Ultraviolet Irradiation of Herpes Simplex Virus: Reactivation Processes and Delay in Virus Multiplication

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SUMMARY

The small-plaque effect induced by exposure of herpes simplex virus to u.v. light was investigated. New results are presented showing that the effect was due to a delay of multiplication of virus in the cells initiating plaques. The delay occurred after entry of virus into the cell and before the replication of virus. The ultimate cause of the delay was probably the formation of thymine dimers, since the small-plaque effect was reversed by photoreactivation in avian cells. Evidence was obtained for co-operative reactivation and host-cell reactivation in infected BHK cells but neither of these phenomena could be related to the small-plaque effect.

INTRODUCTION

We have described (Ross, Wildy & Cameron, 1971) a small-plaque effect resulting from the irradiation of herpes viruses with u.v. light. This effect was predominantly due to a delay in the multiplication of virus in the first cell to be infected in each plaque; it appeared to occur in the early phases of virus multiplication. Since it was likely that the delay represented the period during which damage in the infecting virus genome was repaired, we have investigated different repair mechanisms.

METHODS

Virus. The HFEM strain of herpes simplex virus type 1 was used (Watson et al. 1966). It had been cloned by picking a single plaque and was used after three passes in baby hamster kidney (BHK) cells. Stocks were produced in BHK cells and stored at −70°C (Watson et al. 1966).

Cells. BHK-21 C13 cells (Macpherson & Stoker, 1962) were grown in a modified Eagle’s medium (Vantsis & Wildy, 1962) containing 10% tryptose phosphate broth and 10% calf serum (ETC). Monolayers of BHK cells were prepared by inoculating plastic Petri dishes (50 mm. diameter) with 10^6.5 cells in ETC+0.75% (w/v) CM-cellulose (CMC) and incubating at 37°C in a humidified atmosphere of 5% CO_2 in air for 24 hr. The presence of the CMC ensured even monolayers. Before use, the monolayers were washed twice with ETC. Chick-embryo cell cultures were prepared from 10- to 12-day-old embryos by the method of Paul (1961). Confluent monolayers were prepared from 10- to 12-day-old embryos by the method of Paul (1961). Confluent monolayers were obtained by seeding glass Petri dishes (60 mm. diameter) with 10^6.7 cells in ETC medium and incubating at 37°C for 24 hr. The medium was changed after 12 hr. HeLa cells were grown as monolayers by plating 10^6.5 cells in glass

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Petri dishes with 5 ml. ETC. The cells were incubated at 37° for 48 hr and washed with ETC before use.

Trypsin + versene. A 0.25% solution of trypsin in tris buffer was diluted 1/5 in 0.02% EDTA in buffered saline (Russell, 1962).

Antiserum. Hyperimmune antiserum to RK 13 cells infected with herpes virus was obtained from rabbits after a prolonged period of immunization (Watson et al. 1966).

Virus assay. Virus infectivity was assayed by plaque formation in BHK cells using the suspension method of Russell (1962) with a CMC overlay and incubating at 37° for 72 hr. Monolayers were used in some experiments. Dilutions of virus were added in 0.1 ml. volumes and allowed to adsorb at 37° for 1 hr. The monolayers were then overlaid with 5 ml. ETC + CMC and incubated for 72 hr. In all plaque assays, cell sheets were fixed in 5% formal saline and stained with carbol fuchsin. Three dishes were used for the assay of each virus dilution. When chick cell cultures were used, care was taken to work in subdued light.

Infective centre assay. Infected cells were counted and suitable dilutions plated together with 10^6.3 fresh uninfected cells in 5 ml. ETC + CMC per dish and incubated at 37° for 72 hr. Three dishes were used for each cell dilution.

