Panel of Reactive T Cells as a Measurement of Primed Cellular Alloimmunity in Kidney Transplant Candidates

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Pretransplantation panel reactive antibody (PRA) testing assesses posttransplantation risk for antibody-mediated graft injury. It was postulated analogously that screening for effector/memory alloreactive T cells by “panel of reactive T cells” (PRT) using IFN-γ enzyme-linked immunosorbent spot assays would evaluate independently cellular alloimmunity in transplant candidates. Peripheral blood lymphocytes from 41 hemodialysis patients who were awaiting first renal transplants were tested against a panel of allogeneic stimulator cells. Positive assays were defined arbitrarily as >25 spots/300,000 peripheral blood lymphocytes, and positive PRT was defined as when the responder reacted to 40 or 75% (PRT-75) of the stimulators. Seventeen percent of patients were PRT-75+, whereas 32% were PRA+. Twelve percent of the cohort was PRT-75+/PRA−, and only 5% of the patients were PRA+/PRT-75+, indicating that T cell alloseactivity did not routinely imply B cell sensitization and vice versa. PRT-75+ patients were more likely to be younger (<55 yr) and black. In contrast, a positive PRA was significantly associated with female gender but not race or age. Pretransplantation screening of cellular alloimmunity by enzyme-linked immunosorbent spot-based PRT detects a subset of hemodialysis patients who differ from those that are PRA+. Preliminary correlations with posttransplantation outcome in seven recipients suggest that PRT screening has the potential to aid in risk assessment in renal transplant candidates.

Kidney transplant physicians use a number of pretransplantation tests to assess the risk for posttransplantation rejection and graft loss (1,2). One such approach, HLA matching of donor and recipient at the A, B, and DR loci, has had a marked influence on the outcome of renal transplants. Grafts from deceased donors that are matched at all six loci represent such a significant benefit to the recipient that the United Network for Organ Sharing has adopted a national organ-sharing policy to optimize the use of these organs (3).

In addition to HLA matching, renal transplant candidates all are routinely screened for alloantibodies that are present in their serum (panel reactive antibodies [PRA]) and for antidonor alloantibodies at the time of transplantation (final cross-match) (4). Newer flow cytometry-based techniques for detecting alloantibody have reinforced the findings that sensitized patients with strongly positive PRA and/or positive antidonor alloantibodies are at higher risk for worse posttransplantation outcomes.

The association between a positive cross-match and hyperacute rejection (5), as well as the readily available methods for measuring alloantibodies (reviewed in reference [4]), has led the transplant community to focus on pretransplantation assessment of humoral rather than cellular alloimmunity as a risk factor for posttransplantation outcome. Because T cells are known central mediators of graft injury (6–12), we hypothesized that enumerating the frequency and the breadth of reactivity of activated antidonor T cells in the recipient before transplantation would provide information regarding the patient’s alloimmune repertoire that is supplemental to alloantibody detection. We originally described an adaptation of the cytokine enzyme-linked immunosorbent spot (ELISpot) assay to measure activated/memory alloreactive T cells in humans (13), and we provided strong evidence that the frequency of these T cells pretransplantation was associated directly with worse posttransplantation outcome (14). Other groups have confirmed these findings, and pretransplantation assessments of antidonor T cell immunity are now beginning to influence clinical decision making in a manner analogous to final cross-match testing for alloantibodies (11,15–17).

As another approach to risk stratification for transplant candidates, we postulated that determining the frequency of peripheral T cells that are reactive to a panel of alloantigens would provide a measure of the strength and reactivity of the pre-existing alloreactive T cell repertoire analogous to how PRA testing functions as a measure of pretransplantation humoral allosensitization. If this hypothesis proves to be correct and if cellular alloseactivity measured by cytokine ELISPOT detects a different population of high-risk patients from those with alloantibodies, then assessing a candidate’s cellular response to a panel of allogeneic stimulators (panel of reactive T cells [PRT]) could be used as an adjunctive test for assessing
risk, choosing optimal donors, deciding on immunosuppression regimens, and ultimately improving outcome.

