annealing at 60°C for the first PCR (data not shown). The higher annealing temperature was used to increase the specificity of the reaction for detecting mRNA instead of genomic DNA, as suggested in the original study.

The positive although weaker signal without RT reaction pointed to the presence of genomic DNA, despite sequential centrifugation to avoid cellular contamination. While no signal was seen after the first PCR (Fig. 1A and B), signals in most nested PCR samples became already visible after 5–15 cycles of the nested PCR (data not shown). This explains the relative uniform intensity of the nested RT-PCR bands (Figs. 1 and 2) and of the Southern blot signals after the nested PCR (Fig. 2). Because of the relative overcycling of some reactions, results can just be interpreted as positive or negative. Although the method used by Conrad et al. (9) with Southern blot analysis after the first PCR resulted in different intensities of the bands, all our samples were positive and no specific intensity pattern was seen for either IDDM patients or control subjects (Fig. 2A and B).

It was demonstrated that the nested PCR is more sensitive than Southern blotting of the first PCR reaction. While the signal obtained from plasma samples was relatively insensitive to DNase digestion at room temperature, the RT-PCR resulted in weaker bands when RNase was added (Fig. 3). Increasing the temperature of the DNase reaction and the amount of DNase resulted in a loss of the DNA-derived signal (Fig. 4C), although the RT-PCR signal decreases as well, probably due to Mg-dependent hydrolysis of RNA at higher temperatures (Fig. 4D).

All control plasma samples were positive for IDDMK1,22, but no signal was seen after ultracentrifugation into sucrose gradients at buoyant densities characteristic for retroviruses (Figs. 5 and 6).
However, all RNA and subsequent DNA preparations of the pellet fractions were positive (Fig. 6). Moreover, positive PCR signals from the pellet fraction were obtained with primers for the D-loop of mitochondrial DNA and for genomic primers of β-actin (data not shown).

Increasing the sensitivity of the method by pooling and pelleting possible particles from different fractions did not result in any signal between 1.27 and 1.07 mg/ml even after nested PCR (Fig. 6).

RNA extracted from plasma without ultracentrifugation contained genomic DNA. In contrast, the RNA extracted from the ultracentrifugation fractions was relatively free of DNA. After RNA extraction from pellet fractions, signals were just seen in six out of seven cases if the RT reaction was performed before PCR (data not shown). Furthermore, these signals were sensitive to RNase treatment (Fig. 7).

Positive signals could be obtained with DNA extracted from the pellet fractions without reverse transcription and they were sensitive to DNase digestion at 37°C (Fig. 7).

Therefore, positive signals in the pellet fractions were derived from free genomic DNA and RNA, but not from viral particles.

DISCUSSION

As all plasma samples and cell culture supernatants of patients and control subjects were positive for IDDMK1,22-related sequences by using the same method and primers as used in the original study, we conclude that there is no association between positive signals for IDDMK1,22 and IDDM. After cloning and sequencing of the PCR product, the GeneBank blast search analysis of our sequence yielded the highest homology with IDDMK1,22. There are two possible explanations for the low sequence variation between our sequence and IDDMK1,22, although we have to emphasize that we have used the same primers as Conrad et al. (9). First, there exists a large sequence variation that is not evenly distributed across the viral genome. While the polymerase region is the most highly conserved region, the envelope region is the most variable. The variation of the LTR is somewhat in between (10). If one compares the LTRs of human endogenous retrovirus (HERV)-K18 (GeneBank M12853, M12852) for the region that has been sequenced by us, you end up with a sequence identity of just 92%, which is even lower than the 93.7% reported by us. By looking at the phylogenetic tree of the LTR region of IDDMK1,22 in the work of Conrad et al. (9), one can see that IDDMK1,22 LTR is rather related to HERV-K18, which shows a higher degree of LTR sequence variation (Larsson et al. [11]) than with HERV-K10. Second the IDDMK1,22 clone sequenced by Conrad et al. (9) was derived from supernatants of islet cells. Therefore, one cannot assume that the sequence from plasma necessarily corresponds to the published sequence. The cloning of a nested PCR product and subsequent cycle sequencing, all performed with a conventional Taq polymerase, might further contribute to some sequence variation. Overall, the sequence of the U3 region of IDDMK1,22 might not be best suited for epidemiological studies at all, because a high sequence homology exists to other HERV-K LTRs and to a retroposon integrated into the human genomic DNA (12).

This is the first study searching with sucrose density gradient ultracentrifugation for the existence of viral particles of IDDMK1,22-related sequences in patients with new-onset diabetes and control subjects (n = 12). No viral particles were found in patients and control subjects with a very sensitive nested PCR approach after ultracentrifugation. In particular, signals could not be generated with samples at the buoyant density of 1.13–0.19 mg/ml characteristic for retroviruses. Even the existence of IDDMK1,22-A-type particles in patients with new-onset diabetes and control subjects (n = 12).