Ultraviolet irradiation. The u.v. light source was a Chromatolite 30-w low-pressure mercury discharge lamp in quartz (Engelhard Hanovia Ltd, Slough, Buckinghamshire). It was specified to emit 90% of its energy output at 2537A. Actinometry was done by the ferrioxalate method (Hatchard & Parker, 1956). The crude virus suspension in growth medium was usually diluted 1/10 in buffered saline (Dulbecco & Vogt, 1954) and centrifuged at 4000 rev./min. for 10 min. to remove coarse material before exposure to u.v. light. These virus suspensions contained 0.8 mg. protein/ml. as determined by the method of Lowry et al. (1951). Virus suspensions (1 ml.) were irradiated at room temperature (22°) in shallow (0.35 mm.) layers in glass Petri dishes (60 mm. diameter) at a distance of 43 cm. from the quartz tube; this gave an incident dose rate of 1116 ergs/mm.²/min. The dishes were shaken continuously.

Measurement of plaque-size. The sizes of plaques were determined by projecting images of the plaques on squared paper at known magnification and counting the number of mm. squares within the irregular outline of each plaque. The size was expressed as the square root of the area. Fifty plaques were measured for each determination and the median plaque-size was derived from the distribution. The standard deviation of four replicate estimations was ± 5%. This estimate is appropriate only when the distribution of plaque-sizes is symmetrical. For studies of the kinetics of plaque formation, we used a probit analysis which considers the total number of plaques appearing (Lindenmann & Gifford, 1963).

Single-step growth experiment. BHK cells were infected in suspension by mixing 10^6.4 cells with unirradiated or irradiated virus at an added multiplicity of 5 active p.f.u./cell. The irradiated virus showed 0.18% survival. After continuous agitation at 37° for 1 hr, the cells were washed twice, resuspended in ETC and plated for assay of infective centres. At the same time, samples of 10^5.0 cells in 1 ml. volumes were seeded on dishes made by cementing 18 mm. diameter stainless steel collars on coverglasses. These dishes were incubated at 37° and removed at intervals for disintegration of cells, using an ultrasonic bath, and assay of intracellular infectivity.

Effect of multiplicity of infection on infective centre formation. Samples of 10^5.0 BHK cells were distributed in small screw-capped vials. These were centrifuged at 1000 rev./min. for 15 min. The supernatant fluids were removed carefully with finely-drawn pipettes and the cells infected by adding 0.1 ml. of virus diluted in buffered saline containing 10% ETC. Half-log dilutions of unirradiated and irradiated (8% survival) virus were used. The virus +
cell mixtures were incubated at 37° with occasional shaking for 90 min., when 0.9 ml. trypsin + versene was added and incubation continued for 10 min. Cells were then diluted 100- and 1000-fold into ETC and plated for infective centres. The proportions of cells forming infective centres were 47% and 63% for unirradiated and irradiated virus, respectively. Simultaneous control tests confirmed that the trypsin + versene treatment reduced the infectivity of unadsorbed virus to less than 0.1%.

**Photoreactivation experiments.** Confluent monolayers of chick-embryo cells in glass Petri dishes (60 mm. diameter) were infected with 0.5 ml. volumes of virus suspensions irradiated with different u.v. doses. After adsorption at 4° for 1 hr, with occasional shaking, 5 ml. warm ETC + CMC medium was added and the dishes incubated at 37° for 2 hr. The under surfaces of half of the dishes were shielded with aluminium foil and were illuminated uniformly at a distance of 30 cm. by six white light ‘Osram’ electric bulbs (100 w) at room temperature (22°). A fan blew air between the lamps and dishes and limited temperature rise to less than 0.5°.

**Inhibitor experiments.** Acriflavine (Hopkin & Williams, Ltd, Essex) was kept as a stock solution of 1 mg./ml. in distilled water and pure caffeine (May & Baker, Ltd, Dagenham, Essex) as a stock solution of 20 mg./ml. in saline after Seitz filtration. Monolayer assays were used because these tolerated higher concentrations of acriflavine or caffeine than the suspension assay. The concentration of inhibitor was maintained throughout the incubation period.

