Assessment of Cytotoxic Lymphocyte Gene Expression in the Peripheral Blood of Human Islet Allograft Recipients

Elevation Precedes Clinical Evidence of Rejection

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Studies in nonhuman primates have demonstrated that elevation of the cytotoxic lymphocyte (CL) genes granzyme B, perforin, and Fas ligand in peripheral blood precedes islet allograft rejection. The purpose of this study was to determine whether this approach has utility for prediction of human islet allograft loss. We studied 13 patients who had long-term type 1 diabetes and were treated with steroid-free immunosuppression and given sequential islet cell infusions. All recipients became insulin independent, and eight of them experienced deterioration in glycemic control, followed by reinitiation of insulin therapy. Frequent peripheral blood samples were collected to monitor CL gene mRNA levels with real-time PCR. For the eight back-to-insulin patients, there was a clear elevation of CL gene mRNA levels 25–203 days before the onset of frequent hyperglycemia. Granzyme B was the most reliable indicator of ongoing graft loss. Additional correlations with infection were noted; however, evidence of sensitization in antidonor mixed lymphocyte reaction was observed in seven of eight patients who experienced partial graft loss, whereas this was not seen when upregulated CL gene expression was associated with infection. The results suggest that, when taken into consideration with other clinical parameters, elevated CL gene levels may enable prediction of islet allograft loss. *Diabetes* 53: 2281–2290, 2004

Allogeneic islet transplantation is a promising therapy to restore insulin independence in patients with type 1 diabetes (1–3). Currently, the only indicators of ongoing clinical islet allograft rejection are hyperglycemia and increased insulin requirements. Our studies of nonhuman primate islet allograft recipients demonstrated that it is difficult to rescue significant islet mass unless antirejection therapy is initiated within 1–3 days of observing elevated postprandial glucose, which occurred 2–3 days before observation of elevated fasting glucose (4,5). Reliable and reproducible methods for prediction of islet allograft rejection, before the onset of clinical symptoms, would enable rescue of the graft should effective agents be available.

The cytotoxic lymphocyte (CL) effector molecules granzyme B (GB), perforin, and Fas ligand (FasL) were reported to be actively involved in the process of acute allograft rejection (6–11). Elevated expression of CL gene expression in peripheral blood and urine has been reported to correlate with the occurrence of clinical renal allograft rejection (12–15). Intragraft activation of the genes encoding CL effector molecules was described to precede histological evidence of rejection in human cardiac transplantation (16). Recently, a study of four islet allotransplanted rhesus monkeys that were taken off immune intervention revealed that elevation of CL gene expression in peripheral blood preceded graft loss by 83–197 days, with 2–2.5 months of elevated CL gene messenger RNA (mRNA) levels (8). The purpose of this study was to assess the utility of monitoring CL gene expression levels in peripheral blood for prediction of islet allograft rejection before the onset of clinical symptoms.

RESEARCH DESIGN AND METHODS

Thirteen islet allograft recipients with long-term (>5 years) type 1 diabetes and hypoglycemia unawareness were included in the study. These patients received steroid-free immunosuppression that consisted of sirolimus (Rapamune), tacrolimus (Prograf), and daclizumab (Zenapax). Daclizumab was given intravenously at a dose of 1 mg/kg every 14 days for a total of five doses for every islet infusion and every month in the first year and every 2 months thereafter. Sirolimus and tacrolimus were administered orally to achieve and maintain sirolimus trough levels of 12–15 ng/ml for the first 3 months after islet transplantation (7–10 ng/ml thereafter) and tacrolimus trough levels of 3–5 ng/ml. All protocols were approved by the Institutional Review Board of the University of Miami and the Food and Drug Administration. Each patient gave written informed consent.

Duration of follow-up. Follow-up for the eight patients who experienced partial graft loss was continued for 2 months after resumption of insulin therapy and ranged from postoperative day (POD) 268 to POD 569 (Table 1). For the patients with stable graft function, follow-up ranged from POD 888 to POD 455.

Collection of blood samples. To determine whether elevation of CL gene expression can be used to predict and/or confirm clinical islet allograft rejection, we collected frequent EDTA-anticoagulated peripheral blood sam-

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CL, cytotoxic lymphocyte; FasL, Fas ligand; GB, granzyme B; MLR, mixed leukocyte reaction; PBMC, peripheral blood mononuclear cell; POD, postoperative day.

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TABLE 1
Elevation of CL gene (ECLG) expression relative to graft loss

Event	Patient							
	1	3	6	7	8	10	13	15
Second transplant	99	19	22	33	37	59	76	108
ECLG	193–254	217–285	148–200†	124–185	360–556	307–379	192–256	182–253†
Evidence of sensitization in MLR	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes
Frequent elevation of mean daily blood glucose*	278	320	173	305	461	470	223	385
POD difference between elevated blood glucose and ECLG	85	103	25	181	101	163	31	203
ECLG duration	61	68	52	61	196	72	64	71
Resume insulin	356	399	208	431	508	513	243	509
Follow-up through POD	416	459	268	491	568	573	303	569

Data are expressed as the POD relative to islet cell transplantation on POD 0. *Mean daily blood glucose = mean of pre- and postprandial blood glucose for breakfast, lunch, and dinner and bedtime blood glucose; †There is an elevated CL gene at POD 60–81 for patient 6 and at POD 127–161 for patient 15. Because these elevations occurred very close to the second transplant, it was difficult to distinguish whether the elevated CL gene was evidence of posttransplantation immune activation or of rejection. For these two patients, the PODs for the elevated CL gene shown in the table were chosen because they were clearly distinct from transplant-related activation.

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ples for the assessment of CL gene mRNA levels. On the basis of our nonhuman primate studies, we determined that the ideal interval between sample collections would be 2–3 weeks (8).

RNA isolation and reverse transcription. Total RNA was purified from peripheral blood using the RNA Now-LW kit (Biogentex, Seabrook, TX). Synthesis of first-strand cDNA was performed using SuperScript II Reverse Transcriptase (Life Technologies, Grand Island, NY), as previously described (8).

