Enzyme-Linked Immunosorbent Spot Assay Analysis of Peripheral Blood Lymphocyte Reactivity to Donor HLA-DR Peptides: Potential Novel Assay for Prediction of Outcomes for Renal Transplant Recipients

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Abstract. Chronic allograft dysfunction, which is the most common cause of late allograft failure, is in part caused by an ongoing immune response orchestrated by T lymphocytes primed by the indirect pathway of allorecognition. The low frequencies of such T cells have made it difficult to study indirect alloreactivity by using currently available assays. The development of a sensitive, clinically useful method of measuring indirect alloreactivity among human renal transplant recipients was thus attempted. Furthermore, in a pilot immunologic study, the contribution of the indirect pathway was studied in two groups of renal transplant recipients, i.e., patients with no prior acute rejection episodes and stable renal function (“stable” patients) and patients with at least one previous episode of biopsy-proven acute rejection, who were thus at risk for the development of chronic rejection (“high-risk” patients). The frequencies of type 1 T helper (interferon-γ-producing) and type 2 T helper (interleukin-5- and -10-producing) peripheral blood lymphocytes reactive with a panel of synthetic peptides (corresponding to sequences from donor HLA-DR molecules) were determined for renal transplant recipients and normal control subjects by using an enzyme-linked immunosorbent spot assay (ELISPOT). Among recipients of DR-mismatched allografts, a cut-off value of 60 interferon-γ spots/10^6 cells significantly (P = 0.02) separated stable patients (creatinine concentration, 1.1 ± 0.3 mg/dl) from high-risk patients (creatinine concentration, 2.3 ± 1.7 mg/dl). This is the first demonstration that the enzyme-linked immunosorbent spot assay can be used to monitor indirect alloreactivity to donor HLA-DR peptides among renal transplant recipients. These data provide the rationale for the prospective study of indirect alloreactivity among transplant recipients, to allow predictions of which patients would be at risk for the development of chronic rejection and thus allow appropriate planning of future interventions.

Preventing late allograft loss attributable to chronic allograft dysfunction (CAD) remains one of the most pressing goals in transplant biology. Excluding patients who die with functioning grafts, the most common cause of late graft loss is CAD, and this is a universal occurrence affecting all solid organs (1). Although alloantigen-independent mechanisms contribute to CAD (2), recent clinical data emphasized the importance of ongoing immunologic damage as a major underlying mechanism for this process (3). The orchestrators of this immune response are alloreactive CD4^+ T cells. It is now well established that CD4^+ T cells can recognize alloantigens via two distinct but non-mutually exclusive pathways (4–6). In the direct pathway of allorecognition, T cells recognize intact allogeneic MHC molecules on the surface of donor-derived antigen-presenting cells (APC). Acute allograft rejection may be predominantly mediated via this pathway, because grafts contain a significant number of donor-derived passenger APC that express a high density of allo-MHC molecules. In the indirect pathway of allorecognition, T cells recognize processed alloantigens, including donor MHC antigens, presented on self-APC (7,8). Extensive small-animal studies indicated that indirect allorecognition is sufficient to produce skin allograft rejection (9–11). In vascularized grafts, priming by the indirect pathway promotes the development of chronic allograft rejection, with accelerated vasculopathy, in small-animal (12) and preclinical large-animal (13) models. In human studies, indirect allorecognition of donor HLA antigens has been demonstrated in renal (14), cardiac (15,16), and lung (17) allograft recipients with chronic rejection. Interestingly, in one study of cardiac transplant recipients, chronic rejection occurred despite direct-pathway hyporeactivity, suggesting that the indirect pathway was predominant (18). Another study
demonstrated T cell reactivity to donor HLA-DR peptides among patients with recurring episodes of acute cardiac allograft rejection (19). Most of those studies demonstrated peptide immunodominance, with a shift of T cell responses toward different allopeptides with time, a process termed epitope shifting or spreading (14,15,19). It is thought that this process may lead to continuous recruitment and activation of naive CD4+ T cells, which react to new allopeptides and orchestrate the immune effector mechanisms leading to CAD progression.

Given the potential role of indirect allorecognition in CAD, the measurement and characterization of indirect alloreactivity are of utmost importance, for two reasons, i.e., to predict which patients are at risk for the development of chronic rejection and to monitor responses to therapy after intervention. However, attempts to quantify this pathway have been complicated by the vagaries of limiting dilution analyses used to calculate precursor cell frequencies.

