The Effects of Adjacent Cell Fusion and Ultraviolet Radiation Exposure on Viral Plaque Formation with Herpes Simplex Virus Type I

Rebecca Conner
THE EFFECTS OF ADJACENT CELL FUSION
AND ULTRAVIOLET RADIATION EXPOSURE
ON VIRAL PLAQUE FORMATION
WITH HERPES SIMPLEX VIRUS TYPE I

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Master of Science

by
Rebecca E. Conner
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THE EFFECTS OF ADJACENT CELL FUSION AND ULTRAVIOLET RADIATION EXPOSURE ON VIRAL PLAQUE FORMATION WITH HERPES SIMPLEX VIRUS TYPE 1

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THE EFFECTS OF ADJACENT CELL FUSION AND ULTRAVIOLET RADIATION EXPOSURE ON VIRAL PLAQUE FORMATION WITH HERPES SIMPLEX VIRUS TYPE I

Rebecca E. Conner  December, 1986  37 pages

Directed by: Dr. Thomas P. Coochill, Dr. Larry P. Elliott, and Dr. Frank R. Toman

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In mammalian cell-virus systems, it has been observed that damage caused by exposure of the cell to ultraviolet radiation (UV) will result in an increase in viral plaque development rate. This phenomenon is termed the Large Plaque Effect (LPE). Apparently, viral plaque development increases at a faster rate for Herpes Simplex Virus (HSV) when it is assayed on certain UV-irradiated mammalian cells. The consequence of this increase in plaque development rate is that viral plaques appear larger on irradiated monolayers of cells when compared to plaques that developed on unirradiated cellular monolayers.

The cause of the LPE is not yet understood. It is thought that the enhancement of plaque development, due to UV damage, is a manifestation of the excision repair mechanisms operating on the cellular genome. It is known that agents that act like UV and inhibit DNA synthesis, such as hydroxyurea, caffeine, and the carcinogen N-acetoxy-2-acetylaminofluorene, can produce the LPE. Conversely, cyclohexamide, which inhibits de novo protein synthesis, can completely prevent the LPE caused by UV.
There is also some evidence of a cellular membrane effect involved in generating a non-UV induced LFE as observed in work with dimethyl sulfoxide. In addition, certain syncytial mutants of HSV are known to enhance membrane fusion.
INTRODUCTION

Viral plaque development is faster when unirradiated Herpes Simplex Virus (HSV) is assayed on ultraviolet (UV) irradiated African green monkey kidney cells, a strain of epithelial cells designated CV-1P. This cellular phenomenon is called the Large Plaque Effect (LPE) (Coohill, et al., 1980). It is characterized by the formation of viral plaques that are larger on cells that have been treated with UV when compared to viral plaques on cells that have not been treated with UV. Under the appropriate conditions, the LPE can give rise to a five-fold increase in plaque area (Montes and Taylor, 1986).

Viral plaques are the consequence of infection of cells with virus. The virus enters the cell, takes over some cellular mechanisms, produces viral progeny, produces subsequent cell fusion and, eventually, cell lysis occurs. The viral progeny produced in the initial cell are capable of entering adjacent neighboring cells and beginning a new viral cycle. As each subsequent adjacent cell was infected, the plaque spreads radially outward.

In mammalian cell-virus systems, it has been observed that damage caused by exposure of the cell or the virus or both to UV radiation caused a consistent alteration in
plaque size (Cleaver, 1974; Ross, et al., 1972; Ross, et al., 1971; Coohill, et al., 1980). The mode of detecting this alteration in plaque size was to measure the mean plaque diameter, \( \bar{d} \), at various times after infecting cells with virus. Ross et al. (1971) described a Small Plaque Effect (SPE) with HSV. Here, the virus was pretreated with UV and then inoculated onto cells that had not been pretreated with UV. The resulting viral plaques were smaller than plaques formed by untreated virus (controls). They demonstrated that herpes viruses showed a marked progressive diminution of plaque size with increasing exposure to UV (i.e. the SPE is exposure-dependent).

The standard assay system employed to test for the Large Plaque Effect involved irradiating freshly confluent monolayers, allowing an appropriate delay between cell irradiation and viral inoculation, infecting the monolayers with a low multiplicity of infection (m.o.i.) of HSV, allowing viral plaques to develop, staining the monolayers, and measuring the areas of cell destruction (viral plaques). All of the cells were pretreated with UV at the same time and each cell of a monolayer that contacts an infected cell has been treated identically.

