Evaluation of Anti-Complement Immunofluorescence Test in Cytomegalovirus Infection

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The anti-complement immunofluorescence (ACIF) technique was evaluated for the diagnosis of human cytomegalovirus (CMV) infection in a group of sera derived from renal transplant recipients and donors by comparing it with the indirect immunofluorescence (FA) and complement fixation (CF) tests. The ACIF and FA tests yielded similar results. However, the ACIF test had a distinct advantage over the indirect FA test, since it eliminated the nonspecific cytoplasmic staining that may result in false positive readings in inexperienced hands. Both the indirect FA and ACIF tests were more sensitive than the CF test. In primary CMV infection, the FA and ACIF antibodies appeared earlier and had significantly higher titer than corresponding CF titers. This difference in titers was not seen in seropositive individuals who lacked overt infection. Our previously reported correlation between the seropositivity of the donor and CMV infection in seronegative recipients has been confirmed.

Antibodies to human cytomegalovirus (CMV) have been demonstrated by the neutralization, platelet aggregation, indirect hemagglutination inhibition, complement fixation (CF), and indirect immunofluorescence (FA) tests (1-3, 6, 9, 20). The CF test is specific and practical when large numbers of sera are to be tested, but it does not discriminate between various human strains of CMV (9, 10). There also seems to be some overlapping of CF antigens between simian and human CMV strains (10) but not with other herpesviruses (4). It has been observed that the CF test is not as sensitive as the indirect FA test in pregnant mothers (28) and neonates with CMV infection (19, 27, 31) when the CF antigen was prepared by freeze-thaw disruption of infected cells. Recently, Betts and co-workers showed that, if the antigen was prepared by glycine extraction, the CF test may be as sensitive as the indirect FA method (8). The indirect FA test was thought to be sensitive and specific (9, 23, 25, 28), until Keller et al. (18) and Furukawa et al. (11) showed that CMV-infected cells developed Fc receptors that could bind the immunoglobulin G (IgG) from immune and nonimmune sera, giving rise to nonspecific cytoplasmic staining and resulting in false positive readings.

The anti-complement immunofluorescence test (ACIF) is a modification of the indirect FA method (13). The former requires the addition of complement either during or after the exposure of target cells to the antibody. The bound complement is detected with fluorescein isothiocyanate-conjugated anti-C3. The advantage of ACIF over the indirect FA test is that IgG bound to Fc receptors does not fix complement and will not stain with fluorescein isothiocyanate-conjugated anti-C3, thereby eliminating the nonspecific cytoplasmic fluorescence seen in the indirect FA test as shown with CMV by Keller et al. (18). Fluorescence is restricted to the areas of antigen-antibody reaction in the nucleus. This report evaluates the ACIF technique in a group of human sera derived from a study of CMV infection associated with renal transplantation by comparing it with the indirect FA and CF methods.

MATERIALS AND METHODS

Sera. Sera from 34 renal transplant recipients and their respective donors were examined by the CF, indirect FA, and ACIF tests. Complete series (pretransplantation, donor, 12 posttransplantations at monthly intervals) of sera were not available on all patients.

Antigens. The AD-169 strain of CMV was used as a source of antigen for all three tests. The CF test was performed by the microtiter method of Takatsky (29) and Sever (24), modified to contain 2 U of antigen and 5 U of complement. The end point was the highest dilution of serum giving 75% (3+) fixation. The AD-169 antigen was prepared by freeze-thaw disruption of infected cells (Microbiological Associates, Inc., Bethesda, Md.).
The antigen for the indirect FA and the ACIF tests was prepared as described by Stagno et al. (27). Human fibroblast cultures, passages 5 through 10, were prepared from foreskin of newborns. Confluent monolayers of fibroblasts were grown in 75-cm² plastic bottles (Lux Scientific Corp.) using Eagle modified minimum essential medium (Flow Laboratories, Inc., Rockville, Md.) containing 5% fetal calf serum. Cells were infected with 10 ml of supernatant fluid from CMV-infected cultures at an input multiplicity of approximately 1 for 1 h at 37°C. Cells were detached by exposure to 0.25% trypsin when the cytopathic effect reached 80 to 90%, usually on day 5. The trypsin was neutralized with an equal volume of calf serum. The cells were washed three times with phosphate-buffered saline (PBS; pH 7.4) and mixed with an equal amount of similarly prepared uninfected cells, which served as negative controls. Drops (25 µl) of cell suspension in PBS, containing approximately 20,000 cells, were placed on each of the eight 0.5-cm wells on preprinted slides (Carlson Scientific, Matterson, Ill.). Slides were air dried, fixed in cold acetone for 10 min, and stored at −70°C until use.

