Rapid Detection of Cytomegalovirus by Fluorescent Monoclonal Antibody Staining and In Situ DNA Hybridization in a Dram Vial Cell Culture System

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By using dram vial cell culture methods, three commercially available tests for cytomegalovirus (CMV) detection were compared: direct fluorescent monoclonal antibody staining for CMV-specific early and late antigens (direct FA), indirect fluorescent monoclonal antibody staining for a CMV-specific early antigen (indirect FA), and in situ DNA hybridization with a biotinylated CMV-specific DNA probe kit (DNA probe). Of those tests, only the indirect FA provided consistent, reliable virus detection within the initial 24 h postinfection for serial 10-fold dilutions of CMV AD169 (laboratory strain) and for three selected urine samples. However, when used prospectively, the indirect FA failed to detect virus within the initial 10 days postinfection in 15 of 78 consecutive specimens that were eventually positive by cell culture. Although the indirect FA was more sensitive than the direct FA or DNA probe, its utility appeared limited to specimens with high CMV concentrations. On the basis of these data, we recommend that indirect FA be reserved as an adjunct to standard cell culture for selected samples in diagnostic hospital laboratories.

Cytomegalovirus (CMV) is a significant cause of morbidity and mortality among congenitally infected infants and immunocompromised patients (9, 12). Currently used diagnostic tests for CMV, including serial serologic tests, histopathologic studies for intranuclear inclusions, and tissue culture assays, have been of limited diagnostic utility because they can require several weeks for definitive results. Since antiviral therapy is available for severe CMV infections, rapid detection methods suitable for the clinical laboratory are needed to identify infected patients early so that therapy can be instituted (1, 5).

The objective of this study was to compare the sensitivity of three commercially available systems for the rapid detection of CMV in a dram vial culture system which is readily adaptable for use in diagnostic clinical laboratories. The three commercially available tests for CMV detection used in this study were direct fluorescent monoclonal antibody staining for CMV-specific early and late antigens (direct FA; Whittaker M. A. Bioproducts, Walkersville, Md.), indirect fluorescent monoclonal antibody staining for a CMV-specific early nuclear antigen (indirect FA; New England Nuclear Corp., Beverly, Ma.), and in situ DNA hybridization with a biotinylated DNA probe (Enzo Biochemicals, New York, N.Y.). Their sensitivity was determined by testing a laboratory CMV strain (AD169) at serial 10-fold dilutions, and their applicability for rapid detection of clinical CMV strains was tested by screening multiple clinical specimens. The results presented in this paper suggest that the indirect FA test for the CMV-specific early nuclear antigen is the most sensitive. However, for routine clinical use, the detection of CMV-specific cytopathic effect in standard tissue culture may be as sensitive as the indirect FA technique.

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MATERIALS AND METHODS

Virus strains and clinical specimens. A laboratory CMV strain (AD169) was obtained from the American Type Culture Collection and was prepared to a 50% tissue culture infectious dose of 10^8 infectious particles per ml as described previously (8). The virus was diluted serially with supplemented minimum essential medium with 5% fetal bovine serum, 100 U of penicillin per ml, 100 µg of streptomycin per ml, and 100 mg of amphotericin B per ml (supplemented MEM) to produce multiplicities of infection (MOI) of 0.1, 1.0, and 10 infectious particles per cell. For the prospective study of clinical samples, random throat, urine, sputum, and bronchoalveolar lavage specimens were inoculated into transport medium and either inoculated into cell cultures or dram vials immediately or frozen at -70°C for later study. Urine specimens were diluted with gelatin-Tris-Hanks balanced salts solution, treated with 2.0 ml of 70% sorbitol, and alkalized to pH 7.0 to 7.4 with 7.5% sodium bicarbonate before inoculation of cell culture or freezing. Frozen specimens were thawed at 37°C and diluted at 1:10 with supplemented MEM before viral culture.

Viral culture methods. Confluent monolayers of human fibroblast cell cultures (MRC-5) in glass tubes were inoculated with 0.2 ml of virus or clinical specimen and incubated at 35°C for 1 h, and the inoculum was aspirated, all by standard procedures (14, 15). The monolayers were treated with 1.0 ml of supplemented MEM, maintained on roller drums at 35°C , and examined for characteristic cytopathic effect (CPE) for up to 21 days postinfection (p.i.). The medium was changed on days 1, 7, and 14 p.i.

