JOURNAL OF CLINICAL MICROBIOLOGY, May 1990, p. 965–969
0095-1137/90/050965-05$02.00/0

Vol. 28, No. 5

Enhanced Recovery of Cytomegalovirus in Conventional Tube Cultures with a Spin-Amplified Adsorption†

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Low-speed centrifugation-mediated adsorption was evaluated as an enhancement of infectivity of clinical and laboratory strains of cytomegalovirus (CMV) occurring with cells grown in conventional culture tubes. The time required for reporting of primary isolates of CMV from urine specimens adsorbed onto monolayers of WI-38 cells in culture tubes was calculated. Of 668 specimens adsorbed by the stationary phase (SP) method, 98 were positive by cytopathic effect (CPE) that required an average of 16.8 days for recovery in culture. However, the appearance of CPE required a shorter average time of 11.9 days for 70 CMV strains isolated from 283 specimens adsorbed in tube cultures by the spin-amplified (SA) method. In another phase of clinical CMV recovery, urine specimens were adsorbed by the SA method onto cell cultures grown in both shell vials and test tubes. Of 594 specimens inoculated, a total of 74 were positive by either CPE in test tubes or immunostaining-localized early antigen in shell vials. Approximately one-third of these CMV isolates were recovered only by CPE from specimens adsorbed by the SA method in test-tube cultures. In a related study to further evaluate differences between adsorption methods, the AD-169 laboratory strain of CMV was adsorbed by SP and SA methods onto MRC-5 cells grown in both culture vessels. Early antigen detection by immunomicroscopy was found in the infected cells at least 2 to 4 days prior to the appearance of CPE, regardless of adsorption procedure. In both vessels, the replication of AD-169 virus in cultures adsorbed by the SA method consistently exceeded that of virus adsorbed by the SP procedure. CPE occurred 24 to 48 h earlier and progressed two to four times more extensively; early antigen was expressed two- to fourfold greater within 24 to 48 h postinfection; and foci of infected cells containing late antigen were two to four times greater in number at 1, 2, and 5 days postinfection. Overall, the replication and enhancement of infectivity of laboratory and clinical strains of CMV as determined by CPE and early and late antigen expression occurred most efficiently with specimens adsorbed by the SA method onto cultures grown in conventional tubes or shell vials.

The diagnosis of human cytomegalovirus (CMV) infection has been aided recently with the development of a rapid and sensitive method for recovery of the virus in tissue culture. The use of low-speed centrifugation during adsorption of the virus has been found to enhance the infection of susceptible cells (1). This enhanced infection can be detected with a monoclonal antibody to an early antigen of CMV prior to the development of cytopathic effect (CPE) in tissue culture.

Originally, the centrifuge-mediated adsorption of CMV had been implemented with MRC-5 cells grown on cover slips in shell vials (1). Those authors indicated that 100% of viral isolates derived from urine specimens may be recovered in tissue culture within several days postinoculation. The centrifugation-mediated adsorption of specimens in shell vial cultures, combined with early antigen immunomicroscopy, has enabled this shortened time to detect CMV replication. Some laboratories recover CMV from urine specimens exclusively with the use of shell vial cultures (7a). Other investigators reported the need to inoculate specimens into both shell vial and conventional tube culture vessels for optimal recovery of CMV (3, 4, 7a). However, the adsorption of specimens in shell vials has been centrifugation-mediated, whereas the adsorption with tube cultures has occurred by the routine method.

This laboratory has previously shown that the sensitivity of recovery of herpes simplex virus can also be enhanced with cells grown in standard culture tubes through the use of a modified spin-amplified (SA) adsorption method (5). This report indicates that a similar enhanced and rapid recovery occurs with clinical or laboratory strains of CMV adsorbed with the modified SA procedure onto human embryonic fibroblast cells cultured in conventional tubes. Furthermore, this report is a direct comparison of the effect of SA adsorption upon viral recovery and the detection of early CMV replication in both conventional tube and shell vial cultures.