Whereas the LPE appeared to be produced by damage to the cellular genome, the SPE seemed to be a consequence of damage to the viral genome. It was predominantly due to a delay in the intracellular events in the cell initiating each plaque. It is thought that the delay is due to a postponement in the multiplication of virus in the first
cell to be infected in each plaque (Ross, et al., 1972). The delay in viral production in the initial cell infected by the damaged virus is thought to be due to the time required for cellular mechanisms to repair the invading, damaged virions (i.e. radiation-damaged virus DNA) (Ross, et al., 1972; Ross, et al., 1971). The virus genome must be intact in order to take over the cell and cause the cell to produce viral progeny. Once the virus is repaired it then enters the lytic cycle, producing a delayed, small plaque. After the delay, due to events in the initial cell, healthy virus progeny proceeded, at the same rate, to infect subsequent cells and produce a plaque. Therefore, the SPE is due mainly to a delay during the first round of UV-irradiated virus replication that occurs in the initially-infected cell. The control, unirradiated virus, mean plaque diameter increased linearly with time after a delay of several hours that corresponded to the time required for the first round of viral replication. For irradiated virus, the SPE, the delay time was longer but once plaques appeared they increased in size at the same rate as the controls. Thus events occurring in the first cell of the viral plaque caused the SPE (Ross, et al., 1972; Ross, et al., 1971). Ross et al. (1972) deduced that for the SPE, the most important events occurred in the first cell to be infected in a viral plaque and involved the actual repair of the damaged viral genome. This viral repair was carried out by cellular enzymes. Once the virus DNA was repaired the virus could
begin to replicate, enter the lytic cycle, and ultimately cause formation of a plaque. However, once viral repair was completed in the initial cell infected, subsequent rounds of viral replication in adjacent neighboring cells proceeded at a normal rate. Thus after an initial delay, the final slope for viral plaque development rate was the same for irradiated or unirradiated virus.

In contrast the cause of the LPE is not yet understood. Expression of the LPE requires delayed infection suggesting that it may be an inducible phenomenon (Coohill, et al., 1980; Babich, et al., 1981). However, other evidence suggests that it may not be inducible (Montes and Taylor, 1986; Coohill, unpublished results). The LPE has been exhibited by other strains of HSV-microplaque, e.g. HSV-syn and HSV-KOS. Agents that act like UV and inhibit DNA synthesis, such as hydroxyurea, caffeine, and the carcinogen N-acetoxy-2-acetylaminofluorene (acetoxy-AAF), have been used to produce the LPE (Babich, et al., 1981). Caffeine, which may inhibit DNA synthesis (Fogel, et al., 1979), also gives rise to a LPE for cells infected with either HSV (Babich, et al., 1981) or SV-40 (Cleaver, 1974). Cyclohexamide, which inhibits de novo protein synthesis, can completely prevent the LPE cause by UV (Babich, et al., 1981). Cleaver (1974) also reported that dimethyl sulfoxide (DMSO) gives rise to the LPE with SV-40. DMSO acts to perforate the cellular membrane. This occurrence could allow virus to escape the infected cell prior to lysis of the cell.
Therefore, virus would be available earlier to infect adjacent neighboring cells. It suggests an effect on the cell membrane that enhances the spread of HSV from cell to cell. In fact, one of the early results of cell infection with HSV was the rapid (6 hour) fusion of the infected cell with its surrounding neighbor. Indeed, the syncytial mutants of HSV, the KOS, syn, and macroplaque strains, are known to extensively enhance cell fusion (Person, et al., 1976). Even HSV-wild type increased cell fusion approximately 20% (Person, personal communication). Further evidence for the involvement of cellular membranes in the spread of HSV from cell to cell comes from the work of Harbour et al. (1978). He reported that in the presence of certain prostaglandins (PGs), E₂ and F₂α, there was an increase in the size of HSV plaques. It is thought that PGE₂ does not significantly enhance the growth of virus by increasing the amount of virus produced per cell, but it may enable the virus to spread from cell to cell more efficiently. PGF₂α also increases, to some extent, viral yield.

Coppey and Nocentini (1976) have reported that CV-1 cells, pretreated with low exposures of UV, produced HSV earlier and at a faster rate than cells that were not pretreated with UV, if a 48 hour delay was allowed between cell exposure and viral inoculation. A 48 hour delay is also the optimum delay between cell treatment with UV and viral inoculation for the LFE phenomenon. The delay also has been demonstrated for SV-40 infected BSC-1
cells by Cornelis et al. (1980). The 45 hour delay is similar to the time course of the excision repair process that occurs in UV-irradiated mammalian cells. It is not known if the earlier synthesis of virus in UV-damaged cells contributes to the LPE; however, more rapid infection of surrounding neighboring cells is a possibility.

In analogy to Ross' study of the SPE (Ross, et al., 1972: Ross, et al., 1971), we employed a similar study to investigate the LPE. Our purpose was to determine the following: (1) if the intracellular events in the initially-infected cell of a plaque played the major role in eliciting the LPE, as with the SPE, (2) if the events in the subsequently-infected neighboring cells were the primary cause of the effect, and/or (3) if the LPE was a phenomenon resulting from an acceleration in the intracellular events in both the initially-infected cell and subsequently-infected cells of the viral plaque.

Babich et al. (1981) concluded that a possible cause of the LPE might be a burst of DNA synthesis that occurred after excision repair had taken place resulting in increased viral DNA synthesis and virus production in the first cycle of viral infection. We therefore used an infectious center assay system (Ross, et al., 1972; Lytle, et al., 1980) to observe the result of the separation of events which occurred in the initial cell from events that occurred in subsequent surrounding cells, and to observe the role of each cell in the rate of viral plaque development. Thus
the primary infected cells may form contacts with cells that have been subjected to different exposures of UV for different periods of delay between irradiation and inoculation.

