Enhanced Detection of Cytomegalovirus in Confluent MRC-5 Cells Treated with Dexamethasone and Dimethyl Sulfoxide

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Optimum growth conditions for human cytomegalovirus (HCMV) include the use of subconfluent, actively growing cultures of human embryonic fibroblasts. Many clinical virology laboratories, however, use tissue culture cells from commercial sources. These cells are usually confluent, static cultures that tend to be less sensitive to viral infection. To determine whether dimethyl sulfoxide (DMSO) or dexamethasone (DEX), which are known enhancers of HCMV, facilitates the detection of the virus in confluent cells, we tested both HCMV AD169 and a number of clinical specimens suspected to contain HCMV on MRC-5 cells in both shell vials and conventional tube cultures. We found that, in the shell vial test, treatment of the cultures with either DMSO or DEX before and after inoculation increased the number of cells staining positive by three- to sixfold compared with untreated controls. Best results were obtained by pretreating the cultures with DEX alone and by treating the cultures with a combination of DEX and 1% DMSO postinfection. In the conventional MRC-5 culture tubes, treatment with the reagents resulted in the more rapid appearance of cytopathic effect and a more extensive infection of the cell sheet. The experimental findings indicate that the enhancing effect of DEX is attributable mainly to the increased production of a cellular mRNA during the period preceding viral infection.

The increased awareness of the importance of human cytomegalovirus (HCMV) as a pathogen, particularly in neonates, the elderly, and immunosuppressed patients, has led to the development of new rapid procedures for the detection of this virus in clinical specimens. One of the most sensitive procedures, the centrifugation-enhancement or shell vial method, combines the techniques of centrifugation of specimen inocula onto susceptible cell culture monolayers and incubation of these cultures at 37°C for 20 h, followed by the staining of the monolayers with a monoclonal antibody directed against the products of the immediate early viral genes (IEA) (4). In this test, the nuclei of cells that are positive, that is, infected with HCMV and producing IEA, will stain a bright fluorescent green between 2 and 20 h postinoculation, depending upon the multiplicity of infection. Most clinical specimens have low multiplicities of HCMV infection; therefore, an incubation period of about 20 h is required for detection. This condition is needed especially when confluent, contact-inhibited cultures must be used, as in our laboratory. These cultures, as well as cells metabolically inhibited by UV irradiation or serum starvation, are less sensitive to infection with HCMV than are actively growing nonconfluent cultures (2, 3, 11).

Using a plaquing technique, Tanaka et al. (10) reported an enhancement of HCMV virus production in a semipermissive epithelial cell line and, to a lesser extent, in human diploid fibroblasts, caused by treatment of the cells with dimethyl sulfoxide (DMSO) for 24 h pre- and postinfection, but no increase in the IEA was observed with this treatment. However, an increase in these proteins was detected when both types of cells were treated for 24 h pre- and postinfection with 10⁻⁵ M dexamethasone (DEX), a hormone useful in increasing plating efficiencies in a number of cell lines (11, 12). In all of these experiments, high multiplicities of infection were used.

In this paper, we report the effect on HCMV IEA production of DMSO, DEX, or a combination of both on confluent MRC-5 cells infected with HCMV at low multiplicities. In addition to the centrifugation-enhancement studies, we treated conventional MRC-5 tube cultures and monitored the development of HCMV cytopathic effect in the test specimens and compared the results with those observed for the untreated control tubes. We also investigated the possible mechanisms responsible for the increase in the production of IEA.

MATERIALS AND METHODS

Virus and cell cultures. Stock cultures of HCMV AD169 (ATCC-VR538) were prepared by propagating the virus in MRC-5 cells, disrupting the cells by vortexing with glass beads, removing cell debris by centrifugation, and diluting the supernatant to the required concentrations. Clinical specimens in transport medium and urines were inoculated as received. Approximately 70% of the specimens were urines; the other 30% consisted of throat swabs, pharyngeal washes and tissues. MRC-5 cultures grown on cover slips in shell vials were obtained from Whittaker M. A. Bioproducts, Inc., Walkersville, Md. Eagle minimum essential medium containing 2% fetal bovine serum, 1% glutamine, 50 mg of gentamicin per liter, 50 mg of vancomycin per liter, and 2.5 mg of amphotericin B (Fungizone) per liter was used throughout. The medium, serum, and gentamicin were obtained from M. A. Bioproducts; vancomycin was obtained from Sigma Chemical Co., St. Louis, Mo.; and glutamine and amphotericin B were obtained from Flow Laboratories, Inc., McLean, Va.