**Materials and Methods**

**Study Patients**

The study population consisted of 41 adult hemodialysis (HD) patients who were on the active waiting list for renal transplantation at The Cleveland Clinic Foundation or University Hospitals of Cleveland. Recipients of previous solid organ transplants including kidneys were excluded from the study. The cause of ESRD in this cohort was diabetes (n = 23), hypertension (n = 7), glomerular disease (n = 7), polycystic disease (n = 2), and other/unknown (n = 2). None of the recipients was taking immunosuppressive drugs for their primary renal disease during the period of the study.

Stimulator peripheral blood lymphocytes were obtained from six normal healthy (based on a health questionnaire) volunteers, and stimulator spleen cells were obtained from an additional two randomly chosen deceased donors. Three living donors and one deceased donor were self-described as black. Tissue typing for A, B, and DR alleles was performed by standard molecular techniques for all study patients. All study patients were enrolled under the approved guidelines of the Institutional Review Boards for Human Studies at The Cleveland Clinic Foundation and University Hospitals of Cleveland.

**Sample Preparation**

Peripheral blood samples were obtained in heparinized tubes from HD patients just before the initiation of a dialysis treatment. Aliquots of plasma were stored at −70°C and used for alloantibody testing (see below). Peripheral blood lymphocytes (PBL) were isolated by standard Ficoll density-gradient centrifugation, and live cells were counted using acridine orange/ethidium bromide staining and immunofluorescence microscopy. The isolated PBL were used as responders in the ELISPOT assays as cited below. Stimulator PBL from normal volunteers and stimulator spleen cells from deceased donors were isolated by Ficoll density-gradient centrifugation, and live cells were counted using acridine orange/ethidium bromide staining and immunofluorescence microscopy.

**ELISPOT Assays**

IFN-γ ELISPOT assays were performed as described previously in detail (12–14). A total of 300,000 responder PBL in 200 µl of medium (RPMI 1640 medium;Cambrex, Walkersville, MD) plus 10% human serum, with l-glutamine (Invitrogen, Grand Island, NY), penicillin/streptomycin (Cambrex), and 50 mM 2-mercaptoethanol (Sigma, St. Louis, MO) were placed in 96-well ELISPOT plates (Millipore, Bedford, MA) that were precoated with capture anti–IFN-γ antibody (BD Pharmingen, San Diego, CA). The cells were tested in duplicate or triplicate against medium alone (negative control), the panel of T cell–depleted stimulator cells, and phytohemagglutinin (positive control) at 1 µg/ml medium (Sigma). Because we used unfraccionated PBL, which include antigen-presenting cells as well as T cells as responders, the assay design evaluates total alloreactivity; both the direct (recipient T cells reactive to donor HLA) and indirect (recipient T cells reacting to donor antigens processed and presented by recipient antigen-presenting cells in the ELISPOT wells) allore cognition pathways are assessed together. All study patients were tested against the same panel of stimulators.

The ELISPOT plates then were incubated for 18 to 24 h at 37°C. After washes with PBS and PBS-Tween, a biotinylated anti–IFN-γ detection antibody (Endogen, Woburn, MA) was added and the plates were incubated overnight at 4°C. After an additional wash, streptavidin horseradish peroxidase conjugate (Dako, Carpinteria, CA) was added for 1 h at room temperature. After a final wash, the plates were developed with 3-amino-9-ethylcarbazole (10 mg/ml in N,N-dimethylformamide; Sigma), prepared in 0.1 M sodium acetate buffer (pH 5.0) mixed with H2O2 (200 µl/well).

The resulting spots were counted with a Series 1 Immunospot computer-assisted ELISPOT image analyzer (Cellular Technology, Cleveland, OH). Results were depicted as the mean number of IFN-γ spots per 300,000 recipient PBL on the basis of duplicate or triplicate measurements in a given assay. The frequencies of alloreactive IFN-γ ELISPOT that were presented and used for analyses were obtained after those that were derived from nonstimulated wells (“background”) were subtracted. Previous work has demonstrated that <10 to 15 spots per 300,000 cells represent background reactivity and that >25 spots per 300,000 cells functioned as a threshold that correlated with an increased risk for graft injury posttransplantation (10,12,13,17). We therefore chose >25 spots per 300,000 PBL as an arbitrary threshold to define a positive assay.

