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NONINVASIVE DIAGNOSIS OF ACUTE REJECTION OF SOLID ORGAN TRANSPLANTS

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1. ABSTRACT

Accurate diagnosis of acute rejection remains a formidable challenge in organ transplantation. The current gold standard diagnostic test for acute rejection is histological examination of the transplanted organ. However, biopsy procedures are invasive and complications occur. Furthermore, sampling errors may bias the histological diagnosis. Not uncommonly, empiric anti-rejection therapy has to be provided prior to the availability of a confirmatory histological report. Thus, there is an urgent need for specific and sensitive noninvasive biomarkers of acute rejection. Herein, we review noninvasive strategies for the diagnosis of acute rejection of solid organ transplants.

2. INTRODUCTION

Solid organ transplantation has greatly improved since Gibson and Medawar's seminal skin grafting experiments during World War II (1). With improved understanding of the alloimmune response and the clinical application of new immunosuppressive agents (2), transplantation is now the treatment of choice for most end-stage organ diseases, restoring vital functions and prolonging human lives. One reflection of the significant progress is that in 1998, about 57 patients received a solid organ transplant each day in the United States alone (3).

Despite the advancements in therapeutics and clinical management, acute rejection remains a threat to limit the lifespan of transplanted organs. A major effort in transplantation research is induction of tolerance (4). Tolerance will ensure transplantation without acute rejection. In addition, tolerance will obviate the need for potentially toxic immunosuppressive medications. At the present time, we lack sensitive and specific clinical tests or surrogate biomarkers to replace invasive tissue biopsies to detect acute rejection in allografts. Development of a noninvasive test or assay for rejection will enhance the field of tolerance research as well since it will verify and define tolerance *in vivo* and provide a way of monitoring intragraft immune events (5). As a result of knowing what is going on *in vivo* immunologically, transplant physicians may be able to reduce immunosuppressive medications responsible for numerous side-effects such as malignancies and infections in the transplant recipients (6).

Advances in immunology and molecular biology have led to a better understanding of the pathophysiology of acute rejection (7). These gains in knowledge are being translated and mechanism-based noninvasive tests for acute rejection are being developed and explored in the clinic. Success in the intragraft detection of mRNA encoding cytotoxic T cell attack proteins during acute rejection of renal allografts (8,9) has paved the way for a search for T-cell activation markers in the blood and urine of renal allograft recipients (10). Diagnostic techniques have thus evolved from descriptive cytological analysis or diagnostic imaging studies to molecular and mechanistic studies in renal transplantation. Mechanism based noninvasive tests are also being investigated in other solid organ transplants. Herein, we review some of the significant developments in the area of mechanism based noninvasive tests for the diagnosis of acute rejection of solid organ allografts.

3. NONINVASIVE TESTS FOR RENAL TRANSPLANTATION

The widely used noninvasive method of measuring serum creatinine levels to diagnose acute rejection in renal transplantation lacks sensitivity and specificity. A rise in serum creatinine does not always represent true immunologic basis for graft dysfunction and might as well be due to non-immunologic causes such as infection and/or drug toxicity. Furthermore, subclinical acute rejection occurs without an increase in serum creatinine levels (11). Cytoanalysis of urine to diagnose

