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Comparative Activity of Immunofluorescent Antibody and Complement-Fixing Antibody in Cytomegalovirus Infection

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Three different tests for detection of antibodies to human cytomegalovirus (CMV), complement fixing with antigen prepared by freeze-thaw disruption (CF-FT) or with antigen prepared by extraction with alkaline glycine buffer (CF-GE) and immunofluorescent staining (FA), were compared in renal transplant recipients and their healthy donors. FA and CF-GE tests yielded positive results at an identical and significantly higher frequency than CF-FT in both donors and recipients. CF-GE and FA performed on donors and recipients predicted all virus shedding post-transplant, whereas CF-FT did not. In the individuals who developed primary infection concurrent with the transplanted kidney, FA developed earlier than other antibodies in about one-half and at the same time in the remainder. In addition, the FA test could be completed more quickly and all sera could be interpreted, which made the FA test more useful than the CF-GE, but both of these tests were clearly superior to CF-FT.

It is important that a serological test for detecting past or present virus infection be both sensitive and specific. The complement-fixing (CF) antibody test for human cytomegalovirus (CMV) using an antigen prepared by freeze-thaw disruption (FT) of infected cells has failed to detect antibody in some patients with congenital infection and is therefore considered insensitive (11, 17). In comparative studies, the immunofluorescent-antibody (FA) test has exhibited greater sensitivity than the CF test (2, 13, 15). However, since tissue culture cells infected with CMV develop Fc receptors to which immunoglobulin G (IgG) from immune and nonimmune sera adheres, the specificity of the FA test has been questioned by Furukawa and co-workers (6). The earlier studies of the usefulness of the FA test (2, 13, 15) have not resolved the problem of false-positive serological results.

Another method of preparing a CMV CF antigen, first applied by Krech et al. (10), consists of extracting the antigen from infected cells by incubation with alkaline glycine buffer (pH 9.5) (CF-GE). Recently, Cremer and co-workers compared the activity of the CF antigens prepared by the CF-GE method to that of the commonly used CF-FT antigen. These workers showed that more sera submitted to the California State Laboratory contained CMV antibody detected with CF-GE antigen than with the CF-FT antigen. In addition, most of the positive sera had a higher titer against CF-GE (3). Therefore, both FA and CF-GE appear to be more sensitive than the CF-FT test, but as yet there has been no comparison of the sensitivity of the FA test with that of the CF-GE test. Since complement fixation is more adaptable to widespread use than are FA techniques, the CF-GE test, if proven as sensitive and specific as FA, might be adapted for standard use. Therefore, we compared the FA test with the CF-GE test in a group of hemodialysis patients who underwent renal transplantation.

MATERIALS AND METHODS

Cells and media. Human embryonic lung fibroblasts (WI-38), obtained from Flow Laboratories, were used for both virus isolation and preparation of CF antigens. Human foreskin fibroblasts (HFF) prepared from foreskins obtained from the nursery for newborns were used for preparation of the antigen used in the FA test (2). Cells were maintained as described previously (1).

Virus stock. A strain of Ad-169 was obtained from Flow Laboratories. One other strain of CMV was used: an isolate from a patient that produced typical cytopathic effect in WI-38 cells and was neutralized by hyperimmune guinea pig antisera to Ad-169 (4). Antigen prepared from this strain was more active than Ad-169 antigen. For the CF test, both antigens were used to test each serum. A serum was interpreted as negative only when it did not react with either antigen.

Antigen preparation. The CF-FT antigen was prepared in WI-38 cells as described by Hanshaw (7), and the CF-GE antigen was prepared as described by Cremer and co-workers (3), except that the multiplicity of infection was 10 plaque-forming units per

cell and antigens were harvested at 6 days after infection, a time of maximum development of CF antigen (5). The antigen for the FA test was prepared in HFF by using a multiplicity of infection of 1 plaque-forming unit of Ad-169 per cell and harvesting on day 6.