The aim of this study was to develop a sensitive reproducible assay to measure the frequency and phenotype of indirect-pathway alloreactive T cells against donor HLA-DR peptides, which would be easily applicable for monitoring the clinical progress of transplant recipients. Furthermore, in a pilot study we tested whether this assay could provide a means of identifying patients at risk of developing CAD.

**Materials and Methods**

**Peptides**

Peptides (20- to 25-mers) corresponding to the hypervariable regions of the β-chains of five different HLA-DR molecules (DR0101, DR1501, DR0301, DR0401, and DR0701), based on the most frequent HLA frequencies of donors in the New England Organ Bank (Table 1), were synthesized (Quality Controlled Biochemicals, Hopkinton, MA). These peptides were used to stimulate peripheral blood lymphocytes (PBL) obtained from renal transplant recipients and normal control subjects.

**Study Population**

This study was performed with the approval of the Institutional Review Board for Human Investigation at the Brigham and Women’s Hospital. Blood samples were obtained in heparinized tubes from renal transplant recipients at the Brigham and Women’s Hospital, during routine follow-up visits. HLA phenotypes were determined by using standard serotyping techniques. All renal transplant recipients had undergone transplantation at least 6 mo earlier and were receiving immunosuppressive therapy, consisting of a calcineurin inhibitor (cyclosporin A, prednisolone, and mycophenolate mofetil for seven stable and seven high-risk patients. In addition, one patient from each group received FK506 (with prednisolone or mycophenolate mofetil). One patient in the stable group (sensitized) and three in the high-risk group (of whom only one was sensitized) received azathioprine. Finally, three patients in the stable group and two in the high-risk group received double therapy with mycophenolate mofetil and prednisolone (or cyclosporine).

**Enzyme-Linked Immunosorbent Spot Assay**

PBL were isolated from peripheral blood by standard ficoll density-gradient centrifugation and were either used immediately or frozen for later use. Viable cells were enumerated by using an immunofluorescence microscope, in the presence of acridine orange/ethidium bromide. The enzyme-linked immunosorbent spot assay (ELISPOT) was previously described (21,22) and was adapted to measure interferon-γ (IFN-γ)-secreting cells. ELISpot plates (Cellular Technology, Cleveland, OH) were coated with capture antibodies against IL-5 (PharMingen, San Diego, CA), IL-10, or IFN-γ (Endogen, Woburn, MA), in phosphate-buffered saline (PBS), and were maintained overnight at 4°C. The plates were blocked for 1 h with PBS containing 1% bovine serum albumin and

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino Acid Position</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>0101</td>
<td>6 to 21</td>
<td>RFLWQLKFECFFNGT</td>
</tr>
<tr>
<td>22 to 41</td>
<td>ERVRLLCRIYNQESVRF</td>
<td></td>
</tr>
<tr>
<td>42 to 62</td>
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<tr>
<td>63 to 80</td>
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</tr>
<tr>
<td>81 to 94</td>
<td>HNYGVGVTFTVQRR</td>
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</tr>
<tr>
<td>1501</td>
<td>1 to 20</td>
<td>GDTRPRFLWQPKECHFF(NG)</td>
</tr>
<tr>
<td>21 to 40</td>
<td>TERRFLDRFYQNEVESVF</td>
<td></td>
</tr>
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</tr>
<tr>
<td>61 to 80</td>
<td>WNSQKDLQEQRAAVDTYCR</td>
<td></td>
</tr>
<tr>
<td>81 to 94</td>
<td>HNYGVGVTFTVQRR</td>
<td></td>
</tr>
<tr>
<td>0301</td>
<td>6 to 21</td>
<td>RFLYSTECHFFNGT</td>
</tr>
<tr>
<td>22 to 41</td>
<td>ERVRDLYRHQNEENRF</td>
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</tr>
<tr>
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<td>81 to 94</td>
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were then washed with PBS. A total of $5 \times 10^5$ PBL were added to each well, in 100 μl of complete RPMI 1640 medium [90% RPMI 1640 medium/10% human serum (Sigma Chemical Co., St. Louis, MO) with L-glutamine plus penicillin/streptomycin (BioWhittaker, Walkersville, MD) and 50 mM 2-mercaptoethanol (Sigma)]. Control wells contained responder PBL plus medium alone or an irrelevant peptide (rat MHC class II peptide), as described previously (12). Cells were tested against phytohemagglutinin (PHA) (1 μg/ml; Murex Diagnostics, Dartford, UK), mumps antigen (BioWhittaker), and a panel of HLA-DR peptides (each at 10 μg/ml) corresponding to the donor DR type. After 48 h, the plates were washed, biotinylated detection antibodies were added, and the plates were maintained at 4°C for an additional overnight incubation. After additional washing, horseradish peroxidase conjugate (Dako, Glostrup, Denmark) was added for 2 h at room temperature. Development was with aminoethylcarbazole (10 mg/ml in N,N-dimethylformamide; Pierce Chemicals, Rockford, IL), freshly prepared in 0.1 M sodium acetate buffer (pH 5.0) mixed with 30% H2O2 (200 μl/well). The resulting spots were counted with a computer-assisted ELISAspot image analyzer (Cellular Technology) (Figure 1). The results were then calculated as cytokine-producing cells per $10^6$ PBL.

