Remote activation of host cell DNA synthesis in uninfected cells signalled by infected cells in advance of virus transmission

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Abstract

Viruses modulate cellular processes and metabolism in diverse ways but these are almost universally studied in the infected cell itself. Here we study spatial organisation of DNA synthesis during multi-round transmission of herpes simplex virus (HSV) using pulse-labelling with ethynyl-nucleotides and cycloaddition of azide-fluorophores. We report a hitherto unknown and unexpected outcome of virus-host interaction. Consistent with current understanding during single step growth cycle, HSV suppresses host DNA synthesis and promotes virus DNA synthesis in spatially segregated compartments within the cell. In striking contrast, during progressive rounds of infection initiated at a single cell, we observe that infection induces a clear and pronounced stimulation of cellular DNA replication in remote uninfected cells. This induced DNA synthesis was observed in hundreds of uninfected cells at the extended border, outside the perimeter of the progressing infection. Moreover using pulse chase analysis we show that this activation is maintained, resulting in a propagating wave of host DNA synthesis continually in advance of infection. As the virus reaches and infects these activated cells, host DNA synthesis was then shut off and replaced with virus DNA synthesis. Using non-propagating viruses or conditioned medium we demonstrate a paracrine effector of uninfected cell DNA synthesis in remote cells continually in advance of infection. These findings have significant implications, likely with broad applicability, for our understanding of the ways virus infection manipulates cell processes not only in the infected cell itself but also now in remote uninfected cells, as well as for mechanisms governing host DNA synthesis.

Importance

We show that during infection initiated by a single particle with progressive cell-cell virus transmission (i.e., the normal situation), HSV induces host DNA synthesis in uninfected cells, mediated by a virus induced paracrine effector. The field has had no conception that this process occurs and the work changes our interpretation of virus-host interaction during advancing infection and has implications for understanding controls of host DNA synthesis. Our findings demonstrating the utility of chemical biology techniques in analysis of infection processes, reveal distinct processes when infection is examined in multi-round transmission versus single-step growth curves, reveal a hitherto unknown processes in virus infection, likely relevant for other viruses (and other infectious agents) and for remote signalling of other processes including transcription and protein synthesis.
Introduction

Many viruses inhibit host macromolecular synthesis to suppress cellular antiviral responses or reduce competition from synthesis of host products (1). Viruses also manipulate host autophagic pathways (2), induce and suppress apoptosis (3), and usurp DNA repair pathways (4). The host cell cycle is also modulated by virus infection and can be stimulated or suppressed depending on the virus (5). Small DNA viruses including papillomaviruses and adenoviruses modulate the host G1/S phase transition to stimulate cell-cycle regulated transcription and/or S-phase DNA synthesis and thus support virus genome replication (5-7). On the other hand, large DNA viruses such as the herpesviruses encode their own DNA synthetic apparatus and enzymes for nucleotide production. In the case of herpes simplex virus (HSV), in addition to seven essential replication proteins (8-14), other viral and host proteins localise to segregated replication compartments to promote origin specific virus DNA replication (see review 15). Moreover HSV generally suppresses host cell DNA synthesis or blocks the transition from G1 to S-phase (12) and is thought to interfere with cell cycle at several distinct phases (16-19), reviewed in (20).

All of the events cited above occur within the virus infected cell itself. Generally virus manipulation of the intracellular environment is effected either by early events associated with attachment to the host cell, by structural components of the infecting virus or by de novo expressed virus products. Apart from the well-known processes of paracrine/juxtacrine signalling to uninfected cells in antiviral responses, the prospect that additional modulation of other types of cellular metabolic processes occurs by paracrine signalling to uninfected cells would have significant implications for our general understanding of virus-host interactions and the events which impinge on the outcome of infection.

