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Practical Protocol for Cytomegalovirus Isolation: Use of MRC-5 Cell Monolayers Incubated for 2 Weeks

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Isolation of cytomegalovirus (CMV) in tissue culture is presently the most reliable means of proving active CMV infection. To improve both the cost-effectiveness and clinical usefulness of procedures for the isolation of CMV from fresh clinical specimens, we analyzed results obtained with standard isolation procedures and compared them with results obtained under different conditions. Cell monolayers from commercial sources were inoculated with fresh specimens and then were observed for a cytopathic effect typical of CMV. Of 1,375 specimens submitted over a 12-month period, 6.4% were CMV positive in WI-38 monolayers within 28 days after inoculation. The mean day of CMV detection for 45 urine, 13 cervical-vaginal, and 5 saliva specimens was 6.7 ± 3.1 (mean ± standard deviation), 9.9 ± 3.3, and 7.7 ± 3.3 days, respectively, and 92% were positive within 14 days. When 1,058 subsequent specimens were inoculated in parallel onto WI-38 and MRC-5 cell monolayers, 8.7% were positive for CMV. MRC-5 cells were significantly more sensitive than WI-38 cells: 98% of all positive specimens appropriate for comparison were detected in MRC-5 cultures, but only 85% were detected in WI-38 cells. Although 1 specimen was positive in WI-38 cells only, 38% of all isolates were positive earlier (16 specimens) or only (10 specimens) in MRC-5 cultures. Based on these data, we have developed a practical 2-week protocol for CMV isolation from fresh clinical specimens that includes the use of MRC-5 cell monolayers incubated at 36°C.

Cytomegaloviruses (CMVs) are a group of viruses within the herpesvirus family that infect a wide variety of mammalian species. In humans, most CMV infections are subclinical, but serious infections can occur. Two important examples are congenital infections and infections in the compromised host (2, 3, 5, 12). The diagnosis of CMV infection usually requires laboratory confirmation and cannot be made on clinical grounds alone. At the present time, the most reliable means available for proving active CMV infection is isolation of the virus in tissue culture (9, 13). Human fibroblasts were first used for the isolation of CMV in 1956 (10, 11), and it has since been shown that productive infection generally occurs only in cells of human origin. Monolayers of WI-38 cells, diploid embryonic lung cells, are widely used by diagnostic laboratories, but it is becoming increasingly difficult to obtain this cell line in low passages (14). In a search for an acceptable alternative to WI-38 cells for the detection of CMV, WI-38 cells were compared with another well-characterized commercially available diploid cell line derived from human embryonic lung, MRC-5 cells, for susceptibility to CMV infection and speed of development of cytopathic effect after inoculation with fresh clinical specimens.

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MATERIALS AND METHODS

Cells. Passage 21 to 24 WI-38 cells (6) in 16- by 125-mm tubes were obtained weekly from Flow Laboratories, Inc. (McLean, Va.), and similar cultures of generation 22 to 26 MRC-5 cells (7) were obtained from HEM Research, Inc. (Rockville, Md.), also on a weekly basis. Monolayers of primary human kidney cells, derived from embryonic, fetal, or neonatal tissue, were obtained from Microbiological Associates (Walkersville, Md.). Upon receipt of each shipment, culture medium was replaced with 1.5 ml of modified Eagle medium containing nonessential amino acids, 0.3% sodium bicarbonate, 20 IU of potassium penicillin G (E. R. Squibb & Sons, Princeton, N.J.) per ml, 50 μg of gentamicin sulfate (Schering Corp., Bloomfield, N.J.) per ml, and 10% heat-inactivated fetal bovine serum.