Real-time LightCycler PCR analysis. CL gene mRNA levels in peripheral blood samples were determined by a real-time LightCycler PCR system (Roche, Indianapolis, IN). PCR primers for GB, perforin, and FasL were designed from respective human gene sequences from gene bank (NCBI Entrez, accession no. A26437 for GB, X13224 for perforin, and U08137 for FasL). GB: sense 5′-GGGATCAGAAGTCTCTGAAGAG-3′, antisense 5′-CTTTC-GATCTTCCTGCACTGTC-3′; perforin: sense 5′-CGGCTCACACTCACAGG-3′, antisense 5′-CTGCCGTGGATGCCTATG-3′; FasL: sense 5′-GCCTGTGTCTC-CTTGTGA-3′, antisense 5′-GCCACCCTTCTTATACTT-3′.

Amplification of each sample was performed in a PCR that contained 2 μl of LightCycler DNA Master SYBR Green I, 0.5 μmol/l of each primer, 3 mmol/l $MgCl_2$, 0.056 μmol/l TaqStart antibody, and 2 ml of sample (8). 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, 1 s), annealing (62°C, 10 s), and extension (72°C, 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 88°C for GB, 88°C for perforin, and 84°C for FasL. PCR products were identified by performing melting-curve analysis at the end of each PCR amplification (8). Melting curves were obtained at the end of amplification by cooling the samples 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. Conversion of the melting curves into melting peaks (plot of the negative derivative of florescence versus temperature) enabled identification of each specific gene. The size of each LightCycler PCR amplified fragment (140 bp, 395 bp, and 181 bp for perforin, GB, and FasL, respectively) was confirmed by separating the product on an ethidium bromide–stained agarose gel.

Standard curves for each CL gene were constructed by analysis of serial 10-fold dilutions of CL gene DNA fragments (copy number from 10^7 to 10^2) (8). The efficiency (E) of the PCR was typically >85%, as calculated by the following equation, where S is the slope of the standard curve: $E = 10^{1/-S} - 1$ (17).

β-Actin amplification was used to control for PCR conditions and for 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.

Mixed leukocyte reaction to assess recipient antidonor immunoreactivity. Recipient peripheral blood mononuclear cells (PBMCs) were used as responders against γ-irradiated (3,000 rad) donor or self-PBMC in a one-way mixed leukocyte reaction (MLR). As positive controls, recipient PBMCs were stimulated with ConA and PHA. Culture media consisted of RPMI medium 1640 (Life Technologies) supplemented with 15% normal human serum (Sigma, St. Louis, MO), antibiotics, HEPES, sodium pyruvate, and L-glutamine. Cultures were pulsed with 1 μCi of tritiated thymidine on day 5, were incubated overnight, and were harvested and counted on day 6. Data were expressed as mean counts per minute (cpm) of quadruplicate cultures. Antidonor cpm was obtained from cultures of recipient PBMC versus irradiated donor PBMCs and anti-self cpm from cultures of recipient PBMC versus irradiated recipient PBMCs. Data were expressed as antidonor cpm minus anti-self cpm.

RESULTS

Elevation of CL gene expression in relation to timing of islet transplants, infection, and immune suppression. Twelve of the 13 patients received two islet cell infusions, and 11 became insulin independent immediately after the second transplant. The other patient was intentionally maintained on insulin for the first posttransplantation month and became insulin independent 33 days after the second transplant. There was no deterioration in glycemic control before the second transplant. All of these patients experienced a reduction in insulin requirement after the first islet cell infusion but were not completely off insulin until after the second infusion. For the patient with only one islet infusion, insulin independence was achieved 45 days after transplant. Eight of the 13 patients eventually experienced deterioration in glycemic control and returned to reduced dosages of exogenous insulin (R.A., T. Froud, C.R., et al., unpublished observations).

Representative profiles for the expression of mRNA levels for GB, perforin, and FasL in the peripheral blood of two of the eight recipients who experienced partial islet allograft loss are shown in Figs. 1 and 2. GB mRNA levels for four additional recipients who experienced partial islet allograft loss are shown in Fig. 3. The GB results for four patients who are still insulin independent with stable graft function are shown in Fig. 4. Figures 5–7 detail the relationship among CL gene elevation, blood glucose, C-peptide levels, and return to exogenous insulin. Several key points emerge after analyzing CL gene mRNA data together with patient clinical history.

The majority of patients had minimal to undetectable levels of CL gene expression before initiation of immune suppression and islet transplantation. Elevation of CL gene mRNA in the early posttransplantation period was frequently observed (Figs. 1–7) and was variable before and after the second infusion; these early changes did not