MATERIALS AND METHODS

Clinical specimens and processing. Routine urine specimens submitted for CMV cultures were obtained from patients in various departments including obstetrics and gynecology, internal medicine, pediatrics, renal transplant, and oncology. Specimens were kept on ice during several hours of transportation to the virology laboratory. For processing specimens, a 10-ml volume of urine was centrifuged at 400 × *g* in a tube containing several glass beads. Approximately 7 ml of the urine supernatant was removed, and the remaining urine and sediment were vortexed for 30 to 60 s. The material was then filtered through a 0.45-μm-pore-size syringe filter (Gelman Sciences, Inc., Ann Arbor, Mich.) that was first moistened with 1 ml of maintenance medium (described below). This processed urine specimen, now bacteria free, was used to inoculate tissue culture cells.

Tissue culture reagents and virus stock. Commercially prepared human embryonic fibroblasts were utilized for

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† This report is dedicated to the memory of Sue Hwa Loo for her inspiration, scientific contributions, and dedication.

isolation of CMV in the clinical laboratory. WI-38 cells (M.A. Bioproducts, Walkersville, Md., and ViroMed, Minneapolis, Minn.) and MRC-5 cells (M.A. Bioproducts and Ortho Diagnostics Laboratories, Inc., Raritan, N.J.) were supplied in 16 by 125 mm screw-cap test tubes. Commercially supplied shell vials of MRC-5 were also used in part of the study (M. A. Bioproducts and ViroMed). MRC-5 cells (American Type Culture Collection, Rockville, Md.) were used for the quantitative replication study with CMV. The AD-169 strain of human CMV (American Type Culture Collection) was used in the quantitative replication study. The virus stock had an original titer of 4×10^3 PFU/ml.

Commercially supplied cell cultures used in the clinical studies were held in maintenance medium consisting of Eagle minimal essential medium (EMEM) with 2% fetal calf serum (FCS) as described previously (5). MRC-5 cells used for the quantitative replication study were grown in modified EMEM containing 200 mM glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml (GIBCO Laboratories, Grand Island, N.Y.), and 10% FCS (KC Biologicals, Lenexa, Kans.) for 3 to 5 days at 35°C in shell vials or in 16 by 125 mm screw-cap culture tubes until confluent.

Adsorption of clinical specimens. Culture media were decanted from cell monolayers in tubes or shell vials. The cells were inoculated with 0.2 ml of the processed patient urine specimen. The specimens were adsorbed in tube cultures by the stationary phase (SP) method at $1 \times g$ (9) at 35°C for 1 h or by the SA technique at $750 \times g$ at 30 to 33°C for 45 min (5). Shell vials were centrifuged for 45 min at $750 \times g$ in a swinging bucket rotor at 30 to 33°C. After adsorption, the cultures were washed once with 1 ml of maintenance medium. The tube cultures were incubated with 1 ml of maintenance medium at 35°C and monitored at 1- to 2-day intervals for the appearance of characteristic CPE for 4 weeks. Cultures were refed with 1 ml of maintenance medium at weekly intervals. The cultures in shell vials were fixed with 95% ethanol for 10 min at 3 to 5 days postinfection for later immunostaining for the nuclear-localized early antigen.

The clinical study was composed of three phases. In phase I, all specimens were inoculated in duplicate onto WI-38 cells in culture tubes processed by SP adsorption. All specimens in phase II were inoculated onto duplicate tubes of WI-38 cells and adsorbed by the SA procedure. In phase III, all specimens were adsorbed by the SA procedure onto confluent MRC-5 cells in one shell vial and one tube. SP adsorption of phase I was historically the method used by this laboratory for recovery of all viral isolates in WI-38 cells grown in culture tubes. As a result of work with herpes simplex virus (5) and CMV (unpublished data), urine specimens received in phase II for CMV isolation were only adsorbed by the SA method. In phase III, then, the recovery of CMV following specimen adsorption by the SA method was directly compared in MRC-5 monolayers within the two culture vessels. Specimens were transported, stored, and processed in a similar fashion throughout all phases of the study. Patient populations did not differ between phases. Any isolation of CMV, as determined by characteristic CPE or immunochemical-localized early antigen, was reported to the attending physicians.