The development of azide- or alkyne-derivatised metabolic precursors combined with cycloaddition to biorthogonal capture reagents is increasingly being exploited in various approaches to biological processes and more recently to mechanisms in infection and immunity (21-25). Here we use such techniques to study the spatiotemporal distribution of virus and host cell DNA synthesis when infection is initiated in a single cell and followed by progressive transmission to neighbouring cells. We show that while HSV disrupts host cell DNA synthesis and promotes virus DNA synthesis in the infected cell itself, it also induces paracrine signalling on control of host cell DNA synthesis in distant uninfected cells resulting in a propagating wave of DNA synthesis in advance of virus infection. The results are relevant not only for HSV and potentially for viruses in general, they reveal differences in host cell processes when studied in progressive transmission models versus single step
growth cycles, and could also indicate that similar events occur in other aspects of cellular metabolism e.g., transcription and protein synthesis.

**Materials and Methods**

**Cells and viruses.**

Vero cells were grown in Dulbecco’s modified minimal essential medium (DMEM, Gibco) containing 10% newborn calf serum (NCS) and penicillin/streptomycin. RPE-1 cells, a human telomerase immortalised retinal pigment epithelial line were grown in DMEM/F12 (Sigma) supplemented with 200 mM glutamine, 10% FBS and penicillin/streptomycin. RSC cells were grown in DMEM containing 10% fetal bovine serum (FBS) and penicillin/streptomycin. Virus strains were HSV-1[17] or HSV-1[17].ICP0-YFP a derivative of HSV-1[17] which expresses the immediate early protein ICP0 fused to YFP (26). Other strains included HSV-1 [KOS], [KOS].VP1-2∆NLS which lacks the core NLS of VP1-2 as previously described (27). Routine plaque assays were performed in the presence of pooled neutralising human serum (Sigma) at 2% or clinical grade purified neutralising human immunoglobulin (IVIg, Carimune® NF, Nanofiltered, human immune globulin, CSL Behring) at 2 mg/ml, having demonstrated complete neutralisation of extracellular virus at this dose (>6 log reduction in virus titre). High multiplicity infections were performed at an moi of 5 or 10. For inhibition of virus DNA synthesis phosphonoacetic acid (PAA) was used at a final concentration of 400 µg/ml added as indicated in the figure legends.

For analysis of paracrine signalling (see Figure 7), a dual chamber system (Anapore) was used consisting of an upper insert chamber in which cells (donor cells) were plated on a supporting membrane of defined pore size (0.02 µm), inserted into a lower chamber containing a monolayer of test cells. The upper chamber base membrane with 0.02 µm pore size prevented virus migration through it and in addition the cells were plated in neutralising serum for inhibition of any infectious virus passage through to the bottom chamber. The upper chamber cells were infected at defined multiplicities (Figure 7) or mock infected, placed in the dual chamber system and incubated for 20 hrs. Ethynyl-deoxycytidine (EdC) was then added for a standard labelling period and cells in the lower test chamber were then fixed and processed for analysis of DNA synthesis as described below.

**Immunofluorescence studies.**

Immunofluorescence analysis was performed exactly as described previously (29). Samples were collected at times indicated, washed with PBS, fixed with either paraformaldehyde (4%) followed by permeabilisation with 0.5% Triton X-100, or by methanol (5 mins at -20°C).
Samples were blocked with PBS containing 10% NCS or a mix of 5% goat serum, 5% NCS and 2% BSA, for 1 hr at room temperature. Primary antibodies for the following antigens were; anti-ICP4 antibody (Virusys) used at 1:400; anti-ICP8 antibody used at 1: 400. DNA was stained with DAPI (Sigma) and coverslips mounted in Mowiol supplemented with 2.5% DABCO. Images were taken using an Axiovert 135 TV using Zeiss x10, x40 LD or x63 lenses (Plan-Apochromat, 1.4 numerical aperture), captured using a Retiga 2000R camera with Image Pro Plus software or with a Zeiss Laser Scanning confocal microscope (Zeiss Pascal).

Ethynyl-nucleotide labelling, click-chemistry and image analysis.

