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Cytomegalovirus Replication in Cells Pretreated with 5-Iodo-2'-Deoxyuridine

STEPHEN ST. JEOR AND FRED RAPP

Department of Microbiology, College of Medicine, The Milton S. Hershey Medical Center of The Pennsylvania State University, Hershey, Pennsylvania 17033

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The replication of human cytomegalovirus (CMV) in cells pretreated with 5-iodo-2'-deoxyuridine (IUdR) was studied. Pretreatment of cells with IUdR enhanced several parameters of virus replication. Virus grown in drug-treated cells exhibited a shorter eclipse period and the cells produced more infectious virus sooner than did untreated cells. There was an approximate fivefold increase in virus yield per cell in the drug-treated samples when compared to control cultures. The time required for plaque development was shortened by 6 days in drug-treated cultures. Pretreatment of cells with IUdR also increased plaquing efficiency of the virus by approximately 10-fold. The enhancement of virus replication by IUdR was further demonstrated by varying the multiplicity of infection. In a 7-day period there was a 100-fold increase in sensitivity of the cultures for virus detection when the cells had been previously exposed to IUdR. The data presented indicate the possibility that IUdR interferes with the production of a cellular product inhibitory for CMV replication.

Cytomegaloviruses (CMV) are distinct, usually species specific, herpesviruses (23). In vitro replication of human CMV is characterized by an extremely long eclipse period of 55 h (15), a high particle to infectivity ratio (18), and a dependence on human fibroblasts for replication (19). The reason for the inability of human CMV to initiate an efficient and rapid infection is unknown.

It is known that some thymidine analogues, such as 5-iodo-2'-deoxyuridine (IUdR) and 5-bromodeoxyuridine (BUdR), are able to depress the synthesis of certain cell proteins, including interferon (11, 17, 20). It has also been demonstrated that these same thymidine analogues are capable of inducing C-type particles in malignant cells (1, 13) and increasing the yield of simian virus 40 (SV40) virus from SV40-transformed cells when the transformed cells are pretreated with IUdR and then fused with permissive cells (21). The ability of these compounds to induce the Epstein-Barr herpesvirus in Burkitt lymphoblastoid cells (6, 10) and in somatic hybrids produced with those cells (7, 8) has also been well established.

These results suggested that a possible cell product inhibitory to virus replication might exist in certain cells. Such a product could interfere with CMV replication, and IUdR might depress synthesis of this substance. This could result in a shorter eclipse period as well as an increase in the infective-virus yield. This communication deals with the replication of CMV after treatment of cells with IUdR prior to infection.

MATERIALS AND METHODS

Cell culture and media. Human embryonic lung cells (HEL) were obtained from HEM Laboratories (Rockville, Md.). They were cultured in 16-oz (ca. 440-ml) glass prescription bottles. Eagle medium supplemented with 10% fetal calf serum (FCS), 0.075% sodium bicarbonate, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 100 μg/ml of kanamycin were used to grow the HEL cells.

Maintenance medium consisted of Eagle medium with 5% FCS, 0.15% sodium bicarbonate, and the same concentration of antibiotics employed in the growth medium. HEL cells in unsealed vessels were maintained in a 5% CO_2 atmosphere in a controlled-humidity incubator in Eagle medium with 0.225% sodium bicarbonate, 10% FCS, and the previously mentioned antibiotics.

Viruses. CMV (strain AD 169), obtained from Paul M. Feorino (Center for Disease Control, Atlanta, Ga.), was used in these studies. All virus stocks were prepared in HEL cells in the following manner. Confluent monolayers were infected with a virus-to-cell ratio of approximately 1. Virus was adsorbed for 1 h at 37 C. Maintenance medium was then added and

replenished at 5-day intervals. Maximum cytopathic effects (CPE) were observed in infected cell cultures between 10 to 14 days postinfection. Virus was harvested at this time by freezing and thawing the cultures, sonicating the contents for 2 min in a Branson Sonifier (Cole-Palmer Instrument and Equipment Co.), and clarifying the fluids by centrifugation at 600 × *g* for 5 min. The titer of virus prepared in this manner varied from 8×10^5 to 3×10^6 PFU/ml when titrated by the plaque method in HEL cells. Virus was stored at −65 C for later use.