Statistical Analyses

The Mann-Whitney U test was used to compare serum creatinine levels and mumps responses for the two groups of DR-mismatched transplant recipients. Fisher’s exact test was used to analyze the correlation between IFN-γ-producing cell frequencies and acute rejection.

Results

Normal Control Subjects

First, we used both fresh and frozen PBL from healthy non-transplant-treated control subjects ($n = 5$) to optimize the ELISOT. Cells were stimulated with PHA mitogen, mumps antigen, DR-mismatched irradiated donor cells, and a panel of HLA-DR peptides (Figure 2). Significant responses to mitogen (too numerous to count) and recall antigens (631 ± 480 spots/10^6 cells) were observed. However, although significant responses to allogeneic donor cells were noted (366 ± 68 spots/10^6 cells), mean IFN-γ frequencies in response to donor peptides were extremely low (4 ± 6.8 spots/10^6 cells). The maximal IFN-γ spot count in response to allopeptides was <40 spots/10^6 PBL among the control individuals. Neither IL-5- nor IL-10-producing cells were observed in response to the allopeptides (data not shown). Duplicate wells tested for a single antigen demonstrated <10% variability.

Transplant Recipients

The frequency of IFN-γ-producing cells after PHA incubation was too great to count (>3000 spots/well) in all assays, using both fresh and frozen PBL, indicating adequate viability of these cells. Incubation with medium alone or with irrelevant control peptides produced no detectable IFN-γ spots for any of the studied groups (<20 spots/10^6 cells).

For DR-matched allograft recipients, there was a response to mumps antigen but, as expected, no response to any of the three to five allopeptides of matched donor HLA-DR molecules (Figures 1 and 3). The maximal number of IFN-γ spots in response to any of the matched allopeptides never exceeded 40 spots/10^6 PBL for any of the patients tested.

Among DR-mismatched patients, the responses to mumps antigen were not significantly different in the stable (222 ± 333 spots/10^6 cells) and rejecting (305 ± 120 spots/10^6 cells, $P = 0.21$) groups. IFN-γ-producing cells in response to allopeptides were observed for both groups, with variable responses to individual mismatched peptides (individual patient responses are presented in Figure 4). Although some peptides generated strong responses, other peptides from different parts of the same mismatched DR molecule did not. Such peptide immunodominance is in keeping with previous reports of al-

Figure 1. Representative enzyme-linked immunosorbent spot assay (ELISOT) wells for a DR-matched allograft recipient (a to c) and a DR-mismatched allograft recipient (d to f). Interferon-γ (IFN-γ) production in response to medium alone (a and d), mumps antigen (b and e), or allopeptide (c and f) was measured. The number of IFN-γ spots in each well was determined by computer-assisted image analysis.

Figure 2. IFN-γ ELISOT frequencies for non-transplant-treated control subjects in response to a panel of HLA-DR peptides (subjects 1 to 4), mumps recall antigen (subjects 1 to 4), and irradiated DR-mismatched donor cells (subjects 1 to 3). The HLA-DR peptide frequency for each patient represents the mean response to all tested allopeptides. A fifth subject also demonstrated no response to allopeptides but was not tested with mumps antigen. Duplicate wells were tested whenever cells were available, with <10% variation between wells (see the Materials and Methods section).
matched patients, we used a cut-off value for the maximal basis of the results for healthy control subjects and DR-observed for the group of rejectors (Figure 4). Indeed, on the shown).

response to allopeptides in any of the groups tested (data not stable patients (Figure 3). IFN-γ ELISPOT frequencies for DR-matched allograft recipients (n = 9) in response to mumps antigen and allopeptides of matched donor HLA molecules. The response to HLA-DR peptides for each patient represents the mean response to all matched allopeptides.

lopetide reactivity among human subjects (14–17,19). However, the highest frequencies of IFN-γ-producing cells were observed for the group of rejectors (Figure 4). Indeed, on the basis of the results for healthy control subjects and DR-matched patients, we used a cut-off value for the maximal IFN-γ frequency of 60 spots/10^6 cells, and we observed that this value significantly differentiated the rejectors from the stable patients (P = 0.02).

