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Physiological State of Human Embryonic Lung Cells Affects Their Response to Human Cytomegalovirus

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Cultures of human embryonic lung (HEL) cells in different physiological states were studied for their susceptibility to infection with human cytomegalovirus (CMV) with respect to production of infectious virus, synthesis of viral antigens, and virus-induced stimulation of cellular DNA synthesis. In general, subconfluent, actively growing cells yielded higher amounts of infectious virus than did confluent contact-inhibited cells. The higher yield of infectious virus was correlated with a greater percentage of cells producing viral antigens within the first 48 h after infection. In confluent cultures, 25 to 50% of the cells produced viral antigens within the first 48 h postinfection. This proportion did not change over a 10-fold range of multiplicity of infection, indicating that many of the cells in confluent cultures did not support productive infection. However, virtually all the cells in subconfluent cultures were susceptible. Also, in contrast to herpes simplex virus and pseudorabies virus, infectious CMV is not produced by cells treated with 5-fluorouracil and thymidine. Virus-induced stimulation of cellular DNA synthesis in cells infected at high multiplicities of infection could be detected only in confluent cultures, in which cellular DNA synthesis had been previously suppressed, but could not be detected in similarly treated cultures of subconfluent cells. The lack of detectable stimulation of cellular DNA synthesis in the latter was related to the fact that practically all the cells in the culture synthesized viral antigens within the first 48 h after infection, productive infection and detectable synthesis of cellular DNA being mutually exclusive.

The interactions between human cytomegaloviruses (CMV) with their host cells differ from those of other cytocidal herpesviruses, such as herpes simplex (HSV), pseudorabies (Pr), and equine abortion viruses. Infection of permissive cultures with the latter group of viruses invariably inhibits the synthesis of cellular macromolecules (1). By contrast, infection of permissive cultures with CMV results in a stimulation of the synthesis of cellular macromolecules and of cellular enzymes (3-6, 9, 10).

We have shown previously (3), by means of a technique that combines autoradiography with immunofluorescence, that cellular DNA synthesis is stimulated after infection mainly in those cells in the culture in which no viral antigen synthesis is detectable. Conversely, most of the cells that synthesize either early or late viral antigens do not synthesize detectable amounts of cellular DNA after infection. During the course of these experiments, we observed that the number of cells responding to infection by being stimulated either to synthesize cellular DNA or viral antigens varied from experiment to experiment (using the same virus inoculum) and that the physiological state of the cultures at the time of infection appeared to affect the response of the cells to infection. In the present paper we present the results of an analysis of the effect of the physiological state of human embryonic lung (HEL) cells on their ability to synthesize viral antigens (and infectious virus) and to be stimulated by infection to synthesize cellular DNA.

MATERIALS AND METHODS

Cell cultures and virus. Secondary cultures of HEL cells and stock cultures of human CMV (Davis strain) were prepared and used as described previously (3). Confluent cultures contained approximately 5×10^6 cells, and subconfluent cultures contained approximately 1.5×10^6 cells per 90-mm petri dish. Stock cultures of HSV type 1 were prepared in primary rabbit kidney cells as described previously (8). Assays for infectivity of CMV were carried out according to the method of Wentworth and French (11).

Chemicals and radiochemicals. Cesium chloride, optical grade, was purchased from the Harshaw Chemical Co. 5-Fluorouracil (FU) and thymidine (TdR) were purchased from Calbiochem. [*Methyl*-^{3}H]TdR (specific activity, 67 Ci/mmol) was purchased from Schwarz/Mann.

Treatment of cell cultures to arrest host cell DNA synthesis. Host cell DNA synthesis was arrested before infection either by treatment with FU and TdR (3) or by serum starvation.

(i) FU treatment. Cultures were incubated with FU (10 μg/ml) and TdR (5 μg/ml) for a minimum of 16 h before infection. Control cultures were treated with TdR (5 μg/ml). Unless stated otherwise, FU and TdR were then maintained in the FU-treated cultures, and TdR was maintained in the "untreated" cultures throughout the course of experiments.

(ii) Serum starvation. Cultures were rinsed extensively in serum-free medium and incubated in medium containing 0.2% fetal bovine serum for 3 to 4 days before infection. The culture fluids were set aside, and, after infection (or mock infection) with CMV, the same culture fluid was used for the remainder of the experiment.

