Rapid Publication

Elevation of Cytotoxic Lymphocyte Gene Expression Is Predictive of Islet Allograft Rejection in Nonhuman Primates

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Hyperglycemia and increased insulin requirements are indicators of ongoing islet allograft rejection, but there are no methods to predict or confirm rejection. Elevation of cytotoxic lymphocyte (CL) gene expression in peripheral blood (PB) has been correlated with renal allograft rejection in humans, but no published study has assessed the utility of monitoring these markers as predictors of rejection before the onset of clinical symptoms. We have established quantitative real-time PCR methods to determine the levels of mRNA transcripts for the CL genes granzyme B (GB), perforin, and fas ligand in blood samples from rhesus and cynomolgus monkeys. Four rhesus monkeys with long-term islet allograft function were studied. Antirejection (anti-CD154) therapy was discontinued, and weekly PB samples were obtained to determine whether the levels of mRNA transcripts for CL genes correlated with and/or were predictive of islet allograft rejection, defined as a loss of C-peptide production. For all monkeys, elevation of CL gene expression preceded rejection by 83–197 days, with GB as the best predictor. Elevated mRNA levels were sustained for 2–2.5 months in three of four animals and 1 month in the other, thus suggesting that the testing of these parameters may have practical applications in clinical islet cell transplantation. *Diabetes* 51:562–566, 2002

Transplantation of pancreatic islets is a promising strategy for the treatment of type 1 diabetes (1,2). Hyperglycemia and increased insulin requirements are indicators of ongoing islet allograft rejection; however, there are no methods to predict or confirm rejection before significant loss of islet mass. We have observed that postprandial glucose (PPG) increased 2–3 days before fasting blood glucose (FBG) levels in nonhuman primate islet allograft recipients undergoing rejection; however, unless antirejection therapy is initiated within 1–3 days of the elevation in PPG, it is difficult to rescue significant islet mass (3,4). Activation of transcription of the cytotoxic lymphocyte (CL) genes granzyme B (GB), perforin, and fas ligand (FasL) in transplanted tissue has been reported to be intimately associated with acute renal allograft rejection in humans, particularly when two of the three genes are simultaneously upregulated (5,6). Recently, the elevation of CL gene (ECLG) expression in peripheral blood leukocytes (PBLs) has been correlated with human renal allograft rejection (7,8); however, no studies have been reported that analyze the potential utility of monitoring these markers to predict rejection before the onset of clinical symptoms.

T-cell–dependent immune activation genes (CL) have been implicated as active participants in the process of acute rejection (5,6). GB and perforin are both involved in the apoptotic pathway of DNA fragmentation and cell death (9,10). Perforin, stored and secreted from the granules of cytotoxic effector cells, is a pore-forming protein that polymerizes and perforates target cell membranes, thus causing cell death (11,12). GB, a serine peptidase of the chymotrypsin family, is expressed primarily in activated cytotoxic cells and is a major component of the lytic machinery of cytotoxic cells (13). FasL, a transmembrane protein, belongs to the tumor necrosis factor/nerve growth factor receptor family (14). It is expressed on cytotoxic T-cells and binds with the Fas receptor on target cells, inducing a distinct pathway leading to target cell apoptosis (14–16).

Semiquantitative RT-PCR has been used to detect the early phases of immune activation (7,8,17). Development of the LightCycler PCR instrument enables the user to monitor fluorescence of a PCR continuously in real-time and on-line, and it enables rapid, accurate, and sensitive quantification of nucleic acid. The purposes of this study were to *1*) develop a practical method for monitoring the levels of mRNA transcripts for CL genes in blood samples from nonhuman primate islet allograft recipients using the LightCycler PCR System and *2*) determine whether alterations in CL gene expression in PB samples are predictive and/or indicative of islet rejection, defined as

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CL, cytotoxic lymphocyte; ECLG, elevation of CL gene; FasL, fas ligand; FBG, fasting blood glucose; GB, granzyme B; PB, peripheral blood; PBL, PB leukocytes; POD, postoperative day; PPG, postprandial glucose.

