IDDM Patients Neither Show Humoral Reactivities Against Endogenous Retroviral Envelope Protein Nor Do They Differ in Retroviral mRNA Expression From Healthy Relatives or Normal Individuals

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Recently, human endogenous retrovirus type K (HERV-K [IDDMK$_{1,2}$22]) was isolated from an IDDM patient's β-cell supernatant and shown to be implicated in expression as a superantigen. Furthermore, HERV-K RNA was found in plasma samples from newly diagnosed patients but not in those from healthy control subjects. We had earlier identified the presence of a HERV-K long terminal repeat element of the HLA DQ gene (DQ-LTR) to be positively associated with IDDM, which led us to investigate whether DQ-LTR is related to transcription of the putative retroviral superantigen. Additionally, we sought immunological evidence to determine whether those retroviral antigens could evoke an antibody response. Patients with IDDM (n = 14), Hashimoto's thyroiditits (n = 5), and Graves' disease (n = 12), as well as healthy control subjects (n = 12), were investigated, as were four nuclear families of Graves' disease patients and two of IDDM patients. RNA was isolated from plasma and peripheral blood lymphocytes and subjected to reverse transcription–polymerase chain reaction for transcripts of the env region of the HERV-K (IDDMK$_{1,2}$22) sequence. We identified *env* transcripts in both plasma and peripheral blood lymphocytes in all individuals studied: patients with recent-onset or long-standing IDDM, their relatives, and healthy control subjects, as well as patients with thyroid autoimmune disorders. Furthermore, we screened the sera of patients (n = 62) and control subjects (n = 35) for evidence of humoral immunity against HERV-K by Western blot specific for the ENV protein. Similar frequencies of antibody-positives were observed both in patients with IDDM (29%) and in healthy control subjects (26%). We conclude that neither the ubiquitous HERV-K transcripts nor the comparable percentage of ENV protein antibodies are associated with IDDM. An earlier, presymptomatic antibody response against HERV-K(IDDMK$_{1,22}$22) ENV cannot be ruled out. However, the superantigen hypothesis of an endogenous retrovirus in β-cell autoimmunity awaits confirmation. *Diabetes* 48:215–218, 1999

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bp, base pair; HERV-K, human endogenous retrovirus type K; PCR, polymerase chain reaction; RT, reverse transcription.

IDDM results from β-cell specific T-cell reactivity and autoimmune destruction. Its genetic background has been clarified to some extent, with the major susceptibility loci residing on chromosomes 6p (major histocompatibility complex) and 11p (insulin), as well as on several other loci contributing to or modifying the heritable risk of contracting the disease. It is generally believed that predisposed individuals develop IDDM after a triggering event such as a microbial infection. Population as well as twin studies point to an interaction of genes and environment in the age of onset of IDDM (1). Also, humoral markers of β-cell autoimmunity, although not pathogenetic by themselves, differ among the various age-of-onset subgroups in IDDM, pointing to possible differences in autoimmune mechanisms. Viral infections have long been presumed to be triggering factors, but only few viruses, including rubella (2) and Coxsackie B viral infections (3), have been documented in cases of diabetes manifestation. Because of the long and slow course of β-cell destruction, age of onset will not reveal the time period of initial infection, which may occur as early as in intrauterine development.

Recently, the demonstration of retroviral enzyme activity in supernatants of an IDDM patient's β-cell cultures, as well as the RNA sequence in plasma samples derived from IDDM patients, led to the hypothesis that a human endogenous retrovirus belonging to the human endogenous retrovirus type K (HERV-K) family might express a superantigen initiating or triggering β-cell autoimmunity (4).

Although neither origin nor chromosomal localization of this HERV-K (IDDMK$_{1,2}$22) has been identified as yet, it is structurally related to other retroviruses expressing superantigens, such as the mouse mammary tumor virus (MMTV). We have earlier identified the HERV-K LTR of the HLA DQ region to be associated with IDDM (5). In this study, we addressed the issues of whether the presence of DQ-LTR in patients with IDDM might be related to the transcription of the HERV-K (IDDMK$_{1,2}$22) sequence and whether there is evidence for antibody reactivity toward the ENV protein of this endogenous retrovirus.