Quantitative replication of CMV. The AD-169 strain of CMV stock was serially diluted 10-fold in modified EMEM with 2% FCS. Ice-chilled 10-fold dilutions of virus stock containing 8×10^2 to 8×10^0 PFU/0.2 ml were used to infect MRC-5 cells in both shell vials and culture tubes. Immediately before inoculation, the modified EMEM was decanted from the cells, and 0.2 ml of diluted viral stock was added to each vessel. Virus was adsorbed by either the SP or SA method, as described in the previous section. Mock-infected MRC-5 cultures were included as negative controls. The monolayers were then washed three times, refed with modified EMEM with 2% FCS, and incubated at 35°C, as described for clinical CMV cultures. Tubes and shell vials containing cultures with viral dilutions that were adsorbed by either the SP or SA method were randomly harvested in triplicate and fixed with 95% ethanol for 10 min at 1, 2, 5, and 7 days postinfection (p.i.). Cultures were examined daily in a blinded fashion for CPE prior to harvest, as described previously (5).

Immunostaining. The optimal working dilutions for the anti-CMV monoclonal antibodies against early nuclear and late antigen (catalog no. 9221 and 9220, respectively; DuPont Speciality Diagnostics, Wilmington, Del.) and for the horseradish peroxidase- or fluorescein-conjugated goat anti-mouse immunoglobulin G (Organon-Teknika-Cappel, West Chester, Pa.) were determined in a checkerboard titration on MRC-5 cell monolayers infected with the AD-169 strain of CMV. All antibody incubations occurred at 35 to 36°C for 30 to 60 min, separated by buffer washings ranging from 1 to 5 min.

Previously fixed cell monolayers were rehydrated twice with phosphate-buffered saline (PBS) (pH 7.2). The cells were precoated with 0.2 ml of 10% normal horse serum (GIBCO Laboratories) in PBS for 10 min. Next, 0.150 ml of the monoclonal antibody diluted 1:20 in 10% normal horse serum in PBS was added to the cell monolayer. After incubation, the cells were washed three times with PBS.

The cells in shell vials inoculated with clinical specimens were then treated with fluorescein-conjugated goat anti-mouse immunoglobulin G at a 1:40 to 1:240 dilution in PBS. After incubation, the cells on the cover slips were washed again with PBS, with a final concentration of 1% Evan's blue included in the last washing. For the quantitative replication study, a volume of 0.2 ml of a 10-fold dilution of the horseradish peroxidase conjugate goat anti-mouse immunoglobulin G in 10% normal horse serum in PBS was added to the corresponding tubes or vials. The immunoperoxidase staining progressed as previously described (12). The entire cover slip in the shell vials was examined for the presence of viral antigen-containing cells. Only the cells located within the lower 1-cm butt of the culture tubes were considered for CMV antigen quantitation, because virus-infected cells concentrated almost exclusively in this area of the monolayer. The immunostained cells were examined by inverted microscopy and quantitated for the presence of intensely staining nuclei (early antigen) (see Fig. 2) and cytoplasm (late antigen) (see Table 3).

RESULTS

Clinical recovery of CMV. Recovery of CMV from patient urine specimens was evaluated by both SP and SA adsorption methods (Table 1). In phase I of the study, a total of 90 CMV isolates were recovered from urine specimens by the SP adsorption method with WI-38 cells grown in test tubes. A second series of 70 isolates in phase II was recovered in WI-38 tube cultures adsorbed by the SA method. The mean number of days required for isolates to be reported positive by CPE was determined separately for virus isolated in phase I and phase II. A mean time of 16.8 days was needed for CMV to be reported as recovered from urine specimens processed by SP adsorption in phase I. A shorter mean time

TABLE 1. Recovery of clinical isolates of CMV adsorbed by either the SA or SP method in culture tubes containing WI-38 cells[a]