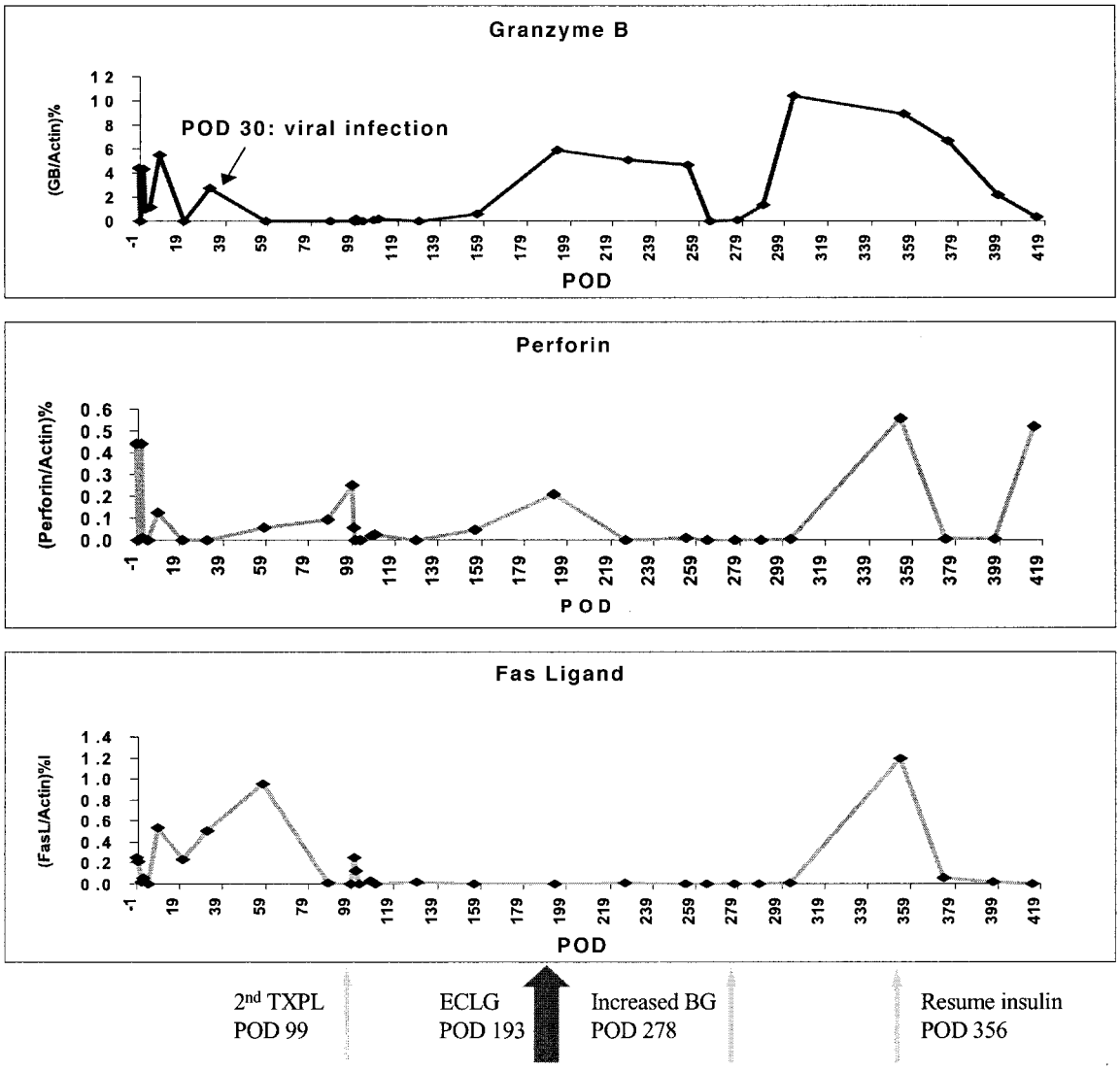

FIG. 1. CL gene expression profile in peripheral blood samples of an islet allograft recipient who experienced partial islet allograft loss: patient 1. The ratio of the copy number of the target gene to the copy number of β-actin was plotted against POD. BG, blood glucose; ECLG, elevated CL gene; TXPL, transplant.

seem to correlate with the loss of graft function. All patients had minimal to undetectable levels of CL gene expression after the posttransplantation changes resolved, and subsequent elevations were easily observed in relation to these negligible values (Figs. 1–7). Infection can clearly contribute to elevation of CL gene levels, as detailed in Figs. 1–7. For example, an elevation of GB mRNA was observed at POD 30 for patient 1 (Fig. 1) and correlated with viral infection. Similarly, increases at POD 78–145 for patient 3 (Fig. 2) were correlated with upper respiratory infection.

Sirolimus and tacrolimus levels were within or above the suggested trough levels at the time of elevated CL gene expression and subsequent partial graft loss; however, for six of eight patients, elevation of CL gene expression occurred in parallel with tapering to maintenance levels (e.g., see GB elevation on POD 192 for patient 13; Fig. 3). Although no correlation between levels of immunosuppression and CL gene elevation was observed for one of the other two patients (patient 8; Fig. 3), the other patient experienced increases in CL gene expression in conjunction with an extended period of low levels of rapamycin (patient 10; Fig. 3).

CL gene expression in the peripheral blood of patients who experienced partial islet allograft loss. For all eight patients who were returned to insulin therapy, we observed a correlation among elevation of CL gene mRNA, eventual onset of hyperglycemia, and reinitiation of insulin therapy. For patient 1 (Fig. 1), two of three CL gene mRNA levels increased at POD 193. GB remained elevated for several weeks in the absence of infection or administration of medications that we can attribute to elevation of CL gene mRNA expression. CL gene elevation occurred 85 days before increases in blood glucose levels and return to insulin therapy. Similarly, elevation of all three CL genes was observed, beginning at POD 217 and extending for several weeks, for patient 3 (Fig. 2). An increase in blood glucose levels and return to insulin therapy occurred 103 days later. Similar patterns were observed in other patients who experienced partial islet allograft loss (Fig. 3 and data not shown). Coincident with hyperglycemia and need for insulin, CL gene mRNA

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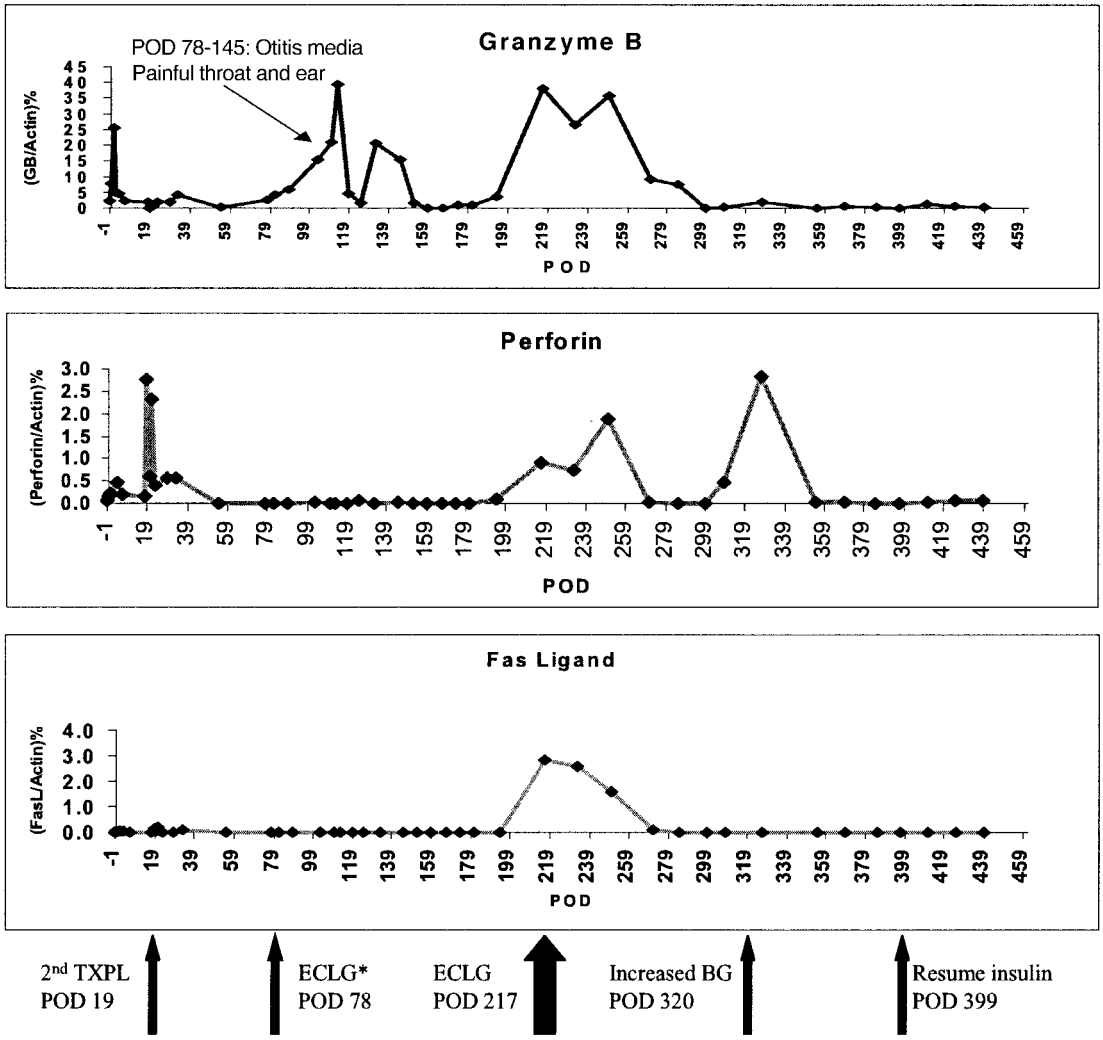