RESEARCH DESIGN AND METHODS

Subjects. The diagnosis of IDDM was based on World Health Organization criteria. Patients with recent onset of disease (duration <3 months, n = 5) and long-standing IDDM (n = 9), first degree relatives of IDDM patients (n = 12), healthy control subjects (n = 12), patients with active Graves' disease (n = 14) and their relatives (n = 13), and six patients with Hashimoto's thyroiditis were studied for

env transcripts. Peripheral blood was taken for fresh lymphocytes and/or plasma samples. Plasma was separated from 10 ml EDTA anticoagulated whole blood by centrifugation at 3,000 rev/min for 10 min, similar to serum that was taken from coagulated samples. Peripheral blood lymphocytes were separated from heparinized whole blood by Ficoll-Hypaque density gradient centrifugation and stored at –80°C or analyzed immediately. RNA was extracted from peripheral blood lymphocytes or from plasma according to standard procedures.

RNA studies. Plasma RNA was prepared using the QIAmp Viral RNA-Kit (Qiagen, Hilden, Germany). RNA isolation from lymphocytes was performed using the Qiashredder and Rneasy-Kit (Qiagen). The RNA yield was subjected to DNase digestion: 50 µl RNA were incubated with 2 U of DNase (Pharmacia Biotech, Freiburg, Germany) in a final volume of 60 µl and subsequently purified. The resultant RNA was reverse transcribed to cDNA with a Not I-d $(T)_{18}$ bifunctional primer of a first strand cDNA synthesis kit (Pharmacia Biotech). The resultant cDNA was used directly (plasma) or diluted 1:10 (lymphocytes).

Reverse transcription–polymerase chain reaction assay. Detection of retroviral transcripts was revealed based on sequence information provided by Conrad et al. (4). The *env* coding region was selectively amplified for a 374–base pair (bp) fragment encompassing the nucleotides 34–408 and using the primers A, 5′-TGCAAAGAAAAGCGCCTCCAC-3′, and B, 5′-GGGCAAGTTTTCCCTTTAGGT-3′, under the following conditions: 25 µl total volume, 2.5 µl cDNA, 2.5 µl 8 mmol/l dNTP, 0.5 µl each primer, 1.25 µl DMSO, 1 U Taq polymerase (Promega, Madison, WI), and 16.13 µl H_2O; this was followed by initial denaturation at 95°C for 2.5 min, denaturation at 95°C for 45 s, annealing at 61°C for 45 s, elongation at 72°C 45 s for 29 cycles, and final extension for 2.5 min. Lymphocyte-derived cDNA was amplified for 30 cycles and cDNA from plasma for 35 cycles.

In addition, we analyzed the presence of HERV-K type 1 or type 2 transcripts (6). The HERV-K *pol-env* transcript in type 1 is 409 bp, and that of type 2 is 701 bp in length (6). Polymerase chain reaction (PCR) conditions were identical to those mentioned above except that 0.8 µl cDNA was used with the primers C, 5′-GGGGAGAGGTTTTGCTTGT-3′ (nucleotide position 6370–6388) (7), and D, 5′-GCTTATGTTTGTCTAAAC-3′ (position 7050–7067), according to the HERV-K10(+) composite sequence (7), which were described by Mayer et al. (8).

