Flow Cytometric Evaluation of Cytotoxic Peripheral Blood Lymphocytes in Acute Renal Graft Rejection

Nagaraka Rap Sridhar, Matthew Blanton, Lynn Whitacre, Kamala Balakrishnan, and M. Roy First

N. Sridhar, M. R. First, Division of Nephrology and Hypertension, University of Cincinnati Medical Center, Cincinnati, OH
M. Blanton, L. Whitacre, K. Balakrishnan, HLA Laboratory, Hoxworth Blood Center, Cincinnati, OH

ABSTRACT

The presence of the S6F1+ epitope on the surface of CD8+ lymphocytes is believed to be uniquely representative of cytotoxic subpopulations. A preliminary study was conducted to evaluate the CD8+ S6F1+ peripheral lymphocytes by flow cytometry in patients undergoing renal allograft biopsy for allograft dysfunction. Lymphocytes, obtained at the time of biopsy, were analyzed by flow cytometry with CD8-FITC/S6F1-PE as the test monoclonal antibody and MlgG-RDI/MlgG-FITC as internal control. A 100% increase in S6F1+ cells over internal control was considered to be positive result. The results were correlated with the histopathologic findings in 14 instances of allograft dysfunction occurring 26.5 ± 11.6 days posttransplantation. The histopathologic diagnosis was acute cellular rejection in eight cases, acute tubular necrosis in four, and cyclosporine nephrotoxicity in two. Flow cytometric detection of an increase in S6F1+ cells yielded a sensitivity of 87.5% and a specificity of 83.3% for the diagnosis of acute rejection. It would appear that the use of a monoclonal antibody to detect increases in the number of CD8+ S6F1+ peripheral lymphocytes is a valuable test for the detection of acute allograft rejection in the initial period after transplantation.

METHODS

Study Population

All patients who underwent a renal allograft biopsy for allograft dysfunction in the first 60 days posttransplantation were studied by flow cytometric analysis of peripheral blood lymphocytes. The protocol was approved by the Institutional Review Board, and written informed consent was obtained. Acute graft dysfunction was diagnosed when there was a 10% or greater increase in serum creatinine over baseline. Fifteen milliliters of peripheral blood was obtained at the time of allograft biopsy. Biopsies were performed between 11:00 and 1:00 pm. Lymphocytes were separated by a density gradient technique with

Key Words: Cytotoxic lymphocytes, CD8+ S6F1+ lymphocytes, flow cytometry, acute cellular rejection
Ficoll-Hypaque. Platelets were removed with chilled ADP and by centrifugation. The lymphocytes were adjusted to a final concentration of 1,000 cells/mL. A cell suspension of 100 mL was stained with MsIgG1-RD1/MsIgG-FITC as internal control (IC), to detect cells coated with mouse anti-human T-cell antibody, and another 100 mL with the test monoclonal antibody (mAb) CD8-FITC/S6F1-RD1, to detect cells expressing the CD8 and the S6F1 antigens in addition to those detected by anti-T-MsIgG1. These mAb were manufactured by Coulter Immunology (Hi- aleah, FL). Both stained cell suspensions were analyzed by dual fluorescence with a Coulter Epics Profile flow cytometer. Figure 1a shows the results of a typical run with dual fluorescence with IC: Zone 3 represents the cells not binding antibody at all. Zone 1 represents cells staining with MsIgG1-RDI, and Zone 4 shows cells staining with MsIgG-FITC. Figure 1b shows the cells stained with the test mAb. Again, Zone 3 represents cells not stained with either the anti-CD8 mAb or the anti-S6F1 mAb. Zone 1 represents cells stained with both MsIgG1-RDI and MsIgG-FITC. Zone 2 in Figure 1a represents cells stained with both anti-CD8 and anti-S6F1 mAb. The cells in each of these zones were counted in the automated analyzer and expressed as a percentage of the total number of cells in all four zones. The increase in the percentage of cells in Zone 2 in Figure 1a over that in Figure 1b represents the total percentage of CD8+ cells expressing the S6F1 antigen, over and above the lymphocytes nonspecifically binding with the mouse anti-human T-cell mAb. In this study, we did not examine serial peripheral blood lymphocyte counts; these have yielded inconsistent results in other studies (2, 3).

