Immunoglobulin M to Cytomegalovirus in Primary and Reactivation Infections in Renal Transplant Recipients

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Two commercially available enzyme immunoassays and one assembled in house were used to measure immunoglobulin M (IgM) antibody to cytomegalovirus (CMV) in a total of 220 serum specimens from 104 renal transplant recipients. All assays included a step in which interfering IgG antibody was removed or complexed. Concordance of results between pairs of assays ranged from 84 to 96%. All sera from patients with recent seroconversion (primary CMV infection) had measurable anti-CMV IgM. Among those already seropositive to CMV when transplanted, 26 to 55% had IgM antibody posttransplant, depending on the assay. This was observed regardless of the CMV serologic status of the kidney donor, indicating that reactivation of endogenous CMV, as well as reinfection, can induce this antibody in transplant recipients. Four cadaver donors known to transmit CMV to eight recipients did not have measurable IgM antibody to CMV.

Detection of immunoglobulin M (IgM) antibody to cytomegalovirus (CMV) has been proposed as a rapid method of diagnosing primary infection with this virus. Studies have shown that IgM antibody to CMV in pregnant women generally signifies recent primary infection, whereas women whose reactivated or recurrent CMV infection seldom have this antibody (7, 13). However, in organ transplant populations, CMV-specific IgM antibody is observed in some persons with nonprimary CMV infections (11, 12) although the frequency and significance of this remains incompletely defined because of the relatively small numbers of sera tested and reported so far. Among previously CMV-seropositive renal transplant recipients, Betts and Schmidt (2) observed that cytolytic IgM antibody to CMV occurred almost exclusively in those with a seropositive kidney donor, suggesting that the antibody response may have reflected reinfection with a donor strain of CMV. Restriction enzyme analysis of viral isolates from pairs of kidney recipients confirms that CMV reinfection does occur when seropositive kidneys are transplanted into seropositive recipients (4). This has prompted us to survey a large number of renal transplant recipients, with emphasis on determining whether the presence of anti-CMV IgM in previously seropositive patients denotes reinfection with a donor CMV strain. We also examined a few serum samples from donors known to have transmitted CMV infection to their recipients, because a previous report indicated that a substantial number of infective transfusion donors appeared to have IgM antibody to CMV (1).

Measurement of IgM antibody has been hampered by a variety of technical problems causing interassay variability (7, 14), including antigen competition by concomitant IgG antibody and false-positive reactions caused by rheumatoid factor (RF). One solution to these problems is to remove IgG antibody in the serum specimen by absorption or binding methods (8). Such a strategy is incorporated into each of the three enzyme-linked immunosorbent assays (ELISAs) used in this study. The first assay kit (Cytomegela-sa-M; MA BioProducts, Walkersville, Md.), has recently been licensed for clinical diagnostic use, and its performance has been examined and compared with radioimmunoassay in several studies (3, 5, 13). It uses staphylococcal protein A to remove IgG from test sera. A second ELISA is commercially available (Enzygnost anti-CMV IgM; Behring Diagnostics, La Jolla, Calif.), for which published performance data have not yet appeared. It uses a sheep hyperimmune anti-human-IgG serum to bind IgG in test sera. No phase separation step is needed. We have assembled a third ELISA in our own laboratories, where the same method is used to bind IgG, but in which the ELISA plates are freshly coated with glycine-extracted strain AD-169 CMV antigen prepared by a standard method (10).

MATERIALS AND METHODS

Sera. Serum samples were collected from renal transplant recipients who were being routinely monitored for CMV serologic changes posttransplant. Classification of sera as CMV seropositive or seronegative was on the basis of an ELISA for IgG antibody to CMV performed as described previously (6), except that plates were coated with CMV and control antigen as described below. We have run over 3,000 assays with this ELISA and have verified its performance with several commercially available assays (data not shown). For this study, 220 serum samples from 104 recipients were chosen; these recipients were at risk for primary CMV infection (seronegative recipient with seropositive donor) or were already seropositive for CMV and presumed to be at risk for CMV reactivation or reinfection or both. Most patients had both pretransplant and posttransplant (6 weeks to 4 months posttransplant) specimens tested. Some of the patients had viral cultures of urine done when the sera were collected. Four additional serum samples were from four cadaver donors that were known to have transmitted CMV to eight recipients. Sera were stored frozen at −70°C. All comparative analyses were done with serum samples that were within two freeze-thaw cycles of each other.