Virus quantitation. The plaque assay method of Wentworth and French (22) was used for detection of infectious virus. HEL cells were grown in 60- by 15-mm plastic tissue culture dishes (Falcon Plastic Co.) and infected as confluent monolayers.

Deoxyribonucleic acid analysis. The method used for DNA analysis was, in general, that described by Crouch and Rapp (5). HEL cells grown in 4-oz (ca. 120-ml) prescription bottles were used for DNA studies. Cells were inoculated with either medium containing CMV with a virus-to-cell ratio of 1 or medium alone. After a 1-h adsorption period, the infecting inoculum was removed, and 5 ml of either maintenance medium or maintenance medium containing 10 μCi of thymidine-*methyl*-^3H (^{3}H-TdR, specific activity 17 Ci/mmol; Schwarz-Mann) were added. Cultures were harvested at daily intervals by using 24-h labeling periods for DNA determinations. Cultures then were analyzed for the amount of ^{3}H-TdR incorporated into acid-insoluble DNA.

Virus growth studies. Duplicate cultures were prepared in conjunction with the DNA studies for analysis of infectious virus by plaque assay as described previously (22).

IUdR treatment. Cells to be treated with IUdR (Calbiochem) were handled in the following manner. Confluent cultures were dispersed with trypsin, the cells were centrifuged, and the cell pellet was suspended to the desired concentration. IUdR then was added, and the cells were distributed to the appropriate containers. DNA replication then would occur with incorporation of IUdR into cellular DNA. Generally, the cultures were exposed to the IUdR for 72 to 96 h. Prior to infecting cells with virus, extracellular IUdR was removed by washing the cultures three times with isotonic Tris buffer (pH 7.4). The cells were kept in the buffer for a total of 4 h to remove as much nonincorporated IUdR as possible.

RESULTS

Effects of IUdR pretreatment of cells on the replication of CMV. Experiments were carried out to determine whether pretreatment of cells with IUdR had an effect on subsequent replication of CMV. The following parameters were examined: latent period, time of initiation of virus DNA synthesis, quantity of virus DNA synthesized, maximum infectious virus yield, and the amount of virus produced per cell. In these experiments, 4-oz (ca. 120-ml) glass prescription bottles were seeded with 7×10^5 to 8×10^5 cells. The bottles contained Eagle medium alone or Eagle medium with 100 μg of IUdR per ml. When the control (untreated) cells reached maximum confluency, usually in 72 h, the cells then were either infected with CMV at a multiplicity of infection of 1 or remained as uninfected controls.

A typical growth curve of CMV under these conditions is presented in Fig. 1. The data obtained from these studies indicate that IUdR pretreatment has no apparent effect on the virus eclipse period. Synthesis of infectious CMV was first detected in both control and treated cells between 2 and 3 days after virus inoculation of the cultures. There was a significant difference in the amount of infectious virus produced in cells pretreated with IUdR as compared with untreated cells. At day 3, the cultures pretreated with IUdR had an infectious virus titer of 1.0×10^5 PFU/ml as compared with 2.8×10^4 PFU/ml in the control cultures.