**Alloantibody Detection**

Plasma anti-HLA antibody was determined by flow cytometry using HLA class I and class II antigen-coated latex beads (FlowPRA Screening Test; One Lambda, Inc., Canoga Park, CA) and then verified by assessing specificity using a panel of HLA-typed specificity beads (One Lambda). The antibody testing was performed according to instructions supplied by the manufacturer. A positive PRA was defined as >10% reactivity (sensitized individuals), and highly sensitized patients were defined as having >50% reactivity.

**Statistical Analyses**

All analyses were performed using SPSS version 11.5. Values are shown as mean ± SD, median (range), or percentage. Categorical variables were compared using the χ² test or Fisher exact test when appropriate. Comparison of mean values was tested using the t test for independent samples (two-tailed) and ANOVA, and median comparison was done using the Mann-Whitney U test. P < 0.05 was considered statistically significant.

**Results**

**Patient Characteristics**

The responder study population consisted of 41 adult patients who had ESRD and were on HD and awaiting renal transplantation (Table 1). Mean time on dialysis was 32 mo. The

<table>
<thead>
<tr>
<th>Table 1. Patient characteristicsa</th>
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<tbody>
<tr>
<td>Total no. of patients</td>
</tr>
<tr>
<td>Age (yr)b</td>
</tr>
<tr>
<td>Black race (n [%])</td>
</tr>
<tr>
<td>Female gender (n [%])</td>
</tr>
<tr>
<td>Time on hemodialysis (mo)b</td>
</tr>
<tr>
<td>HLA mismatches versus stimulatorsc</td>
</tr>
<tr>
<td>No. of patients with positive PRAd</td>
</tr>
</tbody>
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aPRA, panel reactive antibody.
bMean ± SD (minimum to maximum).
cMedian (10th to 90th percentile).
dPositive PRA was defined as >10% alloantibodies.
mean age of the cohort was 56 yr, 36.6% were female, and 53.6% were self-described as black.

Characteristics of the panel of stimulator cells are shown in Table 2. The mean age of these patients was 47 yr. Two were male, and six were female. Fifty percent of the stimulators derived from black patients. The HLA alleles that were expressed by the stimulator panel shown represent approximately 60% of HLA antigens found in the general US population (Table 2; http://www.ashi-hla.org/publicationfiles/archives/prepr/motomi.htm). As might be anticipated as a result of the random selection of responders and stimulators, the median number of HLA mismatches between any responder and any stimulator was high (median 5; range 4 to 6).

Detection of IFN-γ-Secreting PBL Reactive to a Panel of Allogeneic Stimulator Cells

PBL from each responder were tested in IFN-γ ELISPOT assays against the same panel of stimulators. An example of one representative assay is shown in Figure 1A, where PBL from five individual HD patients were tested in duplicate against the eight different stimulators. PBL plus medium alone served as a negative control (Figure 1A, top), and phytohemagglutinin stimulation was used as a positive control (Figure 1A, bottom). On the basis of published studies that defined the coefficient of variance of the ELISPOT assay at 20 to 30% (13) and on the basis of published correlates between frequencies of alloreactive PBL with poor outcome (12), we defined a response of >25 ELISPOT per 300,000 cells as a positive result.

A summary of the detected results from all samples that responded to each individual stimulator, sorted by stimulator, is shown in Figure 1B. Each stimulator induced a wide range of responses. PBL from some patients exhibited positive responses to a given stimulator, ranging from 26 to >150 IFN-γ ELISPOT per 300,000 cells, whereas PBL from other patients did not respond to the same stimulator population. The heterogeneity of the results for each stimulator suggests that the detected frequencies of IFN-γ producers were dependent on characteristics of the responder, rather than the trivial possibility that some stimulators were prepared better (and thus more viable with better stimulating capacity) than others.

The same data are replotted with each responder on the x axis to illustrate the variable reactivity of a given responder to each stimulator.