Immunosuppression

All patients who had a panel reactive antibody >50% received prophylactic OKT3, 5 mg iv daily, from the first day after transplantation for 10 to 15 days (6). Those with a panel reactive antibody <50% received Minnesota antilymphoblast globulin (MALG), 15 to 20 mg/kg iv daily, for the same time period (7). In addition, prednisone, 120 mg/day, was started at the time of transplantation, tapered rapidly to 20 mg/day by the 15th day, and tapered further thereafter. Adjustments in prednisone dosage were made according to clinical circumstances. Typically, the patients were on 10 mg twice a day (8:00 a.m. and 8:00 p.m.) of prednisone on the day of flow cytometric analysis and biopsy, and the morning dose was ingested between 3 and 5 h before the collection of the peripheral blood lymphocytes for analysis. All patients also received azathioprine, 1.5 mg/kg per day, from the first day of transplantation; adjustments were made according to the total white blood cell count. Cyclosporine, 8 mg/kg per day, was started when the serum creatinine declined to less than 4 mg/dL and was adjusted further according to trough whole blood levels.

Figure 1. (a) Zone 1 represents cells stained with MsIgG1-RDI; Zone 2 represents cells stained with MsIgG1-RDI and MsIgG-FITC; Zone 3 represents cells not stained with either control antibody; and Zone 4 represents cells stained with MsIgG-FITC. (b) Zone 1 represents cells stained with S6F1-RD1; Zone 2 represents cells stained with S6F1-RD1 and CD8-FITC; Zone 3 represents cells not stained with either test antibody; Zone 4 represents cells stained with CD8-FITC. Note the lack of increase in CD8+S6F1+ cells over control in an instance of ATN (Zone 2 in panels a and b).
Analysis

For the purpose of this study, the diagnosis of acute rejection was the histopathologic identification of a generalized lymphocytic infiltrate or a localized infiltrate with tubular penetration. The pathologist was unaware of the results of flow cytometric analysis at the time of interpretation of the biopsies. Acute rejection was diagnosed by flow cytometry if a 100% increase occurred in Zone 2 with the test mAb over and above that in Zone 2 with IC. An episode was considered true positive (TP) if there was a 100% increase in CD8+ S6F1 cells over IC by flow cytometry and if the histopathology revealed acute rejection. A true negative (TN) was identified when flow cytometry and histopathology did not diagnose acute rejection. A false positive (FP) was identified when flow cytometry, but not histopathology, diagnosed acute rejection, and a false negative (FN) was identified when histopathology, but not flow cytometry, diagnosed acute rejection. By these interpretations, sensitivity (TP/TP + FN), specificity (TN/TN + FP), positive predictive value (TP/TP + FP), and negative predictive value (TN/TN + FN) were calculated.

An attempt was made to analyze graft-infiltrating cells expressing the CD8 and S6F1 antigens from the last of three or four pieces of tissue obtained at biopsy. However, after digestion of the tissue with collagenase and DNase and filtration through a 100-μm-pore-size nylon mesh, we were unable to obtain a sufficient number of cells for analysis.

RESULTS

A total of 18 biopsies and flow cytometric studies were performed over a 75-day period. Of these, 14 were done for acute allograft dysfunction and 4 were done for the investigation of chronic graft failure with heavy proteinuria. Three patients had more than one biopsy because they had more than one episode of acute graft dysfunction in the study period, and 10 patients had one biopsy each. The age, sex, cause of renal disease necessitating transplantation, the day of occurrence of acute graft dysfunction, biopsy and flow cytometric study results, and interpretation are detailed in Table 1. No patient was on Minnesota antilymphoblast globulin, OKT3, or any other antilymphocyte antibody on the day of the biopsy and flow cytometry. Table 2 provides the overall results of the study and their interpretation. In the 14 instances of acute graft dysfunction, there were 8 instances of acute cellular rejection as diagnosed by biopsy; in 7 of these, flow cytometric identification of a 100% increase in S6F1+ CD8+ cells over IC provided an accurate diagnosis. Although it is theoretically possible that a concurrent viral infection

TABLE 1. Patient demographics, flow cytometry, biopsy results, and interpretationa