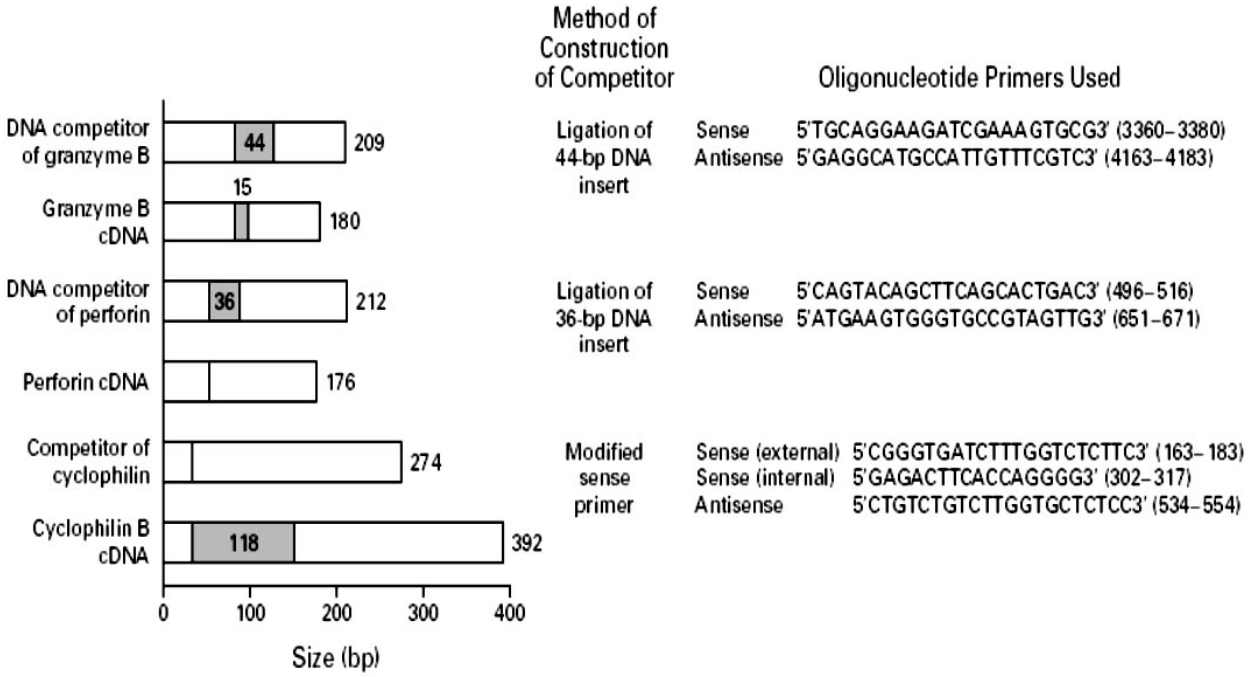

Figure 1. Design and Construction of Competitor DNA Constructs. Granzyme B competitor DNA construct (GB CT) and perforin competitor DNA CT were prepared by digestion of the 180 bp granzyme B wild-type PCR product with *Mse*I and by the digestion of 176 bp perforin wild type PCR product with *Nla*III, and ligation of the respective subfragments with a 44 bp (granzyme B) or a 36 bp (perforin) DNA insert with appropriate cohesive ends at the 5' and 3' ends. The 274 bp cyclophilin B competitor (Cyc B CT) was amplified using a modified sense primer that contains at its 5' end the external sense primer and at its 3' end, a 16 bp sub-fragment internal sense primer corresponding to sequences (302-317) within the wild-type PCR product. (reprinted with permission from New Engl. J. Med., reference 18).

acute rejection has been investigated (12) but may be a lagging indicator subsequent to immunologic damage. A molecular-based approach that incorporates principles of adaptive immunity has the potential to improve the diagnostic accuracy as well as provide mechanistic insights into the immunobiology of allograft rejection.

Immune-based noninvasive tests using urine is appealing to nephrologists since it provides a representative sampling of the entire kidney. Cytokine levels in urine have been measured, at the protein level, with the use of ELISAs (13-15).