CF test. Optimal antigen dilutions were determined by block titration. Complement was obtained from Beckman. The tests were performed by a microtechnique as described (7), with the following exceptions. The sheep erythrocytes were sensitized for 30 min at 37°C instead of 15 min at 25°C, and the final concentration of sheep cells was 2.5% instead of 1.4% (12). Antigen and antibody were added in equal volumes of 0.025 ml, and 0.05 ml of complement containing 6 50% hemolytic complement units was used. To insure complete suspension of the sensitized sheep erythrocytes, the microplates were vibrated on a Thomas vertical vibrator for 60 min at 37°C before centrifugation. A serum was considered negative if it failed to fix complement at a dilution of 1:4.

Immunofluorescent assay. The indirect FA test for IgG was performed according to the modification of the method of Chiang and co-workers (2). Fluorescein-conjugated goat anti-human IgG (gamma chain) (Hyland Laboratories) was used throughout. Reconstituted conjugate of goat anti-human gamma chain was diluted 1:5 in phosphate-buffered saline and absorbed for 18 h at 5°C with rabbit liver powder (Baltimore Biological Laboratories) at a ratio of 100 mg of powder per ml of reconstituted conjugate. The conjugate was then absorbed for 18 h at 5°C with Sephadex G-10 (Pharmacia Fine Chemicals) at a ratio of 100 mg of powder per ml of reconstituted conjugate, after which the Sephadex was removed by centrifugation. The conjugate was used at a final dilution of 1:100.

Infected cells were harvested on day 6 of infection by trypsinization and suspended at a density of 2×10^5 cells/ml, and droplets of 0.025 ml were placed on encircled spaces of glass slides; after attachment of the cells, the suspending medium was carefully aspirated. The dried slides were fixed in acetone at −20°C for 10 min, rinsed, and stored at −70°C until used. The test serum was serially diluted in phosphate-buffered saline from 1:4 to 1:2,048, and the staining procedure was carried out. After staining, the dried slide was covered with 90% glycerin-10% 0.5 M sodium carbonate buffer (pH 8.5) and a coverslip (20 by 50 mm), and examined with the ×40 objective of a Leitz fluorescence microscope. The HBO 200 W/4 lamp, BG 12 excitor filter, and K 530 and BG 38 suppressor filters were used. Sera were coded before being read. The degree of fluorescence was scored at ±1+, 2+, 3+, and 4+. The antibody titer was determined as the highest serum dilution producing a 1+ specific nuclear fluorescence reaction with CMV-infected cells. Care was taken to avoid misinterpretation of nonspecific cytoplasmic inclusion body fluorescence (6). Standard CMV antibody-positive and -negative sera were included in each test as controls. All sera were tested on CMV-infected and uninfected (normal antigen) HFF cell preparations.

Virus isolation. Specimens of urine, oral secretions, and heparinized blood were collected at regular intervals after transplantation, inoculated onto WI-38 cells, and maintained as previously described (1).

RESULTS

Sera obtained just before transplant from 71 hemodialysis patients and from 33 donors to these patients were tested. The data in Table 1 demonstrate that approximately half of the donors and half of the recipients had CMV antibody, but the CF-FT test did not detect antibody in several individuals who showed antibody using the other tests. Eight sera, four from recipients and four from donors, could not be interpreted in the CF test because they were anticomplementary. The frequency of detectable antibody in testable sera was similar for the FA and CF-GE tests. However, one patient had antibody activity in the FA test but not in the CF-GE test, and a donor had antibody in the CF-GE test but not in the FA test.