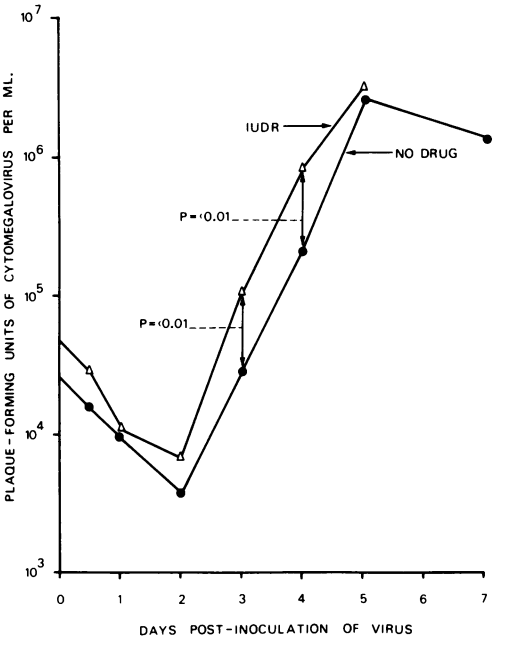

FIG. 1. *Growth curve for human cytomegalovirus in human embryonic lung cells. The triangles represent cultures pretreated with 100 μg of IUdR per ml. The circles represent untreated (but infected) cultures. Cells were infected with a multiplicity of 1 PFU of cytomegalovirus per cell. Virus was adsorbed for 1 h at 37 C. The zero time sample was taken after the 1 h adsorption period. The cultures were harvested at the times indicated, and virus was assayed for infectivity as described in Materials and Methods. The P values obtained were based on determination of 99% confidence limits for plaque counts regarded as samples from a Poisson series.*

This approximate fourfold increase in virus production was observed at days 3 and 4. However, by day 5 there was no significant difference in the amount of infectious virus produced in the IUdR-treated and control cultures.

During the course of these experiments, it was observed that high doses of IUdR appeared to inhibit cell replication, an observation reported earlier by other investigators (16). Consequently, cell counts were performed on cultures during each experiment. These studies indicate that when cells were pretreated with IUdR there was approximately a two-thirds reduction in cell numbers per culture. To further establish the effects of pretreatment of cells with IUdR on CMV replication, the average yield of virus per cell was determined. The maximum virus titer obtained per culture for each sample (Fig. 1) was divided into the average number of cells per 4-oz (ca. 120-ml) bottle for the appropriate sample. Thus, even though the titers obtained at day 5 were only slightly different (Fig. 1) (control, 2.76×10^6; 100 μg of IUdR, 3.30×10^6), the average yield per cell was greatly enhanced in the cells treated with 100 μg of IUdR. The control cells averaged 4.56 PFU per cell as compared with 20.62 PFU per cell in the treated cells. This type of experiment was repeated on eight separate occasions with similar results each time.

The infected cells used in the above experiments also were examined to determine the rate of virus DNA synthesis. Cells were pulsed for 24-h periods with ^{3}H-TdR. Cellular and virus DNA were separated by isospycnic centrifugation. The results of a typical experiment are presented in Fig. 2. The heterogeneity in the density of the DNA in the IUdR-treated cells is due, presumably, to the partial substitution of IUdR for thymidine and the resulting change in density (12). Because the IUdR was washed out when virus was added, there should have been no significant incorporation of IUdR into CMV DNA. The results obtained for the density of CMV and for cellular DNA are in general agreement with reported values (3, 4, 15). The cellular DNA in both infected and noninfected cultures had a density of 1.692 $\pm$ 0.003 g/cm^3. The virus DNA had a density of 1.710 $\pm$ 0.002 g/cm^3. Because there was no change in density in virus DNA in the treated and untreated cells, it must be concluded that there was no substitution of IUdR in virus DNA. A CMV DNA peak appeared as early as 24 to 48 h in the IUdR-treated cells (Fig. 2B, fraction 26), but did not appear until 48 to 72 h in the control cells (Fig. 2C, fraction 25). These results indicate that virus DNA was synthesized earlier when cells were pretreated with IUdR. Figure 3 presents the CMV DNA data plotted as total virus DNA (indicated by counts per minute) versus time in