Specimen handling. Specimens were submitted to our diagnostic laboratory for routine viral studies. Specimens on swabs were placed in tubes containing 2.5 ml of transport medium composed of veal infusion broth (Difco Laboratories, Detroit, Mich.) with 100 IU of potassium penicillin G per ml, 100 μg of streptomycin sulfate (Eli Lilly & Co., Indianapolis, Ind.) per ml,

0.25 μg of amphotericin B (Squibb) per ml, 50 μg of gentamicin sulfate per ml, 6.5 μg of phenol red (Eastman Organic Chemicals, Rochester, N.Y.) per ml, and 0.1% gelatin. Urine and saliva specimens were submitted in specimen cups and diluted 1:1 and 1:2, respectively, with transport medium upon arrival in the laboratory. After vigorous agitation, specimen suspensions were centrifuged (1,200 × *g*, 10 min), and 0.2 ml (0.3 to 0.4 ml for urine specimens) of the resulting supernatant fluid was immediately inoculated onto one monolayer each of WI-38, MRC-5, and primary human kidney cells.

Specimens received on swabs were treated similarly after agitation and compression of the swab to suspend the specimen in transport medium. Heparinized blood specimens were allowed to settle in an inverted syringe. After 1 to 2 h, the plasma layer containing mixed leukocytes was aspirated from the top of the syringe and centrifuged at low speed, and the suspended pellet was used as an inoculum. Tissue specimens, such as liver biopsy material, were homogenized by grinding. The resulting suspension was centrifuged, and the supernatant fluid was used as an inoculum. Tubes inoculated with tissue suspension and blood specimens were washed with fresh medium 2 to 3 h after inoculation. Medium was changed in all culture tubes on day 1 and then every seventh day after set-up.

Organization of study. This prospective study was undertaken in two segments. In the first segment, specimens were inoculated onto a single tube of WI-38 cells and then incubated at 36°C for 28 days. In the second segment, one tube of WI-38 cells was compared with one tube of MRC-5 cells over a 14-day period for susceptibility to CMV infection and speed of development of viral cytopathic effect after inoculation with fresh clinical specimens.

Tightly sealed culture tubes were incubated at 36°C on a roller drum (0.2 rpm). Morphological characteristics of monolayers were examined (×40 magnification) daily for the first 7 days (except Sunday) of incubation, then every second to third day thereafter. Identification of CMV was by typical cytopathic effect (9), and each isolate was confirmed by at least two observers. No CMV-positive specimens produced a cytopathic effect in primary human kidney cells.

Statistical analysis of results was performed, using McNemar's test for correlated proportions (sign test), the paired *t* test, or Fisher's exact test, as appropriate.

RESULTS

CMV isolation in WI-38 cells. Of 1,375 routine specimens submitted for CMV culture in a 12-month period, 88 (6.4%) were positive for CMV in WI-38 cell monolayers. Ninety-two percent were positive within the first 14 days of the 28-day incubation period. Specimens which were slow to become positive (>14 days) are characterized in Table 1.

Comparison of WI-38 cells with MRC-5 cells for CMV isolation. When two tubes of human embryonic lung cell monolayers, one each of WI-38 and MRC-5 cells, were inoculated in parallel with each of 1,058 fresh clinical specimens, CMV was isolated from 92 specimens for an overall isolation rate of 8.7%. However, MRC-5 cells were significantly more sensitive than WI-38 cells for the isolation of CMV (Fig. 1). Ninety-one specimens (99% of all positive specimens) were positive in MRC-5 cells, but only 75 (82%) were positive in WI-38 cells. Seven specimens positive only in MRC-5 cells and 17 specimens positive in both cell lines (24 of the 92 positive specimens) were inappropriate for comparative study due to technical problems: 15 developed bacterial or fungal overgrowth that was controllable by changing the medium in the cultures, 1 MRC-5 positive was paired with an uncontrollably contaminated WI-38 tube, 1 had a second virus isolated, and 7 were set up with multiple tubes of MRC-5 cells. Neither cell line was more susceptible to contamination than the other ($P > 0.05$, McNemar test). Of the 68 positive specimens suitable for comparative analysis, 67 (98%) were positive in MRC-5 cells and only 58 (85%) were positive in WI-38 cells.

Overall, the mean day of initial observation of CMV cytopathic effect was 7.4 days after inoculation. However, the mean day of positive findings varied with the specimen type (Table 2). When all positive results were compared, CMV was detected significantly earlier in MRC-5 cells than in WI-38 cells ($P < 0.001$, McNemar test). When analyzed by specimen type, the observed difference was meaningful only for urine specimens. Positive specimens from sources other than those listed in Table 2 included one nasopharyngeal swab, one urethral swab, and one sputum specimen; these were positive on days 7, 9, and 13, respectively.