We observed no detectable IL-5- or IL-10-producing cells in response to allopeptides in any of the groups tested (data not shown).

Discussion

To prevent the significant degree of graft loss attributable to CAD, transplant physicians must be able to identify patients at risk early during the posttransplant period and monitor them during the course of interventions, either with adjustment of immunosuppressive medications or with novel therapies. Methods of reproducibly quantifying the ongoing immune response to the allograft have thus far proven to be inadequate for clinical usage. The ELISPOT technique represents one of the most sensitive methods for the detection and quantification of antigen-specific responses of in vivo activated T cells (21,22). This method is based on the detection of cytokines secreted by a single cell within a polyclonal population. The level of detection is 1/10^6 cells, a frequency that is 10 to 100 times lower than the precursor frequency of T cells specific for a single peptide (14–16,18,20). Unlike in limiting dilution analyses, T cells are incubated with antigen for only a very short time; consequently, the frequency measured corresponds to the true number of T cells that have encountered a given antigen in vivo, and it does not rely on prolonged culture conditions. In addition, the ELISPOT technique allows precise measurement of the number of type 1 T helper (Th1) and Th2 cells responding to a specific antigen. This assay was previously used to predict renal allograft failure (21,22). Among renal transplant recipients, mitogen-induced PBL IFN-γ/IL-5 frequency ratios of >15, as measured by ELISPOT, were highly predictive of subsequent allograft failure (21). Furthermore, the pretransplant frequency of donor-specific memory cells was correlated with the risk of developing acute rejection after transplantation (22). There have been no reports establishing the use of the ELISPOT technique to monitor indirect alloreactivity to donor HLA peptides in human subjects.

Several human studies have demonstrated indirect alloreactivity to donor peptides among patients with established chronic rejection (14–17). Furthermore, clinical studies have confirmed acute rejection as one of the strongest predictors of subsequent chronic allograft loss among renal transplant recipients (23–28). Importantly, a recent analysis of the extensive United Network for Organ Sharing database demonstrated that there has been a noticeable improvement in long-term allograft survival rates among renal transplant recipients but this improvement has been confined to patients with no episodes of acute rejection (29). In this pilot study, we have demonstrated allosensitization to donor HLA-DR peptides among a cohort of patients with histories of acute rejection. These patients are at risk of developing CAD, and these findings thus represent an in vitro link between acute and chronic rejection that is readily quantifiable.

The assay we established represents a significant advance in our ability to monitor indirect alloimmune responses among transplant recipients. The ability to use both fresh and frozen recipient cells makes this assay very attractive for use in clinical practice. It is a sensitive assay that is capable of detecting subtle changes in the frequencies of responding cells, thus providing a novel tool to monitor the alloreactive T cell frequency with time and to study epitope spreading, which is a poorly understood phenomenon that has been correlated with CAD (14,15,19). This assay is more sensitive than a number of other techniques for measuring alloreactive T cell responses, such as standard thymidine incorporation assays to measure lymphocyte proliferation, enzyme-linked immunosorbent assays performed with supernatants from cultured lymphocytes to measure secreted cytokines, and intracellular cytokine staining. Measurement of gene transcript levels by reverse transcription-PCR is a sensitive method for determining the phenotypes and amounts of cytokines secreted by alloreactive lymphocytes, but this is not a useful assay for measuring the frequency of alloreactive T cells. Although limiting dilution analysis is an accurate method for measuring the frequency of alloreactive T cells, it is a cumbersome assay that involves prolonged in vitro culture systems, which may affect estimations of the true in vivo frequencies. However, ELISPOT results represent a snapshot of the T cell response. Some of the cytokine-producing cells may not proceed to proliferate or undergo activation-induced cell death. However, our data suggest that the assay may allow us to identify, at an early stage, patients for whom significant allosensitization has occurred, who are at risk of developing chronic rejection. Such patients may require a different approach in their immunosuppressive management (30).