Cytotoxic T-cells damage target cells by exuding perforin and granzyme B molecules (16,17). We measured mRNA for cytotoxic proteins, perforin and granzyme B, in the urine of renal allograft recipients with the use of quantitative competitive RT-PCR assay (18). We isolated RNA from urinary cells and reversed transcribed total cellular RNA to cDNA. Modified transcripts of perforin and granzyme B (the competitors) were then co-amplified with the target cDNA using perforin and granzyme B primers (figure 1) in a quantitative competitive polymerase chain reaction assay. Since the amount of competitors were known, the mRNA transcripts of perforin and granzyme B could be quantified and compared in renal transplant recipients with or without acute rejection. A significantly higher levels of perforin and granzyme B mRNA transcripts were amplified in the urine specimens obtained at the time of an episode of acute rejection compared to levels in urine specimens obtained from patients without acute rejection (figure 2). For detecting acute rejection, the sensitivity and specificity of the test using perforin mRNA level were 83 percent whereas for granzyme B mRNA level, they were 79 percent and 64 percent, respectively. Receiver-operating-characteristic (ROC) curve analysis showed that the calculated area under the curve (AUC) was 0.86 for both perforin and granzyme B (figure 3).

We also monitored renal transplant recipients in a longitudinal fashion by collecting serial urine specimens in the first 10 days of transplantation. Our longitudinal studies showed that patients who developed acute rejection had significantly higher levels of mRNA for perforin and granzyme B compared to those who did not manifest an early episode of acute rejection (figure 4). We are now conducting longitudinal studies to investigate the predictive value of serial monitoring in the diagnosis of subclinical rejection of renal allografts.

Severe viral infections such as BK or adenovirus nephritides may increase cytotoxic T-cell effector mRNA levels and affect the diagnosis of acute rejection (data not shown). Additional urinary tests such as urinary electron microscopy or measurement of viral protein mRNA levels in urinary cells (19) may help in distinguishing rejection

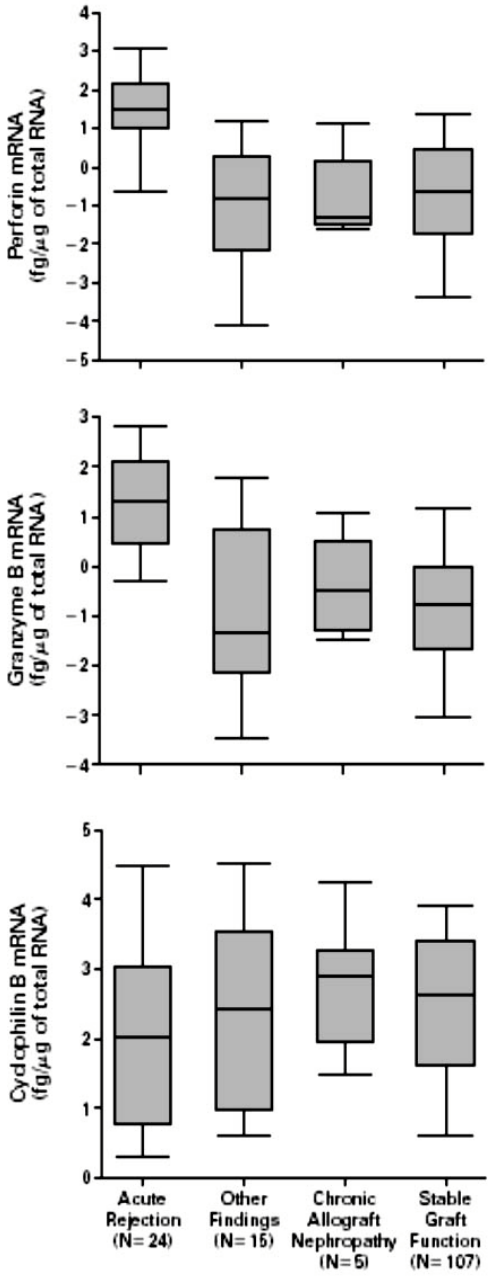

Figure 2. mRNA Levels in Urinary Cells. Box and whisker plots show the 10th, 25th, 50th (median), 75th, 90th percentile mRNA values for perforin mRNA (panel A), granzyme B mRNA (panel B), and cyclophilin B mRNA (panel C) in urine samples from patients classified as having acute rejection; acute tubular necrosis, toxic tubulopathy or non-specific changes (Other); chronic allograft nephropathy (CAN); or the stable post-transplant (Stable) group. The levels of perforin and granzyme B mRNA, but not those of cyclophilin B, were higher in the acute rejection group compared with all other diagnostic categories (P=0.001, one-way mixed-level analysis of variance) (N=number of urine samples quantified for mRNA levels, reprinted with permission from New Engl. J. Med., reference 18).

