Synthesis of Human Cytomegalovirus-specified RNA and Protein in Interferon-treated Cells at Early Times After Infection

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SUMMARY

In human fibroblast cells treated with interferon, cytomegalovirus-specified immediate early RNA was found associated with the polyribosomes at concentrations and size classes similar to the virus RNA found in non-treated cells. Interferon treatment inhibited the translation of the immediate early virus mRNA; the relative rate of virus-specified immediate early protein and antigen synthesis decreased with increasing concentrations of interferon. In addition, the relative amount of virus-specified RNA associated with the polyribosomes at early times after infection was significantly reduced by treatment of the cells with interferon. Inhibition of infectious virus production in interferon-treated cells was primarily due to inhibition of immediate early virus protein synthesis and secondarily to suppression of early virus RNA synthesis. The role of the virus-specified immediate early proteins in regulating subsequent virus gene expression is discussed.

INTRODUCTION

In the human host, infection by cytomegalovirus (CMV) is responsible for a wide spectrum of diseases ranging from the classic cytomegalic inclusion disease to intra-uterine death, prematurity, congenital defects, infectious mononucleosis, post-perfusion syndrome, and interstitial pneumonia in transplantation patients (for reviews, see Weller, 1971; Rapp, 1980). The diseases caused by CMV frequently represent infections after reactivation of latent virus and are usually associated with immunosuppression due to a variety of conditions such as malignant disease and chemotherapy (Weller, 1971; Plummer, 1973). Antiviral agents such as adenine arabinoside and interferon (Chi'en et al., 1974; Alford & Whitley, 1976; Arvin et al., 1976; Cheeseman et al., 1977; Kraemer et al., 1978) have been employed to control CMV infections. Although these agents temporarily suppress virus replication, virus frequently reappears after the treatment is discontinued. This phenomenon is presumably related to the ability of the virus genome to persist in cells for extensive periods of time (Rapp et al., 1975; Boldogh et al., 1977; Mocarski & Stinski, 1979; Williams et al., 1980).

The first event after the reactivation of the virus genome from a quiescent state is presumably immediate early virus gene expression. Immediate early virus RNAs originate from a defined region in the large unique section of the virus genome (0.660 to 0.770 map units) and, subsequently, discrete size classes of virus mRNAs are found associated with the polyribosomes in high abundance (Stinski et al., 1980; Wathen et al., 1981). In vitro translation of the mRNAs originating from the above region of the virus genome indicated that these mRNAs code for the majority of the immediate early virus proteins normally found in the infected cell (M. F. Stinski, unpublished results). After the synthesis of these proteins, a transition from highly restricted transcription to relatively extensive transcription occurs, and
changes occur in the relative abundance of the virus mRNA size classes associated with the polyribosomes (Wathen et al., 1981). Therefore, the immediate early virus proteins are assumed to regulate subsequent virus gene expression. These virus proteins are preferentially synthesized within the first few hours after infection and, subsequently, their relative rates of synthesis decline. This is followed by an early phase of virus gene expression that extends for 24 h or more (Wathen et al., 1981).

To evaluate the role of the virus-specified immediate early proteins in the regulation of CMV gene expression, cells were treated with human interferon for 12 h prior to infection. We demonstrate in this paper that pretreatment with interferon has little effect on the synthesis, relative amount and size classes of the immediate early virus RNAs synthesized in the presence of an inhibitor of protein synthesis. However, interferon treatment induces an inhibition of immediate early mRNA translation. Inhibition of virus gene expression at this stage of infection is related to a suppression in the relative amount of early virus mRNA associated with the polyribosomes and ultimately to the production of infectious virus.

METHODS

Virus and cells. Human CMV (Towne) was plaque-purified and passaged at low multiplicities in human foreskin fibroblast cells as described previously (Stinski, 1978). The amount of infectious virus was determined by plaque assay (Wentworth & French, 1970) or tissue culture infective doses (Furukawa et al., 1973). For cells treated with interferon, the cells were suspended in the extracellular fluid, disrupted by sonication for 1 min and the resulting mixture was assayed for plaques.