As noted above, however, the major differences between the results were with respect to the CF-FT tests. Of the 46 patients who had no detectable antibody using the FT antigen, 12 had antibody using the GE antigen and 13 using the FA technique. The results in these patients were either falsely negative with respect to the CF-FT test or falsely positive with respect to the CF-GE and FA tests. In an attempt to resolve this question, other available sera from these 13 individuals who had antibody by the FA test but not by the CF-FT tests were examined. Depending on how long these patients had been kept on chronic hemodialysis, one to four serum specimens from each patient were tested, one at the time of initial hemodialysis and one at 6- to 12-month intervals before transplantation. A total of 39 sera were tested, four of which were positive by CF-FT. One patient had two sera positive and two negative by this technique, and two other patients had one positive serum and the rest negative. However, all but two sera had antibody activity using the FA test. There were fewer positive sera using the GE antigen because 12 sera had anticomplementary activity. On the other hand, sera selected from the stored specimens of 33 patients whose sera immediately pre-

TABLE 1. *Frequency of antibody detected by different techniques in pretransplant sera and in donors*

Antibody test	Patients	Donors
CF-FT	21/67 (31)[a]	9/29 (31)
CF-GE	33/67 (49)	14/29 (48)
FA	34/71 (48)	16/33 (48)

[a] Numbers in parentheses are percentages.

transplant were negative by all three tests were examined; all 56 were either negative or anticomplementary. Thus, it appears that the CF-FT-negative sera were falsely negative (Table 1).

Studies on anticomplementary sera. There was very close agreement between the reactivity of sera in the FA and CF-GE tests. The major difference was that several sera could not be interpreted by CF because they were anticomplementary. This was true of sera that were FA positive and FA negative. One explanation for this anticomplementary activity is that there are nonspecific sites on the antibody molecule of some patients which fix complement. Therefore, the anticomplementary sera were tested in the following way: a 0.1-ml volume of serum was mixed with 0.1 ml of fresh guinea pig complement. The mixture was incubated at 37°C for 1 h and then at 56°C for 0.5 h. The serum was then retested with the antigen. Of the 20 sera treated, 9 still could not be evaluated, but 11 were no longer anticomplementary (Table 2). Six of these 11, all from patients with FA antibody, demonstrated antibody activity using the GE antigen. The dilution at which they fixed complement in the presence of antigen was low (1:8 to 1:16) after treatment. Five sera, all from patients with no FA activity, were nonreactive. Therefore, approximately half of the previously uninterpretable sera could now be classified as antibody positive or negative using the CF-GE test, and the result agree with that of the FA test.

Antibody titers using different techniques. To test the sensitivity of the different techniques, the serum titers determined by each test on the sera obtained just before transplant were compared. The data in Table 3 demonstrate that (i) the CF-GE and FA tests gave comparable titers in the CF-FT-positive sera, and both tests yielded higher titers than the CF-FT test; and (ii) the FA test yielded significantly higher titers than the CF-GE test in the CF-FT-negative sera.

Comparative value of FA, CF-FT, and CF-GE tests in predicting post-transplant CMV shedding. To explore further whether the CF-FT-negative sera that were FA positive were falsely positive, we studied the relationship of presence of antibody in pretransplant sera and shedding of virus post-transplant. All individuals who were antibody positive by any test (including those whose CF-FT test was negative) either shed virus, lost their kidney, or died in the first month after transplant. Since virus shedding presumably represented reactivation of latent infection in these patients, none of the tests apparently yielded false-positive results. To investigate the question of false negativity of each antibody test, the recipients with negative tests by each method were studied and the influence of serological status of the donor, as measured by the corresponding test, was evaluated (Table 4). Since there were more recipients with negative CF-FT tests than negative CF-GE or FA tests, we had more donors to evaluate (29 versus 25). Most, but not all, seronegative recipients who received kidneys from seropositive donors shed virus. However, if a recipient was seronegative by the FA test and received a kidney from an FA-negative donor or if he was seronegative by the CF-GE test and he received a kidney from a CF-GE-negative donor, he did not shed virus. This was not true when the CF-FT test was evaluated. Virus shedding occurred in seven recipients whose donors were CF-FT

TABLE 2. *Results of testing anticomplementary sera after treatment*

FA antibody	No. of sera	CF results for treated sera		
		Still anticomplementary	CF-GE positive	CF-FT positive
Positive	12	6	6	2
Negative	8	3	0	0

TABLE 3. *Antibody titers in sera as demonstrated by the FA or CF technique*

Status of serum by CF-FT test	Mean (log_2) antibody titer by designated technique	
	FA	CF-GE
Positive (mean 4.2)[a]	6.4[a]	6.2[a]
Negative	5.2[b]	3.5[b]

[a] $P < 0.01$ for FA or GE versus FT.
[b] $P < 0.05$ for FA versus CF-GE.