Adsorption method	No. of isolates/ no. of specimens	Isolation rate (%)	Mean no. of days for CPE development[b]
Phase I (SP)	98/668	14.6	16.8
Phase II (SA)	70/283	24.7	11.9

[a] In phase I, urine specimens were inoculated onto WI-38 cells in culture tubes, with adsorption by the SP method. In phase II, a second group of different urine specimens was inoculated onto WI-38 cells in culture tubes, with adsorption by the SA method. The number of days needed to report CMV recovery as determined by CPE development was totalled for all specimens processed by either adsorption. The mean number of days required for CPE appearance was calculated with this summation total divided by the number of isolates recovered.

[b] A significantly ($P < 0.001$) shorter time occurred for CPE to develop in cultures adsorbed with specimens by the SA as opposed to by the SP method. The statistical analysis was determined by the Student t test.

of 11.9 ($P < 0.001$) days for recovery of CMV by CPE was realized with urine specimens processed by SA adsorption in phase II.

Phase III of the study compared simultaneously the detection of CMV from urine specimens that were adsorbed by the SA method onto MRC-5 cells grown in both shell vials and conventional culture tubes (Table 2). A total of 74 isolates were recovered from urine specimens of patients. For isolates recovered in both vessels, the time required to recognize CPE compatible with CMV in conventional tube cultures was 12.3 days ($n = 42$). The identification of CMV-infected cells cultured in shell vials was routinely determined by immunofluorescence at 3 to 5 days p.i. with the monoclonal antibody to the early antigen of CMV. Over one-third (25 of 74) of the CMV isolates were recovered solely in tube cultures. Another 9.5% (7 of 74) of CMV isolates were recovered only in shell vial cultures. The suggestion that SA adsorption was responsible for the increased recovery of clinical isolates of CMV in tube versus shell vial cultures is addressed in the following section.

Quantitative replication of CMV. A study to show enhanced viral infectivity by SA adsorption in tubes and shell vials was undertaken. The development of CPE in MRC-5 cells inoculated with 10-fold dilutions of a CMV stock adsorbed by either the SP or SA method was recorded in shell vials and test tubes (Fig. 1). Cells that were adsorbed with 8×10^2 PFU/0.2 ml by low-speed centrifugation in shell vials and culture tubes developed CPE 1 to 2 days earlier than those cultures adsorbed by the SP method. The progression of CPE development was determined as a percentage of the cell monolayer affected. Within the first 6 days

TABLE 2. Recovery of CMV from urine specimens processed by SA adsorption with MRC-5 cells grown in tubes and shell vials[a]

No. of specimens	Detection (avg no. of days) of:	
	Early antigen in shell vials	CPE in culture tubes
42	+ (4 days)	+ (12.3 days)
25	—[b]	+ (17.0 days)
7	+ (4 days)	–
520	–	–

[a] A total of 594 specimens were inoculated separately onto cell monolayers in culture tubes and shell vials.

[b] —, Results from four specimens were not interpretable because of toxicity in shell vials.

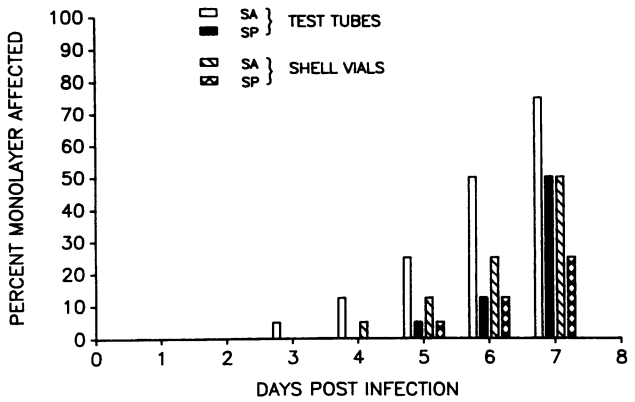

FIG. 1. Effect of SA and SP adsorptions on the replication of CMV as measured by CPE. Culture tubes and shell vials of MRC-5 cells were each inoculated with 800 PFU/0.2 ml of the AD-169 strain of CMV and were processed by either the SP or SA adsorption method. CPE was estimated in a blinded fashion on a daily basis with recordings of 0, <0.5+, 0.5+, 1+, 2+, 3+, and 4+, corresponding to 0, less than 12.5, 12.5, 25, 50, 75, and 100% of cell monolayer involvement, respectively.