**Indirect FA test.** For the indirect FA test, target cells were exposed to serial two- or fourfold dilutions of test serum, incubated for 60 min, and then exposed to fluorescein isothiocyanate-conjugated goat anti-human immunoglobulin, IgG fraction (Cappel Laboratories, Downington, Pa.) diluted 1:60 in PBS. After each step, the slides were washed at least twice in PBS. The slides were then mounted with 90% glycerin-10% PBS and examined using the ×45 objective of an American Optical microscope with model 2070 incidental light fluorescent illuminator. A Schott BG-12 exciter filter and Wratten 15(G) barrier filter were used. The end point was read as the final dilution of serum giving detectable nuclear fluorescence compared with control negative serum.

**ACIF test.** The ACIF test was carried out in three consecutive steps according to Henle et al. (14). (i) The fixed-antigen slides (same as in indirect FA assay) were incubated with dilutions of heat-inactivated (56°C for 30 min) serum for 60 min at 37°C. (ii) Human complement (fresh CMV-negative serum as determined by indirect FA test, diluted 1:30) was applied for 60 min at 37°C. (iii) The cells were then stained with fluorescein isothiocyanate conjugate-labeled goat anti-human complement diluted 1:30 (Behring Laboratories) for 60 min at 37°C. After each staining step and at the end of staining, the slides were washed at least twice with PBS. The slides were mounted and examined as described above. The end point was the final dilution of serum giving detectable nuclear fluorescence. All sera were read under code by one person. All reagents were stored at −70°C before use. Appropriate controls, including conjugate only and antigen treated variously with a known CMV-positive serum, a CMV-negative serum, complement, and conjugate only, were used.

**RESULTS**

**Fluorescence-staining pattern.** In the indirect FA test, two patterns of specific fluorescence were seen with positive sera. At low dilutions, a bright fluorescence involving entire infected cells was evident. With increased dilutions of serum, this fluorescence seemed to be concentrated in the nuclear area. Care was taken to distinguish specific nuclear fluorescence from nonspecific cytoplasmic fluorescence, presumably due to the binding of IgG to the Fc receptor, which appeared in the perinuclear region either as one or two intensely staining polar bodies or as an arcuate structure adjacent to the nucleus (18). Infected cells with the negative control serum showed only the Fc-receptor staining. No fluorescence was seen in uninfected control cells.

Figure 1 illustrates the characteristic staining seen in the ACIF test. Fluorescence was restricted to the nuclear area, which appeared to be packed with coarse granules. Nonspecific Fc-receptor staining was not seen with this technique (18). Specific nuclear fluorescence and the end point of antibody titration were more distinct than with the indirect FA test.

**Frequency of detectable antibodies by CF, indirect FA, and ACIF tests.** Table 1 illustrates the frequency of antibodies detectable by the three methods in 277 sera from renal transplant patients. A serum was defined as positive when the titer was ≥1:4 by any one method. There was little difference between the results obtained by indirect FA and ACIF tests, although in a few instances antibodies were detected by ACIF and not by indirect FA test. There was no instance in which a serum was positive by indirect FA and negative by ACIF test. On the other hand, 32 sera were positive by ACIF but negative by CF. This difference was statistically significant (P < 0.001 by y²).

**Comparison of antibody titers determined by CF, indirect FA, and ACIF.** Figure 2 correlates titers obtained by ACIF and indirect FA tests. In most cases, these titers were similar, except for a few instances in which there was more than a two- or fourfold difference.