The separated purposes of this research was to investigate the kinetics of viral plaque development with HSV. Studies included the possible effects of UV radiation on cell fusion by HSV, rates of plaque development at various times after infection, and by employing the infectious center assay, the contributions of the initial cell and subsequent infected cells to the enhancement of plaque size.
MATERIALS AND METHODS

Mammalian Cell Culture

All experiments utilized a strain of highly contact-inhibited African green monkey kidney cells, clone CV-1P (Mertz and Berg, 1974). This cell line was a gift from Dr. Paul Berg (Stanford University). Cells were grown and maintained in 1X Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/l of glucose, L-glutamine, and sodium pyruvate (Grand Island Biological Company, GIBCO, Grand Island, N.Y.). The buffer system consisted of sodium bicarbonate, 2.0 g/l, N'-2-ethanesulfonic acid (HEPES), 3.57 g/l, N-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), 3.44 g/l, and morpholinosopropanesulfonic acid (MOPS), 3.14 g/l. The medium was adjusted to a pH of 6.8 with 1N HCl or 1N NaOH as needed. It was filter sterilized immediately after preparation using a 0.22 μm Millipore filter (Millipore Corporation, Bedford, MA.) and stored at 4°C. Before use, 450 ml of the medium were supplemented with 55 ml of 10% fetal bovine serum (GIBCO), 5.0 ml of 200 mM L-glutamine (GIBCO) in 0.85% NaOH, and 8.0 ml of an antibiotic-antimycotic mixture containing penicillin (10,000 units/ml), fungizone (25 μg/ml), and streptomycin (10,000 μg/ml) (GIBCO).

Cell cultures were grown at 37°C in a humid atmosphere of 5% CO₂ and 95% air in either closed plastic
tissue culture flasks (Corning Glass Works Co., Corning, N.Y.) or in 60 mm Falcon plastic petri dishes (Becton, Dickinson, and Co., Oxnard, CA.) (Snipes, et al., 1979). Monolayers of confluent cells were divided one to three every three to four days. For cell detachment, a solution of 0.05% trypsin (GIBCO, 1:250), 0.5 mM disodium ethylene diamine tetraacetate (EDTA) in Dulbecco’s phosphate buffered saline (DPBS) pH 7.2 (Dulbecco and Vogt, 1954) was used. Every nine to ten days, stock cultures of cells were trypsined, counted with a hemacytometer, diluted in growth medium, and transferred to 60 mm petri dishes at a density of approximately 3 x 10^5 cells per dish, to which four ml of growth medium were added.

Preparation of Viral Stocks and Virus Assay

A syncytial mutant of Herpes Simplex type I (Herpes hominis), the macroplaque strain, HSV-MP, (Hoggan and Roizman, 1959) was obtained from C.D. Lytle of the Bureau of Radiological Health, Rockville, Maryland. The virus was inoculated onto confluent monolayers of CV-1P cells, harvested and stored in vials at -70°C for use in later experiments (Coohill, et al., 1977). Mammalian cell viral capacity, a DNA-dependent function, was employed as the assay system (Coohill, 1981). When this assay system is used, an important factor that should be taken into consideration is the state of the cells when they are infected with virus (Lytle and Benane, 1974). Also, mean plaque diameters for different macroplaque stocks may vary by a factor of about
three (Babich, unpublished observation). Therefore, it is important to perform LPE assays on cells plated to dishes from the same flasks at the same time, and to infect those cells from the same aliquoted vial of the same virus stocks to insure static results. Daily variations in the same viral stocks are smaller in magnitude (Babich, et al., 1981).

For this assay, viral stocks were diluted into DPBS and inoculated onto freshly confluent monolayers of CV-1P cells. The inoculum consisted of approximately 50 viral plaque-forming units in one ml per petri dish. This titre corresponded to a multiplicity of infection, m.o.i., of about $5 \times 10^{-4}$. After an adsorption period of 60 minutes at 37°C, the viral inoculum was removed and replaced with DMEM containing 0.25% immune serum globulin (Armour Pharmaceutical Company, Kankakee, IL.), which prevented extracellular viral transfer. Viral plaques, areas of cell destruction formed when virus produced in one cell infected neighboring cells, were allowed to develop for 48 hours. Then the experiment was terminated by removing the growth medium, drying the cells, and staining the resulting monolayers with 1% crystal violet. Viral plaques appeared as faint blue or clear areas against a dark blue cellular background.

**Measurement of Plaque Sizes**

Plaque sizes were measured by projecting an image of the cellular monolayer onto a sheet of linear graph paper at a magnification of 4X (Ross, et al., 1971). HSV forms circular plaques on CV-1P cells (Coohill, et al., 1980), and
the outlines of these plaques were drawn. An accurate measurement of plaque size was obtained by measuring the average plaque diameter (i.e. two perpendicular measurements of plaque diameter were determined by measuring the plaque width across the center of the plaque in mm). From these measurements an average value for plaque diameter, $d$, was calculated. All plaque diameters were tabulated and then processed using a specially constructed data program written in Basic computer language on an Apple II computer. The resulting average plaque diameters with corresponding standard deviation ($\bar{d} +/- SD$) were used throughout this thesis.