We initially evaluated the effect of EdC or EdU on the growth and doubling of uninfected cells and on HSV replication. There was a modest effect of EdU on both cell growth and virus yield, but virtually no effect of EdC on plaque numbers and plaque size in multi-cycle analysis or virus yield in single step analysis. We optimised protocols for nucleoside incorporation, click-chemistry and fluorescence detection as follows. Cells on coverslips were mock infected or infected with HSV by normal procedures (moi 10), and incubated in DMEM, 2% NCS. At times indicated, EdC was added at a final concentration of 5 µM for a standard labelling time of 4 hrs unless otherwise stated. The cells were then washed in cold PBS and fixed in 4% paraformaldehyde for 15 mins, quenched with glycine (0.3 M) or ammonium chloride (25 mM) and permeabilised in 0.5% TX100 at room temperature for 5 mins. For localisation of specific antigens, immunofluorescence with primary and secondary antibodies was carried out as above. The samples blocked in 10% calf serum and then subject to click reaction in a buffer prepared freshly in each case (premixed for 2 mins) and containing 10 µM Alexa Fluor 488-azide (Sigma Aldrich); 1 mM CuSO₄; 10 mM sodium ascorbate; 10 mM amino-guanidine and 1 mM Tris(3-hydroxypropyltriazolylmethyl)amine (Sigma Aldrich) in PBS pH 7.4. The reaction was then allowed to proceed by incubation at room temperature in the dark for 90-120 mins. After washing in PBS the cells were counterstained with DAPI (Sigma) and coverslips mounted in Mowiol supplemented with 2.5% DABCO. Images were taken as described above. For quantitation, total cell numbers were enumerated from the nuclear DAPI staining in one channel and EdC positive cells from nuclear staining (above background set in the absence of EdC). Images were quantitated manually (for total numbers and EdC positive numbers) from the images or for more quantitative assessment using the thresholding and segmentation modules of Image Pro Plus. In this workflow, areas of individual objects (nuclei) are defined from the DAPI staining and then total intensity above background reported in the corresponding object area for EdC incorporation. For the data in Figure 7, the range of counts for EdC intensity from minimum
to maximum was binned and the numbers of cells in each bin then returned. For these analyses numerous independent fields and hundreds or thousands of cells were quantified.

In this work, the majority of figures are illustrated using low magnification microscopy for ease of inspection of numerous cells in a field and to discern the main points of the work which are only visible at low magnification. Certain data are illustrated at higher magnification for qualitative points concerning intracellular distribution of DNA and protein components.

**Results**

**DNA synthesis analysed by ethylene-nucleoside incorporation in uninfected and HSV infected cells.**

Analysis of DNA synthesis by labelling with alkyne-derivatised nucleotides and cycloaddition to azide-coupled fluorochromes has been evaluated in several systems (23, 30, 31). In agreement with previous work (23, 32), EdC (ethynyl-deoxycytidine) was at least if not more sensitive for detection than EdU (ethynyl-deoxyuridine) (data not shown). We also found no significant effect of EdC on cell replication at doses required for detection of DNA synthesis (Figure 1a), no effect on virus yields from single step growth curves, nor any effect even over 2-3 days on virus plaque numbers or size (Figure 1b-d). Further experiments were therefore performed with EdC labelling. The majority of experiments were performed in human retinal pigment epithelial (RPE) cells unless otherwise stated. Experiments confirming observations (see below) were also performed in Vero and rabbit skin cells (RSC).