p.i., monolayers of cells adsorbed with virus by the SA technique in culture tubes and shell vials exhibited two to three times more progressive CPE involvement than did monolayers of cells adsorbed with virus by the SP method in either vessel type.

The expression of CMV antigens in MRC-5 cultures infected with 10-fold dilutions of AD-169 stock virus was also determined. During the first 2 days p.i., all SA cultures expressed intranuclear early antigen two to four times greater than did cultures processed by SP adsorption (Fig. 2). The effect of the SA adsorption technique on the expression of late antigen-containing foci of CMV-infected MRC-5 cultures grown in conventional tubes or shell vials was also determined. Again, the SA adsorption enhanced the replication of AD-169 (Table 3). Viral cultures adsorbed by the SA method in either tubes or shell vials at 1, 2, and 5 days p.i., had two to four times more late antigen-containing foci than did comparable cultures processed by SP adsorption. Overall, the virus adsorbed in tube and shell vial cultures consistently exhibited enhanced CPE and greater early and late antigen expression compared with the virus in the same vessels adsorbed by the SP technique.

DISCUSSION

This laboratory has described a modified low-speed centrifugation-mediated adsorption procedure for herpes simplex virus with conventional culture tubes (5). An enhanced recovery of clinical and laboratory strains of herpes simplex virus occurred when specimens were adsorbed by the SA instead of SP method onto cells grown in conventional culture tubes. The sensitivity of recovery of clinical herpes simplex virus isolates was 100% with specimens adsorbed by the SA method, whereas only 88% of isolates were recovered from the identical specimens adsorbed by the SP procedure. This finding with herpes simplex virus led to the current evaluation of the recovery of CMV by the modified SA adsorption procedure in conventional tubes.

The infectivity of CMV was first shown to be enhanced by low-speed centrifugation by Weiss and Dressler (13) and was confirmed later by others (2, 6). Gleaves et al. (1) introduced this adsorption procedure into the clinical virology labora-