Assays. All ELISAs were based on absorption of viral antigen on to a plastic surface and sequential incubations with pretreated test sera, enzyme-linked antibody to human IgM, and enzyme substrate. Commercial ELISA kits for

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assay of CMV IgM antibody were used as directed by the manufacturers. The MA BioProducts assay is done at 1:55 final serum dilution after pretreatment with a staphylococcal IgG absorbent and centrifugation, whereas the Behring assay is done at 1:42 final serum dilution after brief incubation with a sheep hyperimmune anti-human-IgG serum. The sheep anti-IgG serum (8) has a capacity to bind 15 mg of IgG per ml and is standardized by the manufacturer. It forms immune complexes with IgG in test sera, which in turn bind rheumatoid factor. Binding of CMV antigen by specific IgG is also inhibited. The ELISA used in our research laboratory was based on locally prepared CMV-infected cell antigen and is described below. RF in all sera testing positive by one or more ELISAs was assayed with a commercially available qualitative latex agglutination test (Rapi/tex RF Test; Behring Diagnostics).

CMV antigen. Viral antigen was extracted with glycine buffer from human foreskin fibroblasts infected at high multiplicity with CMV strain AD-169 as previously described (10). A 5-ml sample of antigen concentrate was prepared from a 500-cm² roller bottle culture. An uninfected cell control antigen was prepared by the same procedure, but with uninfected monolayers of fibroblasts.

Antigen coating of ELISA plates. A 1:30 dilution of CMV and uninfected cell control antigen, prepared as described above, was made in phosphate-buffered saline (PBS), pH 7.4. Polystyrene 96-well flat-bottom ELISA plates (Immulon-2; Dynatech Laboratories Inc., Alexandria, Va.) were coated in alternate paired columns with 0.05 ml of the diluted CMV and cell control antigens, respectively, per well. Plates were then covered with plastic film and held at 4°C until used, within 10 days of coating. Before use, plates were washed three times with PBS containing 0.05% Tween 20, emptied, and immediately loaded with 0.2 ml of PBS containing 10% horse serum per well. After holding for 2 h at 37°C, the plate was emptied, and test sera were loaded.

ELISA assay procedure. Test sera were diluted 1:20 in PBS–10% horse serum. Sera used as positive controls (from patients with recent primary CMV infection) and negative controls (CMV seronegative) were diluted likewise. To remove rheumatoid factor and competing IgG anti-CMV antibody, 0.05 ml of this serum dilution was added to an equal volume of sheep anti-human IgG serum (RF absorbent; Behring Diagnostics) and mixed. After 1 h at room temperature, the mixture was further diluted with 0.15 ml of PBS–10% horse serum, making a final 1:100 serum dilution. Samples (0.05 ml) of this dilution were loaded in duplicate on to wells previously coated with CMV antigen and with control antigen (four wells per test serum). Plates were then covered with plastic film and held at 37°C for 2 h. Plates were then washed three times with PBS–0.05% Tween 20, emptied, and loaded with 0.05 ml of a 1:2500 dilution of peroxidase-conjugated goat anti-human IgM (Tago, Burlingame, Calif.) per well. After 2 h at 37°C, plates underwent a final washing with 10 cycles of PBS–0.05% Tween 20 and 5 cycles of distilled water. Wells were then loaded with 0.1 ml each of substrate solution consisting of 0.5 mg of 2,2′-azino-bis(3-ethylbenzthiazolin sulfonic acid) (ABTS; Sigma, Chemical Co., St. Louis, Mo.) per ml and 0.03% hydrogen peroxide in 50 mM citrate buffer (pH 4.5). After 15 min, the reaction was stopped with 0.1 ml of a 1% aqueous solution of electrophoresis-grade sodium dodecyl sulfate per well. Absorbance was read at 405 nm on an automated ELISA reader (MR 600; Dynatech). Specific absorbance was calculated by averaging the absorbance in the two CMV antigen wells and subtracting from this the average absorbance of the two cell control antigen wells. A specific absorbance greater than 0.2 was considered positive in this assay.