EdC labelling (5 µM) of asynchronously growing RPE cells for 4 hrs allowed ready detection of DNA synthesis in approximately 25-30% of cells (Figure 2A, Mock). Qualitatively, the pattern of EdC incorporation was similar to that previously reported (23, 30), showing one of a few patterns proposed to reflect the approximate stage within S-phase (Figure 2A,B). An example showing total DNA staining (DAPI) and EdC incorporation is shown in Figure 2B. In contrast, in HSV infected cells (multiplicity of infection, 10), virtually every cell was positive for EdC incorporation by 8 hrs after infection (Figure 2A, HSV). The percentage of EdC positive cells in several hundreds of cells is summarised in Figure 2C, showing ongoing DNA synthesis in virtually every infected cell, compared to the approximately 30% observed in uninfected cells. Moreover the pattern of incorporation in infected cells was qualitatively distinct from that seen in uninfected cells. An example is shown in Fig.2D, showing total cell nuclei (DAPI), ICP8 localisation and EdC incorporation in the same field. The results show the previously documented features of HSV DNA
replication compartments (12, 33, 34), including EdC co-localisation with the DNA replication protein ICP8, and exclusion from DAPI-dense areas of cellular DNA and areas adjacent to the nuclear rim where cellular DNA is marginated (Figure 2B,D, example inset arrowed cell). These features are also clearly observed in the higher magnification image (Figure 2E).

**EdC incorporation into replication compartments after single particle infection.**

We next examined whether virus DNA synthesis could be detected after infection with a single initiating particle, i.e., at an extremely low multiplicity of infection where only one initially infected cell would be expected in several thousand cells. The results demonstrate that early after such infection (within 6-8 hrs), individual infected cells could be identified by the presence of ICP8 (Figure 3A, ICP8, arrowed), surrounded by uninfected cells. The localisation of EdC in such single infected cells was qualitatively distinct. A single focus of EdC incorporation within the nucleus was seen, usually at the periphery, combined with substantially reduced incorporation in the remainder of the nucleus (Figure 3A, EdC, arrowed, and DAPI/EdC panel, inset; see also single focus inset in Figure 7). Such a pattern was only observed in ICP8+ve cells and, consistent with previous data from high multiplicity experiments (12), represents a progressing virus DNA replication compartment, in this case emanating from a single genome beginning to replicate. The general reduction in EdC incorporation, outside the focus of the developing virus replication compartment itself, is consistent with previous data showing that HSV infection blocks cellular G1 to S-phase progression in infected cells (12). However, while our initial intention was to study spatial aspects of DNA synthesis in cells where only a single particle initially infected, observations made during the course of these experiments directed the focus of our investigation to other processes, which are the subject of the remainder of this work.

**HSV infection induces a zone of elevated cellular DNA synthesis in uninfected cells.**

During these experiments we also discovered a distinct feature in the spatial organisation of DNA synthesis. Thus, significantly higher levels of DNA synthesis were routinely observed in uninfected cells surrounding the single initially infected ICP8+ve cells than were observed in more distant cells or in uninfected cells, (more clearly seen in the lower magnification view of a typical experiment, Figure 3B, ICP8, arrowed). This gradient of DNA synthesis could be seen as early as 8 hrs after single particle infections. We repeated these experiments, pulse labelling with EdC at a later time (20-24 hrs) when infection progressed to numerous cells (Figure 4A). The results show a pronounced zone of cells, surrounding the ICP8+ve infected cell boundary, in which elevated levels of DNA synthesis were observed with a gradient extending to distant cells. While levels of ongoing DNA synthesis were significantly increased in this zone, the percentage of cells synthesising DNA remained similar or only
marginally increased compared to that seen in uninfected cell monolayers. This result was obtained independently of the virus antigen used to detect the central infected cells and in several cell types including RSC cells and Vero cells (Figure S1A).

To confirm that the increased EdC incorporation represented cellular DNA synthesis we repeated these experiments, adding the virus specific DNA synthesis inhibitor phosphonoacetic acid (PAA) prior to the EdC pulse (Figure 4B). PAA had no significant effect on the induction of DNA synthesis in the numerous cells surrounding the infected cell boundary, while suppressing DNA synthesis in the central ICP8+ve cells (Figure 4B, DAPI v EdC merged channel and see also EdC single channel). The intracellular patterns of elevated DNA synthesis (see Figures 3 and 4) exhibited a similar spatial distribution of DNA synthesis to that normally seen in uninfected cells, only at higher overall levels. This result was typical of all developing plaques as illustrated by a single tiled image showing a significant portion of the entire monolayer with five developing plaques (Figure 5) and was also observed during infection with HSV-2 (Figure S1B).