Chemicals and reagents. DMSO, DEX, and cordycepin were used at final concentrations of 1%, 10⁻⁵ M, and 30 μg/ml, respectively. These chemicals were obtained from

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Sigma. Mouse anti-early HCMV and mouse anti-late HCMV antisera (diluted 1:10 in saline) were obtained from DuPont-Biotech Research Laboratories, Rockville, Md., and goat anti-mouse fluorescein-conjugated antisera (diluted 1:30 in saline; 0.05% Evans Blue) were from Cappel Laboratories, Malvern, Pa.

**Clinical specimens.** Clinical specimens routinely submitted to our facility were obtained from patients with suspected HCMV infection. These specimens were compared by using the rapid centrifugation and standard culture methods on all treatment groups. Shortage of specimen inocula generally precluded the comparison of all groups at once.

**Rapid centrifugation method.** The medium was removed from MRC-5 vials, and 1 ml of the following solutions was added to specific groups of vials: (i) medium only (control group), (ii) medium + 1% DMSO (DMSO group), (iii) medium + 10^{-5}\ M DEX (DEX group), (iv) medium + 1% DMSO and 10^{-5}\ M DEX (DMSO-DEX group). After 24 h, the appropriate medium was removed and 0.1 ml of virus-containing sample was added. The vials were centrifuged for 1 h (900 \times g; Beckman J-6M) at 36\degree C; the appropriate medium for each group was added, and the vials were incubated at 37\degree C for the required period. The monolayer was then fixed with cold acetone and stained for HCMV nuclear inclusions by the indirect method. A variation of the treatment was also investigated by pretreatment of specimens with DEX alone and posttreatment with a combination of DEX and DMSO. This test, which was done at a later time, was not part of the present study.

**Standard tube culture.** The tubes of MRC-5 cells were treated for 24 h preinoculation with media alone or with media containing 1% DMSO or 10^{-5}\ M DEX. After 24 h, a control tube and a treated tube were inoculated with the specimen and observed daily for 21 days. The treatment was continued for the entire postinoculation test period.

**Virus sensitivity determinations.** The HCMV stock virus was diluted from 10^{-3} through 10^{-6}, and the dilutions were tested in the treated and untreated vials for the presence of immediate-early (20 h of incubation) and late (120 h of incubation) HCMV antigen by the rapid centrifugation method. Six vials per dilution were tested, and the 50% tissue culture infective dose (per 0.1 ml) was calculated by the Reed-Muench method.
RESULTS

Both the laboratory HCMV strain AD169 and a number of clinical specimens were more easily detected in MRC-5 cells treated with 10⁻² M DEX or 1% DMSO, or both (Fig. 1). The inclusions were generally larger and brighter, appeared earlier, and were more numerous at all times between 2 and 20 h in the treated cells (Fig. 2 and 3).

Our dose-response curves for DEX (data not shown) agreed with those of Tanaka et al. (11) and Parks et al. (7, 8) and indicated that a 10⁻² M solution of the hormone was most effective. DMSO was tested at 1, 2, and 3%, but only the 1% concentration was nontoxic to cells under all conditions.

Further experiments with the shell vial technique demonstrated that the greatest enhancement was in cells pretreated with DEX alone and then treated with both DMSO and DEX after the centrifugation period (Fig. 4). Pretreatment with DMSO alone had no extra enhancing effect.

After testing the clinical specimens, we found more positive samples in all treatment categories than we did in untreated control cells (Tables 1 and 2). Moreover, the inclusions were noticeably brighter and at least twofold more numerous on the treated cells, and maximum enhancement was produced in cells pretreated with DEX and posttreated with both DMSO and DEX.

Data on conventional tube cultures for clinical specimens are shown in Table 3. In this group, notable enhancement was observed in terms of earlier and more extensive cytopathic effect.

A previous investigation demonstrated that hormonal enzyme induction by DEX is dependent on the production of one or more cellular mRNAs (9), as is HCMV total virus production in other cell lines (11, 12). To determine whether this cellular mRNA induction also occurs with HCMV IEA enhancement or whether the effect of DEX is primarily on the viral mRNA production taking place after infection (5), we studied the comparative enhancing effect of the drug treatment at (i) 24 h preinfection, (ii) 20 h postinfection, and (iii) 24 h preinfection and then 20 h postinfection. The presence of DEX during the 24 h before infection accounts for the majority of the enhancing effect (Table 4). Increased enhancement was observed with both pre- and postinfection treatments. When cordycepin, an inhibitor of mRNA maturation (1), was added with DEX at preinfection, postinfection, and at both times, the greatest reduction of IEA enhancement was achieved when the inhibitor was present 24 h before infection. Pre- and postinfection treatment with cordycepin alone did not significantly affect IEA production.