On the other hand, when ACIF and indirect FA titers were compared with the corresponding CF titers, there was a distinct difference. Table 2 presents the geometric mean titers of the three groups of patients. Transplant recipients who developed primary CMV infection, defined by seroconversion and virus isolation, showed peak antibody titers of ≥4,096, as measured by ACIF or FA tests, which were significantly higher than the corresponding CF titers. There were seven other patients with primary CMV infection, not shown in Table 2, whose peak CF titers were ≥256. Although lack of material made retesting of these sera impossible, sera collected earlier and later than the peak had CF titers ranging from 32 to 256 and FA and ACIF titers of ≥4,096. Data from these patients were therefore consist
ent with those derived from the seven patients with complete data shown in Table 2.

When the ACIF or FA titers of seropositive kidney donors and preoperative samples of seropositive recipients were compared with the corresponding CF titers, no such difference was noted. Thus, high antibody titers as measured by FA or ACIF appeared to be a feature of acute infection.

Patterns of antibody response in patients with primary CMV infection. Out of 26 recipients tested, 14 were seronegative before the
transplant and developed clinical, virological, and serological evidence of primary infection. The serial sera of these patients were tested by all three serological methods. In 9 out of 14 patients, the indirect FA and ACIF tests became positive at the same time. In two patients, a rise in ACIF titer preceded the rise in indirect FA titer by 4 and 30 days. The rises in ACIF and FA titers preceded the rise in the CF test by 1 to 2 months and were significantly higher. Results from four such patients are shown in Fig. 3. Viruria and/or viremia occurred at about the same time as seroconversion. In the remaining three patients, the CF test became positive on the same day as FA and ACIF. Similar observations with regard to early rise of FA antibodies were reported by Betts et al. (8) and Spencer and Andersen (25).

**Table 1.** Frequency of antibodies detected in 277 sera by CF, indirect FA, and ACIF tests

<table>
<thead>
<tr>
<th>Antibody test</th>
<th>Positive (%)</th>
<th>Negative</th>
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<tbody>
<tr>
<td>CF</td>
<td>170 (61)</td>
<td>107</td>
</tr>
<tr>
<td>FA</td>
<td>199 (72)</td>
<td>78</td>
</tr>
<tr>
<td>ACIF</td>
<td>202 (73)</td>
<td>75</td>
</tr>
</tbody>
</table>

* P < 0.001 for ACIF versus CF by χ².

**Table 2.** Geometric mean CMV titers of patients' sera

<table>
<thead>
<tr>
<th>Patient group</th>
<th>No. in group</th>
<th>Reciprocal of dilutiona</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CF</td>
</tr>
<tr>
<td>Seropositive donors</td>
<td>15</td>
<td>19 (4.25 ± 0.30)</td>
</tr>
<tr>
<td>Seropositive recipients (pre-operative bleed)</td>
<td>9</td>
<td>24 (4.56 ± 0.71)</td>
</tr>
<tr>
<td>Primary CMV, infection (peak titers)</td>
<td>7b</td>
<td>315 (8.25 ± 1.06)</td>
</tr>
</tbody>
</table>

* Log₂ reciprocal ± log standard error. The geometric mean titer and its expression in log₂ titer ± log standard error are given in parentheses.

* In seven other patients, valid comparisons could not be made (see text).

* When CF is compared with either FA or ACIF by the Wilcoxon rank-sum test for multiple samples (32), P < 0.01.
Role of transplant kidney in CMV infection. Using the CF test, we previously reported that CMV infection in seronegative recipients was highly correlated with seropositivity of the donor, indicating that the transplanted kidney may be the source of primary CMV infection in the recipient (17). Sera from these and some additional patients were examined by the indirect FA and ACIF methods. There were 23 recipients who were seronegative before transplantation by the indirect FA and ACIF tests (Table 3). Out of 15 whose donors were $\geq$1:4, 13 (86%) became infected, whereas none of the 8 who received kidneys from donors whose titers were <1:4 became infected. This difference is highly significant ($P < 0.001$ by $\chi^2$), thereby confirming our earlier observation. Similar results by the indirect FA test were obtained by Betts et al. (7).