Control PCRs. Control PCR experiments were performed for the ubiquitously transcribed histone H3 gene (9) to generate a 198-bp fragment from all cDNA samples studied. In addition, a reverse transcription (RT)-PCR experiment for the HLA DQA1 gene was designed using primers that spanned an intron of the HLA DQA1 gene (positions 329–511) to discriminate possible contaminants of genomic DNA by the fragment size of 182 bp for RNA and 625 bp for genomic DNA. Primers were P1, 5′-AGTGCTCCACCTTGCAGTCATA-3′, and P2, 5′-GTCTTGTGGACAACATCTTTCC-3′ (10). RT-PCR was performed with initial denaturation at 94°C for 3 min, in lymphocyte cDNA for 27 cycles, and in plasma cDNA for 35 cycles (94°C for 30 s, 63°C for 30 s, and 72° for 30 s), and in a final elongation step at 72°C for 3 min.

Antibody detection by Western blot. Sera were collected from 58 patients with IDDM and 35 healthy blood donors. Immunoblotting of recombinant HERV-K SU and full-length ENV proteins expressed in insect cells was done as described previously (11). Human sera were diluted 1:100 to 1:10,000. Rabbit anti-ENV sera served as the control (11).

RESULTS

RT-PCR for the *env* mRNA of the HERV-K (IDDMK$_{1,2}$22) sequence. Plasma samples subjected to *env*-specific RT-PCR yielded a cDNA fragment of the expected size (374 bp) in all individuals studied. RNA subjected to control PCR omitting reverse transcriptase failed to produce the expected amplification product (data not shown). No differences in size or intensity of PCR products were observed between the samples (Fig. 1, *lanes 5–8*). RNA prepared from peripheral blood lymphocytes and subjected to *env*-specific PCR also produced the 374 bp fragment (Fig. 1, *lanes 9–13*). A positive control RT-PCR showed a comparable level of transcription for the HLA DQA1 gene in lymphocytes (Fig. 1 *lanes 14–18*), but at a lower level in plasma cDNA (Fig. 1, *lanes 1–4*). No differences were observed between the patients with recent-onset or long-standing IDDM, their healthy relatives, control subjects, or patients with Graves' disease.

To distinguish between transcripts from HERV-K type 1 and type 2 genomes, we used PCR primers encompassing the