Combined autoradiography-immunofluorescence. The procedure for autoradiography combined with immunofluorescence and the human convalescent serum containing antibodies to CMV and other reagents used in indirect immunofluorescence have been described previously (3).

RESULTS

Production of infectious CMV in HEL cells in different physiological states. In these experiments, we compared the ability of three different types of cultures to produce infectious CMV: (i) confluent cultures in which the level of DNA synthesis was relatively low because they are contact inhibited; (ii) subconfluent cultures consisting of actively growing cells; and (iii) subconfluent cultures in which cellular DNA synthesis had been suppressed before infection by pretreatment with FU and TdR (7). Table 1 shows that the cells in subconfluent cultures yielded more (25- to 140-fold) infectious virus than did cells in confluent cultures. (The titer of infectious virus obtained from confluent cultures varied 10-fold between the two experiments. This is probably due to differences in the degree of confluency of these cultures.) Furthermore, cells in subconfluent cultures that had been treated with FU were poor producers of CMV and yielded approximately 700- to 800-fold less infectious virus than did the cells in untreated subconfluent cultures. Thus, CMV does not grow well in contact-inhibited cells and in cells in which DNA synthesis has been inhibited by FU.

The growth of HSV, unlike that of CMV, is not affected by the presence of FU, even when the cells have been incubated with the drug for several days before infection (Table 2). Pr virus also grows well in FU-treated cells (2, 7). Thus, in contrast to other herpesviruses, the growth of CMV is inhibited in FU-treated cells.

The yield of infectious CMV produced by cultures from which FU had been removed before infection and that produced by cultures incubated with the drug throughout the infective process was about the same (Table 2). Although one cannot be certain that no FU was available for incorporation into viral macromolecules, the absence of a significant difference in yields produced by the two sets of cultures indicates that the effect of FU on the production of virus is due, most likely, to the effect of the drug on cellular metabolism before infection.

Number of cells synthesizing viral antigens in cultures in different physiological states. To determine whether the difference in the amount of infectious virus produced by cultures of cells in different physiological states is due to differing numbers of cells in the cultures that are susceptible to CMV, we determined the number of cells producing viral antigens. The results of these experiments are summarized in Table 3.

By 48 h postinfection, approximately 25% of the cells in the confluent cultures contained detectable amounts of viral antigens, both in untreated cultures and in cultures in which cellular DNA synthesis had been suppressed before infection by treatment with FU or by serum starvation. In the subconfluent cultures, however, the number of cells synthesizing viral

TABLE 1. *Production of infectious virus by cells in various physiological states*[a]

State of culture	Expt I		Expt II	
	2 days[b]	6 days	2 days	6 days
Confluent	3.3×10^{1}[c]	2.0×10^{6}	1.0×10^{2}	1.8×10^{5}
Subconfluent	2.9×10^{1}	5.1×10^{7}	3.6×10^{2}	2.5×10^{7}
Subconfluent, FU-treated	1.7×10^{1}	7.1×10^{4}	2.4×10^{3}	1.3×10^{4}

[a] Cultures were infected with CMV at a multiplicity of 1 PFU/cell. Unadsorbed virus was removed by washing. The culture medium was changed daily, and the culture fluids were collected and stored at −70°C. At the indicated times, the cells and culture fluids from individual plates were collected, pooled with previously collected culture fluids from the same plates, and assayed for infectious virus by the plaque technique.

[b] Postinfection.

[c] PFU/10^{6} cells.

TABLE 2. *Effect of FU on the production of infectious CMV and HSV*

Treatment of cultures	HSV[a]		CMV[b]	
	2 h[c]	24 h	1 day	6 days
FU-treated	5.5×10^{4}[d]	1.4×10^{8}	5.3×10^{1}	3.0×10^{4}
FU-pretreated			8.0×10^{1}	6.0×10^{4}
Untreated	1.3×10^{4}	2.2×10^{8}	5.3×10^{1}	3.8×10^{6}

[a] Confluent HEL cells were incubated with FU (10 μg/ml) and TdR (5 μg/ml) (=FU-treated) or with TdR (5 μg/ml) (=untreated) for 72 h before infection. The cultures were then infected with HSV (adsorbed multiplicity, 3 PFU/cell) and further incubated in medium with or without FU. The infected cells were collected at 2 and 24 h postinfection, and the virus yield was assayed as described in Material and Methods.