from infection. To investigate the possibility of contamination from urinary tract infections (UTI), we analyzed granzyme B mRNA levels in urinary cells of patients with UTI (n=15), acute rejection but without UTI (n=29), and without acute rejection or UTI (n=14) (20). We showed that as compared to acute rejection, UTI did not increase granzyme B mRNA levels.

Flow cytometry has been successfully utilized for the cellular profiling of acute rejection. Roberti *et al.* evaluated, with the use of flow cytometry, the urine of renal transplant recipients with acute rejection (21). CD3 and HLA-DR positive cells were significantly higher in the urine specimens obtained from patients with acute rejection compared to those without acute rejection. Viable cells are required for flow cytometry analysis and cell death in urine sediments may affect the sensitivity and specificity of the a flow cytometry based assay.

A novel approach to analyze urine from renal transplant patients with the use of NMR spectroscopy was initially explored by Foxall *et al.* (22) and later refined by Rush *et al.* (23). NMR spectroscopy provides unique biochemical analysis of biological fluids allowing patterns to be recognized for specific clinical conditions such as acute rejection.

Numerous blood-based noninvasive tests for renal rejection have been investigated using T-cell activation markers as the parameter (24-29). Although the process of phlebotomy is more "invasive" than testing urine, they are of high importance in anuric patients with delayed graft function.

The use of flow cytometry to analyze early T-cell activation marker CD69 expression on peripheral blood mononuclear cells (PBMCs) has been explored to diagnose acute rejection in renal transplantation with mixed results. Posselt *et al.* reported elevated CD69 expression on CD3+ and CD8+ subsets of PBMCs in 9 patients with acute rejection (25). In contrast, Karpinski *et al.* did not show any significant elevation in CD69 expression on CD4+ or CD8+ subsets of PBMCs in 13 patients with acute rejection (26).

Vasconcellos *et al.* have used quantitative competitive RT-PCR assay to measure levels of mRNA for cytotoxic effector molecule expression by PBMCs obtained from renal transplant recipients (27). A minimum of 2 to 3 milliliters of blood were required from each patient. In the study, the principle cytotoxic genes analyzed were perforin, granzyme B, and Fas ligand. For detecting acute rejection, perforin had a sensitivity of 82% and specificity of 85%, granzyme B had a sensitivity of 55% and specificity of 85%, and Fas ligand had a sensitivity of 100% and specificity of 75%. Analyzing the combination of genes (perforin, granzyme B, and Fas ligand) of which at least two were positive for acute rejection greatly improved the sensitivity to 100% and specificity to 95%.

An improvement in PCR technology (real-time PCR) enabling PCR amplification to be performed without