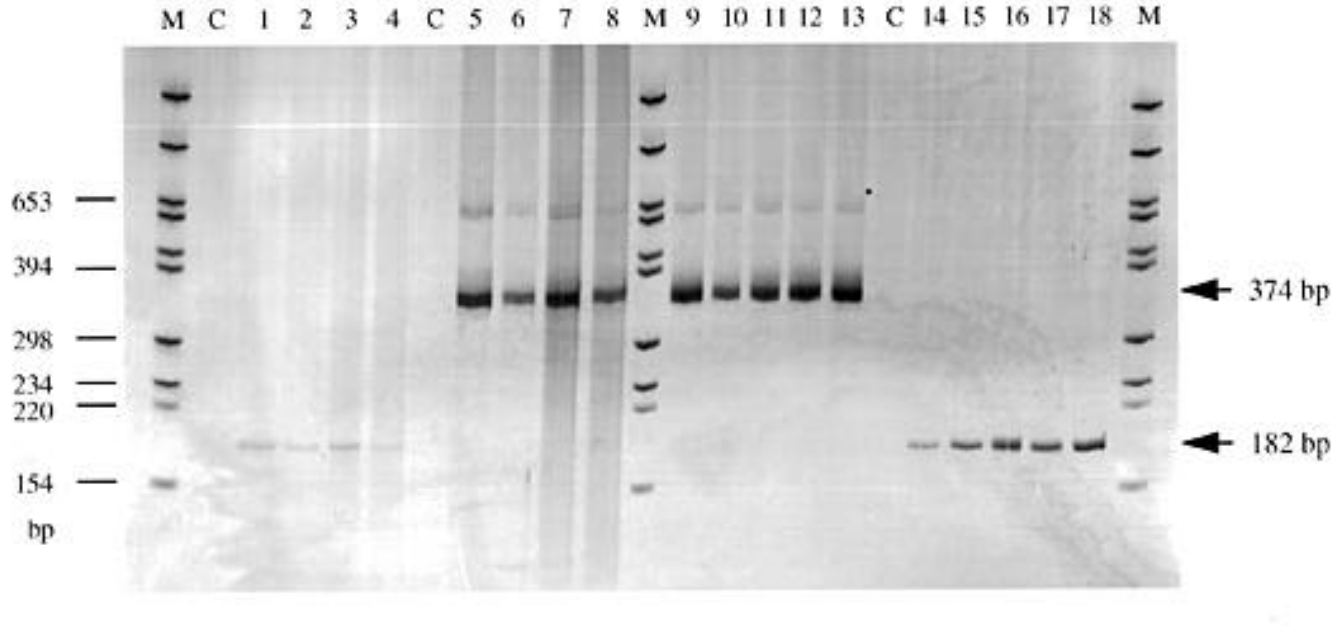

FIG. 1. Retroviral transcripts as detected by RT-PCR for the *env* region, selecting the sequence spanning nucleotide positions 34–408 (374 bp) (4), as well as for HLA DQA1 (cDNA 182 bp, 625-bp gDNA). Total RNA had been prepared from plasma or peripheral blood lymphocytes and reverse transcribed into cDNA. *M lanes* represent molecular weight markers (VI; Boehringer Mannheim, Mannheim, Germany); *C lanes* contain negative control RT-PCR. *Lanes 1–4* show plasma-derived cDNA and 14–18 lymphocyte cDNA for HLA DQA1, whereas *lanes 5–8* represent plasma cDNA, and *lanes 9–13*, lymphocyte cDNA for HERV-K *env* transcripts. *Lanes 1, 2, 5, 6, 9, 10, 13, 14, 15* and *18* represent healthy individuals, and *lanes 3, 4, 7, 8, 11, 12, 16* and *17* are samples from IDDM patients. Electrophoresis was performed in 10% polyacrylamide gels with subsequent silver staining.

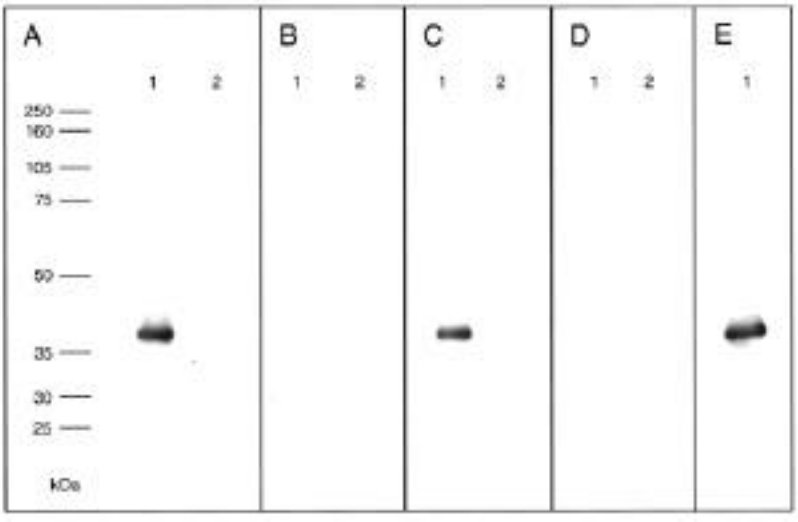

FIG. 2. Representative immunoblot analysis of antibodies against HERV-K ENV proteins. Serum samples used were derived from patients with IDDM (*A* and *B*), from healthy blood donors (*C* and *D*), and from a rabbit immunized with recombinant HERVK SU ENV protein (*E*) (11). Lysates of insect cells infected with recombinant HERV-K *env* baculovirus BacEx23 carrying the outer membrane portion of the ENV protein (*lane 1*, SU-ENV 40-kDa) or infected with wildtype baculovirus (*lane 2*) (11) were separated by SDS-PAGE, and immunoblots were generated as described (11). Serum samples were diluted 1:10,000 (*A*) and 1:1,000 (*B–E*).

HERV-K *pol-env* boundary. Only type 1 transcripts (407 bp) were found (data not shown). No other amplification products were detected.