Interferon. Interferon from human foreskin fibroblast cells was either purchased (Calbiochem-Behring Corp., San Diego, Ca., U.S.A.) or induced with Newcastle disease virus (NDV). NDV induces a mixture of α- and β-interferons in human foreskin fibroblast cells. To induce interferon, human fibroblast cells were infected with NDV at an m.o.i. of 5. After 24 h, the supernatant fluid was harvested and dialysed in sequence against 0.01 M-HCl, phosphate-buffered saline pH 7.4, and minimal essential medium with Earle's salts. The low pH conditions inactivated NDV infectivity. The amount of interferon was determined by infecting interferon-treated and untreated human fibroblast cells with 50 p.f.u. of vesicular stomatitis virus. After 1 day, the plaques were counted. One international unit (IU) of interferon inhibited 50% of the plaques (interferon was calibrated with a human international β-interferon standard obtained from the Research Resources Branch of the National Institute of Allergy and Infectious Diseases, Bethesda, Md., U.S.A.). Mock interferon was prepared following treatment of cells with regular growth medium, harvesting the supernatant fluid, and adding NDV equivalent to the amount used to induce interferon. Treatment of cells with interferon or mock interferon was as indicated.

Virus infection and incubation with radioactive precursors. Cells were infected with CMV at an m.o.i. of 10 to 20, or as indicated. To radioactively label immediate early virus protein, virus was added to cells in the presence of 100 μg/ml cycloheximide to allow for an accumulation of immediate early virus mRNA as described previously (Stinski, 1978; Wathen et al., 1981). After removal of the cycloheximide, the cells were pulse-labelled with [35S]methionine in the presence of hypertonic medium containing 5 μg/ml actinomycin D as described previously (Stinski, 1978). Uninfected cells were treated as described above except the [35S]methionine pulse media contained normal concentrations of sodium chloride.

Polyacrylamide gel electrophoresis (PAGE). Equivalent amounts of radioactivity from cell lysates were analysed by SDS–PAGE in 9% slab gels by a modification (Stinski, 1977) of the method of Laemmli (1970). Molecular weights of virus polypeptides were estimated by the procedure of Weber & Osborn (1969) using standard markers (Stinski, 1976). After electrophoresis, gels were stained with Coomassie Brilliant Blue and destained as described by
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Fairbanks et al. (1971). Autoradiography of dried gels was performed with Kodak Tri-X ortho film at room temperature.

Percentage immediate early virus protein synthesis. The amount of \[^{35}S\]methionine incorporated into immediate early infected cell-specific polypeptides (ICSP) or uninfected cell polypeptides was measured by scanning autoradiograms with a Beckman DU-8 spectrophotometer using the DU-8 wavelength scanning compuset module. The area under each peak was calculated and the percentage of total protein determined. All of the peaks measured by the densitometer were within the linear range of the film.

Antisera and immunofluorescence. Human convalescent serum specific for virus-induced immediate early antigens (M-19) was kindly provided by S. Michelson, Unité de Virologie Medicale, Institut Pasteur, Paris, France. Indirect immunofluorescence according to standard methods (Michelson-Fiske et al., 1977) was performed on methanol-fixed cells using a 1:10 dilution of the antiserum. All immunofluorescent photographs were 1 min exposures.

Isolation of RNA. All reagents and glassware were treated with 0.1% diethyl pyrocarbonate (Sigma) and autoclaved before use. RNA was isolated from polysomal fractions prepared by the method of Palmier (1974). Immediate early virus RNA was isolated at 12 h post-infection from cells infected and maintained in the presence of 200 \( \mu \)g/ml cycloheximide. Early virus RNA was isolated at 12 h post-infection from cells not treated with cycloheximide. Immediate early or early virus RNA was also isolated as described above from cells pretreated for 12 h with 41 to 73 IU/ml interferon.

Gel electrophoresis of denatured polysome-associated RNA. Polysome-associated RNA was fractionated by electrophoresis in 1% agarose slab gels containing 10 mM-methylmercury hydroxide (Alpha, Danvers, Mass., U.S.A.) as described by Bailey & Davidson (1976). Electrophoresis was at 11.5 mA/cm² at 15°C for 4.5 h. Mol. wt. standards were 23S [3.3 kilobase (kb)] and 16S (1.7 kb) Escherichia coli rRNA (Bishop et al., 1967), and 28S (5.3 kb) and 18S (2 kb) human cell rRNA (Anderson et al., 1980). The standards were detected by fluorescence after staining the gel in a solution containing 1 \( \mu \)g/ml ethidium bromide and 0.5 M-ammonium acetate. The sizes of the virus RNAs were interpolated from a standard curve which was prepared for each experiment.