**FIG. 3.** Combined RNase, DNase digest of RNA extracted from plasma samples. Samples were used with (+RT) or without (-RT) prior nested PCR. The intensities of (+RT) and (-RT) cannot be directly compared because they were generated by different sets of experiments. H2O controls were done to exclude false-positive results of the method.

**FIG. 4.** More stringent DNase digest of RNA extracted from plasma samples. The following conditions have been used: control without DNase (A), 2 U of DNase at 25°C (B), 2 U of DNase at 37°C (C), and 15 U of DNase at 37°C (D). The subsequent procedures were the same as those described for Fig. 3. Under more stringent conditions, there is just an RNA-derived signal left (C), but this is also lost (D), probably because of Mg-dependent RNA-hydrolysis, at 37°C.
(density 1.20–1.23 mg/ml) could be excluded. We therefore assume that the IDDMK1,22-related RNA and DNA signals detected in the pellets were derived from destroyed blood cells or culture cells rather than from retroviral particles. This hypothesis is further supported because signals specific for IDDMK1,22, together with those for mitochondrial DNA and β-actin, were obtained after PCR amplification of material derived from the pellet, where free nucleic acids accumulate. The RNase and DNase sensitivity of the amplification product leads to the conclusion that IDDMK1,22 is an endogenous retroviral genome, whose U3 region is transcribed into RNA. The partial transcription of RNA is a common feature of retroelements of the human genome (13,14).

Plasma samples in our cohort were obtained 0.5–24 months after clinical diagnosis of IDDM compared with a 1-month period in the original study (9,15). We therefore examined supernatants from cultured activated PBMCs because cultured splenocytes of IDDM patients were described to produce high RT activity even in the chronic state of the disease (9). Again, no difference between control subjects and IDDM patients could be observed by this method. Retroviral genomes containing an envelope gene are present in cells in much lower copy numbers than other retroelements (13). Therefore, very sensitive methods have to be used to detect their presence or even expressed viral RNA, in particular, in cell-free plasma samples after two preclearing centrifugation steps. Because of the close sequence homology between endogenous retroviruses, the use of an additional nested PCR should also increase the specificity of the detection. This is very important as the primers originally used by Conrad et al. (9) show a very high sequence homology to the HERV K family.

The increased sensitivity of the nested PCR method itself does not produce false-positive results because all water controls readily included in the extraction steps were negative.

IDDMK1,22 could be a defective provirus. The lack of an exogenous counterpart and the two stop codons reported in its reading frame might support the hypothesis. In this context, it is noteworthy that although numerous HERVs of class I and II have been described, human exogenous coun-
terparts have not yet been detected that resemble these simply structured and probably more ancient viruses. Moreover, major variations in copy number and integration sites of full length HERVs have not been observed in the human DNA, indicating a stable provirus integration. On the other hand, no endogenous counterparts of the complex exogenous lentiviridae (like HIV) or spumaviridae have been reported so far in human genomes (13).

We only used sequences specific for the U3 region of the LTR of IDDMK<sub>1,22</sub> using the same primers as in the original study by Conrad et al. (9). Because the U3 region of retroviral genomes is less conserved as the pol-region (10), it is still possible that retroviruses other than IDDMK<sub>1,22</sub> might be found in plasma samples of IDDM patients.

About 5–10% of the mammalian genome consists of elements introduced by reverse transcription (14), and HERV sequences encompass ~1% of the human genome (13). It has been therefore suggested that with increasingly sensitive PCR methods, viros should be purified from cellular nucleic acids before cloning their sequence (16). Isopycnic ultracentrifugation has been successfully used for this purpose in the past (5,6,16). However, we were unable to detect any viral particles in plasma and PBMC supernatants from IDDM patients or control subjects. In contrast, Conrad et al. (9) had performed cloning of the viral genome from cell culture supernatants and the subsequent epidemiological study from plasma samples (9), both of which we showed to be contaminated with genomic DNA and RNA.

Nevertheless, the finding of a HERV with SAG activity (9) is very exciting. Because all humans investigated so far harbor this viral sequence, it might be interesting to investigate if sequence variations in the SAG or differences in protein expression or transactivating activities exist between different endogenous viral isolates.

But we should also keep in mind that some results from the nonobese diabetic mouse model and from epidemiological studies support the idea that viral infections either incite or even protect from IDDM (17,18).

In summary, no correlation between a positive nested PCR signal for IDDMK<sub>1,22</sub> and IDDM could be observed. Our data support the hypothesis that the newly discovered sequence belongs to a member of the HERV family with a lack of an exogenous counterpart.

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REFERENCES
Author Queries (please see Q in margin and underlined text)

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In Figure 8, are “Co, control” and “DM, diabetes” correct?