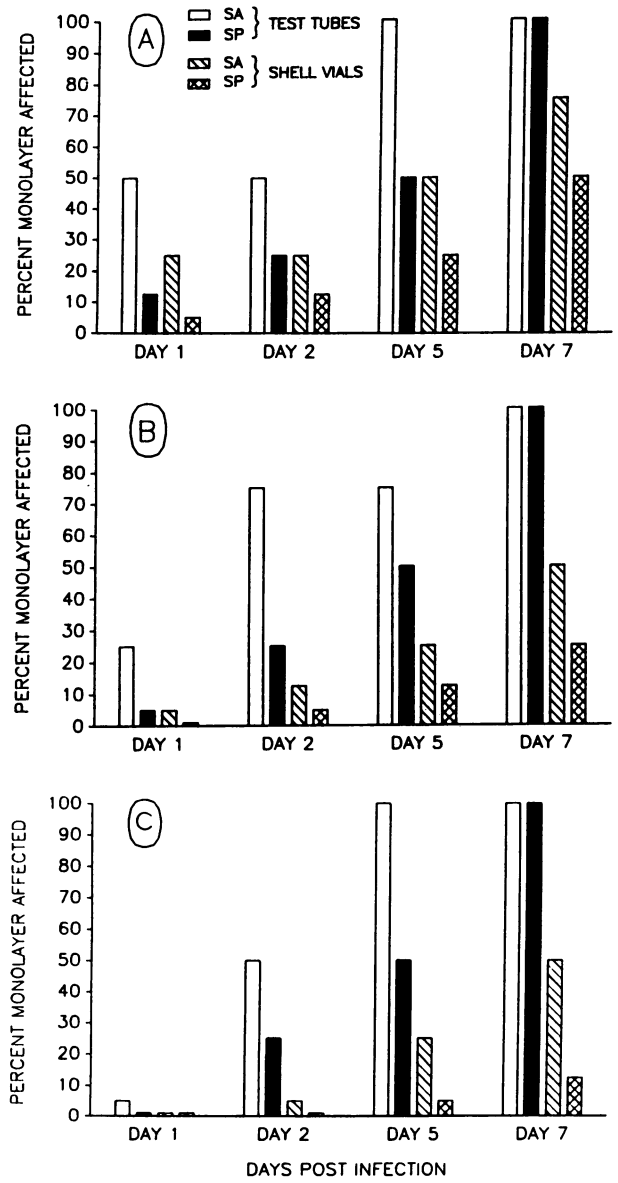

FIG. 2. Effect of SA and SP adsorptions on the replication of CMV as measured by the expression of early antigen. Culture tubes and shell vials of MRC-5 cells were each inoculated with either 800 (A), 80 (B), or 8 (C) PFU of the AD-169 strain of CMV per 0.2 ml and were processed by either the SP or SA adsorption method. At days 1, 2, 5, and 7 p.i., the vessels containing cells processed by either adsorption procedure were fixed in triplicate and stained for the presence of early antigen with the use of monoclonal antibody and indirect immunoperoxidase microsocopy. The viral antigen was quantitated as 0, <0.5+, 0.5+, 1+, 2+, 3+, and 4+ corresponding to 0, less than 12.5, 12.5, 25, 50, 75, or 100% of cell monolayer involvement, respectively.

tory. A monoclonal antibody (10) was used to immunomicroscopically visualize the intranuclear early antigen of CMV within 24 to 48 h p.i. in cells that had been adsorbed by the SA method with urine specimens from infected patients. Others have confirmed these culture findings with urine specimens and have extended the application of recovery of CMV from blood and respiratory specimens (3, 7, 11, 12) inoculated into shell vial cultures.

The recovery of CMV in cell cultures grown in shell vials has gained popularity because of the above-cited studies. However, poor quality or toxicity, sometimes due to bacterial contamination of cell cultures in shell vials, contributes to uninterpretable early antigen detection by immunomicroscopy. Several investigators have reported such a poor quality or toxicity interfering with ≤16.5% of specimens (4, 8, 12). Paya et al. (7) reported that 2% of 1,032 various-site specimens inoculated by SA adsorption into shell vial cultures had deficient cell monolayers. Conversely, the same workers found that bacterial contamination in culture tubes occurred in 8 of 55 (14.5%) specimens that were CMV positive by early antigen in shell vial cultures. Swenson and Kaplan (12) reported 16.5% of 200 shell vial cultures of specimens from various sites developed toxicity whereas only 1.5% of culture tubes inoculated by SP adsorption with the identical specimens exhibited similar toxic effects. In this study, toxicity in tube cultures was inconsequential. In summary, conventional culture tubes are often more refractory to the toxic effects of specimens that have been noted in shell vial cultures.

TABLE 3. Enumeration of CMV late antigen-staining foci localized in MRC-5 cells grown in conventional culture tubes and shell vials following AD-169 virus adsorption by either the SA or SP method[a]

Inoculum titer/ vessel (PFU)	Days p.i.	Adsorption in:			
		Culture tubes		Shell vials	
		SA	SP	SA	SP
8×10^2	1	72	24	24	12
	2	144	48	96	36
	5	TNTC[b]	156	144	60
	7	TNTC	144	144	96
8×10^1	1	36	12	36	12
	2	96	36	48	24
	5	300	72	84	24
	7	TNTC	108	96	36

[a] Foci localized by indirect immunoperoxidase staining were enumerated by taking an average of five or more fields viewed at a total of magnification of ×200. The average number of foci per ×200 magnification field was then multiplied by a factor of 12, since there were 12 such discernable fields in shell vials or culture tubes. Values presented in the table represent a total number of late antigen foci localized by immunostaining in either vessel type.

