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Clinical Experience with Cytomegalovirus Isolation Using Conventional Cell Cultures and Early Antigen Detection in Centrifugation-Enhanced Shell Vial Cultures

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A total of 1,915 clinical samples was inoculated by low-speed centrifugation into shell vials (Bartels Immunodiagnostics, Bellvue, Wash.) containing cover slip monolayers of MRC-5 fibroblasts. At 1 and 2 days postinoculation, one cover slip was stained by an indirect immunofluorescence technique using a monoclonal antibody (Biotech Research Laboratories for Dupont, Billerica, Mass.) to cytomegalovirus (CMV) early antigen (EA). Clinical samples were also inoculated into three MRC-5 or MRHF cell cultures which were observed for 30 days for the appearance of a cytopathic effect (CPE). Of 157 CMV-positive samples, 92 (59%) were identified by centrifugation-enhanced EA (CE-EA) and 131 (83%) produced a CPE. CE-EA was less sensitive than CPE for all types of samples, although 17% of CMV-positive samples were detected by CE-EA alone. Evaluation of the CMV status of patients with CE-EA-positive–CPE-negative samples indicated that these samples likely represented true CMV-positive results. The average elapsed time between culture inoculation and identification of CMV decreased as follows when both CE-EA and CPE, rather than CPE alone, were used: urines, 15 to 7 days; buffy coats, 18 to 9 days; lung samples, 13 to 8 days; throat samples, 18 to 7 days. Although CE-EA was less sensitive than 30-day cell culture, both CE-EA and CPE were identified as valuable in CMV detection, and neither could be discontinued without a decrease in the CMV isolation rate or an increase in the turnaround time.

Cytomegalovirus (CMV) has traditionally presented a challenge to clinical virologists, requiring 14 to 21 days from culture inoculation until virus isolation. In 1984, Gleaves et al. (4) reported rapid isolation of CMV using a shell vial system in which fibroblasts grown on cover slips in shell vials were inoculated by low-speed centrifugation. At 36 and 96 h postinoculation, the cover slips were stained with a monoclonal antibody to CMV early antigen (EA). This system yielded 100% specificity and was more sensitive than traditional cell cultures for isolation of CMV from urine specimens. Similar results were reported in 1985 by Alpert et al. (1), who also tested urine samples by a centrifugation-enhanced EA (CE-EA) detection method in which a different monoclonal antibody and a biotin-avidin-amplified indirect fluorescent stain were used. Further comparison of CE-EA and cell cultures showed that CE-EA was more sensitive than cell culture for CMV detection in all types of clinical samples except buffy coats and identified CE-EA as sufficiently sensitive to allow urine samples to be processed exclusively by this method (5). Recent, more extensive comparisons of the two techniques have yielded data which suggest that both CE-EA and traditional cell cultures should be used for specimens such as blood and lung tissue (7). The specificity of CE-EA, especially with peripheral blood buffy coat samples, has been questioned (J. Englund, C. Edelman, A. Erice, M. C. Jordan, and H. H. Balfour, Jr., Program Abstr. 27th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 1103, 1987).

The objective of this study was to provide an extensive clinical evaluation of CE-EA and 30-day cell cultures for detection of CMV in all types of clinical materials. CMV detection by the two methods was compared, and the CMV status of all patients whose cultures were CE-EA positive and CPE negative was evaluated by review of patient histories and CMV serology results to determine whether this result pattern likely represented false-positive results. The time required for CMV detection with both CE-EA and cytopathic effect (CPE) was compared with isolation time during a previous year in which CMV detection relied solely on CPE production. Both CE-EA and CPE were found to be valuable in CMV isolation from all types of clinical samples.

MATERIALS AND METHODS

Clinical samples for CMV isolation. A total of 1,915 samples received for routine virus culturing in a clinical diagnostic virology facility was tested. Samples were collected from hospitalized patients and outpatients. All urine and peripheral blood buffy coat samples, as well as any sample, regardless of the site of origin, in which CMV was indicated, were cultured in cell culture and examined for CE-EA. Specimens were collected and processed for virus isolation as follows. Blood specimens (10 ml) were collected in heparinized tubes and processed as recommended by the manufacturer by density centrifugation in a Ficoll-Hypaque mixture, Mono-Poly Resolving Medium (Flow Laboratories, Inc., McLean, Va.), to separate mononuclear and polymorphonuclear leukocytes. The separated leukocytes were suspended in 2.5 ml of Eagle minimum essential medium with Earle salts (MEM-E). Urine (10 ml), undiluted and unbuffered, was treated with gentamicin and amphotericin B (Fungizone; Flow Laboratories) for 30 to 45 min and then spun at 1,500 × *g* for 10 min. The pellet, suspended in 3 ml of MEM-E, was used as the inoculum. Tissue samples were placed in 3 to 4 ml of MEM-E and ground with sterile tissue grinders. The mixture was spun at 1,500 × *g* for 10 min, and the supernatant fluid was used. Swab samples (nose, throat,

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etc.) were extracted into 2.5 ml of MEM-E containing gentamicin and amphotericin B and spun at 1,500 × *g* for 10 min, and the supernatant fluid was used.