Immunoblot analysis of antibodies against ENV proteins. Western blot analysis of antibodies directed against HERV-K ENV proteins revealed reactivities in 18 of 62 studied patients with IDDM (29%) and in 9 of 35 tested control sera (26%) (Fig. 2). In the remaining samples, there were no antibodies detectable by immunoblotting. All patients with IDDM that were positive for anti-ENV antibodies in the Western blot were also positive for GAD antibodies.

DISCUSSION

The genetic predisposition to IDDM is a prerequisite for antigen-driven stimulation of autoreactive T-cells that selectively destroy β-cells. Whereas conventional antigen presentation is followed by restricted T-cell stimulation, superantigens may lead to a proliferation of a large panel of T-cells. The demonstration of the Vβ7 T-cell receptor subtype isolated from freshly prepared pancreatic T-cells led to the hypothesis of a superantigen-provoked T-cell response in β-cell autoimmunity (12). The authors later isolated a vertically transmitted retroviral sequence in a patient with IDDM and demonstrated reverse transcriptase activity in lymphocytes, splenocytes, and to a lesser extent, in β-cells (4). Furthermore the superantigen motif identified in the *env* coding region of this HERV-K sequence suggested that this endogenous retrovirus might encode the IDDM superantigen.

In contrast to the data published, we identified transcripts coding for the ENV protein in all samples tested, regardless of whether plasma or lymphocyte RNA was derived from newly diagnosed patients with IDDM, other endocrine autoimmune disorders, healthy relatives, or control subjects. This discrepancy with the findings of Conrad et al. (4) may be due to two reasons. First, the primers we used were designed according to the sequence published by Conrad, which differs slightly from the primers applied in their plasma cDNA samples. We were not able to generate an amplification product using the original primer sequences (data not shown). Also, since the original primer sequence is situated in a highly conserved part of the U3 and R region, it is not specific for the $IDDMK_{1,2}$ sequence (13). Second, there may be heterogeneity in HERV-K transcription that relates to T-cell activation, clinical status, hyperglycemia, or other metabolic parameters. The origin of HERV-K transcripts as detected in plasma and peripheral lymphocytes cannot solely be explained by lytic lymphocytes, since HLA DQA cDNA is detectable at a much lower level in plasma than in lymphocyte-derived cDNA, compared with the HERV-K transcripts. Other blood compartments or vessel wall may thus harbor HERV-K particles. Nevertheless, the origin and nature of HERV-K particles in transcript-positive individuals needs further investigation.

Furthermore, we reasoned that a retroviral infection should mount an antibody response of some sort. Neither by ELISA (data not shown) nor by Western blot analyses could we detect an antibody response that would distinguish patients with IDDM from healthy control subjects. The positive reactivities are similar to those observed in the control population (14), and may be due to humoral immunity against specific HERV-K antigens or cross-reactivities to similar proteins. However, the immune mechanism that generates an antibody response to HERV-K ENV in some individuals but not in others remains elusive.

Retrovirus expression has been implicated in other autoimmune disorders, namely multiple sclerosis and in Graves' disease (15,16). Jaspan et al. (15) identified an association of antibodies against human intracisternal type A retroviral particles with Graves' disease susceptibility genes in families (15). The strongest evidence for retroviral expression has been presented for the multiple sclerosis–associated retrovirus, which has been repeatedly isolated from leptomeningeal, choroid plexus, and Epstein-Barr virus–transformed lymphocytes of patients (16).

Bearing in mind the slow and insidious course of β-cell autoimmunity preceding the manifestation of IDDM, our results do not exclude HERV-K ($IDDMK_{1,2}22$) as a candidate antigen or triggering factor in IDDM. However, we cannot confirm the retroviral transcripts to be unique to IDDM, and humoral immunity to the ENV proteins is not associated with IDDM. Whether other parts of the retroviral sequence are targets of antibodies or T-cells remains to be shown.

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