**Infectious Center Assay**

To determine the effect of neighboring cell treatment on the LPE, the following assay was used. Cellular monolayers, which had been pretreated with UV or had not been pretreated with UV, containing approximately $10^6$ cells per dish were infected with HSV-MP as before (see Preparation of Viral Stocks and Virus Assay). To insure that most cells would be infected, each dish received an inoculum of $10^6$ viral particles in one ml of DPBS. This viral titre corresponded to an m.o.i. of 1.0. After an adsorption period of 60 minutes, the inoculum was removed and replaced by growth medium, containing 15% FCS, for one hour. Infected cells were then trypsinized, collected in a test tube, counted with a hemacytometer, and diluted to an appropriate inoculum in growth medium. These infected cells, referred to as infectious
centers (IC's), were placed on separate cellular monolayers, neighboring cells (C's), at a density of 50 IC's in one ml of growth medium. These neighboring cells had also received UV or not received UV. Cells were returned to 37°C and incubated for an additional 6 hours to allow for IC attachment. Then, an additional four ml of medium were added for cell growth. Again, plaques were allowed to develop for 48 hours from the initial time of infection of the IC.

**Ultraviolet Irradiation of Cells**

Prior to cell treatment, at room temperature, with ultraviolet (UV) light, the growth medium was removed and the cells were rinsed twice with four ml of DPBS. After rinsing, an overlay of two ml of DPBS was added to each dish to provide sufficient moisture for the cells during irradiation (Coohill, et al., 1977). The source of illumination was one 15 Watt General Electric G15T8 germicidal bulb with a principal output (86%) of its energy at 254 nm (Harm, Biological Effects of Ultraviolet Radiation, 1980). This lamp was mounted inside a wooden box equipped with a shutter such that the distance between the center of the bulb and the cell monolayer surface was 40 cm (Figure 1). Exposure rates were determined using a UV-sensitive photovoltaic cell (Jagger meter; Jagger, 1967). The fluence was measured to 2.3 Wm⁻² sec⁻¹. The period of irradiation varied from 0 sec to 11 sec. A minimum of three dishes were irradiated at each exposure. After UV exposure, the DPBS overlay was removed. The cells were then either immediately inoculated with
Figure 1. Schematic diagram of the radiation chamber used in these experiments. The source of UV radiation is a G.E. G15T8 germicidal bulb with 86% of its output at a wavelength of 254 nm. Cells are placed 40 cm away from the center of the bulb. A shutter mechanism controls the amount of UV given to the cells.
unirradiated virus or they were inoculated at various times ranging up to 84 hours after treatment with UV. If inoculation was delayed, four ml of growth medium were placed on the cells and they were incubated until the appropriate time of incubation.
RESULTS

The Large Plaque Effect Assay

Plaque size distributions for HSV assayed on either irradiated or unirradiated cells are shown in Figure 2. The abscissa describes the average plaque diameter measured in mm. The ordinate represents the percentage of the total number of plaques, n, characteristic of the respected diameter. Clearly, these results demonstrated an enhancement in plaque diameter in cells that received 10 Jm$^{-2}$ of UV, histogram b, when compared to cells that received no UV (controls), histogram a. The average plaque diameter (d) for 180 HSV plaques assayed on unirradiated cells was 1.25 mm, whereas d increased to 2.14 mm for 75 plaques assayed on irradiated cells.

The LPE is dose-dependent and time-dependent. The results shown in Figure 3 demonstrated an increase in plaque size as a function of CV-Ip cell exposure. On the abscissa is the exposure, or fluence, of UV given to the cells. The ordinate describes the average, or mean, plaque diameter for HSV-MP plaques assayed 48 hours post inoculation. The results indicated that the LPE increased as the fluence (or exposure) to the cells increased up to a maximum LPE at a fluence of 10 Jm$^{-2}$ to the cells. Fluences above 10 Jm$^{-2}$ diminished maximum plaque size. A fluence of 10 Jm$^{-2}$ did not
Figure 2. Typical histograms of plaque sizes resulting from inoculation of CV-1P cells with Herpes Simplex Virus-Macroplaque strain (HSV-MP). Unirradiated virus was inoculated onto (a) cellular monolayers that were not pretreated with 10 Jm⁻² of germicidal 254 nm UV, 48 hours before inoculation. Average plaque diameter after a 48 hour incubation period is represented by d. The total number of plaques measured is described as n. The standard deviation (s. dev.) is for the respected average plaque diameters. The abscissa is the average plaque diameter measured in mm. Each numerical value is the average of a range of 50 mm (i.e. 0.5 represents plaques ranging in diameter from 0.25 to 0.74). The ordinate represents the percentage of the total number of plaques measured, n, for the respected histogram.
a) Unirradiated cells
\[ \bar{d} = 1.25 \text{ mm} \]
\[ \text{s. dev.} = \pm 0.43 \text{ mm} \]
\[ n = 180 \]