CE-EA detection. Shell vials containing cover slip monolayers of MRC-5 fibroblasts (Bartels Immunodiagnostics, Bellvue, Wash.) were used. Cell culture medium was removed from two vials, and 0.2 ml of a clinical sample processed as described above was used as the inoculum for each vial. Inoculated vials were spun at 700 × *g* for 60 min, excess inoculum was removed, each monolayer was rinsed with phosphate-buffered saline (PBS; pH 7.6), fresh culture medium was added, and the vials were incubated at 35°C. At 1 day (16 to 26 h) and 2 days (40 to 50 h) postinoculation, one cover slip was stained by an indirect immunofluorescence technique with a monoclonal antibody to a 72,000-dalton immediate-early nuclear protein of CMV. This monoclonal antibody was developed by Shuster et al. (8) and is marketed by Biotech Research Laboratories for Dupont (Billerica, Mass.). The staining procedure was performed in the shell vials. Following fixation with cold (4°C) acetone and rinsing with PBS, each cell monolayer was covered with 0.1 ml of undiluted CMV EA antibody. The vials were tilted to ensure that the entire cover slip was covered, and the vials were incubated for 30 min at 35°C and rinsed twice in PBS. Fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (heavy and light chain specific) antiserum (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) routinely diluted 1:100 in PBS, 0.1 ml per vial, was added, and the vials were incubated for 30 min. Following two rinses in PBS, cover slips were mounted on glass microscope slides and viewed with the 40× dry objective of a Zeiss Standard Immunofluorescence Diagnosis Microscope with an IV Fl epifluorescence condenser. Fluorescence of intranuclear inclusions was recorded as a positive result.

Cell culture isolation of CMV. Cell culture medium was removed from three tubes of human fibroblast tissue, either Medical Research Human Foreskin (MRHF) or Medical Research Council lung fibroblasts (MRC-5), purchased from Whittaker M. A. Bioproducts (Walkersville, Md.) and Bartels Immunodiagnostics, respectively. Clinical samples prepared as described above were applied directly to the monolayer. Inoculated monolayers were incubated for 1 h at 35°C, any excess inoculum was removed, fresh culture medium was added, and the cultures were incubated at 35°C in rotating racks. The cultures were observed for 30 days for appearance of a cytopathic effect (CPE) characteristic of CMV.

Calculations. For all samples, a true-positive result was defined as any sample CMV positive by either CE-EA or CPE.

RESULTS

Both CE-EA and CPE readings during the 30 days postinoculation were obtained on 157 CMV-positive specimens. Of these, 92 (59%) were CE-EA positive and 131 (83%) produced a CPE. CMV was detected in 17% of the samples by CE-EA alone and in 41% by CPE alone (Table 1). For all types of specimens, the number of samples positive by CPE only was greater than that positive by CE-EA alone.

Of 92 CE-EA-positive samples, 11 were not tested at both 1 and 2 days because of the poor quality of the cell monolayer in one vial (7 samples), failure to harvest one vial within the specified time interval (3 samples), or breakage of the cover slip during testing (1 sample). For the remaining 81 CE-EA-positive samples, a comparison of detection at 1 and 2 days is shown in Table 2. A total of 57 (70%) was positive at 1 day, and 68 (84%) were positive at 2 days. For all types of specimens, more than half of the CE-EA-positive samples were detected in the 1-day reading. The mean time for CPE production was 12 days for CE-EA-positive samples compared with 17 days for CE-EA-negative samples (data not shown).

TABLE 1. Comparison of CMV detection by CE-EA and CPE in 30-day cell cultures

Specimen type (no. tested)	No. of CMV-positive specimens	No. (%) CMV positive by:		
		CE-EA only	CE-EA and CPE	CPE only
Urine (635)	71	12 (17)	36 (51)	23 (32)
Buffy coat (525)	28	9 (32)	4 (14)	15 (54)
Throat or nose (300)	15	3 (20)	5 (33)	7 (47)
Lung (250)[a]	39	2 (5)	20 (51)	17 (44)
Total (1,915)[b]	157[b]	26 (17)	66 (42)[b]	65 (41)[b]

[a] Includes lung biopsies, autopsies, bronchoalveolar lavage, etc.