Table 2. Normal healthy volunteer characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
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<tbody>
<tr>
<td>Total no. of stimulators</td>
<td>8</td>
</tr>
<tr>
<td>Normal volunteers/deceased donors</td>
<td>6/2</td>
</tr>
<tr>
<td>Age (yr)a</td>
<td>47 ± 13 (35 to 76)</td>
</tr>
<tr>
<td>Black race</td>
<td>4/4</td>
</tr>
<tr>
<td>Male/female gender</td>
<td>2/6</td>
</tr>
<tr>
<td>Alleles present in the stimulator panelb</td>
<td></td>
</tr>
<tr>
<td>HLA A</td>
<td>A2, A3, A11, A24, A28, A30, A31, A33, A34</td>
</tr>
<tr>
<td>HLA B</td>
<td>B18, B35, B51, B8, B13, B14, B44, B45, B50, B53, B60, B62</td>
</tr>
<tr>
<td>HLA DR</td>
<td>DR7, DR1, DR4, DR11, DR13, DR3, DR12, DR16, DR17, DR18</td>
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aMean ± SD (minimum to maximum).

bHLA antigens are listed in order of frequencies expressed in the panel.
each member of the stimulator panel (Figure 1C). Again, note the heterogeneity of the detected frequencies of IFN-γ producers for each responder. PBL from some patients did not respond to any stimulator (e.g., patients 5 and 21), suggesting that they were not reactive to alloantigens. Others responded to some but not all stimulators (e.g., patient 9). PBL from still other patients responded to all stimulators (e.g., patient 37), suggesting a highly alloreactive state.

PRT

To facilitate our analysis of cellular alloimmunity to this panel of alloantigens, we defined a PRT for each responder as the percentage of stimulators (0 to 100%) that induced a positive assay result. As shown in Figure 2A, 46% of the patients had a positive PRT-40 (reacted positively to >40% of the panel, e.g., three of eight stimulators tested), and 17% of the patients had a positive PRT-75 (reacted positively to >75% of the panel, e.g., more than six of eight stimulators tested). In comparison, 32% of the patients in this cohort had a PRA of >10%, whereas 22% of the patients were highly sensitized, as defined by a PRA of >50%.

Whereas there was overlap between those patients with a positive PRT and those with a positive PRA, 12 to 34% of study patients (depending on whether we defined a positive as responding to >75 versus >40% of the stimulators) were PRT positive but PRA negative (Figure 2, B and C). Similarly, 17 to 20% of the study patients exhibited a positive PRA but a negative PRT. Only 5 to 12% of the patients were PRT positive and PRA positive.

Demographic Correlates of a Positive PRT

We next asked whether the presence of a positive PRT correlated with known epidemiologic risk factors, some of which have been associated with an elevated risk for posttransplantation graft dysfunction. As shown in Figure 3, patients who were younger than 55 yr were more likely to exhibit a positive PRT-40 or PRT-75 than older patients (P < 0.05 for each), and there was a strong, albeit not statistically significant, trend for black responders to exhibit a positive PRT-40 (54 versus 38%; Figure 3, middle). This trend became more evident in “strongly alloreactive,” PRT-75+ patients (27 versus 5%; P = 0.09 by Fisher test). There was no statistically significant correlation between the prevalence of positive PRT and male versus female gender (Figure 3, bottom). The incidence of positive PRT-75 was not significantly different in those with a history of multiple blood transfusions (29%) versus those without a history of blood transfusion (30%; P = 0.81; data not shown). Similarly, there was no difference in PRT-75 responses in women with or without history of pregnancies (100 versus 84.6%, respectively; P = 1.0). In contrast, a positive PRA (>10%) was statistically more prevalent in women versus men (P < 0.01; Figure 4, bottom), and this difference remained significant in highly sensitized patients (PRA of >50%; P < 0.01). No significant correlations between a positive PRA and either younger versus older age or black versus nonblack race were detected (Figure 4, top and middle). A strong nonstatistically significant trend to exhibit a positive PRA-50 test was seen in patients with a history of multiple blood transfusions (50 versus 25%; P = 0.15) and in women with a history of pregnancies (100 versus 67%; P = 0.14 by Fisher exact test). Finally, as shown in Table 3, a positive PRT-75 was significantly more prevalent in the population of younger black patients versus older nonblack patients. In contrast, there was no difference between the prevalence of a positive PRA (at either 10 or 50%) between these two subgroups (Table 3). All correlations were independent of HLA matching (data not shown), although the high number of HLA mismatches in all combinations (four to six; Table 2) precluded our ability to assess this variable fully.

