Differentiation of Cytomegalovirus Infection from Acute Rejection Using Renal Allograft Fine Needle Aspirates


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ABSTRACT
Cytomegalovirus (CMV) infection is an important cause of renal allograft dysfunction and may be difficult to distinguish from acute transplant rejection both clinically and histologically. To establish the early diagnosis of CMV infection, we used immunohistochemical staining with antibodies against CMV early nuclear protein (CMV-A) and histocompatibility leukocyte class II antigen (DR) in renal transplant fine needle aspirates. Fifty-eight aspirates from 27 patients were assessed, 53 for CMV-A and 53 for DR. Positive staining was defined as \( \geq 35\% \) stained tubular cells for CMV-A and \( \geq 30\% \) stained tubular cells for DR. Clinical diagnoses were made retrospectively without using the information obtained from aspirate diagnoses. CMV-A staining was negative in 44 aspirates, none at the time of CMV infection. CMV-A was positive in nine aspirates, seven during CMV infection (78%, \( P < 0.00001 \) versus CMV-A negative). DR staining was never present in the absence of acute rejection. All aspirates performed during acute rejection had positive DR staining (\( P < 0.00001 \) versus DR negative). Aspirates with acute rejection comprised 80% of all DR-positive aspirates, whereas those with CMV infection included only 13%. The percent CMV-A staining increased with CMV disease progression; DR staining decreased after successful treatment of acute rejection. These data demonstrate that CMV-A staining is associated with CMV infection whereas DR staining is specifically related to acute rejection. CMV-A and DR staining of fine needle aspirates is a potentially valuable diagnostic tool to distinguish rapidly between CMV infection and acute transplant rejection as the etiology of renal allograft dysfunction.

Key Words: Immunostaining, HLA class II antigen, DR, aspiration biopsy, renal transplantation

Clinically significant cytomegalovirus (CMV) infection commonly occurs in renal transplant recipients (1–3). CMV infection may be difficult to diagnose, and the clinical manifestations may mimic those of acute transplant rejection (ATR). Serologic and culture studies usually give accurate diagnostic results but may not be timely, or, if CMV is unsuspected, cultures may not be performed. Morphologic assessment of the allograft by core renal biopsy rarely yields a specific diagnosis of CMV infection as CMV inclusions are infrequently observed (3–6) and virally induced interstitial inflammation often simulates that occurring in ATR (5).

Cytologic evaluation of fine needle aspirates from renal allografts has been used in an attempt to evaluate CMV infections (7–9). There is, however, frequent overlap of findings in aspirates from patients...
with CMV infection or ATR, including graft and peripheral blood immune activation. This creates difficulty in differentiating between these two etiologies of allograft dysfunction. Additionally, the aspirate findings in patients with viral infection do not specifically identify CMV as the offending virus. We therefore undertook this study using immunohistochemical staining of fine needle aspirates from renal allografts in an attempt to provide a method for (1) the rapid diagnosis of CMV infection and (2) the differentiation of CMV infection from ATR and other causes of renal transplant dysfunction.

**MATERIALS AND METHODS**

**Patients and Diagnosis**

Fifty-eight aspirates were obtained from 27 patients. All patients were treated with cyclosporine and prednisone; azathioprine was added in some patients (10). OKT3 monoclonal antibodies (Ortho Diagnostics, Inc., Raritan, NJ) were used to treat episodes of steroid-resistant rejection. For each aspirate, a retrospective clinical diagnosis was subsequently made on the basis of serologic studies, blood culture results when available, clinical course, and response to therapeutic intervention. The fine needle aspirate routine and immunostaining results were not used in arriving at the retrospective diagnoses. Clinical diagnostic categories included (1) ATR defined as a diagnostic core biopsy response to antirejection therapy, (2) CMV infection defined as a febrile illness associated with elevated immunoglobulin M titers with or without other clinical manifestations, such as pneumonia and leukopenia and improvement after gancyclovir, (3) unexplained self-limited events which resolved without therapy, including febrile illness or transient renal dysfunction, and (4) other findings, including acute tubular necrosis, acute cyclosporine nephrotoxicity, renal artery stenosis, sepsis, and normal aspirate. Donor and recipient CMV status was assessed before transplantation by the complement fixation test (Kolmer method) with a titer of greater than 1:8 considered positive. Renal core biopsy was performed within 4 days of aspiration for 11 of the aspirates. The biopsies were processed for light, immunofluorescent, and electron microscopies in the usual fashion (11); the biopsies and aspirates were assessed independently.