b) 10 Jm\(^{-2}\) to cells
\[ \bar{d} = 2.14 \text{ mm} \]
\[ \text{s. dev.} = \pm 0.55 \text{ mm} \]
\[ n = 75 \]
Figure 3. The Large Plaque Effect (LPE) as a function of CV-1P cell exposure in Jm⁻² to UV. The abscissa represents the exposure of the cells to 254 nm UV. The ordinate is the mean plaque diameter, in mm, for HSV-1P plaques assayed 48 hours after inoculation. The virus was not treated with UV. Each datum point was generated for a delay time between irradiation and inoculation of 48 hours.
significantly affect CV-1P cell survival (Coppey, personal communication) or the cell's ability to host HSV-MF growth (cell capacity) (Coppey and Nocentini, 1976). Figure 4 describes the LPE as a function of the delay time between irradiation of the cells and viral inoculation. Cells that were pretreated with UV radiation received 10 Jm⁻² of germicidal UV. The abscissa represents the delay time, in hours, between irradiation and inoculation of the cells with HSV-MF. On the ordinate is relative plaque diameter, a ratio of plaque diameters on irradiated cells compared to plaque diameters on unirradiated cells. The results demonstrated that a delay time of at least 12 hours is required for plaque sizes in UV-treated monolayers to equal or exceed the plaque sizes in unirradiated cells (controls). The point of emphasis demonstrated in Figure 4 is that the LPE was nearly at a maximum for a delay of 48 hours, and this enhancement was essentially constant for the longest delay tested—84 hours.

**Plaque Development Rate**

Freshly confluent cell monolayers were either irradiated with 10 Jm⁻² of 254 nm UV, the fluence of maximum LPE response, or not irradiated which served as controls for the experiment. After a delay of 48 hours, optimum delay for the LPE, cells were infected with an m.o.i. of approximately 3 x 10⁻⁴, or 30 plaque-forming units, pfu's, per dish. At various times after infection, replicate dishes (i.e. at least three dishes and at the most eight dishes) were fixed
Figure 4. The LPE as a function of the delay time between cellular irradiation and viral inoculation. Cellular monolayers were either pretreated with 10 Jm$^{-2}$ of germicidal UV at a wavelength of 254 nm at time, t=0 or did not receive UV. The virus received no UV radiation. The abscissa represents the delay time, in hours, between irradiation and inoculation. The ordinate represents relative plaque diameter, the ratio of plaque sizes on irradiated cells compared to plaque sizes on unirradiated cells.
and stained. Mean plaque diameter, $\bar{d}$, was measured as a function of time for irradiated and unirradiated cells. Each datum point plotted represents the mean plaque diameter of at least 50 plaques from at least two replicate dishes.

Figure 5 is a description of the results of this study of plaque development rate for CV-1P cells infected with HSV-MP. As shown, $\bar{d}$ was plotted against time, in hours, after viral infection, for cells that were irradiated with 10 Jm$^{-2}$ of germicidal UV and also for cells that received no UV radiation. The abscissa represents the time, in hours, after cells were infected with virus. The ordinate represents mean plaque diameter, $\bar{d}$, measured in mm. Least square slopes and intercepts were calculated for the linear portions of each line. For cells that received UV, the slope of the line generated from the data was 33% greater than the slope of the line generated from the data of the controls, cells that received no UV. Another important point described by this graph is that the X-intercept with the abscissa was 17% less for irradiated cells when compared to unirradiated cells.

These data demonstrated that viral plaques formed in UV-irradiated cellular monolayers developed at a faster rate than the controls, the definition of the Large Plaque Effect. The decreased X-intercept for irradiated cells corresponded to a decrease of approximately 2.6 hours in the time of the first round of viral replication. The occurrence was unlike the events that occurred in the SPE, that was characterized by a delay in the first round of replication (i.e. an increase
Figure 5. Plaque development rate for CV-1P cells infected with HSV-MP at a multiplicity of infection (m.o.i.) of $3 \times 10^{-4}$ at 30 pfu's per dish. Cells that were irradiated received 10 Jm$^{-2}$ of germicidal UV (254nm). Abscissa, time in hours, after cells were infected with virus. Ordinate, mean plaque diameter, measured in mm. (●) represents cells that have received no UV. (▲) represents cells that received an exposure of 10 Jm$^{-2}$ of UV.
MEAN PLAQUE DIAMETER, mm

HOURS AFTER INFECTION

PLAQUE DEVELOPMENT RATE
in the intercept with the abscissa). This shorter time for the initial round of viral replication may contribute to the enhancement of plaque size in the LPE. The slope of plaque formation for irradiated cells remained greater than the controls, again unlike the SPE. Slope differences implied that events in the subsequently-infected cells also contributed to the increased plaque size in monolayers that have received UV. In fact, when all aspects of Figure 5 are considered, not only events in the initial cell but also events in subsequent adjacent cells of the plaque contributed to the enhancement of viral plaque formation. The enhancement occurred in cells that have been treated with ultraviolet light (i.e. each round of viral replication appeared to contribute equally to the LPE).