FIG. 2. CL gene expression profile in peripheral blood samples of an islet allograft recipient who experienced partial islet allograft loss: patient 3. The ratio of the copy number of the target gene to the copy number of β-actin was plotted against POD. *Patient experienced infections and mouth ulcers. BG, blood glucose; ECLG, elevated CL gene; TXPL, transplant.

levels were elevated in most patients (five of eight), as observed in patients 1, 8, and 13 (Figs. 1 and 3). GB was the most consistent indicator, with mRNA levels elevated in all eight patients. mRNA levels of all three CL genes were elevated in three of eight patients, and three patients experienced elevation of two CL genes. A summary of the data is given in Table 1. Elevation of CL gene mRNA occurred 25–203 days before frequent hyperglycemia in the eight patients who experienced partial graft loss. The duration of elevated CL gene mRNA was 52–72 days for seven of eight patients, but patient 8 experienced elevated CL gene expression for 196 days (Table 1).

Figures 5 and 6 illustrate the relationship between GB gene expression in peripheral blood and the mean blood glucose, daily insulin requirements, and C-peptide levels for two patients with partial graft loss. It is clearly seen in the figures that CL genes were elevated several weeks before frequent elevation of blood glucose, followed by initiation of exogenous insulin therapy and decreased C-peptide levels. For all eight patients who experienced partial graft loss, there was no decrease in C-peptide levels at the time of elevated CL gene expression, but C-peptide was decreased eventually, as shown in Figs. 5 and 6. From 69 to 350 days after elevation of CL gene expression, eight of eight patients had C-peptide levels that were reduced by >50%, as compared with the values observed at the beginning of elevated CL gene expression.

MLR and CL gene expression. We consistently observed loss of antidonor MLR in patients treated with steroid-free immunosuppression (data not shown). For seven of eight patients who experienced partial graft loss, we observed evidence of sensitization in antidonor MLR (Table 1) subsequent to increases in CL gene expression. The elevation of CL gene expression associated with viral or bacteria infection, however, did not correlate with sensitization in MLR.

CL gene expression in peripheral blood for patients with stable graft function. Profiles of CL gene mRNA levels in the peripheral blood of four patients who are still insulin independent with stable graft function are shown in Fig. 4. As discussed previously, patients with stable graft function occasionally experienced elevations of CL gene mRNA in conjunction with transplantation or infection. Small spikes at approximately POD 36 for patient 4 and POD 42 for patient 5 were due to a second islet cell infusion. Patient 4 experienced several infections (Fig. 4),

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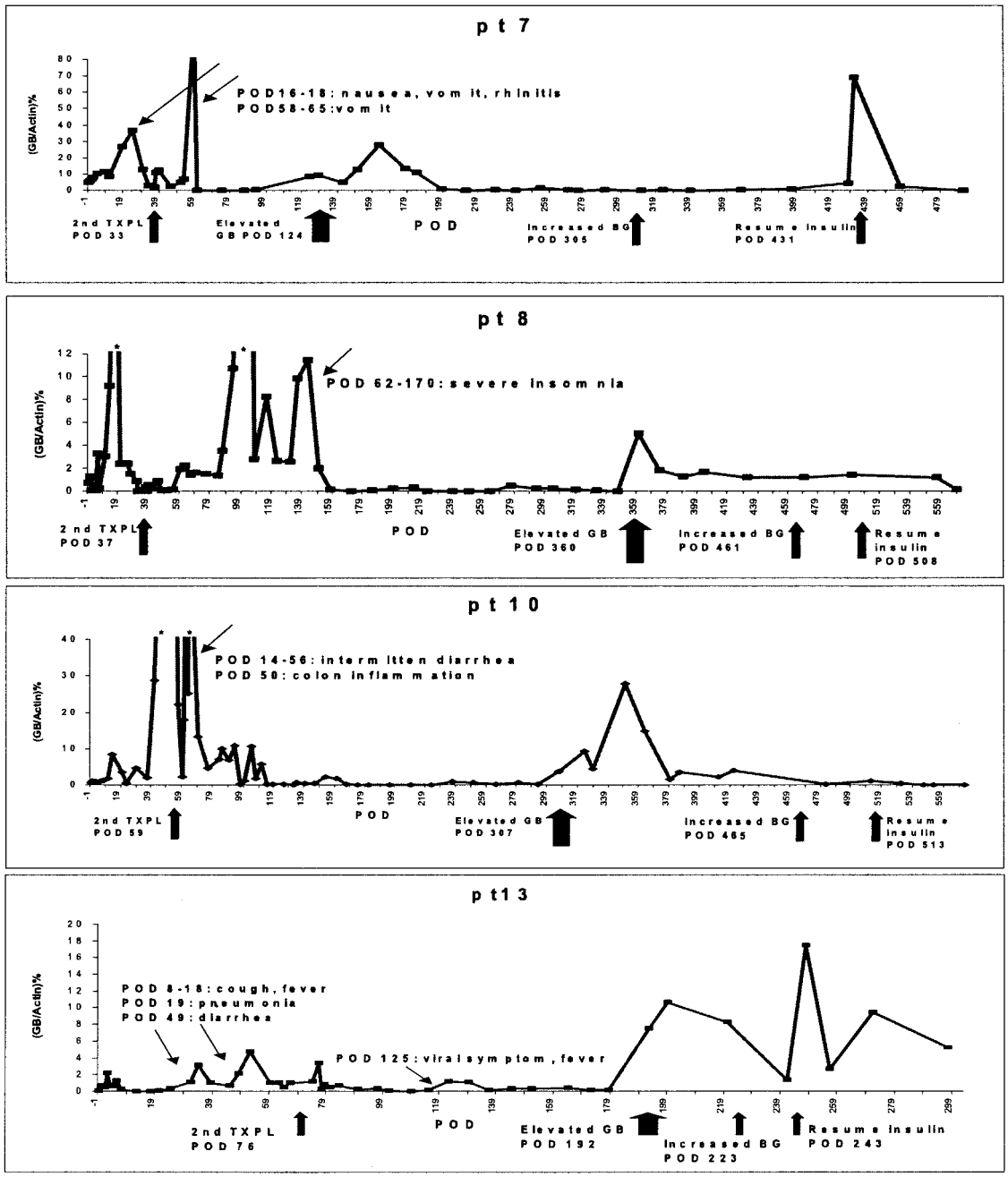