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Episode</th>
<th>Age/Sex</th>
<th>Cause of ESRD</th>
<th>Days Post-transplant</th>
<th>ACR by Flow</th>
<th>Biopsy</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>38/F</td>
<td>DGS</td>
<td>12</td>
<td>No</td>
<td>CN</td>
<td>TN</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>28/F</td>
<td>U</td>
<td>22</td>
<td>No</td>
<td>ATN</td>
<td>TN</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>54/M</td>
<td>CGN</td>
<td>13</td>
<td>No</td>
<td>ATN</td>
<td>TN</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>42/M</td>
<td>CGN</td>
<td>20</td>
<td>Yes</td>
<td>ACR</td>
<td>TP</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>40/M</td>
<td>CGN</td>
<td>35</td>
<td>Yes</td>
<td>ACR</td>
<td>TP</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>45/F</td>
<td>CIN</td>
<td>22</td>
<td>No</td>
<td>CN</td>
<td>TN</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>25/F</td>
<td>DGS</td>
<td>45</td>
<td>Yes</td>
<td>ACR</td>
<td>TP</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>63/M</td>
<td>DGS</td>
<td>22</td>
<td>No</td>
<td>ACR</td>
<td>FN</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>36/F</td>
<td>DGS</td>
<td>43</td>
<td>Yes</td>
<td>ACR</td>
<td>TP</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>42/M</td>
<td>U</td>
<td>15</td>
<td>Yes</td>
<td>ACR</td>
<td>TP</td>
</tr>
</tbody>
</table>

a ACR, acute cellular rejection; CN, cyclosporine nephrotoxicity; CGN, chronic glomerulonephritis; CIN, chronic interstitial nephritis; U, unknown; DGS, diabetic glomerulosclerosis.

TABLE 2. Sensitivity, specificity, and positive and negative predictive values of flow cytometry correlation with histopathologya

<table>
<thead>
<tr>
<th>Results</th>
<th>No. of Instances</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP</td>
<td>7</td>
</tr>
<tr>
<td>TN</td>
<td>5</td>
</tr>
<tr>
<td>FP</td>
<td>1</td>
</tr>
<tr>
<td>FN</td>
<td>1</td>
</tr>
</tbody>
</table>

a Sensitivity (TP/TP + FN), 87.5%; specificity (TN/TN + FP), 83.3%; positive predictive value (TP/TP + FP), 87.5%; negative predictive value (TN/TN + FN), 83.3%.
may have been responsible for some of the increase in S6F1 antigen expression in this series, all seven TP results were seen in patients whose serum creatinine levels returned to baseline with antirejection therapy, an occurrence that would be unlikely with an undiagnosed viral infection. Moreover, these patients had neither clinical features nor laboratory findings suggestive of viral infection at the time of the study. There were four instances of ATN as revealed by biopsy: in three, a 100% increase in CD8+ cells with the S6F1 antigen did not occur. There was one instance where flow cytometry diagnosed acute rejection that was not proven by biopsy (Case 3, Episode 2). This patient had ATN with regenerating tubular epithelium and evidence of focal segmental sclerosis. There was only one instance (Case 8) where histopathology showed mild acute rejection with a patchy infiltrate and there was a decrease in CD8+ S6F1+ cells compared with that in IC. Interestingly, this patient did not show a marked or sustained improvement in renal function with 4 days of methylprednisolone therapy (250 mg/day) and was later found to have obstructive uropathy; the serum creatinine level improved after ureteral repair. One of the 10 patients had three episodes of acute graft dysfunction (Case 2). Two of these were episodes of ATN, and one was acute rejection. Figure 1 shows the lack of a significant increase in cells in Zone 2 with CD8 S6F1 mAb over and above IC during an episode of ATN. Figure 2 shows the results of the same patient during an episode of acute rejection; note the nearly 10-fold increase in cells in Zone 2. Therefore, this new test system appears to have excellent clinical correlation during the first 2 months after transplantation. Sensitivity, specificity, positive predictive value, and negative predictive value were between 83.3 and 87.5%.

**DISCUSSION**

Two distinct lines of investigation have increased our understanding of lymphocyte function and their particular importance in human and experimental allograft rejection. The first is a series of studies done with mAb of the OKT class, derived from a mouse hybridoma. Distinctive antigens were first found on the surface of T cells of humans (8). Later, the functional subsets of these cells were described with other mAb (4, 9) and these cells were characterized as TH2(+) and TH2(−) subsets. The TH2(−) subset reacted with the OKT4 mAb (9), and the TH2(+) subset reacted with the OKT5 mAb (10) and the OKT8 mAb (11). It was further shown that T4+ cells provided the inducer and helper functions in T-T and T-B interactions, to cause cell-mediated lymphotoysis (8), facilitate B-cell proliferation, differentiation, and immunoglobulin synthesis (9), and generate soluble helper factors (12). In contrast, the TH2(+) T5/T8 subset, which accounts for 25 to 30% of peripheral T cells, has been shown to suppress B-cell immunoglobulin production (10, 11). These cells also suppress the antibody response to dinitrophenol linked to keyhole limpet hemocyanin (13). Subsequently, it was shown that the T4+ cells also had cytotoxic potential directed against the Class II MHC antigens, although the major cytotoxic potential came from the