**Fine Needle Aspiration**

Aspirates were performed between 3 days and 19 wk postoperatively for baseline evaluation during the first posttransplant week, at times of renal functional impairment, or during febrile episodes. The aspirates were evaluated by the method of Hayry and Von Willebrand (12-14); routine evaluation included differential leukocyte counts from the aspirate and peripheral blood specimens with quantitation of inflammation by the increment method, parenchymal cell assessment, and immunoblast count. Additional cytospin preparations from each aspirate were air dried and used for immunohistochemical staining.

Monoclonal antibodies against CMV early nuclear protein (CMV-A) (Dupont/NEN Research Products, Boston, MA) and histocompatibility leukocyte antigen (HLA) class II antigen (DR) (Dako, Carpenteria, CA) were used with a modification of an immunoperoxidase method (15). Cytospin preparations were incubated with the primary antibody (CMV-A, 1:6 with BSA; DR, 1:2 with BSA) for 30 to 40 min, and the slides were washed. Secondary linking antibody (Tago, Burlingame, CA) was diluted 1:20 in normal human serum and was added to the slides, and the slides were then incubated for 15 min followed by washing. Color development was accomplished by the addition of AS/AP amplified substrate (Bio/CAN America Inc., Portland, ME); slides were quenched by the addition of levamisole. Counterstaining was with Mayer’s hemalum (EM Pharmaceuticals, Hawthorne, NY). Negative controls were performed by the same technique with substitution of Tris-buffered saline with BSA for the primary antibody. The entire slide was examined, and all positively and negatively stained tubular cells were counted to determine the overall percentage of positively stained epithelial cells. Sample adequacy was defined as a minimum of 50 tubular cells per slide; when clusters of similarly stained tubular cells were present, a maximum of 5 cells were counted for the entire cluster. Slides were examined without knowledge of the clinical symptoms or results of routine aspirate evaluation. The threshold for positive CMV-A staining was based on the analysis of 50 consecutive aspirates from patients without clinical CMV infection, none of whom were included in this study. The mean positive CMV-A staining of tubular cells in patients not infected with CMV was 15.7% with a standard deviation of 9.8%. Therefore, positive staining for CMV-A was defined as ≥35% tubular cell staining (mean +2 SD). The threshold for positive DR staining was based on the analysis of 35 consecutive aspirates from patients without clinical current acute rejection or acute rejection in the preceding or subsequent two wk, when DR can also be elevated (16). Approximately half of these aspirates were included in this study. The mean positive DR staining of tubular cells in these nonrejecting aspirates was 13.8% with a standard deviation of 7.7%. Positive DR staining was therefore defined as ≥30% tubular cell staining, with the upper limit of negative as the mean +2 SD.
Statistics

Results are expressed as mean ± SE where appropriate. Statistical analysis was performed by using analysis of variance and unpaired Student's t test where appropriate.

RESULTS

Fifty-eight aspirates from 27 patients were evaluated during the course of the study, 53 for CMV-A and 53 for DR (Figure 1). Forty-eight aspirates were stained for both antigens. Nineteen patients had ATR, and five had CMV infection; one patient had CMV infection concurrently with ATR. Nineteen aspirates were negative for both CMV-A and DR (Table 1), all of which were from patients without ATR. The majority of these aspirates were from patients with other findings, primarily acute tubular necrosis (nine aspirates) and acute cyclosporine nephrotoxicity (three aspirates). Three were from patients with unexplained events; two of these were febrile illnesses associated with probable unidentified virus infections. Five aspirates were negative for CMV-A, with DR staining not performed (Table 1). Of these, two were obtained at the time of ATR, two were during unexplained events, and one was from a patient with acute tubular necrosis. No aspirates with negative CMV-A staining were from patients with clinical CMV infection.