FIG. 3. GB gene expression profile in peripheral blood samples of islet allograft recipients who experienced partial islet allograft loss. The ratio of the copy number of the target gene to the copy number of β-actin was plotted against POD. *Out of range. Elevations associated with infection and insomnia are depicted on the graphs. BG, blood glucose; TXPL, transplant.

which may have contributed to elevation of CL gene mRNA from POD 44 to 109, 189 to 249, 495 to 557, and 572 to 611. Patient 5 also experienced several infections and concurrent elevations of CL gene expression, as detailed in Fig. 4. For patients 12 and 16, there were elevations of CL gene mRNA within the first 2 months after the first infusion, as we sometimes observed in other patients. The solitary peaks at POD 381 for patient 5 and at POD 358 for patient 12 did not correlate with any known events.

Figure 7 illustrates the GB gene expression profile and mean blood glucose levels of an islet allograft recipient who is still insulin independent but whose blood glucose level has clearly elevated over time. This patient had frequent elevation of blood glucose levels starting on POD 407, and the blood glucose levels were further elevated starting on POD 665. The CL gene mRNA levels were elevated starting on POD 370 and remained elevated until POD 460. We suspect that this patient might have chronic islet rejection. CL gene expression was also clearly shown to elevate before frequent elevation of blood glucose in this case.

DISCUSSION

Hyperglycemia and increased insulin requirements are currently used as indicators of islet rejection; however, once recipients are hyperglycemic and need exogenous

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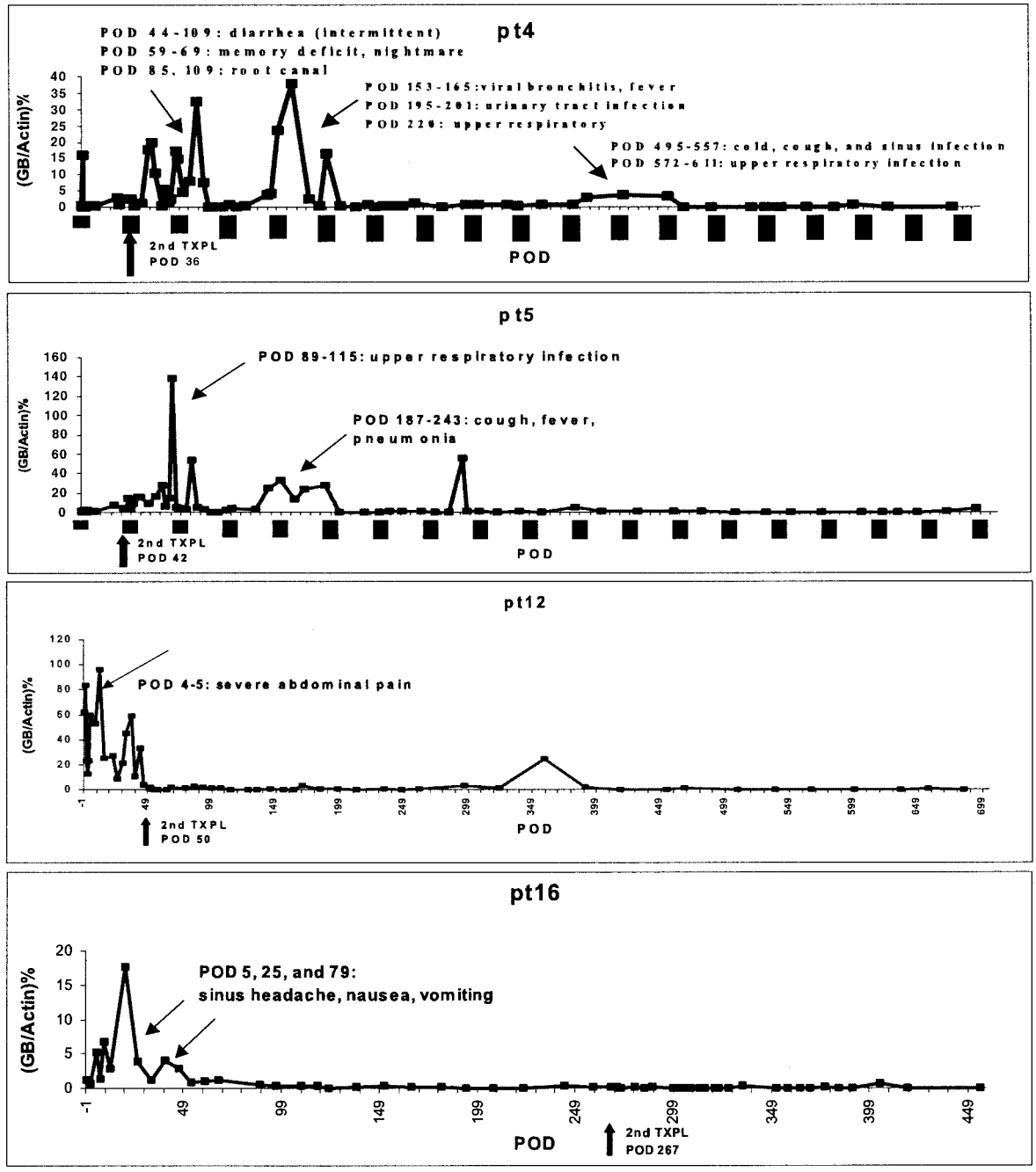