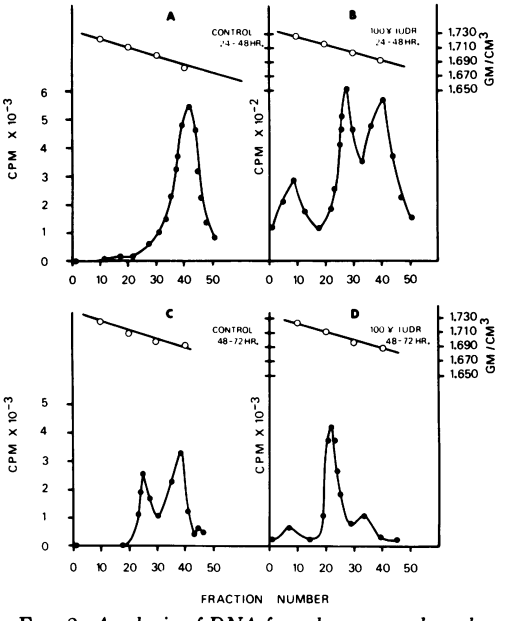

FIG. 2. *Analysis of DNA from human embryo lung cells infected with cytomegalovirus. DNA was labeled with tritium-labeled thymidine for 24-h periods. Cellular and virus DNA were separated by isopycnic centrifugation in CsCl and analyzed for radioactivity. Fig. 2A and B represent the 24- to 48-h period. Fig. 2C and D represent the 48- to 72-h period after infection.*

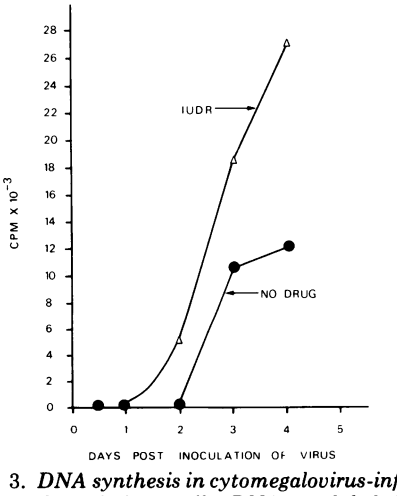

FIG. 3. *DNA synthesis in cytomegalovirus-infected human embryonic lung cells. DNA was labeled with tritium-labeled thymidine for 24-h periods. The triangles represent the IUdR-treated cultures. The circles represent the control cultures. Total virus DNA was determined by separation from cellular DNA by isopycnic centrifugation in CsCl and summation of radioactive counts under typical, virus DNA curves as in Fig. 2.*

days. Virus DNA synthesis occurred about 24 h earlier in the drug-treated cultures than in the control cultures. The specific activities of the ^{3}H-TdR in the control and IUdR-treated cultures were the same. By assuming that the TdR pool sizes were equal, the data indicate that over two times more virus DNA was produced in the drug-treated cells than in the control cells. These results are in agreement with the earlier virus replication studies (Fig. 1).

Effect of IUdR pretreatment on detection of small amounts of CMV in HEL cells. Cells grown in 16-mm multidish disposable trays (Linbro Chemical Co.) were pretreated with various concentrations of IUdR grown to confluency and then infected with various multiplicities of CMV. The cultures were examined at 24-h intervals over a 7-day period. Cultures were considered positive when 50% or more of the cells had developed demonstrable CPE. These data, presented in Table 1, indicate that not only does pretreatment of the cells with IUdR shorten the time required for the appearance of CPE, but it also increases the sensitivity of the system for detection of virus when CPE is used as the indicator. In the untreated cultures, CPE occurred at multiplicities of 1 and 0.1 within 7 days. In the cells pretreated with IUdR, CPE occurred at a multiplicity of infection of 0.001 within 4 days after infection.

Effect of pretreatment of HEL cells with IUdR on the development of CMV plaques. The possibility that pretreatment of cells with IUdR might shorten the length of time required for plaque development by CMV was investigated. Cells grown in 60-mm, plastic petri dishes (Falcon) were either untreated (control cells) or pretreated with 50 or 100 μg of IUdR per ml. The cells were grown to confluency, infected with 10-fold dilutions of CMV, and overlaid with a medium containing agarose. Cells were stained at daily intervals thereafter and examined with the aid of a stereoscopic microscope (American Optical Co.) for plaque development. IUdR treatment enhanced plaque development by use of a number of parameters (Table 2). The early CPE which appeared at day 1 or 2 were in the lower virus dilutions. Plaques could not be distinguished, but typical virus CPE was apparent. Plaques first were observed in the treated cells at day 3; however, the plaques were too small at this time to make an accurate count possible. Nevertheless, the first indication of plaque development in the untreated cultures was 7 days after infection. Furthermore, the final titer obtained in the cells pretreated with 100 μg of IUdR was approximately 10-fold higher than in the control cells.