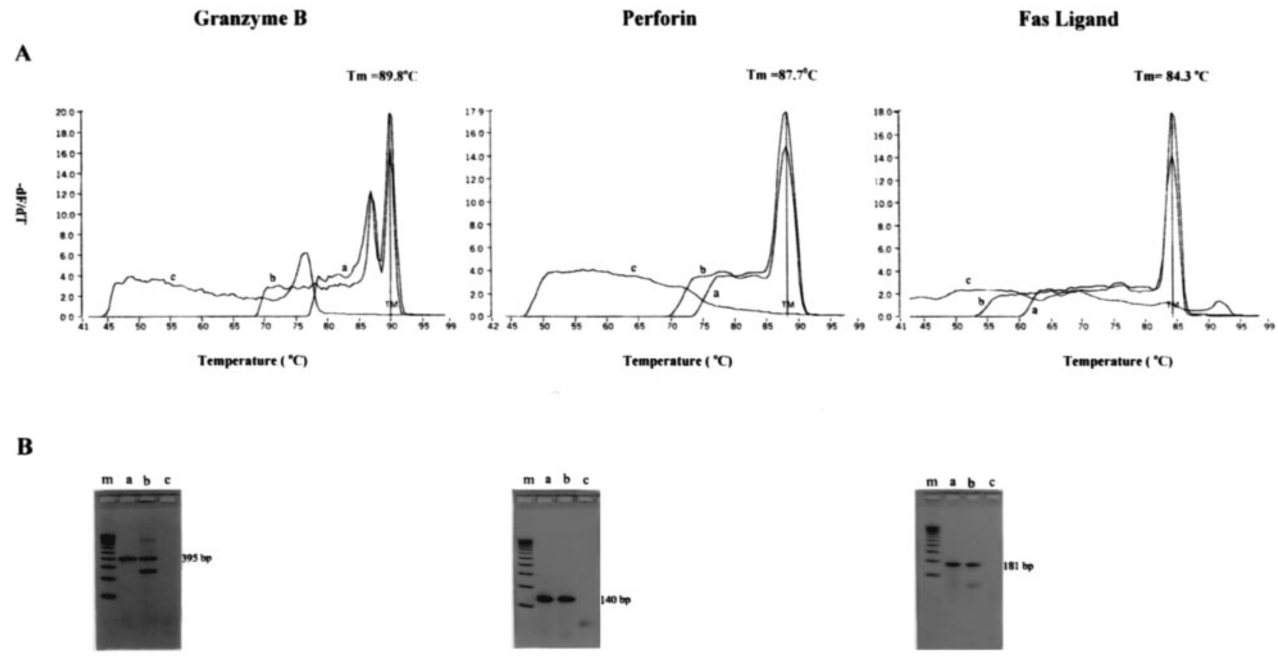

FIG. 1. Melting curve analysis of the specificity of the amplified PCR products in rhesus mRNA. *A*: Melting peak profile of GB, perforin, and FasL of rhesus monkey. a, standard; b, sample; c, negative control. The quantitation of GB, perforin, and FasL was performed acquiring the fluorescence at 88, 85, and 82°C, respectively. *B*. Gel analysis of LightCycler PCR products on a 2% ethidium bromide–stained agarose gel.

loss of C-peptide production, before the onset of clinical symptoms.

RESEARCH DESIGN AND METHODS

Reagents. The RNA purification reagent RNA Now-LW was purchased from Biogentex (Seabrook, TX). The first-strand cDNA synthesis kit SuperScript preamplification system was purchased from Gibco BRL (Grand Island, NY). Oligonucleotides for PCR were synthesized by Keystone Laboratory (Menlo Park, CA). LightCycler DNA Master SYBR Green I was purchased from Roche (Indianapolis, IN). TaqStart antibody was purchased from Clontech (Palo Alto, CA). All other chemicals were of the purest grade available and were obtained from commercial sources.