---

**Figure 2.** For descriptions of cells in all of the zones see legend to Figure 1. Note the nearly 10-fold increase in CD8+ S6F1+ cells over control in an instance of acute cellular rejection (Zone 2 in Figure 2a and b). Figures 1 and 2 represent two different episodes of acute graft dysfunction in the same patient (Case 2). In the first episode (Figure 1), the biopsy revealed ATN; in the second episode (Figure 2) acute cellular rejection was present.
T8+ subset (mainly directed against the Class I MHC antigens) (14). Although the T8 subset showed certain common antigenic properties, it soon became clear that there existed within any T8 subset two different subpopulations that exhibited different phenotypic properties in response to antigenic stimulation (15, 16). An anti-T8 antibody markedly inhibited cell-mediated lympholysis, anti-T8 antibody partially affected it, and anti-T5 antibody had very little effect (17). T8 cells, then, were confirmed to have both suppressor and cytotoxic subgroups that reacted differently to antigenic stimulation in the presence of helper/inducer cells. Until relatively recently, further subcategorization of CD8+ cells by antigen expression was not possible. However, a new epitope on the surface of CD8+ cells was described that appeared to be uniquely representative of the cytotoxic subpopulation (5). It was designated as S6F1, appeared to contain two glycoproteins of relative molecular masses of 180 and 95 kd, and antibody to S6F1 appeared to recognize a new epitope on the LFA-1 antigen. The S6F1 antigen was found on 17% of unfractonated T cells, 14% of CD4+ cells, and 58% of CD8+ cells, null cells, and granulocytes. It reacted with less than 10% of the cells in the B-cell line and macrophages. More importantly, it shows that the CD8+ S6F1+ lymphocyte population exhibited no cytotoxic activity. It was shown that the CD8+ population had cytotoxic (CD8+ S6F1+) cells, suppressor (CD8+ S6F1−) cells, and precursors of both, which were CD8+ S6F1−. It appeared that cytotoxic precursors that were S6F1− acquired the S6F1 antigen during differentiation and proliferation into cytotoxic cells in a mixed lymphocyte reaction (5).

The second series of studies enabled an understanding of the role of lymphocytes in allograft rejection by examining graft infiltrating cells and peripheral lymphocytes (18, 19). Moreau et al. found that of all infiltrating T lymphocytes 95% were T8+ and 40% were T4+ and that at least 35% were both T4+ and T8+ (1). Almost all T8+ clones were cytotoxic against a donor B lymphoblastoid cell line, although both T4+ and T8+ clones proliferated significantly on exposure to this cell line. T4+, but not T8+, clones produced detectable interleukin-2 into the supernatant. It was shown that in human renal allograft rejection, the effector arm of the rejection response rested within the T8+ lymphocyte pool. Other investigators confirmed the dominant role played by the T8+ cells in the cytotoxicity seen in acute allograft rejection (20). However, some studies did not show a significant difference in the ratio of helper/inducer and suppressor/cytotoxic subsets between rejection and nonrejection grafts (21). This was probably because the CD8+ cells in the graft could not be segregated with certainty into the suppressor and cytotoxic subtypes. Interestingly, another study found that during rejection, both suppressor/inducer (CD4+ CD45R+) and suppressor/effectr (CD8+ CD11b+) cells were significantly reduced in number, although the CD8+ suppressor/cytotoxic and the CD16+ NK-like cells formed a larger proportion of the infiltrate in rejection grafts (22). Again, no direct comparison between the suppressor subset and the cytotoxic subset among the graft-infiltrating CD8+ cells appears to have been made. Peripheral blood lymphocyte subset analysis has also yielded mixed results. In one study of 72 cadaver renal allograft recipients in the first 3 months after transplantation, it was found that patients who maintained a T4/T8 ratio of more than 1 had a higher incidence of allograft dysfunction (77%) and graft loss than did those with a T4/T8 ratio less than 1 (allograft dysfunction in 26%), although there was no significant difference in graft survival in subsequent months (23). Another study showed that there was no association between pregraft values of T4/T8 ratios and the occurrence of rejection episodes (2). However, an overall view of the initial studies of T4/T8 ratios in peripheral blood lymphocytes would appear to suggest that a higher ratio is associated with a higher risk of rejection (24–26). A later study investigating the roles of T4 and T8 subsets showed a significant increase of total T8+ cells and of the percentage of the 2H4+ suppressor/effectr subset in 66 stable renal allograft recipients (27).

