Detection of cytomegalovirus (CMV) in culture by the demonstration of CMV immediate-early antigen (IEA) enables much more rapid identification of CMV than standard tube culture (7, 17). Additionally, in vitro infectivity of CMV is enhanced by centrifugation of the clinical specimen onto monolayers of human fibroblasts (8). These two techniques in combination have been extensively investigated for clinical diagnostic use (5, 6, 9, 12, 18–20). The sensitivity and specificity of centrifugation plus IEA detection (commonly called shell vial culture) for CMV are now considered close to and in some reports higher than those for standard tube culture.

More recently, chemical agents, including dimethyl sulfoxide (DMSO), dexamethasone (DXM), and calcium chloride (CaCl₂), have been reported to further enhance the in vitro infectivity of CMV in shell vial culture (13, 15, 21–23). Not all investigations, however, have demonstrated increased identification of CMV directly by treatment with these agents (3, 4). Since in vitro adaptation of a virus can occur even with minimal passaging, we were interested in investigating the effects of these agents on the detection of CMV directly in clinical specimens. We also investigated the effects of epidermal growth factor (EGF), a commonly used in vitro mitogen (1, 14), on enhancement of CMV isolation by the shell vial method because CMV is known to exhibit enhanced replication in actively replicating cells (2).

Preliminary experiments to determine optimal concentrations of DMSO, DXM, CaCl₂, and EGF were performed with CMV strain AD169. Stock cultures of CMV strain AD169 (American Type Culture Collection, Rockville, Md.) were propagated in human foreskin fibroblast cultures and clarified by sonication. The virus preparation was mixed with sorbitol to a final concentration of 25% (vol/vol), aliquoted, and stored at −70°C.

DMSO (Sigma, St. Louis, Mo.) was diluted as needed with Eagle's minimum essential medium (Whittaker Bioproducts, Walkersville, Md.) containing 2% fetal bovine serum (Sigma), nystatin, streptomycin, gentamicin, and penicillin (EMEM). DXM (Sigma) was initially dissolved in absolute ethanol to 10⁻² M and then further diluted to the required concentration in EMEM. CaCl₂ (Sigma) was dissolved in water to 0.5 M and then diluted to the required concentration in EMEM. EGF (Sigma) was initially dissolved in Dulbecco's phosphate-buffered saline (DPBS) and then further diluted to the required concentration in EMEM.

Single lots of mouse monoclonal antibody specific for the IEA of CMV (Dupont-Biotech, Rockville, Md.) and goat anti-mouse immunoglobulin G (heavy- and light-chain specific) fluorescein isothiocyanate conjugate (Cappel, Durham, N.C.) were used throughout. Antibody dilutions were optimized by checkerboard titration prior to use.

The shell vial culture method for CMV was performed as previously described (6) with minor modifications. Briefly, media were decanted from shell vial coverslip cultures of MRC-5 cells (Whittaker), overlayed with 0.2 ml of sample, and centrifuged at 2,000 × g for 45 min at 35°C. Another 1 ml of EMEM was added per vial and the vials were incubated at 37°C for 18 to 20 h. Coverslips were then rinsed with DPBS and fixed in acetone at room temperature for 10 min. Fixed coverslips were rinsed with DPBS, treated with 0.2 ml of anti-IEA monoclonal antibody per vial for 30 min at 37°C, rinsed twice with DPBS, stained with fluorescein isothiocyanate-anti-mouse immunoglobulin G for 30 min at 37°C, rinsed with DPBS, counterstained for a few min with 0.5% Evans blue in DPBS, rinsed, and mounted on slides with buffered glycerol. Stained coverslips were observed by fluorescence microscopy (×100 magnification) for the typical apple-green fluorescent nuclear staining of CMV-infected cells.