Animals and blood samples. Four long-term rhesus monkey islet allograft recipients with stable graft function were used in the study. These monkeys were originally treated with humanized anti-CD154 specific monoclonal antibody (hu5c8, ANTOVA; Biogen, Boston, MA) (3). Three of the monkeys had stable partial graft function, and one was insulin-independent at the time this study was initiated. We have observed rejection 3–5 months after cessation of anti-CD154 therapy in rhesus monkey islet allograft recipients (4). To determine whether ECLG transcripts correlates and/or is predictive of islet rejection, maintenance therapy was discontinued on these four monkeys, and weekly peripheral blood (PB) samples were collected in EDTA-containing tubes to assess CL gene transcript expression. We froze 0.5 ml aliquots of whole blood at −80°C until processing for RNA. The experiments described in this study were conducted according to the principles set forth in the *Guide for Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, Department of Health and Human Services [National Institutes of Health publ. no. 86-23]).

RNA isolation and reverse transcription. Total RNA was extracted from PB using the RNA Now-LW method (Biogentex, Seabrook, TX). RNA concentration was measured by a RiboGreen RNA Quantitation kit (Molecular Probe, Eugene, OR) and a fluorometer (Bio-Rad, Hercules, CA). First-strand cDNA synthesis was performed in a 21-μl reaction volume containing 250 ng of total RNA, 25 ng/μl oligo dT, 0.5 mmol/l of each dNTP, 2.5 mmol/l $MgCl_2$, 10 mmol/l dithiothreitol, and 200 units of SuperScript II RNase H^- reverse transcriptase (Gibco BRL, Grand Island, NY).

PCR primers. PCR primers were originally designed from the gene sequences of human perforin, GB, and FasL. Preliminary experiments to establish the methodologies were performed on RNA samples isolated from phorbol 12-myristate 13-acetate (PMA) and ionomycin-stimulated rhesus monkey PBL by using the human CL primers. The PCR-amplified fragments were cloned in a PCR 2.1 vector (Introgen, San Diego, CA) and confirmed by double-strand DNA sequencing in the DNA core laboratory (Department of Biochemistry, University of Miami, Miami, FL). Nonhuman primate specific primers of the three CL genes were then designed based on the sequence of each of the three nonhuman primate CL fragments as follows: perforin sense 5′-GCCGGC-AACGTGCATGTGTCT-3′, antisense 5′-GTGTGTACCACATGGTAACTG-3′; GB sense 5′-GGGATCAGAAGTCTCTGAAGAG-3′, antisense 5′-CTTTCGATCTTC-CTGCACTGTC-3′; FasL sense 5′-GTAGGATTGGGACTGGGGAT-3′, antisense 5′-AGTTGGGCTTGCCTGTTAAA-3′. β-Actin primer sequences were obtained from A.D. Kirk and colleagues (18). The sequence homologies of the 140-bp perforin, 395-bp GB, and 181-bp FasL fragments between monkey and human were 96.4, 93.4, and 95.0%, respectively.

Real-time LightCycler PCR analysis

Amplification. PCR amplification was performed with the LightCycler DNA Master SYBR Green I kit in a PCR containing 0.5 μmol/l of each primer, 3 mmol/l $MgCl_2$, and 2 μl sample. TaqStart antibody was systematically added to the amplification reaction mixture to block *Taq*DNA polymerase activity during set-up of the PCR at ambient temperature (Clontech, Palo Alto, CA). The amplification and detection were carried out in a LightCycler instrument as follows. The reaction mixture was initially incubated at 96°C for 30 s to inactivate the TaqStart antibody and to denature the DNA. Amplification was performed for 45 cycles, with the following cycle parameters: denaturation (95°C for 1 s), annealing (62°C for 10 s), and extension (72°C for 15 s). The ramp rate was 20°C/s. Fluorescence was acquired at the end of each annealing phase, with the acquiring temperature set at 85°C for perforin, 88°C for GB, and 82°C for FasL.

Melting curves. The melting curves were obtained at the end of amplification by cooling the sample to 60°C at a rate of 20°C/s and then increasing the temperature to 95°C at a rate of 0.2°C/s. Fluorescence was acquired every 0.1°C. The conversion of the melting curves into melting peaks (plot of the negative derivative of florescence versus temperature) allowed identification of each specific gene.