DMSO was most effective when added after the centrifugation (adsorption) step. When added to the cells at 24 h

### TABLE 1. Clinical specimen study by rapid centrifugation

<table>
<thead>
<tr>
<th>Treatmenta</th>
<th>No. of specimens testedb</th>
<th>No. of specimens positive</th>
<th>% with more inclusionsc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>For HCMV</td>
<td>On untreated cells</td>
<td>On treated cells</td>
</tr>
<tr>
<td>DMSO</td>
<td>60</td>
<td>25</td>
<td>17</td>
</tr>
<tr>
<td>DEX</td>
<td>99</td>
<td>46</td>
<td>35</td>
</tr>
<tr>
<td>DEX and DMSO</td>
<td>130</td>
<td>51</td>
<td>40</td>
</tr>
</tbody>
</table>

a Specimens were tested for the presence of HCMV IEA at 20 h postinfection.

b The statistical analysis on the number of isolates from treated and untreated cells was by the McNemar test. $P < 0.01$ for all three groups.

c The number of specimens per treatment varied because of insufficient specimen volume.

d Values are percentages of specimens with more inclusions on treated cells than on untreated cells.

e Values in parentheses are percentages of specimens positive on treated cells only.

### TABLE 2. Comparison of pretreatments by rapid centrifugation

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>No. of specimens positive</th>
<th>% with more inclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEX</td>
<td>27</td>
<td>25 (92)</td>
</tr>
<tr>
<td>DEX and DMSO</td>
<td>26</td>
<td>1 (4)</td>
</tr>
</tbody>
</table>

f For each pretreatment, 32 samples, each with <10 inclusions, were tested. The same specimens were used to compare the treatment groups. All samples were treated with both DEX and DMSO postinfection.
preinfection, DMSO appeared to have a slightly deleterious effect on IEA production. Repeated tests showed that a greater number of cells produced IEA when DMSO was present postinfection only, although its presence during both pre- and postinfection periods still produced slightly more enhancement than that of DEX alone.

**FIG. 4.** Comparison of HCMV AD169 IEA production in untreated control cells, cells treated pre- and postinfection with DMSO and DEX, and cells treated preinfection with DEX alone and postinfection with DMSO and DEX. The multiplicity of infection was 0.001, and each bar represents the mean of six test vials. The preinfection treatment was for 24 h, and the postinfection treatment was for 20 h.

**TABLE 3.** Clinical specimen study by the tube method

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of specimens tested</th>
<th>No. of specimens positive</th>
<th>Avg no. of days for CPE (treated cells/untreated cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>60</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>DEX</td>
<td>21</td>
<td>7</td>
<td>5</td>
</tr>
</tbody>
</table>

* Specimens were observed for HCMV cytopathic effect (CPE) for 21 days.
* Treatment with $10^{-3}$ DEX or 1% DMSO was initiated 24 h before inoculation and continued through the 21-day test period.

**DISCUSSION**

An enhancement of both HCMV IEA and late antigen formation occurs when host MRC-5 cells are treated with DMSO and DEX. This effect was observed even with low multiplicities of infection similar to those seen in clinical specimens. In addition, IEA inclusions were noticeably brighter in treated cells, an observation indicating higher specific antigen levels in these cells, compared with those of untreated controls. The treatment of conventional cell cultures with enhancing agents produced earlier and more extensive cytopathic effect. This fact, together with increased late antigen formation, suggests that the total virus production is increased also.

Of interest to clinical virology laboratories is the finding that approximately 90% of HCMV isolates from clinical specimens were measurably enhanced by the various treatments and that these treatments detected HCMV in 22 to 32% of specimens that were negative in untreated cells.

Experiments with the mRNA inhibitor cordycepin indicate that the enhancement is due predominantly to the presence of a cellular mRNA (and its protein product) elicited by DEX during the 24 h before infection. At this time, we can speculate only on the function of this protein; it may play a regulatory role similar to that of nuclear factor I, which attaches to the viral promoter regions and facilitates increased transcription of the IEA (6). The enhancement after infection may be partly due to the increased production of the viral mRNA itself.

In a similar experiment (data not shown), DMSO did not produce similar results, a fact suggesting that it may operate by a different mechanism. Although further investigation in these areas is necessary, cell treatment with DMSO and DEX, in conjunction with the centrifugation technique, provides a rapid and sensitive HCMV detection system that will enable clinical laboratories using confluent cell cultures to significantly increase their HCMV isolation rates.

**LITERATURE CITED**