The distribution of CMV-positive cultures was analyzed for each of 12 consecutive months. Little seasonal variation was found (data not shown). Thirty-eight specimens, just over half of the total, were positive in both cell lines on the same day after inoculation. However, 38% (26 specimens) yielded positive results earlier or only in MRC-5 cultures. Sixteen specimens were positive an average of 4.8 days (range, 1 to 8 days) earlier in MRC-5 cells. Ten MRC-5-positive specimens were not detected in WI-38 cells within the 14-day incubation period. Three specimens were positive earlier in WI-38 cells by an average of 3.7 days, and only one specimen was positive in WI-38 cells and not in MRC-5 cells. These observed differences were not clustered, and lot-to-lot variability of susceptibility of either cell line was not apparent.

WI-38 and MRC-5 cells were compared for the length of time it took to isolate CMV from urine specimens of different patient groups (Fig. 2). When 15 specimens from 11 infants were cultured, the mean time to initial detection of CMV cytopathic effect was 4.5 days in MRC-5 cells, compared with 5.2 days in WI-38 cells. All

TABLE 1. Summary profiles of six CMV-positive patients whose cultures were not positive within the first 14 days of the 28-day incubation period that followed inoculation of specimens onto WI-38 cell monolayers

Patient age (yrs)	Specimen type	Time to culture-positive results (days after inoculation)	Clinical information
5	Tears	16	Acute lymphocytic leukemia
26	Bronchial brush	17	Renal transplant recipient
44	Urine	16	Renal transplant recipient
58	Urine	21	Lymphoma
	Saliva	17	
22	Urine	24	Mononucleosis syndrome
14	Urine	16	Guillain Barré syndrome

specimens were positive in both cell lines. The mean time to positive results for 12 specimens from eight children was 7.0 days in MRC-5 cells. WI-38 cultures lagged by 1.4 days, and two positive specimens were missed completely. Furthermore, six specimens from six different adults were positive in MRC-5 cells by an average of 6.3 days after inoculation. One positive specimen was missed in WI-38 cells, and the mean day of positive results in WI-38 cells was 3.1 days past the MRC-5 mean.

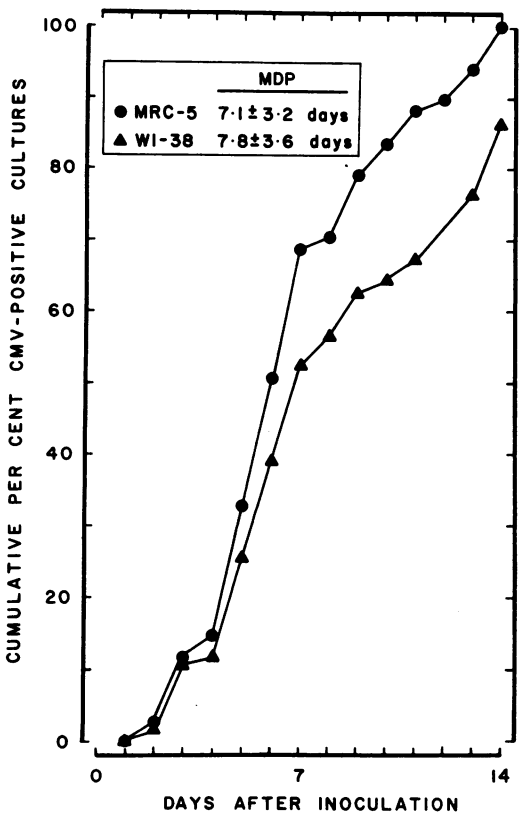

FIG. 1. Comparison of WI-38 and MRC-5 cell monolayers for the isolation of CMV from duplicate samples of fresh clinical specimens submitted over a 12-month period. MDP, Mean time to initial detection of viral cytopathic effect. The observed difference in sensitivity between the two cell lines is significant ($P < 0.01$, McNemar test).