**HSV induces a propagating zone of elevated cellular DNA synthesis.**

The induction of cellular DNA synthesis appeared as an early event in response to even a single infected cell. We wished to address the duration of this mechanism using a combined pulse-chase-pulse regime (Figure 6A). After single particle infection, cells were pulsed with EdC at 20 hrs, washed and incubated for a further 20 hrs to allow virus progression, then pulsed again with EdC and analysed. We anticipated that if induction of DNA synthesis was maintained, we should see a second external zone of elevated DNA synthesis surrounding the infected cells. As shown in Figure 6A, this is exactly what was observed. (The various zones defined below are labelled only on the EdC panel for ease of inspection). Over the course of the 44 hrs, the centre of the plaque showed more extensive cytopathic effect (focus centre) and was lost during processing but the remaining cells from the first zone of elevated DNA synthesis (zone 1) could be seen, now with infected cells (ICP8+ve) extending beyond. Some of the remaining cells in this initial zone 1 could be seen labelled for both cell DNA synthesis and ICP8 (EdC/ICP8, merged panel). Extending beyond this (zone 2), are the infected cells now chased in the absence of EdC, i.e., ICP8 +ve but with little EdC. The cells at the periphery of zone 2 show weak EdC labelling representing viral DNA synthesis during the second pulse-labelling period. However at the extended border beyond zone 2, (beyond the zone of infection marked by ICP8), we now again see elevated DNA synthesis in numerous uninfected cells (zone 3), with a gradient extending to the more distant uninfected cells. Thus at least for up to 2 days, during multiple rounds of infection there is a
A non-replicating virus induces elevated cellular DNA synthesis in uninfected cells.
We could detect elevated cellular DNA synthesis early after a single particle infection in the absence of any significant virus protein synthesis in the surrounding cells and in the presence of PAA outside plaque boundaries. These results indicated that stimulation of DNA synthesis required neither infection nor virus gene expression in activated cells. Nevertheless it remained possible, though unlikely, that events associated with infection could be involved. To rule this out we examined single particle infection with a virus incapable of spreading to surrounding cells (27). This virus, HSV-1.VP1-2ΔNLS, when produced on complementing cells, can infect a cell and make particles, but these particles do not productively infect surrounding cells due to a defect in capsid transport (27). Typical results are shown in Figure 6B where a single mutant virus infected cell is detected expressing ICP8. In this infected cell, the single initiating virus replication compartment can be observed (Figure 6B, EdC, arrowed and inset). This cell is surrounded by cells exhibiting elevated DNA synthesis, with a gradient extending to more distant cells with no virus gene expression and which will not be infected. Since this mutant produces particles albeit ones unable to productively infect cells, we repeated this regime with another disabled virus, HSV-1.ΔVP1-2.VP26.GFP in which the VP1-2 gene is deleted and which does not assemble or release infectious particles (35), with similar results (Figure S1C). Together these data provide strong evidence that the stimulation of DNA synthesis results from a paracrine effect, signalled by infected cells to surrounding uninfected cells.