Virus RNA blots and DNA hybridization. Gels containing immediate early or early polysome-associated RNA were prepared for transfer to diazobenzylloxymethyl (DBM) paper (Schleicher & Schüll, Keene, N.H., U.S.A.) as described previously (Wathen et al., 1981). The DBM paper was activated as described by Alwine et al. (1977). The RNA blots were incubated at 42°C for 24 h in hybridization buffer containing 50% formamide, 0.75 M-NaCl, 0.075 M-sodium citrate, 0.02% (w/v) each of bovine serum albumin, Ficoll and polyvinylpyrrolidone, 2.5 mg/ml sonicated calf thymus DNA, and 1.8% (w/v) glycine. CMV DNA was labelled with \([\alpha^{32}P]dCTP\) by nick translation as described by Rigby et al. (1977). Approximately \(10 \times 10^6\) ct/min/ml \(^{32}P\)-labelled CMV DNA was heated to 100°C for 2 min and then added to the hybridization buffer minus glycine. Hybridization was at 42°C for 48 h. After hybridization, the RNA blots were washed eight times at 45°C for 45 min periods in a solution containing 50% formamide and 5 x SSC (1 x SSC is 0.15 M-NaCl, 0.015 M-sodium citrate) and once in 2 x SSC. Hybridization of the \(^{32}P\)-labelled virus DNA probes to virus-specific RNA was detected by autoradiography.

RESULTS

Effect of interferon on infectious CMV production

To determine the effect of interferon on infectious virus production, human fibroblast cells were treated with various concentrations of interferon for 24 h and then infected with CMV at
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(a) (b)

Fig. 1. Autoradiogram of an SDS–polyacrylamide slab gel containing electrophoretically separated polypeptides from infected or uninfected cells pretreated with various concentrations of interferon. Infected (a) or uninfected (b) cells were treated for 12 h with the following concentrations of interferon (IU/ml). (a) Lane 1, 0; lane 2, 1.2; lane 3, 2.4; lane 4, 6; lane 5, 24; lane 6, 36; lane 7, 73. (b) Lane 1, 0; lane 2, 36; lane 3, 73. The infected cells were treated and pulse-labelled to identify the immediate early virus proteins as indicated in the text. Mol. wt. (x 10^-3) of infected cell-specific polypeptides are shown.

Table 1. Effect of human fibroblast interferon on infectious CMV production

<table>
<thead>
<tr>
<th>Interferon (IU/ml)</th>
<th>Infectious virus (mean p.f.u. x 10^-4)</th>
<th>Reduction at 4 days (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0*</td>
<td>6.3 2900</td>
<td>0</td>
</tr>
<tr>
<td>2.7</td>
<td>6.3 3100</td>
<td>0</td>
</tr>
<tr>
<td>27</td>
<td>7.8 30</td>
<td>98.9</td>
</tr>
<tr>
<td>37</td>
<td>6.8 4.3</td>
<td>99.8</td>
</tr>
</tbody>
</table>

* Mock interferon prepared as described in Methods.

an m.o.i. of approx. 1. After a virus adsorption period of 1 h, medium containing the various concentrations of interferon was added to the cells. The amount of infectious virus associated with the extracellular fluid and cells was quantified at 1 and 4 days after infection as described in Methods. Since it requires 2 to 3 days for virus production (Furukawa et al., 1973; Stinski, 1977), day 1 was considered to represent the inoculum virus. Treatment of the cells with 2.7 IU/ml interferon did not affect infectious virus production at 4 days post-infection. In
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Fig. 2. Percentage of immediate early virus protein synthesis in infected cells pretreated with different concentrations of interferon. Separation of the immediate early ICSP by denaturing gel electrophoresis is illustrated in Fig. 1. The percentage of virus protein synthesis was determined from densitometer scans of autoradiograms. Only the region containing infected cell-specific polypeptides with apparent mol. wt. of $75 \times 10^3$, $72 \times 10^3$ and $68 \times 10^3$ was analysed.

Contrast, 27 to 37 IU/ml interferon reduced infectious virus production by approx. 99% (Table 1).