<table>
<thead>
<tr>
<th>Donor's FA titer</th>
<th>Postoperative CMV infection in recipients*</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Absent</td>
<td>Present (%)</td>
</tr>
<tr>
<td>&lt;1:4</td>
<td>8</td>
<td>0 (0)</td>
</tr>
<tr>
<td>$\geq$1:4</td>
<td>2</td>
<td>13 (86%)</td>
</tr>
<tr>
<td>Totals</td>
<td>10</td>
<td>13 (57%)</td>
</tr>
</tbody>
</table>

*Infection was determined by seroconversion as determined by the FA and ACIF tests and in all cases by virus isolation in the urine.

DISCUSSION

The ACIF test has been extensively used to detect antibodies to Epstein-Barr nuclear antigen (21, 22) and in other viral infections, including those caused by myxoviruses and polioviruses (15, 16). It has been shown to be highly sensitive and specific in detecting complement-fixing antibodies. In our study of renal transplant recipients, it appears to be a good alternative to the indirect FA and CF assays for the diagnosis of CMV infection. Titers obtained by the ACIF method correlated well with titers obtained by the indirect FA test. In some instances, it appeared even more sensitive. The time of appearance of ACIF antibodies in acute infection also paralleled results with the FA test. However, in the indirect FA assay, the disadvantage is that nonspecific cytoplasmic fluorescence due to binding of IgG to Fc receptors may result in false positive readings in inexperienced hands (18). Fluorescence in the ACIF test is restricted to nuclear inclusions, and the serum end points are more distinct. Similar observations have been made by Stagno (personal communication; 26).

The CF test has been used widely, and false positive readings are rare (4), but there has been some question of its sensitivity in pregnant mothers with viruria (28) and in neonates with CMV infection (19, 27). Both indirect FA and ACIF tests detect antibodies more frequently and are more sensitive than the CF test. In patients with primary infection, immunofluorescent antibodies (FA or ACIF) appeared earlier than CF antibodies. Betts et al. (8) and Spencer and Andersen (25) showed similar results with the FA test. That antibodies were always detected by the CF test, although delayed by 4 to 6 weeks, seemed to indicate that similar types of antibodies were measured. However, patients with acute infection showed significantly higher immunofluorescent antibody titers (FA or ACIF) than CF titers. This difference in titers was not seen when FA or ACIF titers were compared with the CF titers of seropositive individuals who lacked overt infection.

It has been reported that CF and ACIF titers parallel each other closely in a number of viral infections, including dengue (5), poliovirus (15), and myxoviruses (16). Since the lack of concordance observed in the present study applied only to sera of patients with active infection, it is possible that the fluorescence methods are detecting antibody to the early CMV antigen (13, 30) which may not be present in the CF antigen preparations.

Our previously reported correlation between CMV infection in seronegative recipients and seropositivity of donors has been confirmed (17). According to the FA and ACIF tests, there was no case of CMV infection in seronegative recipients who received a kidney from seronegative donors. We previously reported three seronegative recipients who got their kidneys from seronegative donors but became infected (17). These results are explained by the more sensitive indirect FA and ACIF tests. By FA and ACIF tests, two of these recipients were initially seronegative. The third patient developed a primary infection, but the donor, seronegative by CF, was shown to be positive by the indirect FA and ACIF tests. Betts and his colleagues (7) also reported that all their patients' primary infections could be attributed to transplantation of kidneys from seropositive donors.

The ACIF test shares the advantages of an indirect FA assay over the CF test in terms of its sensitivity and the speed with which a given sample can be tested. Although the CF test using glycine-extracted antigen seems to be as sensitive as the indirect FA test (8), it is a more laborious procedure which requires an overnight incubation. In addition, sera that have anti-complementary activity can be tested by FA and ACIF tests. Our impression is that, if a single test has to be used to measure antibodies against
CMV, the ACIF technique is quick, sensitive, and easily interpretable.

ACKNOWLEDGMENTS

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