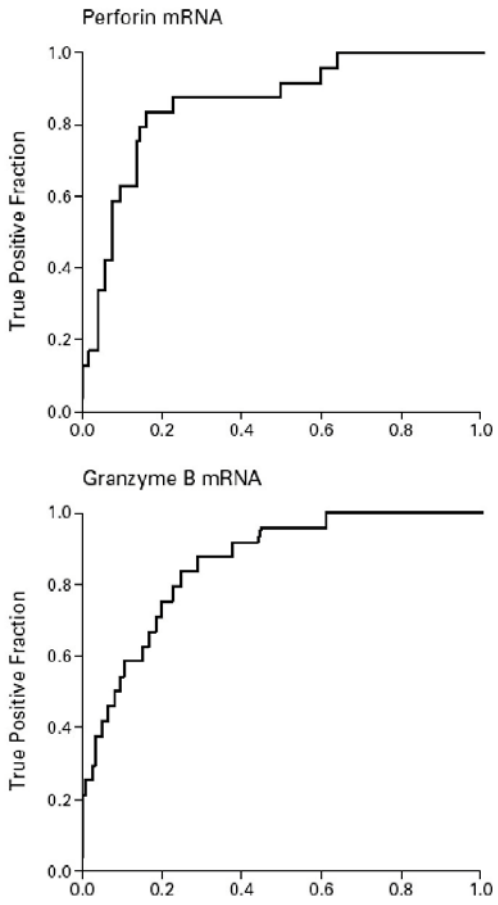

Figure 3. Receiver Operator Characteristic Curve Analysis of mRNA Levels. True positive fraction (sensitivity) and false positive fraction (1-specificity) for perforin mRNA levels (panel A), granzyme B mRNA levels (panel B), and cyclophilin B mRNA levels (panel C) as markers of acute rejection are illustrated. The calculated area under the curve was 0.86 for perforin mRNA levels, 0.86 for granzyme B mRNA levels, and 0.58 for cyclophilin B mRNA levels (0.5 = chance performance and 1.0 = perfect performance, reprinted with permission from New Engl. J. Med., reference 18).

post PCR gel analysis speeds up the process of transcript identification. Sabek *et al.* adopted real-time PCR for the measurement of mRNAs for perforin, granzyme B, and HLA-DR in PBMCs of renal transplant recipients (29). Biopsy proven acute rejection occurred in 8 patients. The sensitivity and specificity of detecting acute rejection were 100% and 74% respectively using any of the three genes (perforin, granzyme B, and HLA-DR). A specificity of 100% was achieved using all three genes as an indicator for acute rejection.

Heeger *et al.* developed an enzyme-linked immunosorbent spot assay (ELISPOT) that can detect alloreactive cytokine producing memory T-cells at single cell level (30). The cytokine ELISPOT assay requires donor PBMCs or spleen cells and permits assessment of the direct recognition pathway for activation as well as the indirect pathway. In this assay, primed memory T-cells vigorously respond to donor antigens and produce cytokines as compared to naïve T-cells. Further refinement of the cytokine ELISPOT assay (31) showed that it is highly sensitive, able to detect a single IFN-γ producing memory T-cell from a pool of 300,000 non-primed PBMCs. To reduce the "noise" from cytokines produced by donor cells, T-cell depleted donor PBMCs or spleen cells are used in the cytokine ELISPOT recall assay. It may be suitable for post-transplant immune monitoring of recipients since the stimulator donor cells can be stored frozen and be thawed without affecting their ability to activate memory T-cells.

Najafian *et al.* have explored the indirect pathway's contribution to allograft dysfunction by using synthetic HLA derived peptides in the ELISPOT assay to stimulate PBMCs of renal transplant recipients (32). In a group of 15 patients (13 with one DR mismatch and 2 with two DR mismatches) who had biopsy proven acute rejection, 11 patients (73%) had positive IFN-γ ELISPOT test against synthetic HLA derived peptides. In contrast, out of 13 patients who were clinically stable, only 3 patients (23%) had a positive IFN-γ ELISPOT test.

4. NONINVASIVE TESTS FOR PANCREAS TRANSPLANTATION

Pancreas transplantation with simultaneous kidney transplantation or following kidney transplantation is an ideal therapy for type 1 diabetic patients with end-stage renal disease (33,35). Since its initial inception in 1966 (34), more than 15,000 pancreas transplants have been performed world-wide. The current one year pancreas survival for simultaneous pancreas-kidney (SPK), pancreas after kidney (PAK), and pancreas transplant alone (PTA) are 84%, 72%, and 71%, respectively (35). The major challenge, especially after PAK and PTA transplants, is the accurate diagnosis of rejection of pancreas allograft prior to the development of hyperglycemia. A substantial number of islet cells have to be damaged for hyperglycemia to manifest. In SPK transplantation, the kidney allograft may act as a "sentinel" for immunologic events occurring in the pancreas allograft. However, rejection may occur independently of the kidney allograft.