Twenty-five aspirates stained positively for DR with 20 negative for CMV-A and CMV-A staining not performed in 5 (Table 2). Twenty-one (84%) were at the time of ATR, and three were performed during unexplained events. One aspirate was obtained at the time of acute tubular necrosis; however, this

Figure 1. Immunoalkaline phosphatase staining for CMV early nuclear protein (CMV-A) or HLA class II antigen (DR) of tubular cells from renal allograft fine needle aspirates. (A) CMV-A-negative aspirate. No staining of tubular cell cytoplasm or nuclei is evident. (B) CMV-A-positive aspirate. There is marked CMV-A staining in the nuclei (arrowheads) with associated cytoplasmic staining; the cytoplasmic staining is possibly a cytopsin artefact. (C) DR-negative aspirate. There is no staining present. (D) DR-positive aspirate. There is cytoplasmic staining without a nuclear component. Magnification, x325.
patient subsequently developed ATR within 2 wk. No aspirates were from patients with CMV infection.

Nine aspirates had positive CMV-A staining, five with positive DR staining and four with negative DR staining (Table 3). Seven of the nine aspirates (78%) were performed at the time of retrospectively diagnosed CMV infection. Of the five CMV-A-positive/DR-positive aspirates, two were from patients with concurrent CMV infections and ATR whereas two had ATR within the preceding 2 wk. The fifth aspirate was from a patient with ATR who developed clinical CMV infection 12 days later. Of the four CMV-A-positive/DR-negative aspirates, three were performed during CMV infection. The other aspirate was obtained during an unexplained febrile illness which resolved spontaneously. After resolution of this event, the CMV-A staining became negative. No CMV-infected patients with negative DR staining had ATR within 3 wk before the diagnostic aspirate. The distributions of CMV-A and DR staining in the various clinical diagnostic categories are shown in Figure 2.

The seven CMV-A-positive aspirates done during CMV infections were from five infected patients. Of these five patients, three had three aspirates each, performed an average of 2.5 wk apart. The average percent tubular cell positivity for CMV-A in these sequential aspirates rose from 13 to 36%, then to 52%, by which time the aspirate was considered positive for CMV-A. The individual diagnostic aspirates (≥35% positively stained tubular cells) coincided with the clinical diagnosis of CMV infection. DR staining was unrelated to CMV infection; one CMV-infected patient with a steroid-responsive ATR had DR staining decrease from 68 to 4%, whereas the corresponding CMV-A staining rose from 18 to 51% on two sequential aspirates performed 3.1 wk apart. There was no consistent relationship between the diagnosis of CMV infection with positive CMV-A staining and the standard aspirate diagnostic criteria

TABLE 1. Clinical diagnoses at the time of CMV-A-negative renal transplant fine needle aspiration

<table>
<thead>
<tr>
<th>Fine Needle Aspirate</th>
<th>Retrospective Clinical Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total aspirates</td>
</tr>
<tr>
<td>CMV-A negative/DR negative</td>
<td>19</td>
</tr>
<tr>
<td>CMV-A negative/DR NA*</td>
<td>5</td>
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</tbody>
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* NA, not available.

TABLE 2. Clinical diagnoses at the time of DR-positive renal transplant fine needle aspiration

<table>
<thead>
<tr>
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<th>Retrospective Clinical Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total aspirates</td>
</tr>
<tr>
<td>DR positive/CMV-A negative</td>
<td>20</td>
</tr>
<tr>
<td>DR positive/CMV-A NA*</td>
<td>5</td>
</tr>
</tbody>
</table>

* N/A, not available.

TABLE 3. Clinical diagnoses at the time of CMV-A-positive renal transplant fine needle aspiration