[b] TNTC, Too numerous to count; representative of >300 foci per culture vessel.

For optimal recovery of CMV, investigators have demonstrated the need to inoculate tissue culture cells in both shell vial and standard tube vessels. The total recovery of primary isolates of CMV from patient urine specimens in this study increased fully one-third when centrifugation-mediated adsorption occurred with cells grown in tubes as opposed to SA adsorption in shell vials. Leland et al. (4) reported a similar finding of 32% of CMV isolates from urine specimens recovered only in tube cultures. They found that another 12 of the 71 (17%) CMV isolates recovered from urine specimens were detected only by early antigen immunostaining in shell vial cultures. Swenson and Kaplan (12) reported 5 of 41 (11%) CMV isolates recovered only in shell vial cultures but not in tube cultures. Paya et al. (7a) concluded that both shell vials and culture tubes were necessary for the most efficient recovery of CMV from patient specimens. Those authors found 30.7% of isolates were recovered only in SA-adsorbed cultures in shell vials while another 15.4% were recovered only in SP-adsorbed tube cultures. Jespersen et al. (3) found that 10 to 20% of isolates would not have been recovered if

either tubes or shell vials had been used as the only culture vessel. Optimal recovery may also be partially due to the number of culture vessels inoculated. Detection of early antigen of CMV from leukocyte specimens was increased 51% if three instead of one shell vial cultures were inoculated by SA adsorption (7). The clinical SA-adsorbed tube cultures described in this report may enable recovery of a subset of infectious virus that is difficult to recover in shell vial cultures immunostained for early antigen detection. Notably, the isolates that were recovered only in SA-adsorbed tube cultures required an average of 5 days longer for development of typical CPE, as compared with recovered isolates in cells processed by SA adsorption in both types of vessels (Table 2). This enhanced recovery in tube cultures adsorbed by the SA method may be explained as a function of low inoculum titer or less-efficient viral replication in shell vial cultures.

Several options now exist for the implementation of SA adsorption of specimens in cell cultures for the improved recovery of CMV. One option is to use shell vial cultures only with adsorption occurring with low-speed centrifugation. Cover slips could be harvested 1 to 3 days later and stained for the detection of early antigen. Gleaves et al. (1) suggest that urine cultures can be terminated by 1 to 2 days p.i. by this procedure. Swenson and Kaplan (12) have reported the recovery of CMV in cells grown in shell vials processed by SA adsorption compared with the recovery in tube cultures adsorbed by the SP method. The specimens adsorbed by the SA method in shell vial cultures exhibited about a 20-fold greater number of nuclei immunostained for early antigen than did identical specimens in tube cultures adsorbed by the SP procedure. As described here, SA adsorption enhances the infectivity and replication of CMV as compared with SP adsorption with cell cultures in tubes. This study is the first to compare the effect of $750 \times g$ versus $1 \times g$ adsorption upon the recovery of CMV simultaneously in shell vial and tube cultures. Further, this study indicates that specimens adsorbed onto cells in tubes by low-speed centrifugation cause the recovery of CMV to equal or exceed that realized with SA cultures in shell vials. These contentions are supported by the following three measurements: CPE (Table 1, Fig. 1), early antigen expression (Table 2, Fig. 2), and late antigen expression (Table 3). Finally, if clinical specimens inoculated onto cells grown in culture tubes can be subjected to SA adsorption followed by immunoperoxidase localization of early antigen or reading for CPE, then the overall sensitivity or rate of isolation of CMV may well be increased.

ACKNOWLEDGMENTS

The support and work contributed by Karen Mills and the technologists at the Hermann Hospital Clinical Microbiology Laboratory are greatly appreciated. We thank Nikky Amin, Annie Rose, and Andre Dixon for their word-processing skills during the preparation of the manuscript.

This work was funded in part by grants from the Gulf Coast Regional Blood Center.

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