FIG. 4. GB gene expression profile in peripheral blood samples of islet allograft recipients who still have stable graft function. The ratio of the copy number of the target gene to the copy number of β-actin was plotted against POD. Elevations associated with infection are depicted on the graphs. TXPL, transplant.

insulin, significant islet mass has been lost. Early detection of ongoing islet allograft rejection is essential for preservation of islet mass. Our study of anti-CD154–treated nonhuman primates indicated that antirejection therapy initiated within 3 days of elevated postprandial glucose enabled reversal of rejection and rescue of partial islet mass (4,5); however, it would be difficult to define the level of postprandial glucose at which antirejection therapy should be undertaken in patients with various degrees of graft function. Our current study demonstrates that elevated CL gene expression in the peripheral blood of clinical islet allograft recipients precedes frequent hyperglycemia by 25–203 days. Levels remained elevated for ~2 months, with one patient experiencing 196 days of elevated CL gene expression. This suggests that elevated expression of CL gene mRNA in peripheral blood might be used as an indicator of early islet allograft loss, thereby facilitating efforts to preserve islet mass.

We have previously observed that hyperglycemia and elevated CL gene expression can go hand in hand (8). This may reflect the observed effects of hyperglycemia itself on serum cytokine levels (18) and the subsequent potential for upregulation of cytotoxic effector molecules. The correlation between elevation of CL gene expression in

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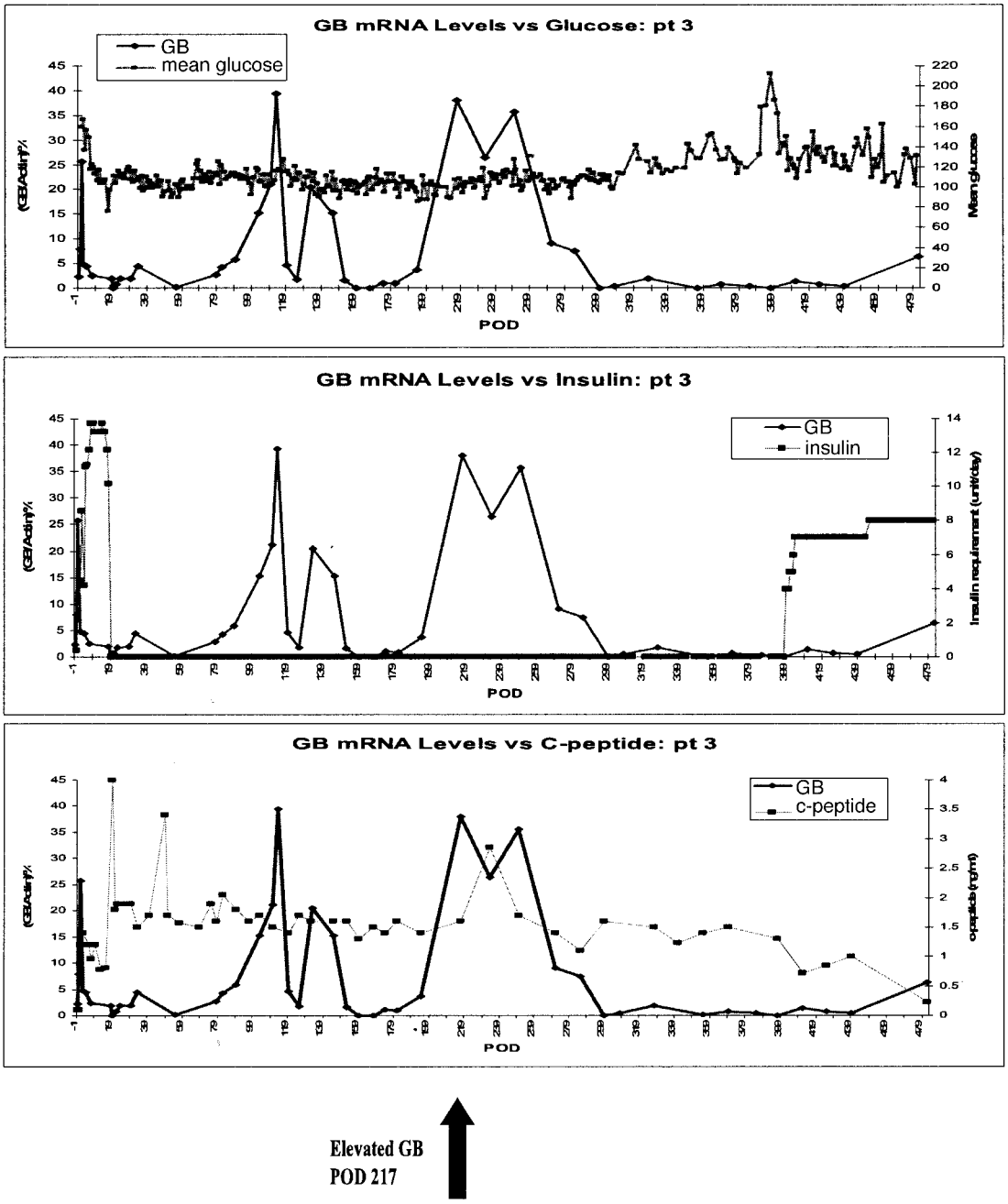

FIG. 5. GB gene expression in peripheral blood samples versus mean glucose (mean of pre- and postprandial blood glucose for breakfast, lunch, and dinner and bedtime blood glucose), daily insulin requirement, and C-peptide level in an islet allograft recipient. Patient had second islet transplant on POD 19. From POD 78 to 145, the patient experienced otitis media and painful throat, ear, and mouth ulcers.

peripheral blood and occurrence of hyperglycemia, as we observed in this study, may also be taken as a confirmation of rejection, as has been shown for kidney transplantation (12–14).