DISCUSSION

A number of different diploid cell lines have been used for the isolation of CMV from clinical specimens (4, 8, 9, 10, 11). Few studies, however, have compared their relative efficiencies for virus isolation. The data presented here indicate that two remarkably similar cell lines were not equally sensitive for the detection of CMV in clinical specimens. As a result of this finding and the results of studies designed to improve the cost-effectiveness and clinical usefulness of CMV isolation, a practical protocol for CMV isolation is presented. The cornerstone of this protocol is the use of a single tube of MRC-5 cells in monolayer incubated at 36°C for 2 weeks.

An incubation period of at least 4 weeks before discarding a negative culture is recommended in many protocols for CMV isolation (4, 9). We found that 92% of 88 CMV isolates were detected in WI-38 cells within the first 2 weeks of incubation. To improve the cost-effectiveness of our protocol, the five tube readings beyond day 14 and the fluid changes on days 14 and 21 were eliminated. This resulted in a reduction of laboratory operating costs and provided the additional time necessary for processing new specimens. At the same time, a sensitivity of greater than 90% was retained for the 14-day procedure when compared with the 28-day procedure in our laboratory.

In an earlier study, Friedman and Koropchak (4) concluded that the speed of CMV cytopathic effect development was the same in WI-38 and MRC-5 cell cultures. That study used cells inoculated with samples of seven frozen urine specimens previously positive in WI-38 cells. Our analysis of 57 specimens positive in both cell lines is essentially in agreement (data not shown). However, the data we present indicate that 16% more positive specimens were detected

TABLE 2. Development of CMV cytopathic effects (CPE) in WI-38 and MRC-5 cell monolayers inoculated with duplicate samples of various types of specimens

Specimen type	No. of specimens	No. of patients	Mean time for development of CPE (days)[a]	Observation of CPE[b]				
				MRC-5 earlier	MRC-5 only	WI-38 earlier	WI-38 only	Both simultaneously
Urine	45	32	6.7 ± 3.1	11/45	5/45	2/45		27/45
Cervical-vaginal	13	9	9.9 ± 3.3	2/13	3/13	1/13	1/13	6/13
Saliva	5	5	7.7 ± 3.3	3/5				2/5
Liver	2	2	3.3		1/2			1/2

[a] Elapsed time (mean number of days ± standard deviation) from inoculation to initial observation of CPE in all positive cultures of each specimen type. The observed differences between these categories are significant (two-tailed t test): urine and cervical-vaginal ($P < 0.01$), urine and liver ($P < 0.02$), cervical-vaginal and liver ($P < 0.001$), and saliva and liver ($P < 0.05$).

[b] Number of cultures in which typical CMV CPE was observed as indicated. Total length of observation was 14 days. The observed difference between MRC-5 and WI-38 cultures is significant for urine ($P < 0.001$, McNemar test), but not for cervical-vaginal specimens ($P = 0.15$, Fisher exact test) or saliva ($P = 0.08$, Fisher exact test).

in MRC-5 cultures than in cultures of WI-38 cells. A trend towards earlier positive results in MRC-5 cells was also observed. These gains can be viewed as distinct contributions to the efficiency of the procedure, and they may even offset the 8% reduction in positive results sustained by restricting the incubation period to 14 days. Overall, 38% of 68 isolates were positive earlier or only in MRC-5 cells. Furthermore, the 26 specimens that yielded positive results earlier or only in MRC-5 cultures were well distributed throughout the year, reducing the probability of extreme variability in susceptibility of cells in particular commercial shipments. The lack of seasonal variation in CMV isolation is consistent with what is known about the epidemiology and transmission of this virus (1–3).

The apparent differences in mean day to first cytopathic effect observed in our study versus that of Friedman and Koropchak (4) may not be real differences and may only reflect the timing of culture reading, the type of specimens examined, or the effects of storage. They reported a mean day of onset of cytopathic effect of 10.4 and 8.4 days for WI-38 and MRC-5 cells, respectively, when cultures were examined twice a week (4). In comparison, when we analyzed the temporal appearance of cytopathic effect for 45 isolates of CMV from fresh urine specimens, the mean day of first appearance in the same two cell lines was 7.2 and 6.3 days, respectively. Our tubes were read daily for the first week because approximately 50% of positive specimens were found within the first 7 days of incubation, a period when results might be expected to be of maximal clinical value.