To determine the optimal concentrations of the agents for enhancement of CMV IEA in shell vial culture, human CMV AD169 was inoculated at a concentration previously determined to produce approximately 100 fluorescent foci per coverslip culture in the absence of cell treatments. Treatments were performed on five replicate cultures, and the total numbers of foci per coverslip were counted under epifluorescence microscopy and averaged for each treatment. The results showed that the addition of either DXM, DMSO, CaCl₂, or EGF in the overlay medium of the shell vials increased the number of fluorescent foci when cultures were stained for CMV IEA. Although maximal focus counts were obtained with 2% DMSO (data not shown), as has been previously reported (22), this concentration was occasionally toxic. Therefore, a 1% concentration of DMSO was chosen as optimum.
levels of enhancement were noted with all concentrations of DXM from $10^{-4}$ to $10^{-6}$ M (data not shown); $10^{-6}$ M DXM was therefore selected for use in all further studies. Enhancement with CaCl$_2$ treatment was greatest at 5 mM, and EGF enhancement was greatest at 50 ng/ml (data not shown).

It has been reported that the enhancement effect of DXM in CMV shell vials is optimized when the vials are treated prior to inoculation as well as after inoculation of virus (13, 21, 22). We confirmed that pretreatment of shell vials with DXM within 24 h of inoculation as well as the inclusion of DXM in the overlay after inoculation (posttreatment) resulted in a higher fluorescent-focus count than posttreatment inclusion of DXM alone (data not shown). However, while pretreatment with EGF 3 h prior to inoculation produced greater enhancement than EGF posttreatment alone, overnight pretreatment with EGF was inhibitory (Fig. 1). This is consistent with findings for EGF and CMV in standard culture (11). Since a 3-h pretreatment step is impractical in our diagnostic laboratory, EGF was used only postinoculation.

The agents were then tested individually and in combination on 14 low-passage (in vitro) clinical isolates of CMV. The isolates ranged from passage 3 to passage 5 and had been isolated from specimens of urine, buffy coat, liver biopsy tissue, duodenal biopsy tissue, and bronchoalveolar lavage fluid obtained from adult solid-organ transplant recipients. Isolates were cultured, harvested, and stored as for CMV AD169. Viruses were inoculated for the enhancement experiments at a concentration previously determined to produce approximately 100 fluorescent foci per coverslip in the absence of cell treatments. Treatments were performed on five replicate cultures of each CMV isolate, and the total numbers of foci per coverslip were counted under epifluorescence microscopy and averaged for each treatment.

For each virus, the percent change in fluorescent-focus count with each treatment compared with untreated controls was calculated, and then the average percent change was calculated for the five low-passage clinical isolates. When these agents were added in combination, the enhancing effect was greater than when they were used individually (Fig. 2). Since the inclusion of EGF in this enhancing treatment gave no further elevation of fluorescent-focus count above that observed with the combination of DMSO, DXM, and CaCl$_2$, EGF was not used in the treatment studies with clinical specimens.

Following these initial experiments to investigate reported enhancement effects and to determine optimal concentrations of the agents, a combination treatment of DXM, DMSO, and CaCl$_2$ was applied to clinical specimens. Sequential clinical specimens obtained primarily (>95%) from adult solid-organ transplant recipients, with the remainder (<5%) from adult AIDS patients, were inoculated into two shell vials of MRC-5 cells. One culture was inoculated, incubated, and processed as usual (untreated). For the matching treated culture of each specimen, the overlying medium on the shell vial was changed the day before inoculation to EMEM containing $10^{-6}$ M DXM. Following specimen inoculation and centrifugation, the treated cultures were overlaid with EMEM containing $10^{-6}$ M DXM, 1% DMSO, and 5 mM CaCl$_2$. Treated and untreated cultures were then incubated, fixed, stained, and examined for
the total numbers of fluorescent foci per coverslip in the positive cultures.
A total of 1,579 specimens were tested, consisting of 459 buffy coat samples, 356 urine specimens, 290 throat wash specimens, 341 tissue biopsy samples, 62 bronchoalveolar lavage specimens, 41 sputum samples, 12 cerebrospinal fluid samples, 12 stool samples, and 6 pleural fluid specimens. Of the 1,579 specimens, 133 were positive in shell vial culture for CMV. For 100 of the positive specimens, accurate counts of fluorescent foci in both treated and untreated vials were possible. In 62 of these specimens, focus counts were elevated in the treated vial compared with those in the untreated vial (\(P < 0.01\), Wilcoxon signed-rank test), while counts for 33 specimens were decreased by the treatment and counts for 5 specimens were unchanged.