Initial Cell and Adjacent Cell Contribution to the LPE

Results of Figure 5, the description of the plaque development rate, indicated that not only the initial cell to be infected in the development of a plaque but also surrounding subsequently infected cells in the plaque may contribute to the LPE. To test this hypothesis, the viral assay system was modified so that the initial cell infected was separated from the surrounding subsequently infected, adjacent cells of the plaque. Therefore, the contribution of the initial cell to the acceleration in plaque development and the effect of adjacent cell exposure to UV in the development of the LPE could be made. Finally, the above hypothesis could be further substantiated.
In the assay system, termed the Infectious Center (IC) Assay, cellular monolayers were treated with 10 Jm\(^{-2}\) of UV light. They were allowed to incubate for 48 hours. Then they were inoculated with HSV-MP at an m.o.i. of 1.0 and treated as described in Materials and Methods. Upon treatment with trypsin, individual cells were taken from one dish and served as single cell infectious centers (ICs). These ICs, or first cells, were utilized to inoculate dishes containing monolayers of cells (C) which served as "new neighbors" to these primary infected cells. Each dish was inoculated with approximately 50 ICs, corresponding to an m.o.i. of 5.0 \(\times\) 10\(^{-4}\). Thus it was possible to change the adjacent cell neighbors of a UV-treated IC or a control IC (an IC that received no UV treatment). The new adjacent cell neighbors may have been pretreated with UV light or not pretreated with UV light. Therefore by altering the irradiation schedule of an IC from the irradiation schedule of its "new neighbors," a determination of the contribution of the events in each cell in the plaque to the LPE could be made.

The histograms in Figure 6 present the results of this assay. The ordinate represents the average plaque diameter, \(\bar{d}\), of at least 74 plaques measured in four separate experiments. The mode of cell exposure to UV radiation is noted on the abscissa. A plus sign (+) denotes cells (C) or ICs that were irradiated. A minus sign (-) denotes unirradiated Cs or ICs. The designation C refers to cells that were not used as ICs,
Figure 6. Histograms of plaque diameters of CV-1P cells treated by means of the infectious center assay. All cells that were irradiated received 10 Jm$^{-2}$ of germicidal UV (254 nm). Abscissa, method of irradiation of cells. (-) denotes cells that received no UV. (+) denotes cells that received UV. Ordinate, mean plaque diameter measured in mm for plaques in cell monolayers. C represents neighboring (adjacent) cells. IC represents infectious centers (primary infected cells). Con represents cellular monolayers that received no UV and were infected with virus without the use of an IC assay. LPE denotes cells that were irradiated and infected with virus after a delay of 48 hours and not subjected to an IC assay. ICs were either irradiated or not irradiated. After a 48 hour delay, they were infected with HSV-MP at a m.o.i. of 1.0. Neighboring cells were either irradiated or not irradiated as noted. After a delay time of approximately 48 hours, after neighboring cell treatment, these monolayers were infected with approximately 50 primary infected cells per petri dish.
MEAN PLAQUE DIAMETER, mm

INFECTION CENTER ASSAY

RADIATION EXPOSURE = 10Jm⁻²
the "new neighbor" cells.

The first histogram describes cells that were not irradiated and were not subjected to an IC assay. They were identical to the controls for previous LPE studies and are designated Con. A second control for the IC assay system was cells that received UV, were not treated as ICs, and were identical to the monolayers used to demonstrate the Large Plaque Effect. These monolayers are designated LPE. The standard LPE measured in this experiment was an increase in plaque diameter of 1.67X (or 67%) over the Cons. A third control in this assay was an infectious center control in which ICs were not irradiated and were inoculated onto adjacent cells that received no UV radiation (C-/IC-). Note the presence of a small LPE, 1.28X over Con due to the use of the assay system alone. The reasons for this slight enhancement of plaque size are not yet understood. However, further study of these histograms demonstrated that the small increase in plaque size did not inhibit the overall effect demonstrated in the assay system.

To test the relative contributions of primary and adjacent cells to the LPE, the following experiments, described in Figure 6, were conducted using the IC assay. First the infectious centers were irradiated, but the subsequent adjacent cells (C-/IC+) were not irradiated. The results of the mode of irradiation is described in the fourth histogram. A LPE of 1.40X was obtained over the controls (Con). In the second instance, the subsequent adjacent cells
received UV radiation, but the infectious centers did not receive UV radiation (C+/IC-). The treatment, as shown by the fifth histogram, yielded an increase in plaque size of 1.89X over the Con. Also, the LPE in this instance, C+/IC-, was greater than the standard LPE. Finally, the infectious center and the adjacent neighboring cells (C+/IC+) were treated with 10 Jm\(^{-2}\) of UV radiation. A large Plaque Effect of 2.07X over the Con occurred. The method of radiation exposure resulted in an even larger LPE than (C+/IC-). Of course, this was also a large enhancement of plaque size over the C-/IC- (28% greater than the Con) and the Con, themselves.