Early cytopathic effects of CMV were easier to detect in MRC-5 cells than in WI-38 cells, and it is tempting to speculate that MRC-5 cells are more efficient for the detection of low numbers of infectious viruses than are WI-38 cells. Studies are in progress to test this hypothesis, although the lesser sensitivity of WI-38 cells may simply be a function of their age. Indeed, the present utility of the MRC-5 line may diminish with increasing passage.

Maiatico et al. (Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, C36, p. 268) found little benefit from inoculating multiple tubes of the same cell type for the isolation of a variety of viruses. Only 3 of 107 isolates from 535 fresh clinical specimens would have been missed had a single tube rather than multiple tubes been used. In our study, two cell lines that are similar but not

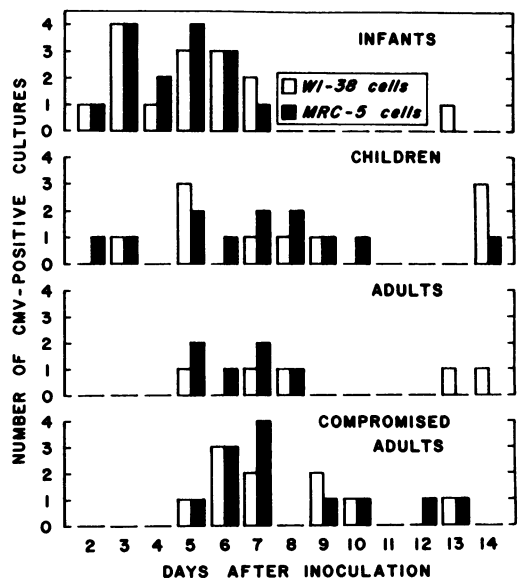

FIG. 2. Comparison of the time to initial detection of CMV cytopathic effect in WI-38 or MRC-5 cell monolayers that had been inoculated with fresh urine specimens from different patient groups.

identical (WI-38 and MRC-5) were compared, and one line (MRC-5) was found to be superior for CMV isolation. The apparent difference in positive rates in the data presented here, 6.4% for one tube of WI-38 cells incubated 28 days versus 8.7% for two tubes (different cell lines) incubated for 14 days, probably reflects differences in specimens processed during the two segments of the study. However, sampling an increased volume of specimen by inoculating multiple tubes may provide additional sensitivity in detecting CMV, particularly for specimens that contain low titers of virus. Additional tubes might also enhance sensitivity if they escape the toxic effects of bacterial or fungal contamination, although contamination can often be controlled by changing the medium in the tubes as soon as contaminants appear. Nevertheless, a second tube of WI-38 cells adds little to the sensitivity and speed of detection of CMV when MRC-5 cells are in the primary tube (Fig. 1 and Table 2). Our experience, albeit limited, with duplicate tubes of identical cells (data not shown) was consistent with the observations of Maiatico et al. (Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, C36, p. 268). We therefore chose to eliminate the WI-38 tube from our setup rather than replace it with an additional tube of MRC-5 cells.

Decreasing the incubation time (from 28 to 14 days) and the number of tubes inoculated (from 2 to 1) reduced the cost to our laboratory of culturing specimens for CMV by approximately 40% without greatly affecting the overall efficiency of the procedure. The savings realized were in terms of technologist time, the cost of cell culture tubes, and the materials used for culture maintenance. Each laboratory should review its CMV isolation results before making significant procedural changes since a number of variables including age and immune status of the patient, the type of specimen, and storage may influence the speed and rate of CMV recovery. It appears reasonable, however, for diagnostic virology laboratories to consider using single tube cultures of MRC-5 cells, incubated for 2 weeks at 36°C, for the routine isolation of CMV. Attempts to isolate other viruses should include other protocols, as appropriate.

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