<table>
<thead>
<tr>
<th>Fine Needle Aspirate</th>
<th>Retrospective Clinical Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total aspirates</td>
</tr>
<tr>
<td>CMV-A positive/DR positive</td>
<td>5</td>
</tr>
<tr>
<td>CMV-A positive/DR negative</td>
<td>4</td>
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° Two aspirates were at the time of concomitant CMV infection and ATR.
Therefore, the usual criteria for diagnosing viral increases in immunoblast/plasma cell infiltrates. Aspirates with monocytosis had concomitant in-
and three with aspirate monocytosis. These findings
three aspirates have similar degrees of DR staining. Two of the
ion. In contrast, CMV-infected and noninfected patient
significantly more DR staining than do those without rejec-
tion. (B) DR staining. Aspirates from rejecting patients have
CMV infec-
rejection column, the aspirate with 44% positive CMV-A
staining than do those without infection. In the acute
episode within 2 wk (70%).
patients (52% each); the other preceded an acute rejection
for viral infection (increased aspirate and peripheral
blood large granular lymphocytes and activated pe-
ripheral blood) (7–9). Of the seven CMV positive as-
pirates, there were three with increased numbers of
peripheral blood large granular lymphocytes, two
with immunoblasts and/or plasma cells in the pe-
ripheral blood, three with these findings in the aspir-
ate, three with elevated total corrected increment,
and three with aspirate monocytosis. These findings
varied among the samples; for example, none of the
aspirates with monocytosis had concomitant in-
creases in immunoblast/plasma cell infiltrates.
Therefore, the usual criteria for diagnosing viral in-
fecions were not helpful in evaluating these aspir-
ates.
Donor and recipient CMV status before transplan-
tation was reviewed. Of the five patients who devel-
oped CMV infection, four were donor +/recipient−
(80%) and one was donor +/recipient + (20%). In
contrast, only 2 of 22 patients who were CMV-A
negative without CMV disease were donor +/recipient−
(9%) (P < 0.02 versus CMV infected) before transplan-
tation. The remaining patients were evenly
divided among the other donor/recipient combina-
tions. Patients with ATR had no predominant donor/
recipient CMV status represented.
Eleven core renal biopsies were performed within
4 days before or 4 days after a specific fine needle
aspirate. None of these were in CMV-infected pa-
tients or were in conjunction with CMV-A-positive
aspirates. Six biopsies demonstrated ATR, and all
responding aspirates had DR-positive tubular cell
staining. The remaining five biopsies showed mild
focal interstitial inflammation and/or tubular cell
injury consistent with cyclosporine administration
and/or acute tubular necrosis at the time of CMV-A-
and DR-negative aspirates.
The various clinical diagnoses were examined rela-
tive to the postransplantation time of aspiration.
CMV infection with CMV-A-positive aspirates was
observed from 5.7 to 10.7 wk postoperatively, with
an average time of 7.4 ± 0.9 wk. This was similar to
the average time of the unexplained events (7.3 ± 1.6
wk). In our cohort of patients, CMV infections oc-
curred later than did ATR (4.9 ± 0.4 wk; P < 0.02).
Other findings, which consisted primarily of acute
tubular necrosis and acute cyclosporine nephrotox-
ity, occurred even earlier in the postransplant
course (3.3 ± 1.0 wk).

**DISCUSSION**

This study has examined the expression of HLA
class II antigen (DR) and presence of CMV early nu-
clear protein (CMV-A) in tubular cells from renal
allograft fine needle aspirates. CMV-A staining was
found in nine aspirates, seven of which were per-
formed at the time of CMV infection. No CMV infec-
tion was present in the absence of CMV-A staining.
DR-positive tubular cell staining was associated sig-
nificantly with ATR. Absent DR staining was never
observed in the presence of ATR.
It has been suggested that CMV infection in human
renal allografts is associated with the upregulation
of HLA class II antigen in lymphocytes and renal
parenchymal cells [17,18]. Our study failed to dem-
onstrate any specific correlation between tubular cell
DR expression and CMV infection. In contrast, we
found DR positivity to be a reliable way of discrimi-
nating between CMV infection and ATR. Many in-
vestigators have documented the association of elevated tubular cell DR expression with ATR (16,19-22). In the experience of Von Willebrand et al., 12 of 14 CMV infected patients with DR-positive aspirates had overt ATR before CMV infection (17). Other investigators have demonstrated that CMV infection in renal transplant recipients has a strong temporal association with ATR (22-24) and that ATR only is associated with elevated DR (22). Our data suggest that ATR frequently precedes CMV infection and that the rejection is responsible for the increase in tubular cell DR staining associated with CMV infection.