[b] Confluent HEL cells were incubated with FU (10 μg/ml) and TdR (5 μg/ml) (=FU-treated) or with TdR (5 μg/ml) (=untreated) for 24 h before infection. Part of the FU-treated cultures was washed extensively to remove FU before infection (=FU-pretreated). The cultures were infected (adsorbed multiplicity, 1 PFU/cell) and further incubated in medium containing either FU and TdR (FU-treated) or TdR alone (FU-pretreated and untreated). The cells and culture fluids were collected 1 and 6 days after infection and virus yield was assayed as described in Materials and Methods.

[c] Postinfection.

[d] PFU/10^{6} cells.

TABLE 3. *Synthesis of viral antigens and of cellular DNA by CMV-infected cultures in different physiological states*[a]

Treatment of culture	Uninfected	Infected	
	Cellular DNA (%)	Cellular DNA (%)	Viral antigens[e] (%)
Confluent cells			
Untreated[b]	11.0	11.9	25.9
FU-treated[c]	0.1	0.7	25.8
Serum-starved[d]	1.3	11.7	21.7
Subconfluent cells			
Untreated[b]	78.0	35.0	58.4
FU-treated[c]	0.2	0.1	94.3
Serum-starved[d]	1.5	2.7	85.0

[a] Cover slip cultures of HEL cells in various physiological states were infected at a multiplicity of 3 PFU/cell and labeled with [^{3}H]TdR (1 μCi/ml) between 24 and 48 h postinfection. The cells were examined by combined immunofluorescence-autoradiography, and the number of cells that had synthesized either cellular DNA or viral antigens was determined.

[b] Cultures were treated 16 h before infection with medium containing TdR (5 μg/ml). After infection, the cultures were incubated further in medium containing TdR.

[c] Cultures were treated 16 h before infection with medium containing FU (10 μg/ml) and TdR (5 μg/ml). After infection, the cultures were incubated further in medium containing FU and TdR.

[d] Cultures were incubated in medium containing 0.2% serum for 4 days before infection. This medium was saved and replaced on the cells after the adsorption period.

[e] The antisera used reacted with both early and late CMV antigens (3).

antigens within the first 48 h after infection was about 58% in untreated, 94% in FU-treated, and 85% in serum-starved cultures. Thus, a much larger proportion of the cells in the subconfluent than in the confluent cultures synthesized viral antigens.

The smaller number of cells synthesizing viral antigens in confluent than in subconfluent cultures might be due (despite our attempts to control this factor) to a greater multiplicity of infection in the latter cultures. Consequently, the effect of stepwise dilution of the virus inoculum on the number of cells synthesizing viral antigens in confluent and in subconfluent cultures was tested. The results, illustrated in Fig. 1, show that less than 50% of the cells in the confluent cultures (both serum-starved and FU-treated) fluoresced by 48 h postinfection, and no appreciable difference in the number of fluorescing cells was observed when the multiplicity was varied between 1 and 10 PFU/cell. In subconfluent cultures, however, 90% of the cells fluoresced, even at a multiplicity as low as 2 PFU/cell. In this experiment, the cells were examined by indirect (antiglobulin) fluorescence at 48 h postinfection. Essentially similar results were also obtained when the cells were examined at 72 h postinfection or when they were examined by anticomplementary fluorescence. Thus, practically all the cells in the subconfluent cultures were susceptible to CMV infection; however, this was not the case for the cells in confluent cultures.

The absence of synthesis of CMV antigens in a large proportion of the cells in confluent cultures is not due to interference by crowding. All the cells in confluent HEL cultures infected with HSV at a multiplicity of 10 PFU/cell pro-