Quantification. Respective cloned CL plasmids, with serial dilutions from copy number 10,000,000 to 100, with 10-fold intervals, were used as standards to construct each standard curve in the LightCycler PCR quantification. A reaction mixture without the template and a reaction mixture without SuperScript II reverse transcriptase were used as negative controls. Quantification was carried out using LightCycler analysis software. Background fluorescence was removed by setting a noise band. The log-linear portion of the standard's amplification curve was identified, and the crossing point was the intersection of the best-fit line through the log-linear region and the noise band (19). The standard curve was constructed by plotting the log of copy number versus the crossing points. β-Actin amplification was used to rule out failure in each RNA purification, reverse transcriptase reaction, and PCR

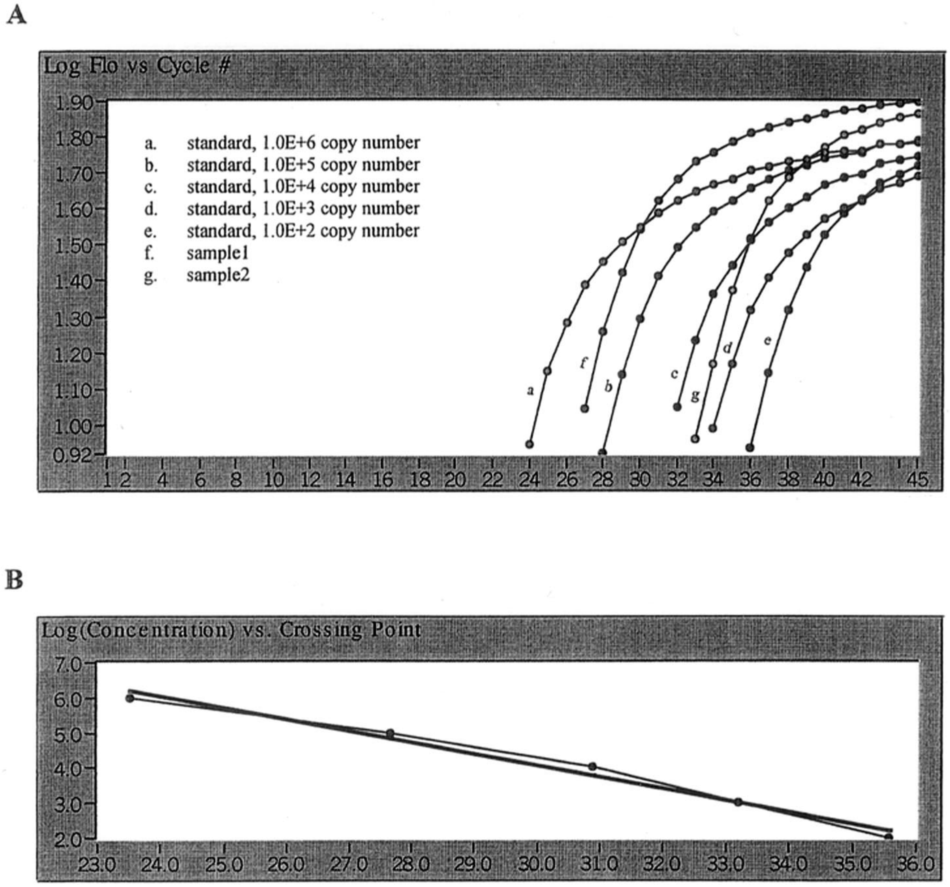

FIG. 2. Quantitation of PCR products. *A*: Logarithmic plot of fluorescence versus cycle number of perforin of rhesus monkey. *B*: Standard curve constructed with the perforin standards from 10^6 to 10^2 copies. The standard curve was constructed by plotting the logarithmic concentration of the standard versus the crossing points (cycle number). The error of the standard curve (ϵ) = 3.4353E-3 (ϵ = sum of squares of deviation/number of measuring points).

amplification reaction and to control variation in cDNA quantity among samples. All results were expressed as the ratio of the copy number of the target gene to the copy number of β-actin.