MRC-5 cells (10^6 /ml) were inoculated into 1-dram (ca. 3.7-ml) vials containing 12-mm circular cover slips and were

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maintained with 1.0 ml of supplemented MEM until a confluent monolayer of approximately 10^5 cells formed. The MEM was then aspirated, and 0.2 ml of specimen was added. The vials were centrifuged at $700 \times g$ for 60 min (Sorvall GLC-2B centrifuge; Du Pont Co., Wilmington, Del.), the fluid was aspirated, 1.0 ml of supplemented MEM was added, and cultures were incubated at 37°C. At various times p.i. the monolayers were fixed by removing the MEM, washing three times with cold phosphate-buffered saline, air drying, and treating with acetone at -20°C for 10 min. Three vials were fixed per specimen (except for two urine specimens) for each time point to allow simultaneous testing with the various assays. The cell monolayers were examined for CPE immediately after virus inoculation and centrifugation (time point zero), at 16 h p.i., and every 22 to 24 h p.i. Time points were fixed during the intervening 24-h intervals for specimens having positive results with one of the assays within the initial 24 h p.i. DNA probe testing was only done on the samples having positive FA staining within 5 days p.i. It was necessary to perform sequential FA staining, followed immediately by DNA hybridization for certain specimens in which less than three vials had been fixed per time point.

Direct FA staining assay. One drop of fluorescein isothiocyanate-labeled murine monoclonal anti-CMV immunoglobulin G, specific for early and late antigens (Whittaker M.A. Bioproducts), was added per cover slip. This reagent detects a 67-kilodalton early protein, which accumulates in the nucleus, and a late cytoplasmic protein (specific characterization unavailable). The cover slips were incubated at 37°C for 40 to 45 min, washed twice with 0.01 M phosphate-buffered saline and once with deionized water, air dried, and mounted on glass slides with 90% glycerol. Fluorescence microscopy (Olympus fluorescence microscope; maximum excitation wavelength, 400 to 490 nm) was used to detect distinct intranuclear and cytoplasmic staining in at least five cells per monolayer for a positive test.

Indirect FA staining assay. A 150- μ l sample of a 1:20 dilution of unlabeled anti-CMV early nuclear protein immunoglobulin G2a (New England Nuclear) was added per cover slip. This reagent detects a 72-kilodalton early nuclear protein. The cover slips were then incubated at 37°C for 30 min. The monolayers were washed twice with 0.01 M phosphate-buffered saline and once with deionized water and air dried. Then 150 μ l of 1:50 dilution of fluorescein-conjugated F(ab')₂ fragment goat anti-mouse immunoglobulin G (Cappel Co., Cochranville, Pa.) was added per cover slip. Cover slips were incubated at 37°C for 30 min, and processing was completed as described above for direct FA staining. Distinct intranuclear staining in at least five cells per monolayer was considered a positive test.

DNA hybridization. The CMV Pathogene kit (Enzo Biochemicals, New York, N.Y.) was used for specimen processing. Cover slips containing infected cell monolayers were removed from the dram vials, placed on glass microscope slides, treated with 1 drop of DNA probe solution, covered with a glass cover slip, heated on a block at 90 to 95°C for 1 min, and incubated at 37°C for 10 min. After cover slips were removed, each monolayer was treated with 4 drops of probe wash, incubated at 37°C for 5 min, rinsed with wash buffer, treated with 3 drops of detection complex, and incubated at 37°C for 10 min. After a rinse with wash buffer, each monolayer was treated with 4 drops of a solution containing aminoethylcarbazole-H₂O₂-acetate, incubated at 37°C for 10 min, rinsed with deionized water, treated with 3 drops of counterstain for 2 min, washed again with deionized water, and viewed with a light microscope with deionized water as

a mounting medium. Although room temperature was recommended by the manufacturer, 37°C was used for all incubation steps. Brick red deposits in cells seen with light microscopy indicated a positive reaction for hybridized DNA. In instances where DNA hybridization was performed after FA staining on the same monolayer, the glycerol FA mounting solution was removed by washing the monolayer with 0.01 M phosphate-buffered saline and deionized water, followed by air drying.

RESULTS

Comparison of methods for CMV AD169 detection. To determine the sensitivities of the various methods for detecting CMV in tissue culture cells, the AD169 strain of CMV was inoculated into dram vials at various MOIs and tested for the presence of viral antigen, viral DNA, or infectious viruses at various times p.i. Table 1 illustrates the sensitivities of the direct FA, indirect FA, and DNA probe tests compared to standard CPE for CMV AD169. At an MOI of 0.1, indirect FA was detected at 1 day (24 h) p.i., direct FA staining was detected at 5 days (100 h) p.i., DNA hybridization was noted at 5 days (120 h) p.i., and CPE was detected at 8 days (190 h) p.i. At an MOI of 1.0, indirect FA staining was noted at 1 day (24 h) p.i., direct FA was noted at 3 days (50 h) p.i., DNA hybridization was noted at 4 days (84 h) p.i., and CPE was detected at 7 days (150 h) p.i. Direct and indirect FA were present at 1 day (3 h) p.i. at an MOI of 10, with DNA hybridization noted at 1 day (23 h) p.i. and CPE noted at 4 days (96 h) p.i.