Elevation of CL gene expression during the first 2 months after islet infusion suggests that many inflammatory events are occurring in the early posttransplantation period that are not repressed by current immune suppression strategies. It is also possible, as has been suggested by one group, that such early elevations—not observed in renal allograft recipients who receive steroids but seen in those on steroid-free immune suppression—may be indicative of immune reactions that are essential for beneficial adaptation to the graft (19). Current techniques for monitoring of immunity in the clinical setting do not allow us to distinguish this possibility.

CL gene data on eight patients with partial islet allograft loss showed that GB is the most consistent indicator of early islet allograft loss, whereas elevation of perforin and FasL can be used to confirm further this indication. Activation of T-cells, triggered by antigen and subsequent infiltration of activated $CD4^+$ and $CD8^+$ T-cells, macrophages, and natural killer cells into the graft, is the major event in acute allograft rejection (12). GB and perforin are proteins that are present in the granules of cytotoxic T-cells and in natural killer cells (20–22), whereas FasL is

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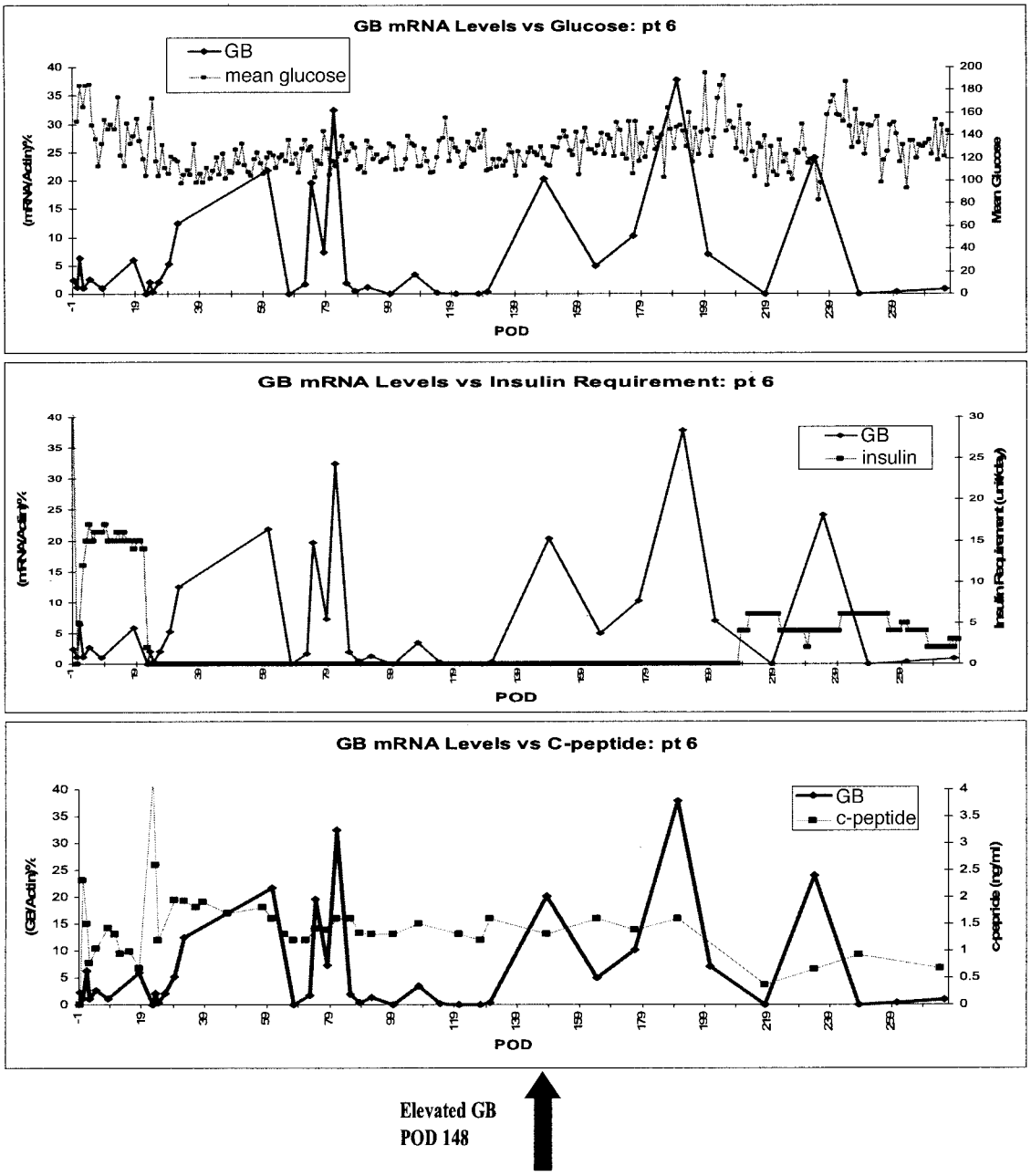

FIG. 6. GB gene expression in peripheral blood samples versus mean glucose (mean of pre- and postprandial blood glucose for breakfast, lunch, and dinner and bedtime blood glucose), daily insulin requirement, and C-peptide level in an islet allograft recipient. Patient had second islet transplant on POD 22. This patient had urinary tract infection on POD 42.

a transmembrane protein and is expressed on cytotoxic T-cells (23). Peripheral blood GB mRNA levels in patients with long-term type 1 diabetes were found by us to be no different from those of normal control subjects, whereas the expression of perforin and FasL in patients was significantly lower than controls (D.H., J.L., R.A., W. Bolton, C.R., N.S.K., unpublished observation). GB may be most sensitive and informative because it is not depressed in type 1 patients as compared with healthy control subjects, whereas perforin and FasL are. GB has been reported to mediate extracellular biologic effects independent of perforin (24) and to enter the target cell independent of perforin via high-affinity binding sites (25,26). GB and perforin are differentially expressed during primary activation of native $CD8^+$ T-cells, kinetically and at the single-cell level (27). The genes encoding perforin and GB may be differentially regulated in activated $CD8^+$ T-cells, perhaps reflecting previously unrecognized specialization of cytotoxic T-cell function (28). These differences in GB expression among naive and activated CD8 T-cells may contribute to differential elevation of gene expression among GB, perforin and FasL in our patients.