CMV infection can be followed by ATR (25,26) with viral induction of γ-interferon and subsequent HLA class II expression as a suggested mechanism (27-29). However, some studies have failed to demonstrate elevated DR expression in transplanted islet cells after γ-interferon treatment (30,31) or any relation between DR increases and subsequent ATR (31,32). The use of cyclosporine may also modify the production of γ-interferon (33). Additionally, γ-interferon and viral infection enhance the expression of HLA class I antigens on epithelial cells, possibly influencing development of ATR by this means (30,34). Alternatively, the decreased immunosuppression usually administered during viral infection may predispose patients to ATR, irrespective of HLA presentation. Our findings imply that DR induction in tubular cells is not the mechanism underlying the association of CMV infection and ATR, but that DR positivity is a useful means of identifying the onset of ATR in the face of infection.

Several aspirates were done at the time of unexplained events. Although the exact causes of these transient episodes of fever and/or renal dysfunction cannot be proven, several etiologies are possible. CMV infection is often a self-limited illness in transplant recipients, causing minimal systemic symptoms (35). Additionally, CMV serologies may be unreliable in allograft recipients as antibody production can be compromised in these patients (30,34). Therefore, it is possible that undiagnosable CMV infection was present in the CMV-A-positive patient with an unexplained febrile illness and normal serologies. As is the case with CMV infection, ATR may occur as a self-limited event. This could have accounted for the three unexplained events at the time of DR-positive aspirates (Table 2). Functional or hemodynamic factors related to drug effects or other abnormalities can also result in an elevated serum creatinine (38) and may have been responsible for episodes of transient renal dysfunction associated with CMV-A and DR-negative aspirates.

The observed associations of specific donor and recipient CMV status with CMV infection, and time of onset of CMV disease, are in agreement with those reported in the literature. Positive donor CMV titers portend a higher likelihood of new or reactivated CMV infections, ranging from 33 to 90% (39-41). We found no CMV infections in patients receiving CMV-negative donor kidneys; however, in larger studies, CMV infection has occurred in this group of recipients (40,41). CMV infections most often occur between 4 and 16 wk posttransplantation (24,35,42). The average onset of disease in our patients was 7.3 wk after engraftment, well within this time frame. The onset of ATR is generally within the first 4 to 6 wk posttransplantation, as was the case in our patients. The other findings occurred even sooner (3.3 wk) and largely represented postoperative acute tubular necrosis and acute cyclosporine nephrotoxicity. Interestingly, the unexplained events occurred in a similar time frame as did the CMV infections. This might suggest that they represent CMV or other viral infections, although ATR and functional abnormalities remain as possibilities.

The optimal use of renal transplant fine needle aspiration in a serial fashion is underscored by the results of this study. In those patients with CMV infection, the percentage of CMV-A-positive tubular cells increased in sequential aspirates until the clinical diagnosis of CMV infection was made. Repeated aspiration of allografts may predict the onset of significant CMV infection and allow therapy to be altered or initiated accordingly. Additionally, the degree of tubular cell staining may correlate with disease severity. CMV-A-positive patients who had previously treated ATR had concomitant reductions in the percentage of DR-positive epithelium; DR staining decreases by 2 wk after successfully treated ATR (16,43). Therefore, DR status can be evaluated to assess the possible recrudescence of ATR, affording the transplant physician specific information to aid in the successful management of the patient. Fine needle aspiration is best used in a serial fashion, to follow closely alterations in renal inflammation, parenchymal cell injury, and response to therapy (12,44). The evaluation of CMV-A and DR antigens in aspirate material expands the advantages of the close monitoring of renal transplants with aspiration cytology.

The assessment of CMV-A and DR antigens in allograft fine needle aspirates is a rapid, easy technique for differentiation of CMV infection from ATR. The concomitant evaluation of CMV-A and DR antigens, particularly in a serial fashion, may also show when ATR and CMV infection coexist. Although this method is not 100% sensitive, no patients with CMV-A-negative epithelium had a CMV infection diagnosed by other methodologies. Therefore, the specificity is 100% in our small sample. Larger studies should be done to determine more accurate sensitivity and specificity levels and to evaluate further the use of CMV-A and DR staining in clinical settings.

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The application of these antibodies to allograft aspiration material is potentially a powerful tool for the transplant physician in distinguishing between ATR and CMV infection or other etiologies of allograft dysfunction. This distinction will allow for more rapid initiation of appropriate therapy and may positively impact graft and patient survival.

REFERENCES