Paracrine signalling from infected cells stimulates cellular DNA synthesis.
To next pursue the possibility of a paracrine mechanism initiating from the infected cell, we used an insert culture system in which the infected effector cells were not in contact with the test cells. Cells grown in an insert containing a membrane with 20 nm pores were infected or mock-infected (i.e., donor cells) and incubated with the recipient test cells in the lower chamber (Figure 7A). Approximately 20 hrs after infection the inserts were removed and the test cells pulse-labelled with EdC. The results (Figure 7B) demonstrate a significant increase in test cell DNA synthesis when exposed to HSV-infected donor medium compared to uninfected cell donor medium. This increase also showed a dose response in relation to the numbers of initially infected cells in the donor cell population (c.f., test cell DNA synthesis when donor cells infected at moi 0.001, 0.01, 0.1). The results were quantified and expressed in numerous test cells, binned into ranges and plotted for mock-infected or HSV-
infected donor cells (Figure 7C). The results show a substantial increase in EdC incorporation in test cells exposed to donor medium from infected cells. This result cannot be explained by virus infection per se in the lower test chamber. Firstly, infection would yield a focus of increased DNA synthesis emanating from an infected cell. This was not observed. Secondly, HSV will not pass through a 20 nm pore membrane. Thirdly, the cultures were incubated in the presence of neutralising antibody. Finally, no virus infected cells were detected in the test monolayer. Taken altogether, our results indicate that for induction of host DNA synthesis during progressive rounds of infection, the activated cells do not need to be in contact with infected cells and that a paracrine mechanism operates whereby signal(s) even from a single infected cell, promote elevated DNA synthesis in surrounding uninfected cells.

Discussion

The results of this work have several implications specifically, for processes involved in HSV replication and generally for consideration of mechanisms involved in virus replication. Such processes are frequently studied and deduced from single step growth analysis and, based on this work, may be qualitatively distinct when studied during progressive rounds of transmission where the environment of a susceptible uninfected cell is modified by exposure to infected cells.

Previous work from high multiplicity analysis convincingly shows that HSV actively blocks various stages of the cell cycle including G1-S transition and mitosis, although if cells are infected during active S-phase, continued DNA synthesis may not be blocked (12, 17). It has been concluded that HSV infection is independent of the cell cycle and if anything infection suppresses cell cycle progression presumably to promote an optimal environment for virus replication. In the context of the infected cell itself our results are in agreement with these observations. Nevertheless what has not previously been demonstrated nor appreciated, are the results shown here of paracrine stimulation mediated by infected cells to surrounding uninfected cells. Two questions present themselves, firstly on the implication for the outcome of virus infection and secondly on the possible mechanisms involved.

With regard to the first question the most reasonable proposition would be that activation of host DNA synthesis might promote virus infection by any of a number of not mutually exclusive mechanisms including production of cellular replication components or establishing particular intranuclear niches. We have attempted to show, e.g., by increased plaque numbers or faster spread that conditioned medium promotes more efficient infection but
have not observed a significant increase in these parameters. It is possible that such an outcome may be demonstrated only under sub-optimal conditions e.g., with debilitated or poorly replicating viral mutants where the efficiency of initiation of infection or the kinetics of spread may reveal an augmented outcome. It is also possible that any effect may not be readily demonstrable in the otherwise very permissive system of tissue culture infection by HSV. It may be that the physiological relevance of this paracrine effect is reflected more in the situation of initial infection in vivo or of reactivation in neuronal cells which are not normally transiting through S-phase or surrounding supporting cells in the three dimensional environment. Ultimately investigation of the influence on the outcome of infection will require knowledge of the virus and/or host components involved in order to interfere with the pathway and explore the consequences. We note also that notwithstanding HSV repression of S-phase progression many lines of evidence indicate a positive association between the cell cycle/cellular DNA synthesis and the outcome of herpesvirus replication. For example cell-cycle dependent kinases are known to be required for replication of several herpesviruses including HSV and HCMV (36, 37). HCMV manipulates quiescent cells producing a pseudo S-phase environment to support viral replication (38, 39) and certain viral proteins from EBV can actively stimulate E2F responsive transcription and S-phase progression (40). More recently the whole way we will... The help file a,

With regard to the complex question of mechanism, while direct cell-cell communication remains possible, our results indicate a paracrine effect on cellular DNA synthesis produced from infected cells. Such an effect could be due to a virus or host cell encoded product which activates host DNA synthesis. Whether this is a conventional type of growth factor and whether it is secreted as an individual component or e.g., as some form of vesicle such as an exosome, awaits further mechanistic and biochemical studies. In terms of the processes within an infected cell itself to elicit the response in uninfected cells, preliminary data where single particle infection was carried out in the presence of PAA, added from the time of infection, did not result in zones of elevated DNA synthesis surrounding the single infected cell. This indicates that infection per se is insufficient and late events or accumulating early events are required.