**Production of infective virus in the presence of bromodeoxyuridine (BUDR).** A suspension of 10⁸ BHK cells was deposited, washed in buffered saline and resuspended in Eagle's medium + 1% calf serum (EC medium). Virus was added at a multiplicity of 5 p.f.u./cell and the mixture stirred for 1 hr at 37°. The cells were sedimented by centrifuging, washed in EC medium and samples of 10⁸ cells transferred to 20 oz medical flat bottles, together with 40 ml. EC medium + 1 μM BUDR (Koch–Light Laboratories). After 24 hr at 32°, the cells were scraped off, washed 3 times in ETC, pooled and disrupted by ultrasonic treatment in ETC. Released virus was stored at −70°. The virus yield was 0.8 p.f.u./cell and the particle to infectivity ratio 3000:1.

Incorporation of BUDR in virus particles was verified by infecting BHK cells as described above and incubating at 37° in EC medium +2 μM-[³H] BUDR (400 mc/m-mole). The radioactivity of the medium was 1 μC/ml. The virus obtained was purified by differential centrifugation, treated with DNase + RNase (Koch–Light Laboratories: 50 μg./ml. of each overnight at 4°) and centrifuged to equilibrium (40 hr) in CsCl + 0.1% bovine serum albumin (Armour Pharmaceuticals Ltd) at 35,000 rev./min. in a SW20 rotor of the Omega II Christ ultracentrifuge. Control virus was treated in parallel. Both the radioactive and the control preparations gave clear peaks of infectivity with a poor recovery of 4%. With the BUDR virus, the peak of radioactivity (recovery 80%) coincided with that of infectivity and virus from this peak was twice as sensitive to u.v. light as control virus when assayed in BHK cells.

**‘Holding’ experiments.** Virus was assayed on BHK monolayers with or without 0.2 μg. acriflavine/ml. The dishes were kept in a humidified atmosphere of 5% CO₂ in air in sealed plastic boxes and either incubated at 37° for 72 hr, or held at room temperature (22°) in the dark for 48 hr, before transfer to 37° for further incubation for 72 hr.
RESULTS

The kinetics of plaque formation

We have shown (Ross et al. 1971) that the small-plaque effect is related to delay in virus multiplication in the cells initiating plaques. We now present evidence that the previous estimates of median plaque-size provide only an approximate measure of this delay. A number of Petri dishes was set up as for infectivity assays using unirradiated and irradiated (1.3% survival) virus. At intervals of 24 hr, three dishes from each dilution were removed from the incubator and the infectivities assayed. The results (Fig. 1a) indicate that constant numbers of plaques were scored after 3 days for the unirradiated virus and after 5 days for the irradiated virus. Measurements of plaque-size were made using the same dishes. These estimates of the median size of recognized plaques suggest that the plaques developed at an approximately constant rate for up to 5 days (Fig. 1b; points), with the plaques of irradiated virus increasing in size more slowly. This result is determined by the large proportion of the developing plaques which were too small to be detected during the first 3 days. Correction was made for this by using a more refined estimate of median plaque-size (Lindenmann...
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Fig. 2. Escape of infective virus from the neutralizing effect of antiserum added at different times after mixing irradiated (0.03 % survival) or unirradiated virus with BHK cells in suspension. ●, unirradiated virus; ○, irradiated virus.

& Gifford, 1963); the plaque-sizes for irradiated and unirradiated virus, after different latent periods, increased at constant and equal rate up to the 5th day. Moreover, there was high correlation between the sizes of irradiated and unirradiated virus plaques at all equivalent time points ($r = +0.98, P < 0.01$). The previous interpretation that the small-plaque effect was caused by a delay in the initiation of the plaques is therefore upheld. Although it is clear that the earlier estimation of median plaque-size was less satisfactory when size distributions were asymmetrical, the method was so much less cumbersome than the refined method that we have used it in all other experiments.