**RESULTS**

Concordance of ELISA results. Sera testing positive for anti-CMV IgM had an average (standard deviation) specific absorbance of 1.175 (0.587), 0.494 (0.241), and 0.683 (0.484) for the MA, Behring, and in-house assay, respectively. With 105 serum samples tested using both the commercial assays, there was an 85% concordance of results when the positive cutoff criteria specified by the manufacturer were used in each case (Table 1). We chose a positive cutoff of 0.2 specific absorbance units for our in-house assay. This gave the best correlation with the MA assay and was also well above values obtained with CMV-seronegative sera; 30 of these serum samples had an average signal of 0.009 absorbance units, with a range of 0.002 to 0.026. Using a positive cutoff of 0.1 on our assay would have given a positive result with 18 additional serum samples, none of which represented primary infection; 4 were associated with known concurrent viruria, an additional 2 were from patients who shed CMV some time in the first 3 months posttransplant, and 6 each were pre- and posttransplant specimens from asymptomatic seropositive recipients. Most of the discordant results were encountered with serum samples from CMV-seropositive recipients, which registered a positive result with the MA and in-house assays but negative on the Behring assay. Two of three serum samples, IgM-negative on the MA assay but positive on the Behring assay, were negative on repeat testing with the latter assay; these samples were from seropositive patients who did not seroconvert after several further months of follow-up. One serum sample from a patient with primary infection was initially negative with the Behring kit because of high reactivity with uninfected cell antigen; on repeat testing, reactivity with control antigen decreased enough to give a negative result. With 102 serum samples tested with the MA kit and the in-house assay, there was a 96% concordance of results (Table 2). The only discordant results involved two pretransplant and two posttransplant serum samples from seropositive recipients, which were positive only on the MA assay. One of these four serum samples involved a patient reinfected with a donor strain of CMV (signal 0.112 with in-house assay); with the other three
serum samples there was no evidence of active CMV infection at the time. Concordance of the Behring assay with the in-house assay (203 specimens, 84% concordance, Table 3) was similar to that with the MA assay. The in-house assay and the Behring kit used the same RF removal method; the main difference is the antigen coating on the ELISA plates, although the serum dilution, the enzyme-conjugated second antibody, and the substrate system were also different, and all specimens were loaded in duplicate on our in-house assay.

RF. A total of 82 serum specimens were positive for IgM to CMV on one or more of the three ELISAs. Eight of these were positive for RF, and the rest were negative. Of the serum samples positive for RF, five were from patients with primary CMV infection as documented by a recent seroconversion and compatible clinical illness. All IgM ELISAs on these five serum samples with two or all three assays, were strongly positive, as appropriate for a primary CMV infection. The remaining three samples were from seropositive transplant recipients 2 months posttransplant; one of these recorded positive on the Behring assay, and all three were positive with the in-house assay. These represent a small fraction of the positive results recorded with sera in this recipient subset (Table 4). The adequacy of the RF-removal steps used in these assays has been previously documented (8).

IgM antibody in recipient groups. Prevalence of IgM antibody in seropositive and seronegative recipients is shown in Table 4. Seronegative recipients were negative for IgM antibody at time of transplant and at follow-up, unless they acquired primary infection, in which case 100% developed IgM antibody. Primary infections were defined by seroconversion (negative to positive by IgG ELISA [6]), and all were confirmed by detection of viruria or viremia.

Pretransplant serum samples from seropositive recipients had a 5 to 25% prevalence of IgM antibody, depending on the assay. Three of these samples were from patients who were known to have had a primary CMV infection (seroconversion) within the past year; the other serum samples testing positive did not appear to be associated with active CMV infection. The one specimen in this study known to come from a viruric pretransplant patient tested negative on all three assays.

Posttransplant serum samples on seropositive recipients showed that 26 to 55% (depending on the assay) had IgM antibody. This occurred whether the organ donor was seropositive or seronegative. Seropositive recipients of seropositive kidneys had anti-CMV IgM more frequently than recipients of seronegative kidneys, but the difference was not statistically significant and could have been related to the prevalence of viral shedding in the two groups. Taking only patients known to be shedding virus at the time of specimen collection, 3 of 5 recipients of a seronegative kidney, and 7 of 12 recipients of a seropositive kidney had CMV IgM by the in-house assay. Posttransplant specimens were available on three recipients with molecular epidemiologic evidence of reinfection (4). One was highly reactive in all three assays, one was positive on two assays (MA and in-house), and the third was positive only on the MA assay.

The intensity of positive signals tended to be highest among those with primary infection, intermediate among seropositive recipients of seropositive kidneys, and lowest among seropositive recipients of seronegative kidneys, but there was a considerable overlap among these groups. For example, with our in-house assay, the average (standard deviation) positive signal was 0.836 (0.407), 0.744 (0.527), and 0.541 (0.450) absorbance units for the three patient groups, respectively.

IgM antibody in donor sera. Serum samples from four seropositive kidney donors who transmitted CMV to eight recipients (confirmed by restriction enzyme analyses) were assayed for IgM with the in-house ELISA; none was positive. One was tested with all three ELISAs and was negative on all of them.

**DISCUSSION**

Using three ELISAs, we found specific IgM antibody to CMV in all instances of posttransplant primary CMV infection where IgG seroconversion had occurred. We also found the antibody in a some CMV IgG-seropositive recipients at time of transplant and considerably more of them at posttransplant follow-up, regardless of the serologic status of the donor. The observation of IgM antibody in up to 44% of seropositive recipients of a seronegative kidney, including some known to be viruric, implies that reactivation of endogenous virus is sufficient to induce this antibody. Seropositive recipients of seropositive kidneys tended to have IgM antibody more frequently and with stronger signals than those who received seronegative kidneys, but these differences were not statistically significant. It remains possible that reinfection with a donor strain frequently induces high levels of IgM antibody, similar to primary infection, but we have already encountered a reinfeected recipient where IgM antibody was detectable on only one of the three assays used. The presence of IgM in a given seropositive recipient, as measured with these ELISAs, cannot be considered suggestive of reinfection with a donor strain. This is in contrast to a previous study (2), where nearly all seropositive recipients with cytolytic IgM antibody posttransplant had received a seropositive kidney and might therefore be reinfeected with a new viral strain. Whether this relates to a difference in the type or quantity of antibody