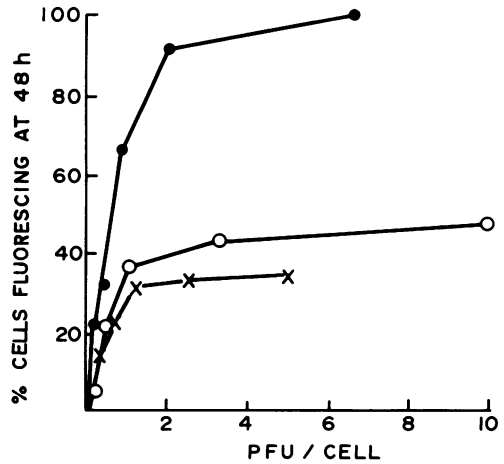

FIG. 1. *HEL cells responding to infection in cultures in different physiological states. Cultures of HEL cells on cover slips were infected with different multiplicities of CMV. At 48 h postinfection, the cultures were fixed in acetone and stained by indirect immunofluorescence, and the numbers of cells exhibiting viral antigens were counted. Symbols: (●) Subconfluent, FU-treated cells; (○) confluent FU-treated cells; (×) confluent serum-starved cells.*

duce HSV viral antigens by 7 h postinfection (unpublished observation). We conclude, therefore, that the physiological state of the cells at the time of infection affects their ability to synthesize detectable amounts of CMV antigens and to support CMV growth.

The experiment illustrated in Fig. 1 shows that not only do virtually all the cells in the subconfluent cultures respond to infection by producing viral antigens but that the proportion of cells producing viral antigens at a given multiplicity was higher than expected on the basis of the multiplicity of infection. This is due to either of the following: (i) the plaque assay method underestimates the number of infectious virions in the virus stocks; (ii) some of the particles in the stocks are unable to give rise to plaques but will induce the synthesis of viral antigens in the cells they infect abortively.

Synthesis of cellular and viral DNA in CMV-infected HEL cells in different physiological states. Those cells that have synthesized cellular DNA and those that have synthesized viral DNA can be differentiated easily from each other by autoradiography-immunofluorescence (3). A cell that has synthesized cellular DNA during a 24-h labeling period incorporates considerably more [^{3}H]TdR than does a cell that has synthesized viral DNA. By autoradiography one can, therefore, detect, after a short exposure time, only those cells in the culture that have synthesized cellular DNA. The nuclei of such cells are covered evenly with grains and look identical to the nuclei of cells that have synthesized DNA in uninfected cultures, labeled under identical conditions. Cells that have synthesized viral DNA can be detected by autoradiography only after longer exposure times; these cells contain inclusion-like bodies covered sparsely with grains (after a time of exposure that completely blackens the emulsion over cells that have synthesized cellular DNA) and contain viral antigens as well. Furthermore, by performing the experiments at relatively early times after infection, when little viral DNA is synthesized, one can be confident that the cells with grains are those that have synthesized cellular DNA. It should be mentioned that this method would not detect the synthesis of only a small amount of cellular DNA in some of the cells. However, it allows one to identify those cells in the culture in which cellular DNA synthesis has been massively induced by infection.

Table 3 shows that, in confluent cultures, there is an increase in the number of cells synthesizing cellular DNA after infection, but this increase occurs only in those cultures in which cellular DNA synthesis has been suppressed before infection by treatment with FU or incubation with low serum. This result is consistent with earlier reports showing that the CMV-induced stimulation of cellular DNA synthesis can be detected only in cells in which cellular DNA synthesis has been previously suppressed (3, 9).

In subconfluent cultures no increase in the number of cells synthesizing cellular DNA was apparent, even after pretreatment of cultures with FU (Table 3). In fact, the number of cells synthesizing cellular DNA was suppressed by infection even below the small number of residual cells that did synthesize cellular DNA in the uninfected FU-treated cultures. We have reported earlier that stimulation of cellular DNA synthesis by CMV is not detectable in those cells in the cultures in which a detectable amount of viral antigen is synthesized (3). It was possible, therefore, that stimulation of cellular DNA was not detected in subconfluent CMV-infected FU-treated cultures because practically all the cells in these cultures synthesized viral antigens. To determine whether this was the case, both confluent and subconfluent cultures were infected with CMV at two different multiplicities, and the number of cells stimulated to synthesize cellular DNA was determined. The results of this experiment are summarized in Table 4.

In confluent FU-treated cultures infected at a

TABLE 4. *Effect of the multiplicity of infection on stimulation of cellular DNA synthesis in confluent and subconfluent cells*[a]

Multiplicity of infection (PFU/cell)	State of culture			
	Confluent		Subconfluent	
	Viral antigens	Cellular DNA	Viral Antigens	Cellular DNA
10	43.1[b]	1.1[c]	99.4	0.1
1	36.9	0.4	66.6	0.6
Mock-infected		0.1		0.2

[a] Confluent and subconfluent cultures of FU-treated HEL cells on cover slips were infected with virus at the indicated multiplicities. The cultures were labeled with [^{3}H]TdR between 24 and 48 h and analyzed by the combined autoradiography-immunofluorescence technique for the number of cells synthesizing cellular DNA and viral antigens after infection.