TABLE 4. *Influence of the serological status of donor on post-transplant virus shedding in seronegative recipients*

Serological status of donors by given test	No. of donors	No. of recipients shedding[a]
FA		
Positive	11	9
Negative	14	0
CF-GE		
Positive	12	9
Negative	13	0
CF-FT		
Positive	9	7
Negative	20	7

[a] All were seronegative before transplant by each test.

negative. These data suggest that in those seven, either the donor's or the recipient's CF-FT test was a false negative. Altogether, virus shedding after transplant occurred in 43 of our 71 patients. Shedding could be related to antibody status in 40 using the FA test and 39 using the CF-GE test on the donor and recipient. The shedding in one patient could not be accounted for using the CF-GE test because the recipient's serum was anticomplementary. For the remaining three shedders, donor sera were not available.

Use of antibody tests to detect early infection in known seronegative individuals. There were 12 patients for whom one to four sera tested before transplant were negative by every test who shed virus after transplant, and presumably had acquired a primary infection concurrent with the transplanted kidney. All 12 developed antibody post-transplant. To determine which of the three antibody tests gave the earliest positive result in primary infection, serial sera collected post-transplant from these 12 patients were tested. The time of detection of antibodies by each of the three tests is shown for these 12 patients in Fig. 1. In four patients, the FA and CF-GE tests became positive on the same day. In the other eight patients, the FA test was positive from 2 to 14 days (mean 10.1, median 11) before the CF-GE test. In all 12, the CF-FT test either was the last test to become positive, sometimes by as long as 24 days after the other tests, or became positive on the same day as the FA and CF-GE tests (three patients). Although not shown in this figure, antibody measured by CF-FT remained low (positive only at a 1:8 serum dilution) in two patients for up to 3 months, but antibody titers measured by the FA or CF-GE technique rose rapidly in these two and in the other ten patients shown in the figure, to mean values of $\log_2$ 11 and 12, respectively.

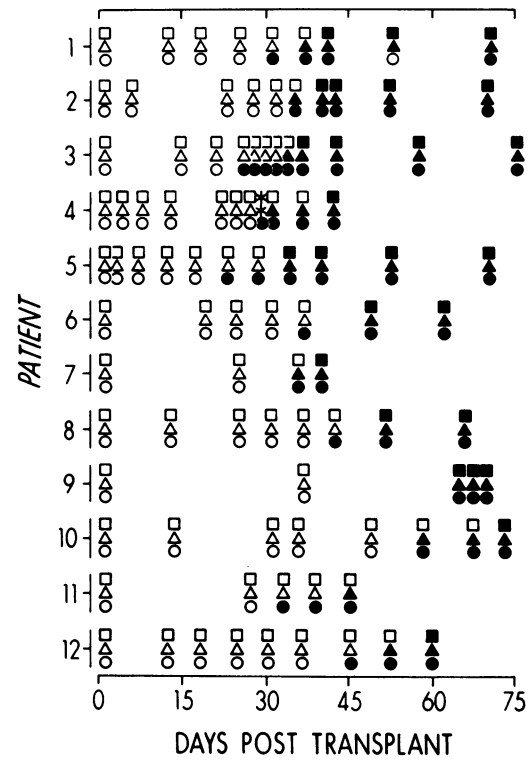

FIG. 1. *Time (days post-transplant) of first detectable antibody in 12 patients with primary CMV infection. Symbols: (□) CF-FT negative; (■) CF-FT positive; (△) CF-GE negative; (▲) CF-GE positive; (○) FA negative; (●) FA positive. Asterisk indicates anticomplementarity.*

DISCUSSION

This study of CMV serology in hemodialysis and transplant patients confirms the greater sensitivity of the FA test as compared with the CF-FT test, which has also been shown for women tested pre- and post-delivery (15). The greater sensitivity of the CF-GE test as compared with the CF-FT test in our dialysis patients and normal donors was similar to that shown previously in sera submitted for diagnostic purposes (3). It was also demonstrated that the CF-GE test is quite similar in its sensitivity to the FA test. An individual with apparent latent infection who had antibody to CMV by the FA technique almost always demonstrated antibody in the CF-GE test.