The quality of the indirect FA stain was excellent at all time points tested, with clear intranuclear apple-green fluorescence noted against a dark background. There was considerable variability of the direct FA stain for early time points (72 to 120 h p.i.), with many cells having nonspecific cytoplasmic staining that interfered with the adequate visualization of nuclear staining that is required for an accurate interpretation of the assay. Optimal staining with the direct FA technique was observed at 5 to 6 days p.i. (depending on the MOI), when apple-green intranuclear fluorescence against a red background (Evans blue counterstain) was evident. The red color of the hybridized DNA complexes became progressively dull brown at late time points (>168 h p.i.) making interpretation of those assays difficult. To enhance the color intensity, the incubation temperature was changed from room temperature to 37°C.

Comparison of methods for detection of CMV in clinical specimens. To determine the sensitivity of these methods for

TABLE 1. CMV AD169-infected MRC-5 cell monolayers at specific MOIs

Days p.i.	Test results at the indicated MOI											
	CPE			Direct FA			Indirect FA			DNA probe		
	0.1	1.0	10	0.1	1.0	10	0.1	1.0	10	0.1	1.0	10
0	-	-	-	-	-	-	-	-	-	-	-	-
1	-	-	-	-	+	+	+	+	+	-	-	+
2	-	-	-	-	+	+	+	+	+	-	-	+
3	-	-	-	-	+	+	+	+	+	-	-	+
4	-	-	+	-	+	+	+	+	+	-	+	+
5	-	-	NT ^a	+	+	NT	+	+	NT	+	+	NT
6	-	-	NT	+	+	NT	+	+	NT	+	+	NT
7	-	+	NT	+	+	NT	+	+	NT	+	+	NT
8	+	+	NT	+	+	NT	+	+	NT	+	+	NT
9	+	NT	NT	+	NT	NT	+	NT	NT	+	NT	NT

^a NT, Not tested.

TABLE 2. Clinical urine specimens in dram vial system

Days p.i.	Test results with samples from the indicated patients ^a											
	CPE			Direct FA			Indirect FA			DNA probe		
	a	b	c	a	b	c	a	b	c	a	b	c
0	-	-	-	-	NT ^b	-	-	-	-	NT	-	-
1	-	-	-	-	NT	+	+	+	+	NT	-	-
2	-	-	+	-	NT	+	+	+	+	NT	-	+
3	-	-	+	+	NT	+	+	+	+	NT	+	+
4	-	-	+	+	NT	+	+	+	+	NT	+	+
5	-	-	+	+	NT	+	+	+	+	NT	+	+
6	+	-	+	+	NT	+	+	+	+	NT	+	+
7	+	+	+	+	NT	+	+	+	+	NT	+	+
8	+	+	+	+	NT	+	+	+	+	NT	+	+

^a a, Homosexual man with Kaposi's sarcoma; b, infant with congenital CMV disease; c, infant with congenital CMV disease.

^b NT, not tested.

detecting CMV in clinical samples, three urine samples known to be positive by tissue culture assay were inoculated into dram vials and analyzed. Table 2 illustrates the results of testing three urine specimens for clinical CMV strains compared with standard CPE. For specimen a, indirect FA staining was detected at 1 day (17 h) p.i., direct FA was detected at 3 days (65 h) p.i., and CPE was detected at 6 days (144 h) p.i. For specimen b, indirect FA was noted at 1 day (22 h) p.i., DNA hybridization was noted at 3 days (72 h) p.i., and CPE was detected at 7 days (168 h) p.i. Specimen c had positive direct and indirect FA staining at 1 day (16 h) p.i., with DNA hybridization and CPE appearing at 2 days (45 h) p.i. The difficulties described above with early direct FA staining (<4 days p.i.) and late hybridized complex color visualization (>6 days p.i.) were also encountered in evaluating the clinical specimens. No adverse interactions were noted in visualizing the hybridized complexes when the DNA probe was applied after FA staining reagents had been applied to the cell monolayers. There were no detectable CMV-specific early antigens or hybridized DNA complexes immediately after dram vial inoculation and centrifugation (time point zero) with the three assays. This was a consistent finding among the CMV AD169 and clinical urine specimens tested. These results indicate that the indirect FA technique utilizing a monoclonal antibody to an early nuclear antigen of CMV is a useful procedure for detecting CMV in clinical specimens that have a significant amount of infectious virus.