Interestingly it has been shown in models of ocular pathogenesis that HSV induces the secretion of cytokines including e.g. VEGF which are implicated in blood vessel migration but also capable of promoting growth in non-endothelial cells (41). It is possible that these processes are linked and that the stimulation in DNA synthesis observed here is integrated with other responses in vivo. Nonetheless, by quantitatively evaluating the percentage of S-phase cells in the zones exhibiting increased synthesis compared to total cell numbers in the
zones (data not shown), we find little alteration from the percentage of S-phase cells in the uninfected cell population. The results may indicate that the process is not one advancing the G1 to S onset or duration but rather one influencing the overall abundance of DNA synthesis in the cell. Such a mechanism might involve an increase in the efficiency of origin firing or in the suppression of certain cell cycle controls limiting DNA replication. It is noteworthy that adenovirus infection which stimulates S-phase DNA synthesis has been suggested to involve uncontrolled firing of DNA replication origins by suppressing cyclin A and geminin which otherwise suppress genome re-replication (5, 42). It is possible therefore that stimulation represents a perturbation of cell control mechanisms e.g., in timing of origin firing or re-replication, rather than a stimulation of normal DNA synthesis. Other processes including e.g. DNA damage/repair could also be involved.

While a full mechanistic understanding awaits further analysis, these results on the process itself are immediately relevant for HSV, for herpesvirus biology and potentially for other classes of viruses. They also reveal distinct differences in host cell processes studied in progressive transmission models versus single step growth cycles, and could also indicate that similar events take place in other aspects of cellular metabolism including for example transcription and protein synthesis. We propose therefore that these observations advance our fundamental concepts about processes involved in perturbation of host processes during virus infection. They are likely to be highly relevant in the broader virology context and while raising many questions open new lines of investigation on the diverse pathways by which viruses manipulate host processes to influence infection outcome and disease progression.

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Figure 1. Lack of effect of EdC on cell growth and virus replication.

(A) RPE cells (approximately 5x10^4) were counted and plated in duplicate in normal growth medium containing 10% NCS. After 24 hrs, medium without or with increasing concentrations of EdC was added as indicated. After a further 48 hrs, cells were harvested and viable cell numbers evaluated using trypan blue exclusion EdC labelling for spatial analysis of uninfected and infected cell DNA synthesis.

(B) RPE cells were infected with HSV at low moi with approximately 40 pfu per well of a 12-well cluster plate. After 2-3 hrs the medium was removed and replaced with medium containing increasing doses of EdC as indicated and infected cultures incubated in the continued presence of the label. After 48 hrs, cells were fixed, and plaque number and size evaluated. A typical individual plaque is shown in panel B, with average plaque area and number indicated in C and D respectively.
Figure 2

(A) Typical fields showing incorporation of EdC from 4-8 hrs after mock-infection or HSV infection (moi 5) in RPE cells. Cells were counter-stained with DAPI. (B) Higher magnification image (x63 objective) showing qualitative features of the localisation of EdC incorporation in uninfected or infected cells compared to total DNA. (C) Quantitative analysis of EdC incorporation in mock-infected or HSV infected cells as a percentage of total cell count. (D) A typical field of HSV infected cells simultaneously analysed for total cell DNA (DAPI), EdC incorporation, and ICP8 localisation. The insert shows a higher magnification of a cell showing typical features of bulk DNA margination, ICP8 distribution and EdC incorporation as discussed in the text. (E) A single confocal section taken with an x63 objective and zoom 4 showing EdC incorporation into HSV replication compartments and colocalisation with ICP8 as discussed in the text.

Figure 2. EdC labelling of host and virus DNA synthesis in uninfected and infected cells.
Figure 3. EdC labelling of virus DNA replication compartments initiated after infection in a single cell.