The current gold standard test for diagnosing rejection is percutaneous biopsy of the pancreas allograft via ultrasound guidance. Noninvasive serum tests such as amylase, lipase, anodal trypsinogen, and other biochemical markers lack sensitivity and specificity for detecting acute rejection (36). Urinary amylase levels may be helpful in detecting rejection in bladder-drained pancreas transplants (37). However, due to the high morbidity of draining the exocrine secretions via the bladder, more pancreas transplants are performed via enteric drainage (38).

Radio *et al.* investigated SPK recipients with exocrine drainage via the bladder to evaluate the utility of noninvasive cytoanalysis for evaluation of allograft rejection. However, the authors cited several shortcomings

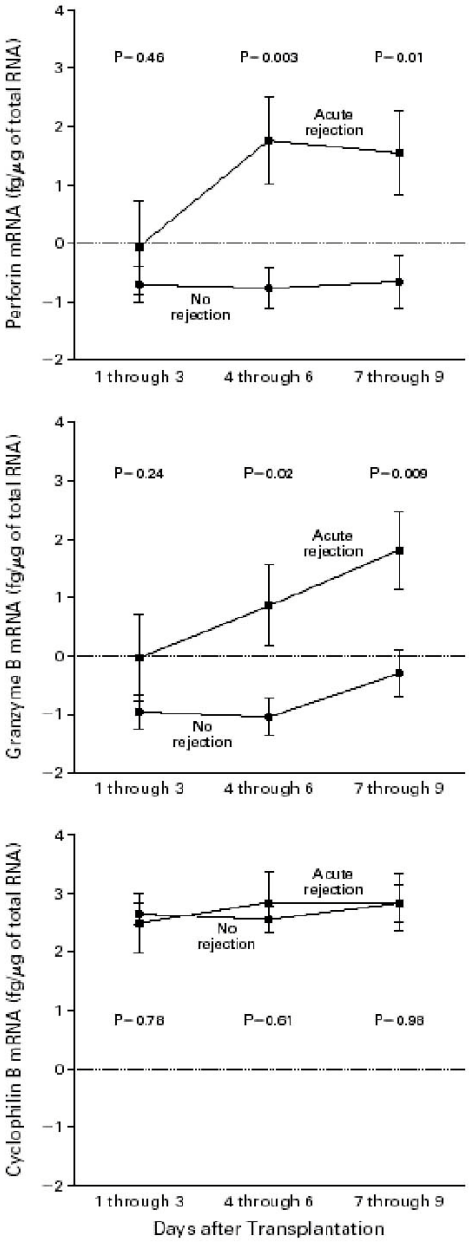

Figure 4. mRNA Levels in Sequential Urine Samples. Perforin (panel A), granzyme B (panel B) or cyclophilin B (panel C) mRNAs were measured in urine samples obtained in the first 10 days after transplantation. The levels of perforin or granzyme B mRNA but not those of cyclophilin B were higher in the 8 patients who developed acute rejection within the first 10 days after transplantation (OC O, number of samples from post-transplantation days 1,2 or 3 = 6; days 4, 5, or 6 = 5; and days 7, 8, or 9 = 6) as compared with 29 patients who did not develop acute rejection within the first 10 days after transplantation (MC M, number of samples from post-transplantation days 1,2 or 3 = 43; days 4, 5, or 6 = 26; and days 7,8, or 9 = 14). Means, standard errors, and P values were estimated using a mixed-level two-way analysis of variance. (reprinted with permission from New Engl. J. Med., reference 18).

such as low sensitivity, possible bacterial contamination, and laborious turnaround time for the test (39).

A noninvasive test that would predict acute rejection of pancreas allograft prior to the development of hyperglycemia is urgently needed and when developed would be of significant help in the management of not only pancreas graft recipients but also islet graft recipients.