Suppression of immediate early virus protein synthesis by pretreatment with interferon

To determine the effect of interferon on virus-induced immediate early protein synthesis, cells were treated with different concentrations of interferon for 12 h and then infected. *De novo* protein synthesis was inhibited for a period of 8 h with 200 µg/ml cycloheximide to allow for an accumulation of virus mRNA. After removal of the cycloheximide, the cells were pulse-labelled for 2 h with $[^{35}S]$methionine containing actinomycin D. These are the standard conditions for detecting the synthesis of CMV-induced immediate early proteins (Stinski, 1978). The proteins synthesized in interferon-treated or untreated cells infected with CMV or left uninfected were fractionated by SDS–PAGE as described in Methods. Fig. 1 illustrates that the relative amount of synthesis of the immediate early ICSP was reduced with increasing concentrations of interferon treatment. The major immediate early ICSP in untreated cells had an apparent mol. wt. of $72 \times 10^3$. Other ICSP at lower relative concentrations had apparent mol. wt. of $75, 68, 59, 56, 46, 42, 39, 27$ and $6.7$ (all $\times 10^3$). Virus mRNAs isolated from infected cells maintained in the presence of an inhibitor of protein synthesis code for many proteins in an *in vitro* translation system with the same apparent mol. wt. (Wathen et al., 1981) and, therefore, the majority of immediate early ICSP are considered to be virus-specified proteins. The relative suppression of immediate early ICSP synthesis was greater with increasing concentrations of interferon (Fig. 1). In contrast, high concentrations of interferon (36 to 73 IU/ml) did not suppress uninfected cell protein synthesis (Fig. 1 b).

Relative amount of immediate early virus protein synthesis after treatment with various concentrations of interferon

The predominant immediate early virus polypeptide has an apparent mol. wt. of $72 \times 10^3$ (Stinski, 1978; Wathen, et al., 1981). Two immediate early ICSP synthesized in lower relative amounts have mol. wt. of $75 \times 10^3$ and $68 \times 10^3$ (Stinski, 1978). The relative amount of synthesis of these proteins was measured by densitometric scans of the autoradiogram as described in Methods. Fig. 2 illustrates the relationship between percentage immediate early virus protein synthesis and the concentration of interferon used to treat the cells before infection. Treatment with 2.4 IU/ml interferon reduced immediate early virus protein synthesis by approx. 57%. Treatment with high concentrations of interferon (36 to 73 IU/ml) reduced immediate early virus protein synthesis by 80% or greater. Under these conditions,
approx. 20% of the cells had cytopathic effect which is characteristic of early virus gene expression (Furukawa et al., 1973).

Inhibition of immediate early virus protein synthesis was linear at low concentrations of interferon followed by a decrease in the rate of inhibition at high concentrations of interferon. A small amount of immediate early virus protein synthesis occurred even at relatively high concentrations of interferon and presumably a small number of cells entered the early stage of virus gene expression. These data suggest that either a small number of cells were not affected by the interferon treatment or a small percentage of the immediate early virus gene expression is insensitive to the treatment with interferon. Nevertheless, treatment of uninfected cells with relatively high concentrations of interferon did not inhibit cellular protein synthesis as determined by densitometric scanning (data not shown).

**Effect of interferon treatment on virus-specific immediate early antigen synthesis**

To test the effect of interferon on virus protein synthesis in cells not treated with cycloheximide, cells were treated for 12 h with various concentrations of interferon or left untreated. At 3 h post-infection the cells were fixed and stained for immunofluorescence using a human convalescent serum specific for the virus immediate early antigens as described in Methods. Fig. 3 illustrates that pretreatment of the cells with interferon suppressed the relative amount of virus-specific immediate early antigen detected in the nucleus. This effect was dependent upon the concentration of interferon. When cells were pretreated with as much as 36 or 73 IU/ml interferon, relatively low amounts of virus-specified antigen were detected (Fig. 3).