TABLE 1. *Effect of 5-iodo-2'-deoxyuridine (IUdR) pretreatment of cells on development of cytopathic effects (CPE) in human embryo lung cells infected with cytomegalovirus (CMV)*[a]

Multiplicity of infection	Days before appearance of CPE after CMV infection					
	IUdR concn					
	0	20γ	40γ	60γ	80γ	100γ
1.0	2	1	1	1	1	1
0.1	7	2	2	2	2	2
0.01		5	5	3	3	3
0.001			6	4	4	4
0.0001						

[a] Cells were considered positive when 50% of the cells in a culture exhibited CPE. All samples were done in triplicate in 16-mm tissue culture wells (Linbro multidish disposable trays [Linbro Chemical Co.]) The study was concluded at day 7.

TABLE 2. *Effect of IUdR on plaque formation in human embryo lung cells by cytomegalovirus*

Determination	Control	50 μg of IUdR	100 μg of IUdR
Day CPE first appeared	2	1	1
Day plaques first were observed[a]	7	3	3
Day first readable plaques were observed[b]	9	6	6
Maximum titer observed	1.0×10^4	5.0×10^4	1.3×10^5

[a] The plaques observed at this time were too small to count. However, they were observable under a stereoscopic microscope at ×15 magnification.

[b] This is the day at which the first accurate estimate of virus titer could be accomplished. Virus titer is reported in PFU per milliliter.

DISCUSSION

The results presented in this communication indicate that treatment of cells with IUdR enhances the replication of CMV. The growth studies indicate that, when cells are pretreated with IUdR prior to infection, greater amounts of infectious virus per cell are produced. Even though the final virus titer obtained per culture was not significantly different, the virus yield per cell was enhanced approximately fivefold. The results of the DNA studies correlated with the infectious virus data. It is significant that not only was there a greater amount of virus DNA produced in the IUdR-treated cells, but also virus DNA synthesis occurred 24-h earlier in the drug-treated cells.

The results obtained in the studies involving the effects of IUdR on the development of virus CPE indicated that when cells are pretreated with IUdR, productive infection occurs more rapidly in drug-treated than in control cells. This is of interest from both a biological and a clinical viewpoint. A distinct problem in the isolation of CMV from clinical material is that small amounts of virus, as are often encountered when inoculating clinical specimens into tissue culture (2), require an extended period of time for CPE to be manifested. If cells are first pretreated with IUdR prior to inoculation with a clinical specimen, the time required for virus isolation could be shortened dramatically.

The enhancement of the plaque assay by IUdR supports the other results presented in this paper. The toxicity of IUdR poses a problem in conducting this assay, but this problem is not prohibitive and again shortens the time required for virus assay.

The reason for the enhancement of CMV replication by IUdR is unknown. However, there are several explanations for the results reported in this communication. It has been reported that IUdR treatment of cells will inhibit their ability to produce interferon (11). If interferon was inhibiting CMV replication in HEL cells, this would be an obvious explanation of the observed results. However, it has been reported that CMV are resistant to the antivirus effects of interferon, are poor inducers of interferon, and can depress interferon formation in vivo (9, 14).

From an examination of the present information, it would appear that the most logical explanation is that there is a cell factor other than interferon which is inhibiting virus replication. It is known that when cells are treated with IUdR, a cellular phenotypic change occurs which results in the elimination or faulty production of a cellular product (6, 11). It is possible that the effects observed can be related to the observations which have demonstrated that IUdR can activate synthesis of both RNA- and DNA-containing viruses (1, 6–8, 10, 13, 21). Whether or not these phenomenon have similar molecular mechanisms is unknown.

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