The greatest increment in plaque diameter between the different modes of irradiation occurred in the (C+/IC-), i.e. treatment of the adjacent neighboring cell alone with 10 Jm\(^{-2}\) of UV. There was an increment of 0.49X, or 49%, between (C+/IC-) and irradiation of the first cell only (C-/IC+). Thus the irradiation of the subsequent neighboring cells contributed more to the LPE than did the primary infected cells in the viral plaque. These data further substantiated the findings of the plaque development rate assays, Figure 5, from which it may be concluded that there is a concurrent increase in each subsequent round of viral replication in each cell as the plaque spreads radially outward.

It should also be noted that the average plaque diameters listed in each histogram of Figure 6 correspond to even larger changes in area. Consider the following:

\[
r = \frac{1}{2} d
\]
where $\bar{r}$ is average plaque radius.

$$\bar{A} = \pi \bar{r}^2$$

where $\bar{A}$ is average plaque area.

Since plaques are two-dimensional, a more accurate description of size is average plaque Area, $\bar{A}$, which is a two-dimensional measurement. Changes in plaque area corresponded to an increase in number of cells destroyed in each round of viral replication (i.e. subsequent viral lytic cycles) as the plaque spreads radially outward. The 2.07 fold increase of $(C+/IC+)$ in diameter over the controls (Con) converted to a larger increase in plaque area of $4.31X$ over Con. It also represented an increase in area of $1.53X$ that of the standard LPE, and an increase in area of $2.62X$ over that of the IC control $(C-/IC-)$. 
DISCUSSION

The results shown in Figure 6 involving the Infectious Center Assay demonstrated that, at relatively low exposures, UV radiation damage to the cell enhanced the rate of virus production in the initially-infected cell but more substantially in the subsequently-infected, neighboring cells of the developing viral plaque. The conclusion is in agreement with the results of Figure 5 concerning plaque development rate. The latter results showed a progressive enhancement in viral plaque diameter as the time between cellular irradiation and viral inoculation was increased. In fact, the definition of the Large Plaque Effect (LPE) is that plaque development is faster when untreated Herpes Simplex Virus (HSV) is assayed on UV-irradiated CV-1 cells.

Faster plaque development resulting from UV treatment of cells yielded an observable increase in plaque size over plaque size in non-UV treated cells. As described in Figure 2, the Large Plaque Effect was maximum at a fluence of 10 Jm\(^{-2}\). Fluences above 10 Jm\(^{-2}\) decreased the LPE. A delay between irradiation and inoculation was required to achieve an enhancement in plaque size. That delay had to be at least 12 hours. The LPE was maximum for a delay of 48 hours. It remained at a maximum for a delay as long as 84 hours.

The plaque development rate study, Figure 5, also
axes of the line generated for irradiated cells was less than
picture 5 also showed that the intercept of the horizontal
contrast to the phenomenon of the SPF. The results of
replication (i.e., initial cell injected in the plaque)
controls. In other words, only the first round of viral
increase in plaque diameter that was at the same rate as
viral production for irradiated viruses and was followed by
in the SPF, that was characterized by an initial delay in
with ultraviolet light. These results were in direct contrast
of viral plaque formation in cells that have been treated
adjacent cells of the plaque contributed to the enhancement
events in the initial cell but also events in subsequent
fact, when considering all aspects of picture 5, not only
replication must have contributed equally to the LDF.
Since these results yielded a linear plot, each round of
cell contributed to the plaque as it spread radially outward.
each round of viral replication, each subsequent injected
cellular events which gave rise to the LDF occurred during
must have received UV. It can be interpreted to mean that the
contrasted to the increase in plaque size in monolayers that
impacted those events in the subsequently-infected cells also
unirradiated controls (i.e., cells that received
recovered 10 days of UV radiation was uniformly higher than
unirradiated cells. The slope of the line for cells that
monoclonally higher for irradiated cells than for
indicated that the rate of HSVG plaque development was
that for unirradiated cells. The decrease in the X-intercept for irradiated cells corresponded to a decrease of approximately 2.6 hours in the time of the first round of viral replication. The occurrence was unlike the events that occurred in the SPE, which was characterized by a delay in the first round of replication (i.e. an increase in the intercept with the abscissa) (Ross, et al., 1972). It indicated that a shorter time for the first round of viral replication may have contributed to the enhancement of plaque size in the LPE.

In the mammalian cell viral capacity assay system, virus has to traverse the membranes of the initially infected cell and perhaps subsequent adjacent cells to infect those adjacent cells. Mutant variations of HSV-1 are known to enhance membrane fusion (Person, et al., 1976; Roizman, 1962; Hoggan and Roizman, 1959). In fact, it has recently been observed that HSV-1 wild-type increases membrane fusion at least 20% (Person, personal communication). In a confluent monolayer of mammalian cells infected with a mutant of HSV-1, the entire monolayer of cells may ultimately become one giant multinucleated syncytium. It is important to note that viral progeny may be able to travel through channels created by membrane.