There was no threshold level of expression that correlated with increased risk of developing hyperglycemia and insulin dependence in the transplanted patients. Rather, increases from a baseline of minimal to undetectable gene expression were observed and were variable in degree for each patient.

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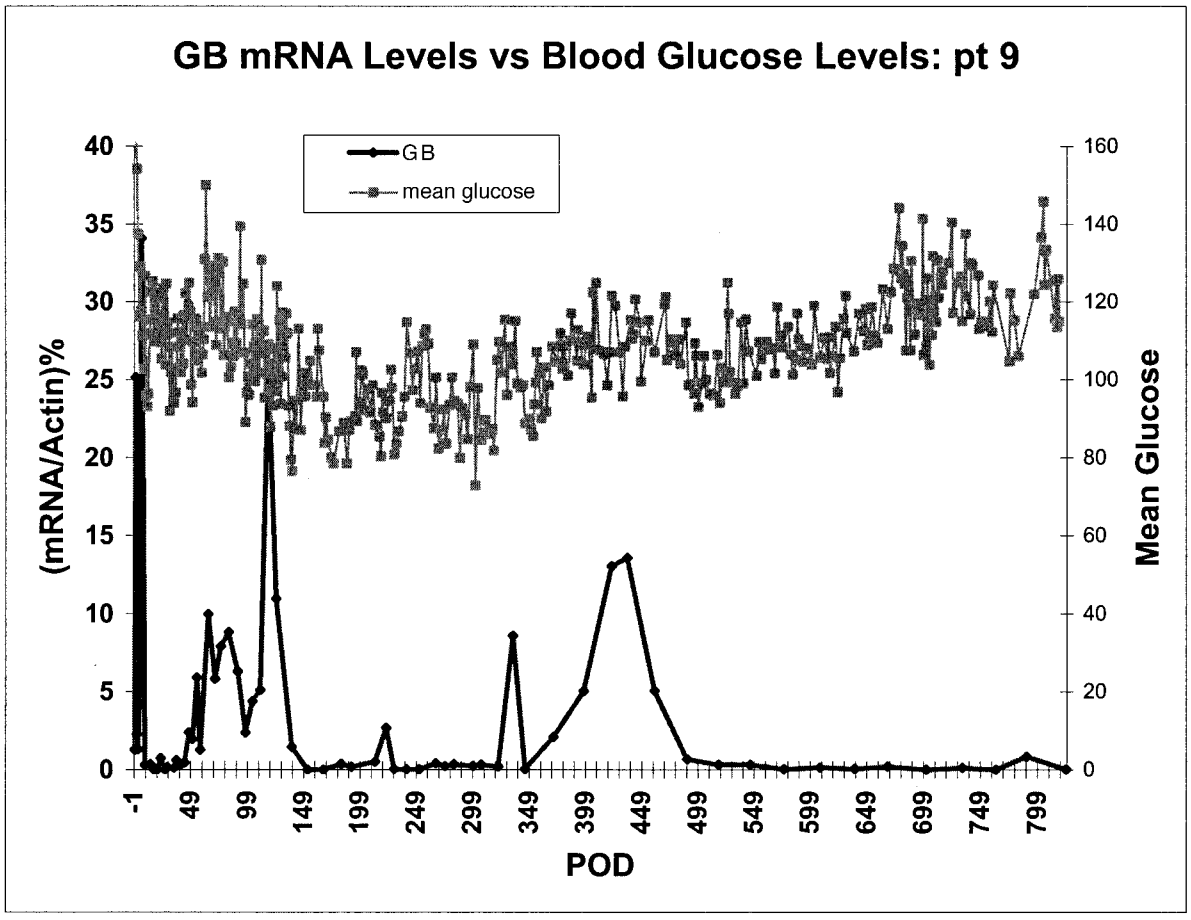

FIG. 7. GB gene expression profile in peripheral blood samples versus mean blood glucose level (mean of pre- and postprandial blood glucose for breakfast, lunch, and dinner and bedtime blood glucose) of an islet allograft recipient who might have chronic islet rejection. This patient had fever and was admitted to the hospital on POD 61. Patient had Neupogen for neutropenia on POD 62, 70, and 77. Patient had nodules in lymph in posterior head on POD 97 and skin infection from POD 105 to 188.

MLR results reflect the level of immune responsiveness of recipient T-cells to donor antigen. In our study, patients who were treated with steroid-free immunosuppression routinely became nonreactive to donor cells in MLR. Reactivity to third party was variable and difficult to interpret because of the degree of sharing of HLA antigens between multiple donors, the recipient, and third-party cells. The prediction of early graft loss by elevation of CL gene expression was further confirmed by observation of sensitization in MLR. MLR was tested relatively infrequently, and the temporal relationship between elevated CL gene expression and increased MLR could not be defined clearly; however, it seemed that sensitization in MLR was not predictive but rather correlated with loss of graft function.

Our results suggest that evidence of immune activation in the form of elevated CL gene and return of antidonor MLR seems to be linked to inadequate immune suppression. Utilization of these assays may enable identification of patients who require higher levels of maintenance of immune suppression to prevent rejection. Alternatively, the data suggest that the tapering period needs to be extended, as CL gene elevation occurred during this time frame in six of eight patients.

We have obtained antibodies to assess intracellular expression of perforin and GB by flow cytometry and are developing multiparameter assays that will enable us to assess the expression of CL gene proteins in different cell populations (e.g., natural killer cells versus $CD8^+$ CLs). Our preliminary findings support elevation of these proteins specifically in natural killer and $CD8^+$ cell subpopulations. We plan to analyze patients in ongoing studies with both PCR- and flow-based techniques to determine whether both protein expression (flow) and mRNA expression are effective indicators of rejection and islet loss. Ultimately, it would be ideal to assess the functional status of CL gene–positive recipient cells and to incorporate, e.g., by tetramer technology, to enable us to distinguish between donor-specific, autoimmune, and infectious immune responses.

In summary, analysis of CL gene expression in peripheral blood, especially GB, may allow for prediction of islet allograft loss before the onset of clinical symptoms. To be useful in the clinical setting, it will be necessary to define and exclude carefully other events, such as infection/inflammation and administration of medications and metabolic challenges, that may also result in elevated CL gene expression.

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