Positive specimens were then divided according to the type of specimen, and the effect of the agents was assessed for each (Table 1), since the enhancing effect of these agents has been suggested to be dependent on specimen type (16). Significant increases were seen in urine and throat wash specimens only. While specimen numbers were too low for conclusions about the effects of these agents on bronchoalveolar lavage or sputum samples to be drawn, the agents did not appear to produce significant enhancement in CMV shell vial culture of tissue biopsy (\(P > 0.05\)) or buffy coat (\(P > 0.05\)) specimens.

We found no increase in the number of positive specimens when the enhancing agents were applied to clinical specimens, in contrast to other workers’ findings (22, 23). Fourteen specimens that were negative in untreated vials were positive in treated vials, and 14 specimens that were positive in untreated vials were negative in treated vials. Furthermore, no correlation was seen with specimen type.

Use of the enhancing mixture was found to result in an increased incidence of specimen toxicity. While the majority of specimens (93.6%) produced no toxic reaction in culture, 1.3% produced toxic reactions in both treated and untreated vials, and 1.2% produced toxic reactions in the untreated vial, but 3.9% of specimens produced toxic reactions only in the treated vial. Such increased susceptibility to specimen toxicity by enhancing agents has been reported previously by other researchers (4).

It has been shown that actively growing cells are more susceptible to CMV replication than static cultures (2). Additionally, the mitogenic effects of DMSO and CaCl\(_2\) have been related to enhanced detection of CMV in shell vial culture (23). Likewise, we found that the addition of EGF, a fibroblast cell mitogen (1, 14), also enhanced CMV detection in shell vial culture. While this effect was striking when EGF was applied in the absence of the other agents, no increase was observed beyond that already produced by treatment with the combination of DMSO, DXM, and CaCl\(_2\). It is possible that some antagonism occurred between the calcium and EGF with regard to mitogenic stimulation (10) or that increased CMV detection by mitogenic stimulation of the cell cultures plateau at a certain level and further enhancement of cellular replication beyond this point produces no increase in viral detection.

In conclusion, application of the combination treatment of DMSO, DXM, and CaCl\(_2\) to clinical specimens was not found to increase the overall number of positive specimens identified. We did, however, confirm previous findings of West and Baker (23) of a significant increase in the number of fluorescent foci for positive urine and throat wash specimens. This enhancement was most striking in urine specimens, in contrast to the findings of Espy et al. (3) and Fedorko et al. (4). In the latter studies, however, a different source of MRC-5 shell vials was used (Viromed Laboratories, Minnetonka, Minnesota); these shell vials have been reported to be significantly less responsive to the enhancing agents than those from Whittaker (24) that were used in our study. Finally, we saw no enhancement effect on tissue biopsy or buffy coat specimens. In conclusion, these chemical treatments increase the incidence of toxic reactions to specimens and do not enhance detection of CMV in tissue specimens or buffy coats. Additionally, increased fluorescent-focus counts in positive urine and throat wash specimens are of questionable clinical significance. We therefore do not recommend their use for diagnostic CMV shell vial cultures of clinical specimens.

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REFERENCES


### TABLE 1. Effect of enhancing agents on detection of CMV in clinical specimens

<table>
<thead>
<tr>
<th>CMV-positive specimen (n)</th>
<th>No. (%) of specimens with indicated change in CMV fluorescent-focus count</th>
<th>(P) value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Increase</td>
<td>Decrease</td>
</tr>
<tr>
<td>Urine (48)</td>
<td>33 (69)</td>
<td>13 (27)</td>
</tr>
<tr>
<td>Tissue biopsy (16)</td>
<td>8 (50)</td>
<td>8 (50)</td>
</tr>
<tr>
<td>Throat wash (15)</td>
<td>10 (67)</td>
<td>4 (27)</td>
</tr>
<tr>
<td>Buffy coat (9)</td>
<td>4 (44)</td>
<td>4 (44)</td>
</tr>
<tr>
<td>Bronchoalveolar lavage (6)</td>
<td>4 (66)</td>
<td>1 (17)</td>
</tr>
<tr>
<td>Sputum (6)</td>
<td>3 (50)</td>
<td>3 (50)</td>
</tr>
<tr>
<td>Total (100)</td>
<td>62</td>
<td>33</td>
</tr>
</tbody>
</table>

* Wilcoxon signed-rank test.

**NS**, not significant.