**Effect of interferon on virus-specified RNA associated with the polyribosomes at immediate early and early times**

In the CMV-infected cell, there is a temporal regulation of virus-specified RNA synthesis at immediate early and early times. In the absence of de novo protein synthesis, immediate early virus transcription originates from restricted regions of the virus genome and only three mRNA size classes are associated with the polyribosomes in intermediate to high abundance (Stinski et al., 1980; Wathen et al., 1981). If de novo protein synthesis occurs, a transition from highly restricted transcription to relatively extensive transcription occurs and additional size classes of virus mRNA in high abundance are found associated with the polyribosomes (Wathen et al., 1981). These virus RNA size classes are detected in the cell at approx. 6 h post-infection. Since they are also detected in high abundance in the absence of virus DNA replication, they are considered to represent early virus mRNAs. Therefore, it is postulated that the immediate early virus proteins regulate CMV transcription (Wathen et al., 1981).

To determine the effect of interferon treatment on virus-specified RNA associated with the polyribosomes, immediate early and early polysome-associated RNA was isolated from infected cells and fractionated by electrophoresis in agarose gels containing methylmercury
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Fig. 4. Autoradiographs of CMV–specified RNA associated with the polysomes at (a, b) immediate early and (c, d) early times after infection of cells treated with interferon. Infected cells were pretreated for 12 h with interferon at concentrations of 73 IU/ml (b) or 41 IU/ml (d) or left untreated (a, c). Polysome–associated RNA was isolated at immediate early and early times after infection and then 10 μg RNA (lanes 1) or 40 μg RNA (lanes 2) was fractionated according to size in denaturing 1% agarose gels as described in the text. The standard RNAs were 3.3 and 1.7 kb E. coli ribosomal RNA and 5.3 and 2 kb human cell ribosomal RNA. A standard curve was prepared after electrophoresis and used to interpolate the sizes of the virus–specified RNAs. The virus RNA was transferred to activated DBM paper and detected by hybridization of 32P–labelled CMV DNA followed by autoradiography as described in the text. Virus–specified RNA is designated according to kb size.

hydroxide as described in Methods. After immobilization of the RNA on to DBM paper, virus–specified RNA was detected by hybridization of 32P–labelled virus DNA followed by autoradiography.

In infected cells not treated with interferon, the typical immediate early virus RNA size classes of 4.8, 2.2 and 1.9 kb were detected (Fig. 4 a) as previously described by Wathen et al. (1981). In infected cells pretreated for 12 h with 73 IU/ml interferon (Fig. 4 b), the same size classes of RNA were detected and there was no evidence for virus mRNA degradation. The 4.8 kb mRNA appeared to be present in the same relative abundance but there was a significant increase in relative amount of the 2.2 kb size class. In addition, there appeared to be a slight decrease in the 1.9 kb size class. When the amount of the polysome–associated RNA for gel electrophoresis was increased fourfold, the amount of virus–specified immediate early RNA was judged to have increased approx. fourfold (Fig. 4 b, lane 2).

When polysome–associated RNA from untreated infected cells was analysed at early times as described in Methods, the typical virus RNA size classes as described by Wathen et al. (1981) were detected, i.e. the abundant virus mRNA size classes of 2.7 and 1.2 kb were present (Fig. 4 c). In infected cells pretreated with 41 IU/ml interferon, there was a suppression in virus RNA associated with the polyribosomes. For example, when 10 μg of polysome–associated RNA was analysed, little to no virus–specified RNA was detected (Fig. 4 d, lane 1). When the concentration of RNA was increased fourfold, virus RNA size classes characteristic of early virus RNA (2.7 and 1.2 kb) as well as immediate early RNA (4.8 and
1.9 kb) were detected (Fig. 4d, lane 2). It was estimated that treatment with 41 IU/ml interferon suppressed the amount of early virus RNA associated with the polyribosomes approx. fivefold. Although pretreatment with interferon had little effect on the synthesis of immediate early virus mRNA, there was an inhibiting effect on the synthesis of early virus mRNA.

**DISCUSSION**

Like other herpesviruses, CMV has regulated phases of transcription (DeMarchi *et al.*, 1980; Stinski *et al.*, 1980; DeMarchi, 1981; Wathen *et al.*, 1981) and translation (Stinski, 1978; Wathen *et al.*, 1981). Treatment of cells with interferon did not inhibit CMV-specified immediate early transcription as judged by the relative amount and size classes of virus-specified RNA associated with the polyribosomes. The major effect induced by interferon was suppression of immediate early virus protein synthesis. However, a small amount of immediate early virus protein synthesis was detected as either virus-induced ICSP or virus antigen after treatment with relatively high concentrations of interferon. The kinetics of inhibition of immediate early virus protein synthesis suggested that a small amount of virus protein synthesis would occur after treatment with relatively higher concentrations of interferon.