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**TABLE 3.** Results of testing 203 sera for IgM antibody to CMV with the Behring ELISA kit and an in-house ELISA

<table>
<thead>
<tr>
<th>Behring ELISA (cutoff, 0.2 absorbance unit)</th>
<th>In-house ELISA (cutoff, 0.2 absorbance unit)</th>
<th>No. negative</th>
<th>No. positive</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. negative</td>
<td>139</td>
<td>6</td>
<td>145</td>
<td></td>
</tr>
<tr>
<td>No. positive</td>
<td>26</td>
<td>32</td>
<td>58</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>165</td>
<td>38</td>
<td>203</td>
<td></td>
</tr>
</tbody>
</table>

* Assay concordance was 84%.

**TABLE 4.** Prevalence of IgM antibody to CMV in transplant recipient subsets as measured by three ELISAs

<table>
<thead>
<tr>
<th>Kidney recipient group</th>
<th>ELISA IgM, no. positive/no. studied (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seronegative at transplant</td>
<td>MA Behring In house</td>
</tr>
<tr>
<td>Pretransplant specimens</td>
<td>0/20 (0) 12/23 (52) 12/12 (100) 0/11 (0)</td>
</tr>
<tr>
<td>Primary infection</td>
<td>0/20 (0) 12/23 (52) 12/12 (100) 0/11 (0)</td>
</tr>
<tr>
<td>No infection</td>
<td>0/20 (0) 12/23 (52) 12/12 (100) 0/11 (0)</td>
</tr>
<tr>
<td>Seropositive at transplant</td>
<td>Pretransplant specimens</td>
</tr>
<tr>
<td>Posttransplant specimens</td>
<td>22/40 (55) 23/90 (26) 42/91 (46)</td>
</tr>
<tr>
<td>Seronegative donor</td>
<td>7/16 (44) 9/42 (21) 15/41 (37) *</td>
</tr>
<tr>
<td>Seropositive donor</td>
<td>15/24 (63) 14/48 (29) 27/50 (54) *</td>
</tr>
</tbody>
</table>

* P = 0.074; seronegative versus seropositive donor, Fisher exact test.
measured or to some difference in the patient populations remains unclear.

Only about half of reactivation infections, as defined by viral shedding in a seropositive recipient, are associated with measurable IgM antibody. This is consistent with previously published data (9, 11). We also did not find IgM antibody to CMV in four cadaver donors who transmitted CMV to eight recipients. Thus, lack of this antibody in prospective donors does not exclude CMV infectivity and is not an adequate donor screening procedure, although a previous study showed that 12 of 28 patients developing posttransfusion CMV infection had received blood containing CMV IgM (1).

Published experience with CMV-specific IgM assays, as well as our own experience in assembling an ELISA, indicate the need for a strategy to eliminate false-positive reactions due to RF and to reduce signal suppression due to IgG antibody competition for the same antigen. We have found the methods used in the present assays for removal of reactive IgG to be attractive because they allow the use of an assay protocol closely analogous to that used for measuring IgG antibody, a much more frequently used test. Use of sheep anti-human-IgG serum, as in the Behring assay and our in-house assay, is especially convenient since this eliminates a time-consuming centrifugation step needed with staphylococcal absorption in the MA assay.

In comparing the two commercial assays, both did well in identifying IgM in those with recent primary infection with seroconversion, but the Behring assay, which appears to be less sensitive, identified fewer of those with reactivation or reinfection or both. Our in-house assay, which uses a different antigen from the Behring kit but is otherwise similar, gave many more positive results with the same sera, and was closely concordant with the MA kit. Deciding the proper cutoff level for a positive result is problematic, since there is no generally recognized reference assay, and there is a tendency for borderline results with sera from seropositive recipients, in whom evidence of active infection is often inconclusive. A recent study (5) suggested that the correlation of the MA assay with radioimmunoassay was best when the ELISA cutoff was increased from 0.3 absorbance unit, as suggested by the manufacturer, to 0.6 absorbance unit, indicating that the MA assay may be overly sensitive. This could account for some of the pretransplant sera that tested positive on the MA assay, in recipients who did not appear to have active CMV infection.

ACKNOWLEDGMENTS

We thank Julie Serra for assistance with referral of clinical specimens and Behring Diagnostics for donating ELISA test kits and materials.

This work was supported by Veterans Administration Research Funds.

LITERATURE CITED