Among our cohort of patients with acute rejection, significant allosensitization to peptides occurred with 11 of 15 DR mismatches (IFN-γ spots, >60/10^6 PBL). With only four of 15
Figure 4. IFN-γ ELISPOT frequencies for individual DR mismatches among “stable” allograft recipients (n = 13) (A) and “high-risk” allograft recipients (n = 15) (B). Each graph represents the reactivity of patient cells (frequency of IFN-γ-producing cells per 10^6 peripheral blood mononuclear cells on the y-axis) to individual peptides (on the x-axis) for each mismatched DR antigen. Between three and five peptides were tested for each DR mismatch. Significant responses seemed to be limited to one or two immunodominant peptides, which varied among patients for any given mismatch. In the stable group of patients, only three of 13 mismatches resulted in IFN-γ frequencies of >60/10^6 cells. In contrast, in the high-risk group, 11 of 15 mismatches resulted in IFN-γ frequencies of >60/10^6 cells. This cut-off value of 60 IFN-γ spots/10^6 cells significantly separated the two groups of patients (P = 0.02).
Figure 4B.
did acute rejection not lead to allosensitization to HLA-DR peptides (Figures 4B and 5B). Clinical studies have suggested that not all patients with acute rejection experience progression to chronic rejection (28). Several factors, including the number, severity, and timing of acute rejection episodes (24,25,28,31,32), have been demonstrated to alter the risk of development of chronic rejection. Other authors demonstrated that there was no increased incidence of chronic rejection if graft function returned to normal by 1 yr (33). It remains to be determined in formal prospective studies, with large numbers of patients, whether our novel assay can differentiate between patients with histories of acute rejection and those who are truly at risk of developing CAD.

Among our cohort of stable patients, only three of 13 mismatches resulted in >60 IFN-γ spots/10⁶ cells, representing allosensitization to the indirect pathway (Figures 4A and 5A). Although none of these patients experienced clinical rejection, we cannot rule out the possibility of subclinical rejection some time in the past, leading to allosensitization. However, allograft recipients with no history of acute rejection can experience progression to chronic rejection (27,34). Early protocol biopsies may reveal mild tubulitis in some of these patients, which may be predictive of the development of chronic rejection (35).

We observed no evidence of cytokine switching among rejectors or stable patients, on the basis of a classic Th1/Th2 paradigm, suggesting that such a switch does not contribute to long-term graft acceptance. Whether a cytokine switch does indeed occur during episodes of epitope switching/spreading remains to be determined. Furthermore, we have confirmed the clinical observations that, for some patients, conventional immunosuppressive therapy is inadequate to regulate the indirect pathway of allore cognition. However, inadequate immunosuppression is not the factor differentiating patients with and without antidonor alloreactivity, because reactivities to the recall mumps antigen were similar for stable patients and rejectors.

We realize that our pilot assay development and immunologic study describes preliminary results for a relatively small number of patients. In addition, the prevalence of chronic rejection was not specifically assessed in this study, although the high-risk patients exhibited worse renal transplant function, as indicated by serum creatinine levels. However, our novel data provide the rationale for proceeding with a large prospective study that is being planned as part of a newly funded National Institutes of Health program. The ability to monitor donor peptide alloreactivity among our transplant recipients may have tremendous effects on their immunosuppressive therapy. The presence of HLA peptide alloreactivity may predict allograft rejection and thus indicate the need to increase or modify immunosuppressive therapy. Alternatively, a lack of HLA peptide alloreactivity may enable us to identify patients who may be eligible for tapering of certain immunosuppressive drugs, with close monitoring of their alloreactivity with our assay. Given the cost of currently available drugs, their side effects and toxicities (36), and their possible contributions to CAD (27), the availability of such an assay could revolutionize the immunosuppressive treatment of our patients.

Acknowledgments

Dr. Najafian is the recipient of a National Kidney Foundation fellowship grant; Dr. Salama is the recipient of a traveling fellowship from the British Renal Association and the Peel Medical Research Trust. This work was supported by National Institutes of Health Grants U01-AI46135 and RO1-A133100. We thank Helen Mah and the staff of the Tissue Typing Laboratory, Brigham and Women’s Hospital, for performing the HLA serotyping and Dr. Nelson Goes for helping with the collection of clinical samples.

References