An intracytoplasmic IgG receptor which develops in cells infected with CMV could, as suggested by Furukawa and co-workers (6), result in a false-positive FA interpretation. However, using nuclear fluorescence as an indication of a positive reaction, we found no evidence of false-positive results in this study. Although the presence of the cytoplasmic receptor sites was confirmed this did not interfere with interpretation of the test.

Thus, the FA test appears to be a highly sensitive and specific test which proved in this study to have striking advantage over the CF-FT test. Not only were some sera uninterpretable in the CF test, due to anticomplementary activity, but also the CF-FT test gave a significant number of false-negative tests and the results occasionally fluctuated from negative to positive and then negative again. If the CF-FT test was used in both donors and recipients, virus shedding could be related to antibody status in only 25 of 32 shedders (excluding 10 shedders whose donor's serology was un-

known). This frequency was quite similar to that reported by Ho and his colleagues (8). However, the FA test could always be interpreted and rarely gave false-negative results, and when the FA test was used in donors and recipients, all virus shedding post-transplant could be related to antibody status, with the exception of three patients whose donor's serology was unknown. Furthermore, all patients who had antibody by the FA test either shed virus, died, or underwent nephrectomy in the first 6 weeks post-transplant. There were 14 patients who were seronegative and whose donor serology was known to be negative; none of these shed virus. This strongly suggests that this test did not give false-positive or false-negative results, but detected all individuals who had latent infection with CMV.

There were two seronegative recipients who received a kidney from separate seropositive donors but did not become infected. These donors were seropositive by CF-FT, CF-GE, and FA tests and had relatively high antibody titers. It seems reasonable to assume that the potential infection failed to be transmitted from these two donors rather than that the antibody results in these two donors were false positives.

The advantages of the FA test over the CF-GE test were less striking. The CF-GE test accounted for all virus shedding post-transplant, similar to the FA test. The differences in titers between the FA and CF-GE tests in these CF-FT-negative patients were not striking. Except for anticomplementary sera, the CF-GE test was positive when the FA was positive and negative when the FA test was negative in both donors and recipients. For some reason we did not see fluctuation of the CF-GE from positive to negative and then positive again as described by Waner and colleagues (16), although this did happen with the CF-FT test. The major advantages of the FA test were: (i) all sera could be interpreted, as compared with approximately 15% of the sera that were anticomplementary and could not be interpreted by the CF-GE test; (ii) the FA test could completed in about 2 h, as compared with overnight for the CF-GE test; and (iii) FA antibody developed earlier than CF-GE antibody in primary infection in about one-half of the patients.

All three of these advantages are important. First, although it may be possible to develop a method to eliminate anticomplementary activity, no entirely satisfactory method is now available. Second, if it eventually proves important to select kidneys from seronegative donors for antibody-negative potential recipients, a 2-h test has a definite advantage over an 18-h test. Third, the earlier development of FA antibody in primary infection could prove to be useful in the evaluation of patients. Development of viral cytopathic effect after inoculation of specimens onto tissue culture often takes 3 to 6 weeks (4), and antibody in our patients was detectable before viral cytopathic effect was recognized in inoculated tubes in every instance. The drawback of the FA test is the expense of the reagents and the microscope, but the cost is justified by the other factors mentioned.

One other important conclusion of this study is that both the FA and the CF-GE tests probably identify all individuals who have been infected with CMV, and thus both tests are far more useful than the conventional CF-FT test. Either test could prove useful in epidemiological studies to define the antibody status of study populations. They could also be used to determine who should be vaccinated, should a safe and effective vaccine become available (14).

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