The stage at which delay occurs in the multiplication of u.v. irradiated virus

Effect of irradiation on the entry of herpes virus into the cell

Since preliminary experiments designed to measure the rate of virus attachment to cells showed no demonstrable difference between irradiated and unirradiated virus (Ross, 1969), we determined the rates at which infective virus, added to cells, became resistant to the action of neutralizing antibody. Samples of $10^7$ cells were shaken at 37° in 4 ml. volumes containing about 400 p.f.u. of irradiated (0.03 % survival) and unirradiated virus. At intervals, one sample from each series was diluted with 16 ml. ETC supplemented with 10% pooled human antiserum and 1/100 hyperimmune serum and maintained at 37° until 2 hr after the experiment had begun. Each sample was then plated in 4 Petri dishes and incubated for 4 days. The rate of uptake of irradiated or unirradiated virus was the same (Fig. 2) and, further, the sizes of the plaques were not influenced by the time at which antibody was added (results not shown). We conclude that the delay occurred at a stage after virus had entered the cell.

Kinetics of growth of irradiated herpes virus

Single-step, high-multiplicity growth experiments were done by infecting $10^6$ BHK cells with unirradiated and irradiated virus (0.18 % survival) using an added multiplicity of 5 active p.f.u./cell. The results (Fig. 3a) amply confirmed the delay in virus production. The
delay in the eclipse of virus infectivity is unexplained but the large number of u.v. inactivated particles may have undergone co-operative reactivation in the assay system. However, the slow increase in infective virus after 14 hr was almost certainly due to newly-made progeny originating in a proportion of the infected cells. This conclusion is reinforced by the increase in plaque-size observed in assays at this time. Until 14 hr the plaques were as small as in assays of the inoculum, but from 14 hr onwards they were of nearly normal size (Fig. 3b).

The results indicate that in multiply-infected cells, the growth of irradiated virus was delayed at some time between entry into the cell and formation of new infective virus. Also, the formation of plaques of normal size by progeny virus supports the earlier finding that the small-plaque effect is not heritable (Ross et al. 1971).

**Effect of multiplicity of infection on plaque-size**

Early experiments (Ross, 1969) showed that the numbers of plaques obtained in assays of irradiated herpes virus were not always proportional to virus dilution. This suggested that co-operative reactivation occurred and influenced the delay of virus multiplication. It was therefore desirable to observe single-cycle growth under conditions precluding co-operation between particles. Although this was technically difficult, it was possible to study the relationship between delay and co-operative reactivation using plaque-size as an index of delay.

In the first experiment, 0.2 log dilutions of virus were assayed in suspension after different doses of irradiation. After incubation for 3 days, the resulting plaques were counted and measured. The log (p.f.u./dish) showed straight line relationships with log concentration (Fig. 4a). Deviations were found where plaques were too crowded on the dishes. The slopes of the straight portions of the curves were calculated. Unirradiated virus, and virus irradiated with a dose of 1116 ergs/mm.², gave slopes of unity. Only with larger u.v. doses was there a progressive increase in slope, indicating co-operative reactivation. The plaque-sizes diminished markedly with small u.v. doses (Fig. 4b), reaching less than half the control value at 1116 ergs/mm.² (Fig. 4c). Thus, the delay was independent of co-operative action.
In the second experiment (see Methods), BHK cells were infected with unirradiated or irradiated virus (8% survival) at multiplicities from 0.02 to 20 p.f.u./cell and were assayed as infective centres (Fig. 5). The multiplicity values shown in the figure were calculated from the plaque counts obtained at the highest dilutions when the multiplicity of infection was assumed to be low. The measured plaque-sizes were unrelated to the multiplicity of infection.

We conclude that co-operative reactivation occurred but that it did not affect plaque-size (or delay).

Reversal of the small-plaque effect by photoreactivation

Photoreactivation of pseudorabies and herpes simplex viruses was reported by Pfefferkorn, Rutstein & Burge (1965), Pfefferkorn, Burge & Coady (1966) and Pfefferkorn & Coady (1968). Using their protocol (see Methods), we found that the effect was demonstrable in avian cells but not in BHK cells (Fig. 6a). The small-plaque effect was clearly demonstrable in chick-embryo cell monolayers and after illumination with white light the effect was reversed; plaques were increased in both size and number (Fig. 6b). We conclude that delay in the growth of irradiated virus was overcome by photoreactivation. It follows that the lesions causing the delay were probably in the nucleic acid of the virus.
Host-cell reactivation and the small-plaque effect

We have attempted to determine by a number of indirect tests whether the small-plaque effect is concerned with host-cell reactivation. Taken together, these indicate that u.v. irradiated herpes virus can be reactivated but there is no evidence that procedures which modify the survival of virus infectivity influence plaque-size.