5. NONINVASIVE TESTS FOR CARDIAC TRANSPLANTATION

Cardiac transplantation was first pioneered by Barnard in 1967 (40). It is the third most common solid organ transplanted in the United States after kidney and liver transplants (3). Although HLA matching is not routinely performed in cardiac transplantation, it may be beneficial for long term allograft survival (41). Acute rejection has a negative effect on short-term patient survival (42, 43). Persistent rejection may lead to cardiac allograft vasculopathy which is the main cause of mortality in cardiac transplant recipients (44).

The current gold standard test for detecting acute rejection is performance of an endomyocardial biopsy and histological evaluation. However, complications and risks associated with the endomyocardial biopsies have prompted the development of noninvasive methods. Mehra *et al.* recently examined this topic in detail (45). We will highlight the immune-based tests in our review.

Flow cytometry analysis has been used to study early T-cell activation marker CD69 expression on PBMCs of cardiac transplant recipients. Schowengerdt *et al.* (46) investigated 22 pediatric patients (mean age of 15.9 years). An average of 1 to 3 milliliters of blood were collected during cardiac catheterization and endomyocardial biopsy. Flow cytometry analysis for CD69 expression by CD4+ and CD8+ T-cells indicated a higher percentage of expression in patients with histologic evidence of rejection compared to those without acute rejection. Serial data from 3 patients suggested an upward trend in the level of expression of CD69 prior to rejection and a downward trend following resolution of rejection with anti-rejection therapy. Creemers *et al.* tested 62 cardiac transplant recipients and found heightened expression of CD69 on CD8+ subset of PBMCs in patients with moderate to severe rejection (47).

Several investigators have evaluated the role of imaging radiolabeled annexin-V in diagnosing acute rejection (48,49). Annexin-V is a human protein that binds phosphatidylserines which become shifted to the outer lipid bilayer in cells undergoing apoptosis. Radiolabeling annexin-V allows noninvasive imaging to pick up cells undergoing apoptosis *in vivo* during cardiac allograft rejection.

Morgun *et al.* have correlated intragraft expression of mRNAs for cytokines involved in T-cell activation (T-cell immune response cDNA 7, TIRC7) with their expression in PBMCs (50). mRNA levels of IL-8,

TNF-α, IFN-γ, granzyme B, and perforin were also measured in this study of 9 subjects. Interestingly, during an episode of endomyocardial biopsy confirmed acute rejection , mRNA levels of TNF-α and IL-8 were elevated whereas mRNA levels of TIRC7, IFN-γ, granzyme B, and perforin were reduced in PBMCs. Intragraft expression of mRNA for TIRC7 was elevated in patients with acute rejection.

6. NONINVASIVE TESTS FOR LIVER TRANSPLANTATION

Liver transplantation was pioneered by Starzl in 1963 (51). Starzl *et al.* have also discovered the role of microchimerism in inducing tolerance in recipients of liver allografts (52). Although the liver allograft is considered to be less susceptible to acute rejection as compared to renal and cardiac allografts, acute rejection can occur and may lead to allograft failure (53).

Clinically, serum liver tests (bilirubin, alkaline phosphatase, and AST) may suggest the presence of acute rejection but are non-specific. The current gold standard test for diagnosing rejection is percutaneous biopsy of the liver allograft. However, the biopsy procedure may be complicated by bleeding in patients with coagulopathy.

Umeshita *et al.* measured IL-6 levels in recipients of liver allografts. Fifty-one patients were studied and bile specimens were collected from T-tube drainage daily after engraftment and until clamping of the T-tube. Sera were also collected from 22 of the 51 patients. ELISA was used to measure IL-6 levels. It was found that IL-6 levels were elevated in the bile of patients with allograft dysfunction due to rejection whereas decreased levels correlated with response to treatment with steroids or OKT3. Interestingly, serum IL-6 levels were not a correlate of acute rejection (54).