[b] Totals include 205 additional specimens of various types that were tested. Four of these were CMV positive; one gastric biopsy was CE-EA and CPE positive, and one bone marrow, one liver biopsy, and one eye sample were positive by CPE alone.

The turnaround time (time from culture inoculation until CMV was identified) for the 157 CMV-positive cultures identified by CE-EA or CPE during the 15-month study period was compared with the turnaround time for 74 CMV-positive cultures identified during the 12 months before this study, when only CPE was used for CMV isolation. When both CE-EA and CPE were used, the turnaround times decreased from 15 to 7 days for urine samples, from 18 to 9 days for buffy coats, from 13 to 8 days for lung samples, and from 18 to 7 days for throat samples. The overall mean turnaround time decreased from 16 to 8 days.

Twenty-five samples from 20 patients were CE-EA positive and CPE negative. Of the 20 patients, 10 (50%), responsible for 15 (60%) of the specimens, had clinical evidence consistent with CMV infection; 8 had CMV isolated from other sites or specimens, and 2 were positive for CMV immunoglobulin M. Six (30%) of the patients responsible for 6 (24%) of the CE-EA-positive and CPE-negative samples were CMV antibody positive by latex agglutination or belonged to risk groups (patients with acquired immunodefi-

TABLE 2. CMV CE-EA detection in 81 CE-EA-positive samples at 1 versus 2 days postinoculation[a]

Specimen type/no. CE-EA positive	No. (%) CE-EA positive at:	
	1 day	2 days
Urine/43	29 (67)	39 (91)
Buffy coat/9	6 (67)	7 (78)
Throat or nose/8	6 (75)	6 (75)
Lung[b]/20	15 (75)	15 (75)
Total (n = 81)[c]	57 (70)[c]	68 (84)[c]

[a] Data from 11 additional CE-EA-positive samples are not included here. These were not tested at both 1 and 2 days because of the poor quality of the cell monolayer in one of the vials (seven samples), failure to harvest one vial during the specified time interval (three samples), or breakage of a cover slip (one sample).

[b] Includes lung biopsies, autopsies, bronchoalveolar lavage, etc.

[c] Includes one gastric biopsy specimen that was CE-EA positive at both 1 and 2 days.

TABLE 3. Comparison of CMV detection by CE-EA and CPE in 14-day cell cultures

Specimen type	No. of CMV-positive specimens	No. (%) CMV positive by:		
		CE-EA only	CE-EA and CPE	CPE only
Urine	55	20 (36)	26 (47)	9 (16)
Buffy coat	23	10 (43)	3 (13)	10 (44)
Throat or nose	13	4 (31)	4 (31)	5 (38)
Lung[a]	30	4 (13)	17 (57)	9 (30)
Total	123[b]	38 (31)	51 (41)[b]	34 (28)[b]

[a] Includes lung biopsies, autopsies, bronchoalveolar lavage, etc.
[b] Totals include one gastric biopsy specimen positive by CE-EA and CPE and one liver biopsy specimen positive by CPE alone.

ciency syndrome, transplant recipients, and patients with multiple congenital anomalies) in which CMV infection is common. Four (20%) of the patients with CE-EA-positive–CPE-negative samples were lost to follow-up. In 9 (36%) of the 25 CE-EA-positive–CPE-negative samples, problems involving the cell culture monolayer were noted during the 30-day incubation period; in 4, the cell cultures deteriorated, and in 5, there was bacterial or fungal contamination in the cell culture tubes.

Data from Table 1 were recalculated to compare CE-EA with CPE produced within 14 days postinoculation (Table 3). Of 123 CMV-positive samples, 89 (72%) were detected by CE-EA, and 85 (69%) produced a CPE. CMV was detected in 31% of these by CE-EA alone, and in 28% it was detected by CPE alone. For urine samples, the number of samples detected by CE-EA alone exceeded that detected by CPE alone. For all other types of samples, CPE detected as many or more positive cultures.

DISCUSSION

We found CMV detection by CE-EA to be a valuable addition to the routine CMV isolation protocol used in our laboratory. Seventeen percent of our CMV-positive samples were identified by CE-EA alone. These samples were from known CMV-positive individuals, from CMV-seropositive individuals, or from those belonging to CMV risk groups and were accepted as true-positive results for CE-EA and false-negative results by CPE.

Seventy percent of the CE-EA-positive samples were identified at 1 day, and 84% were positive at the 2-day reading. The increased sensitivity at 2 days may have been due to increased incubation time. However, Paya et al. (6), who inoculated and simultaneously stained two vials per clinical sample, reported that use of two vials rather than one improved the positivity rate of CMV detection by 7, 10, and 5% for urine, tissue, and bronchoalveolar lavage specimens, respectively, and by 20% when three vials rather than two were inoculated for buffy coat samples.