Because studies have shown that the strength of PRA tests

![Figure 2](image-url)
can vary in a given patient when tested at different time points (4), we tested whether cellular alloreactivity as assessed by PRT varies over time. We performed repeat PRT testing in 17 of the 41 patients 6 to 20 mo after the initial assay and using the same panel of stimulator cells. As shown in Figure 5, PRT reactivity did change in a number of the tested patients between the two time points, although only one of 14 patients became newly highly reactive (new + PRT-75).

Correlation between Positive PRT and Posttransplantation Clinical Outcome

Although the correlations between PRT, PRA, and epidemiologic risk factors for posttransplantation graft injury are intriguing, the clinically relevant issue is whether ELISPOT PRT values correlate independently with posttransplantation graft injury. Correlations between PRT results and clinical outcomes for the seven patients who underwent kidney transplantation are shown in Table 4 (all seven of these patients underwent single PRT testing within 6 to 12 mo of transplantation). Although the numbers are small, the results show that the five patients with a negative PRT-75 remain free of renal rejection episodes, whereas one of the two patients with a positive PRT-75 had biopsy-confirmed acute cellular rejection of the transplanted kidney within the initial 8 mo posttransplantation. Notably, all seven patients were PRA negative.

Discussion

In a manner analogous to PRA testing to detect alloantibody-based sensitization in transplant candidates, ELISPOT PRT testing provides information regarding the strength of the effector/memory cellular alloimmune repertoire. Because cellular alloimmunity, specifically the frequency of effector/memory alloreactive T cells, has been implicated as an important mediator of graft injury (6,9,10,17), this new ability to quantify such T cells in the peripheral blood of transplant candidates has the potential to function as an adjunctive tool for clinical risk assessment. The assay is technically straightforward and can be accomplished in <24 h, making it feasible for large-scale clinical application. Our analysis of ELISPOT PRT assay results in this cohort of 41 transplant candidates provides several new pieces of information.
First, we found that human reactivity to alloantigens can be manifest through expression of alloantibodies or alloreactive T cells or both cellular and humoral immunity, a finding that extends previously published work by our group (10). We found that only 12% of our cohort exhibited a positive PRT and a positive PRA, whereas 34% of patients had a positive PRT but a negative PRA, and 20% exhibited a positive PRA but a negative PRT (Figure 2). Thus, the information obtained from simultaneously testing PRA and ELISPOT PRT provides complementary information regarding the nature of the primed, human alloimmune repertoire.

Although it would be of interest to define and compare specificities of the alloreactive T cells and alloantibodies in this cohort, the relatively small number of responders that were both PRA and PRT positive (12%), the limited number of stimulators (eight), and the highly polymorphic nature of HLA molecules prevented us from being able to make definitive conclusions on this issue. One long-term goal is to expand the

![Figure 4](image.png)

**Figure 4.** Demographic correlates with PRA+ status. Univariate analysis of the prevalence of PRA+ (10%) patients (A) or highly sensitized PRA+ (50%) patients (B) with age (top), race (middle), and gender (bottom).

| Table 3. Demographic correlates of positive PRT\(^a\) and PRA |
|-------------------|-------------------|-------------------|
|                   | Black Patients <55 Years | Nonblack Patients ≥55 Years | \(P\) Value\(^b\) |
| PRT-40 (n [%])    | 7/11 (63.6)         | 4/15 (26.7)         | 0.11 |
| PRT-75 (n [%])    | 5/11 (45.5)         | 0/15 (0)            | <0.01 |
| PRA-10 (n [%])    | 4/11 (36.4)         | 4/15 (26.7)         | 0.68 |
| PRA-50 (n [%])    | 2/11 (18.2)         | 4/15 (26.7)         | 1.0  |

\(^a\)PRT, panel of reactive T cells.  
\(^b\)By Fisher exact test.
stimulator panel to include a large number of HLA haplotypes that ultimately would permit us to define the specificity of the cellular alloimmune response, an approach that is beyond the scope of this work.

Our approach using PBL as responders detects the total alloresponse but does not differentiate T cells that respond through the direct versus the indirect allorecognition pathways. Whereas using HLA-derived synthetic peptides can be used to assess indirect responses after graft injury is manifest (9,10), there is no evidence that PBL from patients who have not received a transplant respond to indirectly presented HLA-derived peptides. The extensive polymorphic nature of the HLA complex makes peptide-based screening impractical, and preliminary studies with isolated T cells from two individuals showed that the reactivity occurred through the direct pathway (data not shown). More important, studies that provided strong correlates between pretransplantation immunity and posttransplantation outcomes used unfractionated PBL as responders (10,12–14,17). Future work to assess pretransplantation reactivity to indirectly presented peptides as well as pretransplantation PRT and correlating results with posttransplantation outcome need to be performed to address the importance of pretransplantation indirect reactivity in more detail.