Against this background, we hypothesized that although CD8+ cells were recognized to be the principal cytotoxic cells in the effector limb of the immune response in acute cellular rejection, the reason why the total T4/T8 ratios are higher in acute rejection or are unreliable is because investigators have not been able to segregate the cytotoxic cells among all CD8+ cells. With the availability of the S6F1 antigen, we believe this issue could be reevaluated. Therefore, we conducted this preliminary study using standard histopathology as the yardstick for comparison with the results of flow cytometric analysis and attempted to study the usefulness of this new mAb in identifying patients with acute rejection from among all other causes of acute graft dysfunction. It is possible that NK cells expressing both the CD8 and S6F1 antigens may be present in zone 2 in our flow cytometric analysis because triple fluorescence to segregate the CD3+ non-NK cells was not performed; however, we feel that the proportion of NK cells in this zone should be small, because only approximately 10% of NK cells express the CD8 antigen (5). In addition, the importance of flow cytometric identification of NK cells without a functional assay of killer activity has been reported as being unreliable (28). Further studies should include three-color fluorescence to exclude CD3− NK cells. Our initial experience with this system has been very encouraging and leads us to be-
lieve that this test system affords the clinician an opportunity to make a more definite clinical diagnosis of acute rejection before proceeding to biopsy. The sensitivity, specificity, and positive and negative predictive values of this test system were 83 to 88%. If further studies with serial blood samples at regular intervals indicate that a rise in CD8+ S6F1+ cells heralds the onset of biopsy-confirmed acute rejection, this new mAb will have added substantially to our ability to diagnose acute rejection, noninvasively, by immunologic monitoring. At present, it takes about 3 h of working time to obtain the results of this test. Reductions in work time should be possible in the future with automated lymphocyte separation.

ACKNOWLEDGMENTS
This work was supported by a grant from the Kidney Foundation of Greater Cincinnati.

REFERENCES
1. Moreau JP, Bonneville M, Peyrat MA, et al.: T lymphocytes cloning from rejected kidney allografts; growth, frequency and functional/phe-
4. Rheinherz EL, Kung PC, Goldstein G, Schloss-
man SF: Separation of functional subsets of hu-
man T cells by a monoclonal antibody. Proc Natl Acad Sci USA 1979;76:4061-4065.
6. Schroeder TJ, First MR, Mansour ME, Alex-
8. Kung PC, Goldstein G, Rheinherz EL, Schloss-
man SF: Monoclonal antibodies defining distinct-
9. Rheinherz EL, Kung PC, Goldstein G, Schloss-
man SF: Further characterization of the human inducer T cell subset defined by monoclonal an-
10. Rheinherz EL, Kung PC, Goldstein G, Schloss-
man SF: Monoclonal antibody reactive with the human cytotoxic/suppressor T cell subset pre-
viously defined by a hetero antigen termed

11. Rheinherz EL, Kung PC, Goldstein G, Levey
12. Rheinherz EL, Kung PC, Goldstein G, Breard
14. Meuer SC, Schlossman SF, Rheinherz EL: Clonal analysis of human cytotoxic T lymphocytes: T4+ and T8+ effector cells recognize produc-
tnts of different major histocompatibility com-
15. Damle NK, Mohagheghpour N, Engleman EG: Soluble mitogen primed inducer T cells activate antigen specific suppressor T cells in the absence of antigen pulsed accessory cells: Pheno-
typic definition of suppressor-induced and sup-
16. Morimoto C, Letvin NL, Distaso JA, Aldrich
WR, Schlossman SF: The isolation and charac-
17. Rheinherz EL, Hussey RE, Fitzgerald K, Snow P, Terhorst C, Schlossman SF: Antibody di-
18. Loveland BE, Hogarth PM, Ceredig R, Mc-
19. Kerman RH, Van Buren CT, Payne W, Flechner
22. Tufveson G, Alexapoulous E, Raftery MJ, Hartley
1176–1177.