Less time was required for CPE production by CE-EA-positive samples than by CE-EA-negative samples and, overall, for cultures identified as CMV positive by CE-EA and CPE together rather than by CPE alone. This rapidity in CMV reporting was invaluable in providing excellent clinical service and was the most valuable aspect of CE-EA use for us. CE-EA was less sensitive for CMV detection than was CPE production in cell cultures incubated for 30 days. This was true for all specimen types, including urine specimens. On the basis of these data, we cannot recommend using CE-EA alone for CMV detection in any single type of specimen.

In previous investigations (4, 5), CE-EA was reported as more sensitive than cell culture for isolation of CMV from urine specimens, and CMV detection by this method alone was recommended. In these studies, the cell culture incubation period was only 14 days. Upon recalculation of our data to compare CE-EA with 14-day cell culture incubation, CE-EA was more sensitive overall than 14-day cell culture. Urine was the only type of sample in which CE-EA detected more positive samples than a 14-day CPE; all other specimen types had equal or greater numbers of positive cultures by 14-day CPE than by CE-EA. These results compare favorably with those of Paya et al. (7), who recommended that both CE-EA and 14-day cell cultures be used for all types of specimens except urine. These investigators reported that 31% of their clinical samples were positive by CE-EA alone. Our data on 14-day cell culture showed that 31% of all specimens were positive by CE-EA alone.

In studies in which the incidence of CMV is high and buffy coat samples are excluded, CMV detection rates by CE-EA may be higher than those reported in this study. Gleaves et al. (3), who used a fluorescein-conjugated monoclonal antibody with a direct staining technique to detect CMV EA, isolated CMV from nearly 50% of their specimens (no buffy coats included). They detected 89 (99%) of 90 CMV-positive samples by CE-EA at 16 h postinoculation and found CE-EA more sensitive than 35-day cell culture. Likewise, DeGirolami et al. (2), who isolated CMV from 20% of their samples (no buffy coats included), reported that CE-EA was more sensitive than 28-day cell culture. In the present investigation, the overall CMV isolation rate was <10% (157 of 1,915). Our data indicate that 30-day cell culture is sensitive and productive in CMV isolation from all types of clinical samples and may be especially valuable for culturing of buffy coats and of specimens from low-incidence general populations.

Variations in cell culture quality (i.e., commercial preparation versus in-house preparation) and inoculation methods may also affect the relative sensitivities of CE-EA and CPE. The absorption type inoculation used here for cell culture inoculation for all types of samples may enhance isolation of CMV. This type of cell culture inoculation was not described by others who have compared CE-EA and CPE for CMV isolation. Also, the use of commercially purchased shell vials may contribute to decreased CE-EA detection. DeGirolami et al. (P. C. DeGirolami, W. L. Drew, C. A. Gleaves, P. A. Hanff, and A. L. Warford, *Procedure Manual for the detection of CMV and HSV in Shell-Vial Cultures*, Syva Microtrak, Palo Alto, Calif., 1988) reported reduced 16- to 24-h sensitivity for CE-EA detection in purchased vials compared with vials seeded in house. In the present study, cell culture isolation may have been enhanced relative to CE-EA detection by use of three cell cultures tubes when only two vials were tested for CE-EA. The use of only 0.2 ml of inoculum per shell vial, rather than the 0.3 ml used in other investigations (2, 5) may have additionally decreased CE-EA detection.

The cell monolayers in the shell vials were less susceptible to contamination or deterioration than the cell culture monolayers. In 9 of 25 CE-EA-positive–CPE-negative samples, the cell culture monolayer was lost before the end of the 30-day incubation period because of bacterial or fungal contamination or deterioration of cell monolayers. However, problems with cell monolayers were not confined to cell culture tubes. In 7 of 11 CE-EA-positive samples in which both 1- and 2-day readings were not obtained, the samples had shell vial monolayers that were not acceptable

for CE-EA testing. In their procedure manual, DeGirolami et al. reported that lot-to-lot variations in the quality of purchased cells occur and, regardless of the vendor, approximately 30% of vials have poor monolayers. Stirk and Griffiths (9), in an extensive comparison of a CMV EA detection method and traditional cell culturing, reported that, because of contamination or deterioration of the cell monolayers, their rapid method was unable to produce a result in 130 (6.5%) of 1,955 specimens, while traditional cell cultures failed in 206 (10.5%) of 1,955 specimens.

Although CMV detection by CE-EA is not sufficiently sensitive relative to 30-day CPE to allow CE-EA alone to be used, we believe its use is helpful in CMV detection in all types of clinical specimens. CMV detection by both CE-EA and CPE is requisite for optimal CMV sensitivity and timely reporting.

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