A secondary inhibitory effect of treatment with interferon was on the early virus mRNA. These data support the interpretation that synthesis of the virus-specified immediate early proteins is necessary for efficient early virus gene expression and, that the immediate early virus proteins are regulatory proteins. However, the role of host cell proteins in regulating the switch from immediate early to early virus gene transcription is presently not understood.

Several mechanisms for inhibition of virus protein synthesis in interferon-treated cells have been described (Lebleu *et al.*, 1976; Roberts *et al.*, 1976; Samuel *et al.*, 1977; Farrell *et al.*, 1978; Epstein & Samuel, 1978; Zilberstein *et al.*, 1978; Ratner *et al.*, 1978; Samuel, 1979; Baglioni, 1979; Baglioni & Maroney, 1980; Miyamoto & Samuel, 1980). Interferon induces a 2',5'-oligo(A) synthetase and a protein kinase. These enzymes require double-stranded RNA and ATP. The synthetase, indirectly by production of an effector molecule, activates a nuclease which degrades mRNA. However, the 2',5'-oligo(A) synthetase activity is unstable and is possibly subject to regulatory mechanisms (Krishnan & Baglioni, 1981). It is possible that the cycloheximide treatment for accumulation of immediate early virus RNA affects the relative amount of 2',5'-oligo(A) synthetase and, consequently, virus mRNA is not degraded. The protein kinase phosphorylates an initiation factor (eIF-2) which inhibits the initiation of mRNA translation. In the CMV-cell system, the primary level of virus genome expression inhibited as a result of interferon treatment appeared to be translation of the immediate early virus mRNA. This block in virus gene expression could be due to phosphorylation of the eIF-2 initiation factor. An alternative possibility for inhibition of immediate early virus protein synthesis could be impairment of the virus mRNA due to improper splicing or cap methylation (Desrosiers & Lengyel, 1979). The increase in the relative amount of the 2.2 kb immediate early virus RNA size class and the slight decrease in the 1.9 kb size class in the interferon-treated cells is presently not understood.

It is proposed that the decrease in the relative amount of early virus RNA associated with the polyribosomes in interferon-treated cells is due primarily to a suppression in the synthesis of immediate early virus proteins which are necessary for regulation of early virus RNA transcription. However, only 80% of the immediate early virus protein synthesis was inhibited and, consequently, it is estimated that some of the infected cells entered the early stage of virus gene expression. Approximately 20% of the infected cells showed cytopathic effects. The early virus RNA detected in interferon-treated cells presumably originated from the above cells in which immediate early virus protein synthesis was not inhibited. An alternative explanation is that interferon induced a decrease in the amount of virus-specified
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early RNA associated with the polysomes. When whole cell RNA was analysed by Southern blot hybridization at early times after infection as described previously (Wathen et al., 1981), there was a qualitative reduction in the amount of virus RNA present in the interferon-treated cells (M. F. Stinski et al., unpublished results). This effect was assumed to be due to a reduction in early virus RNA since in cells not treated with interferon the relative amount of immediate early virus RNA is normally reduced at this time after infection (Wathen et al., 1981; Wathen & Stinski, 1982). Lastly, it is possible that 2',5'-oligo(A) synthetase induced nuclelease activity under the conditions defined for early virus RNA. However, the presence of degraded RNA was not detected in the methylmercury hydroxide gels.

The inhibition of virus mRNA translation without adversely affecting the translation of most cellular mRNAs was observed in this series of experiments. The biochemical reasons for this discriminatory regulation in gene expression remain unclear. Human CMV replicates as cellular macromolecular synthesis continues (Stinski, 1977) and, consequently, this virus may either depend upon or prefer an actively metabolizing cell. This phenomenon coupled with the fact that the virus can persist in non-productive cells for a long period of time (Rapp et al., 1975; Boldogh et al., 1977; Mocarski & Stinski, 1979) suggest that CMV replication would resume after the disappearance of the effects of interferon. Therefore, interferon could delay infectious CMV replication but, without additional defence mechanisms, infectious virus production would resume.

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