Comparative assays in different types of cell

Lytle (1971) used parallel assays in different host cells to demonstrate host-cell reactivation of herpes virus. We assayed irradiated and unirradiated virus in BHK cells and chick-embryo cells (Fig. 6) and in BHK cells and HeLa cells (Fig. 7) and found survivals of infectivity which differed in different hosts. The results suggest that repair is more efficient in HeLa and chick-embryo cells than in BHK cells.

Effect of acriflavine and caffeine

These compounds are known to inhibit host-cell reactivation (Cleaver, 1969) and have been used with pseudorabies virus (Závadová & Závada, 1968). We examined the effects of these inhibitors on plaque formation in monolayer cultures of BHK cells and found that both drugs reduced the survival of virus infectivity (Table 1). However, these inhibitors did not influence plaque-size.
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Fig. 6. Photoreactivation of irradiated virus assayed in chick-embryo cells: the effect on (a) survival of infectivity and (b) plaque-size. (a) O—O, chick-embryo cells exposed to white light; •—•, chick-embryo cells shielded from white light; △—△, BHK cells exposed to white light; ▲—▲, BHK cells shielded from white light.

(b) O—O, chick-embryo cells exposed to white light (virus irradiated at 11,160 ergs/mm.²); •—•, chick-embryo cells shielded from white light (virus irradiated at 11,160 ergs/mm.²); ▲—▲, chick-embryo cells infected with unirradiated virus.

Fig. 7. Comparison of survival of virus infectivity in BHK cells (O—O) and HeLa cells (●—●).

Effect of incorporating BUdR in the virus

Sauerbier (1961) and Szybalski (1961) showed that the incorporation of BUdR into DNA increased its sensitivity to u. v. irradiation. There is evidence that part of this may be due to inhibition of host-cell reactivation. Schneweis (1969) found that the u. v. sensitivity of herpes virus increased when BUdR was incorporated in DNA. With virus prepared in the presence of BUdR (see Methods), u. v. irradiation gave decreased survivals of infectivity compared with normal virus (Fig. 8). Moreover, when assayed in the presence of 0.2 µg acriflavine/ml, the survival of infectivity was not decreased further and was comparable with that obtained
Table 1. Effect of caffeine or acriflavine on the survival of infectivity after irradiation with u.v. light

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>u.v. dose, ergs/mm²</th>
<th>% survival of infectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>558</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
<td>11.2</td>
</tr>
<tr>
<td>Caffeine, 1.0 mg./ml.</td>
<td>100</td>
<td>3.5</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
<td>ND</td>
</tr>
<tr>
<td>Acriflavine 0.2 µg./ml.</td>
<td>100</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND = Not done.

Fig. 8. Effect of u.v. light on survival of virus infectivity with and without incorporated BUdR, assayed in the absence or presence of 0.2 µg. acriflavine/ml. ●—●, normal virus without acriflavine; ○—○, normal virus with acriflavine; △—△, BUdR virus without acriflavine; ▲—▲, BUdR virus with acriflavine.

when normal virus was assayed in the presence of acriflavine. Although plaque-sizes were not measured, no obvious differences were noted for any particular dose of irradiation. We conclude that the incorporated BUdR inhibited host-cell reactivation since acriflavine had no further effect on survival of infectivity.

'Holding' experiments

When irradiated bacteria are plated and 'held' under conditions precluding growth ('liquid holding'), their survival is apparently increased (Harm, 1966). In a similar experiment we 'held' infected BHK cells for 2 days at 22°, a temperature at which virus multiplication was negligible. The results (Fig. 9) show a marked increase in the survival of infectivity. It is
clear that both 'holding' and acriflavine affected survival of infectivity whether applied separately or together. Neither acriflavine nor 'holding' influenced the proportional diminution of plaque-size by u.v. light.