RESULTS

Quantification of perforin, GB, and FasL genes in PB. We carried out a preliminary melting curve analysis to determine the proper temperature for the acquisition of the fluorescent data. The melting curve analyses for the GB, perforin, and FasL genes of the rhesus monkey are shown in Fig. 1*A*. The center of the melting peak is the melting temperature (T_m) of the DNA product. The target DNA product can be uniquely identified from other fragments based on these melting peaks. Nonspecific amplification products tend to melt at much lower temperatures and over a broader range. The melting temperatures for rhesus GB, perforin, and FasL were 89.8, 87.7, and 84.3°C, respectively. Therefore, the quantitation of GB, perforin, and FasL was performed, acquiring the fluorescence at, respectively, 88, 85, and 82°C, temperatures at which any nonspecific fluorescence is excluded. The size of each LightCycler PCR amplified fragment was confirmed by separating the products on an ethidium bromide–stained agarose gel (395, 140, and 181 bp for GB, perforin, and FasL, respectively) (Fig. 1*B*).

A representative amplification curve for the perforin PCR product is shown in Fig. 2*A*. After completion of PCR, the LightCycler software generates the amplification curve and sets a baseline x-axis. The baseline identifies the cycle in which the log-linear signal can be distinguished from the background for each sample. The plot of the x-axis "crossing point" for each standard against the logarithm of standard concentration generates the standard curve (Fig. 2*B*), from which the concentration of the target sequence in the sample can be extrapolated. The primers used for analysis of these genes in rhesus monkeys work equally well in a cynomolgus monkey model (data not shown).

Perforin, GB, and FasL mRNA expression in PB of rhesus monkeys. Figure 3 shows the expression of mRNA levels of GB, perforin, and FasL in the PB of four rhesus monkey islet allograft recipients subsequent to discontinuation of anti-CD154 therapy, and the date relative to postoperative day (POD) is summarized in Table 1. ECLG expression preceded islet allograft rejection, defined as a loss of C-peptide production, by 83–197 days in the four monkeys studied (Table 1). ECLG occurred 165–176 days after discontinuation of 5c8 therapy in three monkeys and lasted 2–2.5 months. For the other monkey, ECLG occurred 33 days after cessation of the monoclonal antibody therapy and lasted 25 days (Table 1). Comparing the POD for ECLG duration with the POD for elevation of