We next attempted to assess the usefulness of the indirect FA technique for detecting CMV in routine clinical samples submitted to our clinical microbiology laboratory. In a prospective, blinded study of 78 consecutive clinical samples submitted for CMV culture, 15 were positive for CMV. They required an average of 12.2 days to appearance of CPE. None of the 78 samples had positive indirect FA staining after 10 days p.i., including those that subsequently developed CPE on culture. These results indicate that the indirect FA may not be useful for routine clinical work.

DISCUSSION

Numerous rapid detection methods have been described in which monoclonal antibodies or DNA probes are used to screen clinical specimens for CMV (2-4, 6, 7, 11, 13, 14, 16-18). In this comparative study of three commercially available tests, indirect FA staining for a CMV-specific early antigen was the most rapid and reliable modality for detecting CMV AD169 in a dram vial cell culture system. How-

ever, when applied to clinical specimens, it was most efficacious only for evaluating those with high virus concentrations.

Characteristic CPE in tube cell culture has been considered the standard test for CMV detection (12), although it may not appear for up to 8 weeks p.i. Recent studies have utilized dram (shell) vials containing MRC-5 cell monolayers with specimens centrifuged onto the monolayer to enhance viral adsorption and infection (6, 7, 17). Excellent viral recovery compared with that in tube culture has been noted with that method (6, 7, 13, 17).

In this study, rapid detection of CMV was demonstrated in clinical urine specimens as early as 16 h p.i. with the indirect FA test in a dram vial assay. This test can be performed in most clinical laboratories and requires minimal technician time and expertise. The superior quality and reproducibility of the indirect FA stain compared with the visualization difficulties experienced with the direct FA technique make it the preferred FA rapid testing procedure.

Molecular hybridization assays (isotopic and biotinylated) for CMV with nitrocellulose filters have been assessed in recent studies (2, 3, 10, 11, 16, 18). However, there have been no previous publications describing the application of DNA probe techniques to dram vial cell culture systems. In situ DNA hybridization assays were performed successfully in this study with dram vial cell monolayers, and no antagonistic interactions were evident when FA staining and DNA probe testing were performed sequentially on the same monolayers. Although good sensitivity has been reported with the biotinylated DNA probes, hybridized DNA complexes were not detected for long time periods after early nuclear antigens were detectable with the FA stains (2, 10, 11). Among the clinical urine specimens, the earliest time that CMV DNA was noted was at 45 h p.i. with the probe kit compared with early antigen detection at 16 h p.i. with the FA stains.

Since neither CMV early antigens nor DNA was noted at time point zero, it is likely that sufficient time periods are necessary for adequate early antigen and DNA production to occur before a positive test result is generated. The longer time interval required for hybridized DNA complex detection is likely related to the sequential events of the CMV replicative cycle, in which early proteins are synthesized before the initiation of viral DNA replication (12). Active CMV protein production and DNA replication appear crucial for successful application of the tests evaluated in this study. Their suitability for detecting latent virus remains uncertain.

The limitations of indirect FA staining for rapid CMV detection are illustrated through the prospective evaluation of clinical specimens. Positive indirect FA was noted only for the three selected urine samples with rapid time to development of CPE in tube culture (<7 days p.i.), indicating relatively high virus concentrations. It offered no advantage to tube culture for CMV detection when blinded, random specimens having widely varied virus concentrations were screened (average time to CPE development, 12.2 days). It is possible that freeze-thawing of some specimens followed by 1:10 dilution may have altered the infectivity of any CMV present. Thus, although it is adaptable for use in hospital laboratories, the indirect FA test does not appear to have sufficient sensitivity at low virus concentrations to be relied upon in place of standard tube cell culture for CMV detection.

In summary, there are two principal conclusions from this investigation. First, indirect FA provides better results than

direct FA or DNA probe for rapid CMV detection. It is suitable for the dram vial cell culture system and is reliable for clinical samples with high virus concentrations. Second, in the routine hospital laboratory, the indirect FA test is not significantly better than standard tissue culture alone for detecting CMV. When applied to specimens with unpredictable viral concentrations, it is best considered an adjunctive procedure to screen for samples with high virus concentrations. Unless the population under study has a propensity for high CMV excretion, it may be more useful for detecting viruses with more rapid replicative cycles, such as herpes simplex and influenza viruses. The FA technique, either direct or indirect, is still very useful for culture confirmation.

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