(A) Cells were infected at an extremely low moi, approximately 1/4,000 cells infected, labelled with EdC from 4-8 hrs and processed for total cell number and DNA (DAPI), ICP8 expression and EdC incorporation. A single initially infected ICP8+ve cell is detected (arrowed) surrounded by numerous uninfected cells. In this cell a single focus of EdC is observed. (B) A field now imaged at low magnification (x10 objective) in order to show a single infected cell (ICP8+ve) in a field of approximately 700-800 cells. The single ICP8+ve cell is immediately surrounded by cells showing elevated levels of EdC incorporation as discussed in the text.
Figure 4. HSV infection induces a zone of elevated host cell DNA synthesis in uninfected cells.

(A) Cells were infected at an extremely low moi, approximately 1/4,000 cells infected, labelled with EdC from 20-24 hrs and processed for total cell number and DNA (DAPI), ICP8 expression and EdC incorporation. The panels show different combinations of channels as labelled for ease of inspection of the results. (B) As for (A) but with the prior addition of the virus DNA synthesis inhibitor PAA (400 μg/ml), 1 hr before addition of EdC.
Figure 5. HSV induction of elevated host cell DNA synthesis in progressing plaques. RPE cells were infected as in Figure 4, labelled with EdC from 20-24 hrs and processed for ICP8 expression and EdC incorporation. Multiple images were captured on a motorised stage and tiled together to one image covering a quarter of the entire dish and showing 5 developing foci of virus spread, all exhibiting elevated DNA synthesis in the extended boundary beyond virus infection.
Figure 6. HSV infection induces a propagating wave of elevated DNA synthesis in uninfected cells.

(A) Cells were infected at low moi, pulsed with EdC at 20 hrs, chased in the absence of EdC, and then pulsed again at 40 hrs. The progressing infection can be assigned to different zones of activity as discussed in the text. (B) Cells were infected at low moi with the mutant HSV-1 VP1-2∆NLS, pulsed labelled with EdC from 20-24 hrs post-infection and processed. A single infected ICP8+ve cell (arrowed) is detected, within which is a single focus of viral EdC incorporation (see inset, arrowed cell, EdC panel). This cell was surrounded by numerous uninfected cells exhibiting elevated DNA synthesis.
Figure 7. HSV infected cells induce a paracrine mediator of elevated cellular DNA synthesis. (A) Diagram of a dual chamber system to examine paracrine stimulation of DNA synthesis. (B) The donor cell chamber was mock-infected or infected with HSV at different mois as indicated and 20 hrs later the donor chamber removed and EdC pulse labelled in the test cells for 4 hrs. Two individual fields, (low magnification, x10 objective), are illustrated for each condition. (C) Quantitative analysis of EdC incorporation test cells in medium from mock infected or HSV infected donor cells performed as described in materials and methods.
Figure S1. HSV infection induces elevated DNA synthesis in multiple cell types uninfected cells.

(A) Vero cells or RSC cells were infected with HSV-1 at low moi, pulsed with EdC from 20-24 hrs and processed for ICP8 expression and EdC incorporation, shown in grey scale and merged in colour (ICP8, red channel; EdC green channel). Representative uninfected areas distant from the focus of infection are illustrated in the left hand panels. (B) Cells were infected with HSV-2 [186] and pulsed with EdC from 20-24 hrs. In this case the cells were not stained for ICP8 expression. HSV-2 plaques generally progressed quicker than HSV-1, and late incorporation of EdC into infected cells within the plaque was at a lower level. However clear, pronounced elevated EdC incorporation in ring zones surrounding the central area of cpe was obvious. (C) Cells were infected at low moi with the mutant HSV-1ΔVP1-2, pulsed labelled with EdC from 20-24 hrs post-infection and processed. A single infected ICP8+ve cell is detected (red channel, arrowed), surrounded by numerous uninfected cells exhibiting elevated DNA synthesis in a gradient to more distant cells (green channel).