Pfeifer *et al.* measured plasma C3a and C4a levels in 63 liver transplant recipients following transplantation (55). Serial levels of C3a and C4a were available in 10 patients for correlation with clinical events such as rejection or viral infection. Elevated levels of C4a more than C3a were seen in 1 patient with well defined rejection episode. The authors suggested using complement degradation products as a monitoring tool for rejection, although viral infections (CMV, HCV) may affect the specificity of the test.

Lun *et al.* (56) measured soluble IL-2 receptor (sIL-2R) levels in the peripheral blood of liver transplant patients with the use of ELISA. Flow cytometry analysis was used in this study to detect cell surface expression of IL-2 receptor on CD4+ T-cells and CD8+ T cells. A total of 119 patients were monitored and 69 of the 119 patients developed acute rejection between days 5 and 11 after engraftment whereas the remaining 50 patients were stable during the postoperative follow-up period. On the day of rejection, using a cutoff point of 3850 IU/mL, the ROC curve for measuring sIL-2R had an AUC of 0.897 (sensitivity, 58% and specificity,96%) with the positive predictive value of 83% and negative predictive value of 85% respectively. Whereas both serum alkaline phosphatase and bilirubin had AUCs of 0.688 (cutoff point of 118 IU/L) and 0.627 (cutoff point of 4mg/dL) respectively with poor positive and negative predictive values. As expected, IL-2 receptor expression was significantly higher in patients who developed acute rejection compare to those without acute rejection.

Warle *et al.* (57) investigated the usefulness of serum and bile levels of ICAM-1, sIL-2R, sTNF-RII, IL-6, IL-8, and IL-10 to distinguish infection from acute rejection early after liver transplantation. The specimens were collected daily for a month after transplant from serum and bile (T-tube drainage) of 45 patients. sIL2-R and IL-10 were excluded from analysis due to basiliximab induction therapy. Among the parameters measured, serum sICAM-1 (mean 1,401±157 ng/mL) and biliary IL-8 (11,623±4,255 pg/mL) were significantly elevated at the beginning of acute rejection. The serum sICAM-1 level significantly correlated with biliary IL-8 level (ANOVA) and both cytokines were independently associated with acute rejection (multivariate Cox-regression). The authors concluded that measurement of IL-8 in the bile was not superior to the measurement of sICAM-1 in the serum for diagnosing acute rejection early after liver transplantation.

7. NONINVASIVE TESTS FOR LUNG TRANSPLANTATION

Lung transplantation was first performed in the late 1960s by Hardy (58). With enhancement in surgical technique and immunosuppressive medications, patient survival rate has improved but is still lagging behind that of liver, cardiac, and renal transplants (59). Chronic rejection manifests as bronchiolitis obliterans and accounts for the poor graft and patient survival rates in lung transplantation. Acute rejection has been implicated as a significant risk factor for the development of bronchiolitis obliterans (60).

The most accurate method for diagnosing acute rejection is by performing bronchoscopy and transbronchial lung biopsy and histological evaluation. Guilinger *et al.* have reported that only 66% of clinical diagnosis of rejection is validated by biopsy findings (61).

Noninvasive methods of diagnosing rejection have been tested on animal models using radiolabeled annexin-V (62). An immune-based noninvasive test on humans has yet to be developed.

8. PERSPECTIVE

Our review is focused on immune and mechanism based noninvasive tests for diagnosing acute rejection of solid organ transplants. It is reasonable to posit that an episode of acute rejection is actually a continuum with the clinical manifestation (that is, graft dysfunction) is accompanied as well as preceded by cellular events (that is, histological rejection) that in turn is preceded as well as predicted by molecular signatures. With technological refinements in resolving the genomics and proteomics of

allograft rejection/acceptance, we are well poised to further develop specific and sensitive noninvasive tests for acute rejection that provide mechanistic insights as well as help individualize therapy.

9. ACKNOWLEDGEMENTS

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