We found several intriguing correlations between the PRT positivity and other epidemiologic factors associated with posttransplantation graft injury. Black transplant candidates, in particular, younger black candidates, had a higher incidence of positive PRT than the other tested subgroups (Figure 3, Table 3). From epidemiologic studies, it is well known that this group of transplant candidates is at high risk for posttransplantation acute rejection and graft failure, raising the possibility that pretransplantation effector/memory alloreactive T cells contribute to the high-risk state (18–22). The additional observation that PRT positivity is less frequent in older transplant candidates (Figure 3) is interesting. Published studies suggest inherently diminished immune reactivity as patients age (23,24). Specifically, in the transplant setting, epidemiologic data reveal a lower incidence of acute rejection in older transplant recipients (23–25). The lower incidence of PRT positivity in this >55 yr old population suggests a functional basis for these previously identified clinical associations.

Importantly, we did not detect a correlation between the prevalence of alloantibody sensitization (+ PRA) and age or race, providing further support for the speculation that the effector/memory cellular alloimmune repertoire contributes to the higher risk for posttransplantation graft injury. Pregnancy and multiple blood transfusions were associated with alloantibody sensitization (but not with PRT+), verifying previous associations found in the literature (4).

Our data provide preliminary evidence from seven patients implicating a relationship between PRT results and posttransplantation outcome, independent of the presence of alloantibodies. We and others have shown that the frequency of pretransplantation IFN-γ ELISPOT that are reactive to donor cells correlates with early acute rejection episodes and posttransplantation renal function independent of other recipient and donor factors that are known to influence these outcomes (11,14,17). If additional prospective analyses show that the PRT proves to have similar predictive value, then the relationship between the final pretransplantation donor-specific IFN-γ ELISPOT assay and PRT as measures of T cell reactivity may prove to be analogous to the relationship between the “final cross-match” and PRA in assessing alloantibody sensitization.

The data presented herein suggest that a high frequency of
alloreactive T cells as assessed by PRT will be predictive of posttransplantation outcome. However, because the threshold for defining a positive response in this cohort was chosen arbitrarily, it is possible that different/higher thresholds will improve the predictive value of the PRT. Evaluating frequencies of T cell alloreactivity in large numbers of normal volunteers may also aid in better delineating clinically relevant thresholds for predicting outcome. Regardless, these findings support the contention that ELISPOT screening for PRT status will provide different information than screening tests that are focused on detection of alloantibodies. Prospective studies that analyze larger numbers of patients with posttransplantation follow-up will be required to make definitive conclusions regarding these hypotheses.

The variability in detected PRT responses that are found in some patients (Figure 5) is not surprising. From a technical standpoint, this test has a known coefficient of variability of approximately 20% (13), and because we used only eight distinct stimulators, a change in reactivity to a single stimulator approximately 20% (13), and because we used only eight distinct stimulators, a change in reactivity to a single stimulator might reflect environmental influence such as undiagnosed intercurrent infections, alterations in medications, recent immunizations, etc., that can influence significantly the cellular alloimmune response (27). None of the patients with significant alterations in PRT from our cohort has received a transplant, so we cannot comment on whether the detected alterations in PRT are clinically important. This issue is being addressed specifically in ongoing clinical trials.

**Conclusion**

We have defined a novel approach for evaluating and screening cellular alloimmune reactivity in transplant candidates. If these findings are verified to correlate with posttransplantation outcomes, independent of PRA and HLA matching, then pretransplantation PRT testing may become a useful adjunctive clinical tool for pretransplantation risk assessment. It is anticipated that through clearer identification of high-risk patients on the basis of functional measures of immune reactivity, physicians ultimately will be able to make individualized therapeutic decisions to optimize outcome and avoid the potential pitfalls of using epidemiologic data to define treatment regimens empirically.

**Acknowledgments**

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Access to UpToDate on-line is available for additional clinical information at http://www.jASN.org/