Results of cell fusion kinetics experiments clearly demonstrated that the more rapid spread of HSV particles to adjacent neighboring cells in irradiated monolayers was not due to any change in the fusion kinetics of the infected cells. Whether the cellular monolayers were UV-damaged or
not UV-damaged, infected cells fused with their adjacent neighbors at the same time, 6 hours post infection. Subsequent cell fusion also occurred at the same rate. Therefore, the earlier occurrence of the initiation of plaque formation in cells pretreated with UV, as described in Figure 5, was not caused by events that rely on the fusion responses of adjacent cells (Coohill, personal communication).

Studies using the Infectious Center Assay demonstrated that UV damage to the cells that were adjacent to the initially-infected cell contributed more to the subsequent rate of viral plaque development than UV damage to the initial cell, as depicted in Figure 6. Changes in plaque size due to the varying methods of irradiation of the initial cell (IC) and subsequent neighboring cells (C) were described.

The IC Assay system, in itself, gave rise to a slight enhancement of plaque size. The phenomenon is not understood. One possible cause of this enhancement could be the treatment of the initial cells, or infectious centers, IC with the enzyme trypsin. In order to transfer the ICs onto the monolayers of the subsequent neighbor cells, C, trypsin was used to enzymatically cut the ICs from the petri dish. Another parameter of the IC Assay method that may contribute to the increase in plaque size is the high m.o.i. of \(1.0 \times 10^6\) viral particles per \(10^6\) ICs. Inoculation of such a high concentration of virus per dish may allow a cell to become infected with more than one virus. High inoculation could
cause an increase in the viral progeny produced in this single cell. Plaque formation could begin earlier in cells producing a larger quantity of virus. Thus, an increase in plaque size could occur. A third possibility that could contribute to this slight but unexplained enhancement is the formation of plaques in doublets (i.e. two plaques forming next to each other in the cellular monolayer). Preliminary investigations have indicated that neither trypsinization of the IC, inoculation of the IC with a high m.o.i., nor doublet formation contributed to the slight increase in plaque size of C-/IC-. Further research should be conducted on this particular aspect.

The greatest LPE occurred when both the IC and the adjacent cell, C, were UV-damaged. The observation correlated with the conclusions drawn from the results of Figure 5. Both the initial cell and subsequent adjacent cells of the plaque contributed to the Large Plaque Effect resulting from the UV damage of these cells. The most important observation described by Figure 6 was the increase in plaque diameter from irradiation of the initial cell, C- /IC+, to the irradiation of the subsequent neighboring cells, C+/IC-. Irradiation of this neighboring cell caused a greater increase in viral replication occurring in that cell compared to the slight increase in viral replication in the initial cell. An increase in viral replication as the plaque spreads from cell to cell radially outward was the most important conclusion drawn from these data.
The conclusions drawn from these data on the kinetics of viral plaque development are as follows:

(1) The Large Plaque Effect observed in CV-1P cells infected with HSV-MP is maximum at a fluence of 10 Jm$^{-2}$ and at a delay between irradiation and inoculation of at least 48 hours.

(2) The fusion kinetics of cells infected with HSV-MP were not influenced by UV damage to those cells. Therefore, the LPE was not caused by an enhancement of cell fusion by HSV-MP due to UV treatment of the cell.

(3) Events in the initially-infected cell contribute to the enhancement in plaque size. However, unlike the SPE, the LPE cannot be explained by events occurring only in the initially-infected cell.

(4) The events in neighboring cells that become infected as the viral plaque spreads radially outward determine, for the most part, the increase in plaque size observed in the LPE.

(5) The rate of plaque development observed in the LPE, as substantiated by the IC Assay suggests that each round of viral replication contributed to the increase in the LPE.

In fact, one possible explanation of the LPE is a decrease in the time required for each subsequent round of virus replication. It could be explained by Babich's hypothesis (Babich, et al., 1981) of the UV-induced LPE being caused by a burst of DNA synthesis occurring after excision repair had
taken place. Even though both the IC and C were infected after a 48 hour delay, each subsequently-infected cell of the plaque had to wait for an additional 18 to 24 hours longer for viral progeny to arrive from the previously-lysed cell. The most logical interpretation of the data presented in this thesis is that each round of virus replication is shorter in UV-damaged cells.

The actual cellular events that cause the LPE have yet to be deduced. One obvious hypothesis is the fact that UV-damaged cells, inoculated with virus after an appropriate delay, produce complete viral particles earlier than do irradiated cells (Coppey and Nocentini, 1976). The earlier synthesis of complete viral particles suggested that newly synthesized virus may then be available to infect neighboring cells earlier. The hypothesis is supported by the fact that virally-induced cell fusion begins before complete viral particles are made (i.e. 6 hours after infection). As the viral infection spreads from cell to cell, each newly infected, UV-damaged cell also makes virus earlier. Thus infection of its neighbor cells occurs faster as the plaque develops. The hypothesis is also supported by the conclusions drawn from the IC Assay. Whether it is the major method of propagation of the Large Plaque Effect awaits further studies.
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