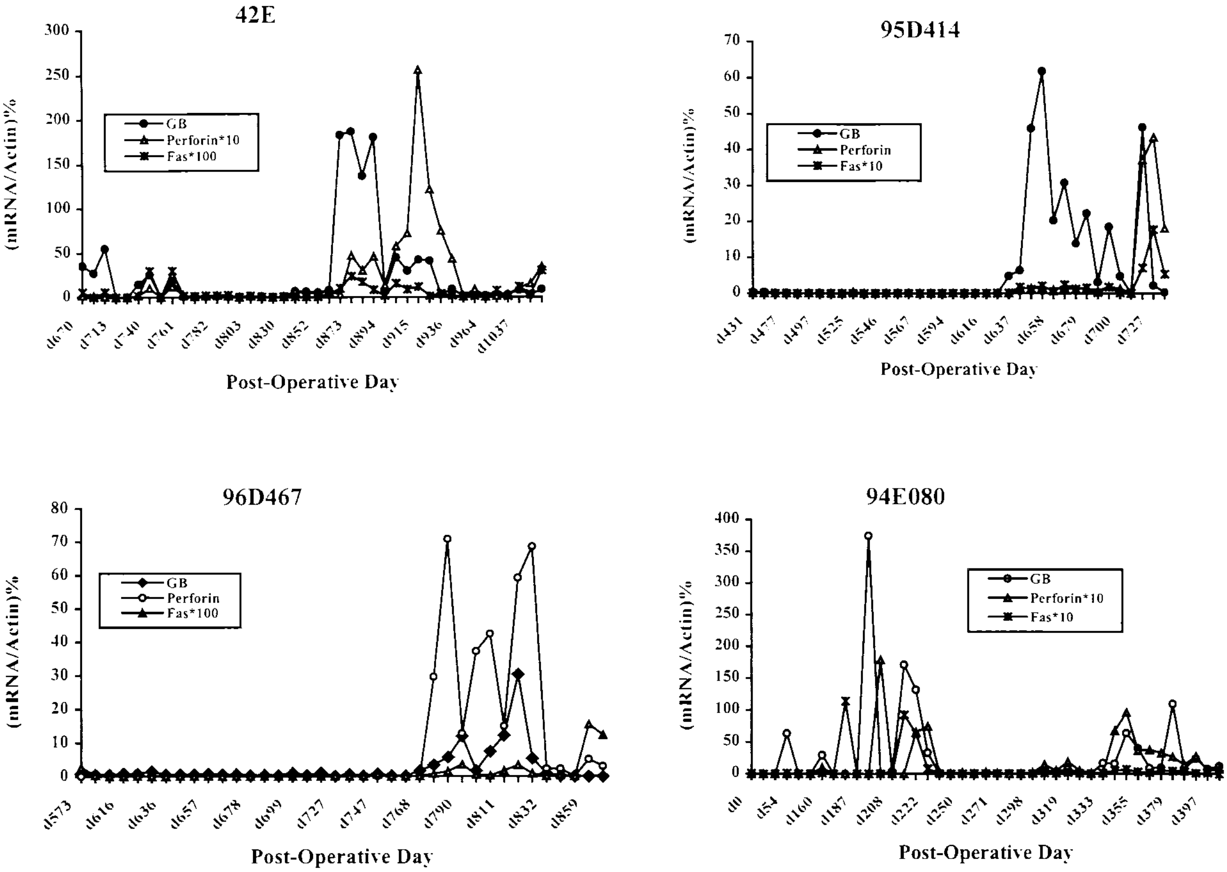

FIG. 3. Quantitative analysis of GB, perforin, and FasL gene expression in PB samples from rhesus monkey islet allograft recipients (real-time LightCycler PCR analysis). The ratio of the copy number of the target gene to the copy number of β-actin was plotted against POD. For some data sets, the result was multiplied by 10 or 100 (indicated as *10 or *100, respectively) to make it possible to see the pattern of gene expression in the figure.

2-h PB glucose (Table 1), it can be observed that ECLG expression correlated with the elevation of 2-h postprandial blood glucose in two of the four monkeys.

DISCUSSION

Hyperglycemia and increased insulin requirements are currently used as indicators of islet rejection; however, these parameters are not predictive and occur at the time when significant islet mass has been lost. Our study demonstrates that ECLG expression in PB precedes islet graft rejection, defined as a loss of C-peptide production, by 2.5–6.5 months, with a duration of 1–2.5 months. Antirejection therapy initiated within 3 days of elevated PPG enabled reversal of rejection and rescue of partial islet mass in anti-CD154–treated nonhuman primates (3,4); however, it would be difficult to define the level of PPG at which antirejection therapy should be undertaken in patients with varying degrees of graft function. It will be important to conduct further studies to demonstrate that detection of elevated CL gene expression enables reversal of rejection episodes in a clinically feasible time frame and results in reproducible maintenance of islet mass.

Competitive quantitative RT-PCR techniques have been widely used to detect gene expression in biological sam-

TABLE 1
Elevation of CL gene expression relative to rejection

	Monkey no.			
Event	42E	95D414	96D467	94E080
Last day using 5c8	689	464	603	164
ECLG	865	637	768	197
Negative C-peptide	1,062	720	866	333
Elevation of 2-h postprandial blood glucose	1,004	632	764	325
$POD_{Negative\ C\text{-}peptide}$-$POD_{ECLG}$	197	83	98	136
POD_{ECLG}-$POD_{Last\ day\ using\ 5c8}$	176	173	165	33
$POD_{Negative\ C\text{-}peptide}$-$POD_{Last\ day\ using\ 5c8}$	373	256	263	169
ECLG duration	71 (936–865)	63 (700–637)	64 (832–768)	25 (222–197) 70 (403–333)

Data are expressed as the POD relative to islet cell transplant on POD 0 or days (POD range).

ples. The use of the LightCycler PCR instrument enables monitoring of the fluorescence of a PCR continuously and on-line. The subsequent ease of identification of the log linear cycles of a PCR allows for rapid, accurate, and sensitive quantification of nucleic acid.

In summary, analysis of CL gene expression in PB samples may allow for detection of islet allograft rejection several weeks before loss of C-peptide production. The maintenance of increased expression for a period of 1–2.5 months suggests that testing of these parameters may have practical applications in clinical islet cell transplantation.

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