[b] Percentage of cells with viral antigens.

[c] Percentage of cells with grains.

multiplicity of 10 PFU/cell, there was an 11-fold increase in the number of cells synthesizing cellular DNA, whereas in subconfluent cultures infected at the same multiplicity, there was a decrease in the number of cells synthesizing cellular DNA. However, when the cells were infected at a multiplicity of 1 PFU/cell, a stimulation of cellular DNA synthesis was observed in both the confluent and the subconfluent cultures. These results indicate that the lack of CMV-induced stimulation of cellular DNA synthesis observed in subconfluent cells is related to the large proportion of the cells in these cultures that are susceptible to productive infection. These results support our previous conclusions that productive infection and detectable stimulation of cellular DNA synthesis are mutually exclusive events and occur in different cells in the culture (3).

As Table 3 shows, only a small proportion (0.1%) of the infected cells in FU-treated subconfluent cultures synthesized cellular DNA, as determined by autoradiography. In some experiments (not shown), this percentage was even lower. It seemed possible, however, that cellular DNA synthesis occurs in most of the infected cells but at a level below the range of detection by autoradiography under the conditions used. Consequently, the question whether any cellular DNA was synthesized in FU-treated, infected subconfluent cultures was answered more directly. The infected cells were incubated with [^{3}H]TdR, cellular DNA was separated from viral DNA by isopycnic centrifugation in CsCl, and the amount of label incorporated into cellular DNA was determined (Table 5). In untreated subconfluent cultures, the amount of [^{3}H]TdR incorporated into cellular DNA decreased fivefold between 24 and 48 h postinfection. In FU-treated subconfluent cultures, however, no detectable amount of [^{3}H]TdR was incorporated into cellular DNA between 24 and 48 h postinfection (a time when FU-treated confluent cultures are stimulated to synthesize cellular DNA most markedly [3]). A small amount of incorporation of [^{3}H]TdR into cellular DNA in the FU-treated cultures was observed between 0 and 24 h postinfection, but it was below the residual amount incorporated into FU-treated uninfected cells. Thus, it is clear that infection does not stimulate cellular DNA synthesis in cells of subconfluent FU-treated cultures and that if cellular DNA is synthesized by these cells beyond 24 h postinfection, the amount is too small to be detected by the methods used.

Table 5 shows also that viral DNA is synthesized in FU-treated subconfluent cultures, although to a somewhat lesser extent than in untreated cells. Thus, viral DNA synthesis can occur in the absence of detectable amounts of cellular DNA synthesis. Viral antigens are also synthesized in virtually all the cells in these cultures (Table 3). The production of infectious CMV after treatment of cultures with FU is, however, decreased 700-fold (Table 1). The nature of the block induced by FU treatment, which results in a lack of formation of infectious CMV (a block that does not occur with Pr virus or HSV), is unknown at present.

DISCUSSION

This paper shows that the susceptibility of

TABLE 5. *[^{3}H]TdR incorporation into viral and cellular DNA in CMV-infected subconfluent cells*[a]

Labeling period (h)	FU-treated cells			Untreated cells		
	Infected		Uninfected cellular	Infected		Uninfected cellular
	Viral	Cellular		Viral	Cellular	
0–24	0	3.8[b]	4.6			
24–48	16.6	0	3.8	23.6	43.6	214.6
48–72	29.0	0	3.0	86.1	14.8	318.9

[a] FU-treated and untreated subconfluent cultures were infected with CMV (approximately 5 PFU/cell) and labeled with [^{3}H]TdR (specific activity 1 μCi/5 μg). At the end of the labeling periods, the culture fluids were removed and the cells were scraped into SSC (0.15 M NaCl plus 0.015 M sodium citrate) + 1% sodium sarkosinate. The amount of radioactivity incorporated into viral and cellular DNA was determined after isopycnic centrifugation in CsCl.

[b] Counts per minute $\times 10^{-3}$ per 10^6 cells.