**DISCUSSION**

In a previous paper (Ross et al. 1971), we reported that irradiation of three herpes viruses resulted in the formation of small plaques. The effect was non-heritable and reflected a delay of the events in the first cell to be infected in each developing plaque. Further, under conditions in which single separated plaques were formed, immunofluorescence studies showed that the delay occurred before the formation of detectable virus-specific antigen. It was thought that the primary cause of the delay was a u.v. induced lesion in the DNA of the virus since treatments such as heat, which would damage protein, failed to cause the small-plaque effect (Ross, 1969). We show here that the delay also occurred when cells were multiply-infected (Fig. 3) and occurred after the infecting virus had entered the cell (Fig. 2). The delay was independent of multiplicity of infection even when the infectivity assays showed that co-operative effects were occurring (Fig. 4, 5). It follows therefore that delay must have taken place before phenotypic expression, which is necessary for complementation, and before DNA replication, which is necessary for multiplicity reactivation.

Even though our u.v. source emitted a mixture of wavelengths, it is likely that the predominant damage to the virus DNA was dimerization of adjacent thymine units by the 254 nm. band (Setlow, 1961). We have strong, indirect evidence that these thymine dimers were the primary cause of the delay since the effect was reversed in avian cells by photo-
reactivation, as shown by measurement of plaque-sizes (Fig. 6). It is known that the photo-reactivating enzyme of avian cells can split thymine dimers (Pfefferkorn & Coady, 1968).

We conclude that the delay arises as a result of u.v. induced thymine dimers in the virus nucleic acid which prevents virus multiplication. Such damage may have one (or both) of two results. Firstly, it may completely prevent phenotypic expression and DNA replication unless repaired (lethal damage). Secondly, it may impair these functions but not prevent them completely (non-lethal damage). Harm (1965) made this distinction when discussing the behaviour of the delay in the growth of irradiated T T and λ bacteriophages in host-cell-reactivating and non-reactivating bacteria. We are unable at present to establish whether either of these processes can explain our results.

Vertebrate cells are known to possess repair mechanisms (see review by Regan, 1969). These have been demonstrated directly (e.g. isolation of excised thymine dimers, demonstration of repair of single strand breaks in irradiated cells). They have also been demonstrated indirectly (e.g. by use of inhibitors of host-cell reactivation (Arlett, 1969)). In general, indirect methods may be less reliable since they may have unrecognized side effects.

Host-cell reactivation of herpes viruses has been demonstrated indirectly by the use of inhibitors. Caffeine reduced the survival of pseudorabies virus growing in chick cells (Závadová & Závada, 1968). Different rates of inactivation of herpes simplex virus by u.v. irradiation have been demonstrated in different host cells (Lytle, 1971; Rabson, Tyrrell & Legallis, 1969), including \textit{Xeroderma pigmentosum} cells in which repair of u.v. induced DNA damage is undetectable (Cleaver, 1968; Setlow et al. 1969; Regan, Setlow & Ley, 1971). The replacement of thymine by bromouracil in herpes virus DNA rendered it more sensitive to u.v. irradiation (Wacker, Reinhardt & Cramer, 1965; Schneweis, 1969). This effect is probably due, partly at least, to impairment of repair processes (Setlow & Boyce, 1963). We used these and other approaches, sometimes in combination, and conclude that host-cell reactivation of u.v. treated herpes simplex virus occurred under our experimental conditions. If the lesion causing the delay in virus multiplication were ‘lethal’, then the sizes of surviving plaques should increase when host-cell reactivation was restrained; thus, in the absence of host-cell reactivation, the surviving plaques must represent undamaged genomes. In fact, we observed no modification of plaque-size under conditions that altered plaque survival. Although this result accords with the notion that delay is due to ‘non-lethal’ damage, it does not constitute positive evidence for it. More information is needed to elucidate precisely the cause of the delay.

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