HEL cells to productive CMV infection is influenced by the physiological state of the cells at the time of infection. Subconfluent, actively growing cultures yield higher amounts of infectious CMV than do confluent, contact-inhibited cultures. Furthermore, within the first 72 h postinfection, a greater proportion of the cells in subconfluent than in confluent cultures produces viral antigens. The lack of susceptibility to CMV of a portion of the cells in confluent cultures is demonstrated clearly by the fact that the proportion of cells producing viral antigens does not change appreciably over a 10-fold range of multiplicity (Fig. 1). A similar effect of the physiological state of HEL cells on the growth of HSV was not observed, and practically all the cells in confluent HEL cultures infected with HSV produced viral antigens.

The lack of susceptibility of some of the cells in confluent HEL cultures to CMV does not imply that these cells will not eventually become productively infected and yield virus. Whereas only part of the cells (25 to 50%, depending on the experiment) synthesize viral antigens up to 4 days after infection, by 6 to 8 days, all the cells in the cultures contain viral antigens and cellular degeneration is widespread. By 4 days, however, the first cycle of viral replication has been completed, and it is likely, therefore, that the degeneration of some of the cells in the cultures affects the physiological state of the remainder of the cells which, as a consequence, become susceptible to CMV infection. The yield of virus per cell in confluent cultures is, however, considerably lower than in subconfluent cultures (Table 1).

The nature of the functions required for the synthesis of viral antigens that are provided by cells in subconfluent cultures, but not in confluent cultures, is unclear. What is clear, however, is that cellular DNA synthesis is probably not required for viral DNA or antigen synthesis. We conclude this from the following. In subconfluent, FU-treated cultures, almost 100% of the cells synthesize virus-specific antigens within the first 48 h after infection. The number of cells synthesizing cellular DNA in these cultures (Table 3), as well as the incorporation of [^{3}H]TdR into cellular DNA (Table 5) (as determined after separation of cellular and viral DNA by isopycnic centrifugation in CsCl), is low.

The inability of some of the cells in the confluent cultures to synthesize viral antigens is, therefore, probably not related to the low level of cellular DNA synthesis in these cultures; other factors must be involved.

The synthesis of cellular DNA in the infected cells is also not necessary for viral DNA synthesis to occur. The results in Table 5 show that viral DNA is synthesized by subconfluent FU-treated cultures, even though little cellular DNA is synthesized by these cultures. Thus, we conclude that, although the physiological state of the cells affects their ability to respond to infection with CMV, the functions required for the growth of CMV are not those provided by cells in S phase.

Although viral antigen and DNA synthesis occurs in the cells of FU-treated cultures, production of infectious virus is reduced drastically. The nature of the block induced by treatment of the cells with FU on CMV production is not known. It is likely that the inhibitory effect of the drug is through host cell metabolism rather than directly on the expression of viral functions. Evidence that this may be the case comes from the fact that when cultures are treated with the drug, which is then removed before infection, the yield of infectious virus is not significantly higher than when the drug is allowed to remain in the cultures throughout the infective process (Table 2). Thus, CMV differs from other herpesviruses (HSV [Table 2] and Pr virus [2, 7]), which grow well in FU-treated cells.

One important point to emerge from the results of this as well as a previous study (3) is that detectable virus-induced stimulation of cellular DNA synthesis and the synthesis of viral antigens appear to be mutually exclusive events. Thus, in subconfluent, FU-treated cultures, practically all the cells synthesize viral antigens when infected at a multiplicity of 10 PFU/cell. No stimulation of cellular DNA synthesis can be detected under these conditions of infection. If the infecting virus inoculum is diluted so that fewer of the cells in the cultures produce viral antigens, an increase in the number of cells synthesizing cellular DNA can be detected (Table 4). These results are consistent with the hypothesis that productive CMV infection inhibits the synthesis of detectable amounts of cellular DNA, as does productive infection with other herpesviruses (1). Induction of cellular DNA synthesis, thus, probably occurs in abortively infected cells in which the synthesis of viral antigens detectable with convalescent human sera does not occur. Because of the greater susceptibility of the cells in subconfluent cultures to productive CMV infection, the detection of a stimulation of cellular DNA synthesis is less likely in these cultures than in confluent cultures.

Our results indicate that the conditions that govern the outcome of CMV infection in HEL cell cultures are complex. The physiological state of the cells may be only one of a number of

factors that may affect this outcome.

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