Production of Tubular Structures in Vero Cells Infected with Herpes Simplex Virus Type 2: Effects of Ultraviolet Light Irradiation and Antimetabolites

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SUMMARY

In order to investigate the nature of tubular structures specifically found in herpes simplex virus type 2 (HSV-2)-infected cells, the multiplication of HSV-2 was studied in Vero cells cultured in the presence of varying concentrations of cytosine arabinoside (Ara-C) and cycloheximide (CH), inhibitors of DNA synthesis and protein synthesis respectively. Ara-C, at a concentration of 60 μg/ml, inhibited the multiplication of HSV-2 by more than 99% and also prevented the appearance of tubular structures and virus particles in the nuclei of infected cells. Nevertheless, the synthesis of virus specific surface antigens of HSV-2-infected Vero cells was not reduced, as revealed by the fluorescent antibody technique. On the other hand, 10 μg/ml of CH inhibited both the appearance of tubular structures and virus particles and the synthesis of virus specific surface antigens by more than 99%. These observations strongly suggest that the appearance of tubular structures is one of the late events in the process of virus multiplication.

To measure the comparative genome size needed to produce membrane antigens, tubular structures and infectious centres, the effect of u.v.-inactivation of HSV-2 on these processes was studied. After u.v.-irradiation, the capacity to induce tubular structures was inactivated at a slower rate than the capacity to form infectious centres, but at a faster rate than the induction of surface antigens. Furthermore, more tubular structures could be induced by u.v.-inactivated virus than by the non-irradiated virus which was diluted to the same infectivity as the u.v.-irradiated virus. These results indicate that expression of the entire genome is not required for the production of tubular structures.

INTRODUCTION

Tubular structures or filamentous structures found in herpes simplex virus (HSV)-infected cells were first reported by Murphy et al. (1967) in mouse brain infected with HSV and many reports have been made since then (Couch & Nahmias, 1969; Schwartz & Roizman, 1969; Aurelian et al. 1971; Nii & Katsume, 1971; Mori et al. 1973; Young et al. 1977). All of these reports have presented observations that tubular structures are specifically observed in herpes simplex virus type 2 (HSV-2)-infected cells, but not in herpes simplex virus type 1 (HSV-1)-infected cells. Therefore, tubular structures are considered to be one of the morphological markers that can differentiate HSV-2 from HSV-1, but their nature is not known.
We previously reported that the tubular structures appeared as early as 6 h p.i. and their production reached a maximum after 12 h, while the infectivity reached a maximum at 24 h p.i. (Oda & Mori, 1976). This observation suggested the possibility that the tubular structures are materials that are either related to virus structural subunits or are structures required for the maturation of the virions.

The results of previous studies did not allow us to decide whether the appearance of tubular structures is a late event or whether expression of the whole genome of HSV-2 is required for the production of tubular structures. To delineate these points, we examine in this paper the effects of antimetabolites affecting different steps in the virus replication cycle, and the effects of u.v. light inactivation on the synthesis of infectious virus, the appearance of virus-specific antigens and the appearance of tubular structures.

**METHODS**

**Cells.** Monolayer cultures of Vero cells were grown in Eagle’s minimal essential medium (MEM) supplemented with 10% calf serum.

**Virus.** The YS-4 strain of HSV-2 was used. The isolation and properties of this strain have been described (Tasaki et al. 1975). A virus stock was obtained by growing the virus in a monolayer of Vero cells in a roller bottle using Eagle’s MEM supplemented with 2% calf serum. The virus was harvested by sonication of the infectious culture fluid after one freeze-thaw cycle when maximum cytopathic effect was observed. Centrifugation at 3000 rev/min for 15 min was used to remove cell debris. Samples were stored at -70°C.

**Antimetabolites.** The following drugs were used at concentrations listed below. Cytosine arabinoside (Ara-C; Sigma Chemical Co., Missouri, U.S.A.): 0.3, 1.0, 3.0, 10, 30 and 60 µg/ml. Cycloheximide (CH; Wako Pure Chemical Industries, Osaka, Japan): 0.2, 0.5, 1.0 and 10 µg/ml.

**Treatment with antimetabolites.** Monolayer cultures were infected with HSV at a multiplicity of 7 p.f.u./cell. The inoculum was left at 37°C for 1 h to allow virus adsorption. Unadsorbed virus was removed by three cycles of washing with MEM. MEM supplemented with 2% calf serum with or without above-mentioned drugs was then added as maintenance medium.

**Ultraviolet irradiation.** The virus suspension (2.0 ml) in 60 mm Petri dishes was exposed to u.v. light (Toshiba germicidal lamp, GL 15) at a distance of 57 cm (10 erg/mm²/s) for varying times. Irradiated as well as non-irradiated preparations were assayed for their capacity to produce virus-specific surface antigens, tubular structures and infectious centres in Vero cells. A representative preparation of HSV-2 had a titre of 8.5 x 10⁷ p.f.u./ml before u.v.-irradiation and the infectivities after varying doses of u.v.-irradiation were 8.1 x 10⁶ p.f.u./ml (600 erg/mm²), 3.0 x 10⁶ p.f.u./ml (1200 erg/mm²), 1.2 x 10⁶ p.f.u./ml (2400 erg/mm²) and 3.2 x 10⁵ p.f.u./ml (4800 erg/mm²).

**Determination and quantification of virus-specific surface antigens.** For the detection of HSV-2 specific surface antigens, immunofluorescent staining was used. Infected or non-infected monolayers were scrapped off and the cells were centrifuged at 1000 rev/min for 5 min, washed with PBS and stained with the indirect fluorescent antibody technique. Hyperimmune serum against HSV-2 was prepared in a rabbit. A white rabbit was injected by the intracutaneous route with an HSV-2 strain, YS-4, previously passaged in rabbit kidney cell (RK-13) cultures. Serum was collected 7 days after the last injection and was inactivated at 56°C for 30 min. The appearance of virus-specific surface antigens was quantified as the percentage of the fluorescent-positive cells. At least 200 cells were counted.

**Preparation of cells for electron microscopy.** The cells were scraped from the glass at the
time indicated, pelleted by centrifugation, fixed in 2% glutaraldehyde for 30 min, thoroughly washed and post-fixed in 1% osmium tetroxide for 60 min. Following dehydration in graded dilutions of ethanol, the cells were embedded in epoxy resin (Epon 812). Thin sections were stained with uranyl acetate and lead citrate, and examined with JEM 100C or JEM T8 electron microscope.

Titration of infectivity. Infected monolayers of Vero cells were frozen at the times indicated and kept at −70 °C until used for titrations. After three cycles of freezing and thawing, they were centrifuged to remove the large cellular debris, and the supernatants were used for the infectivity titrations. Monolayer cultures of Vero cells were grown in 30 mm plastic Petri dishes (Falcon Plastics, no. 3001) and infected with 0.1 ml of tenfold serial dilutions of the material. After adsorption at 37 °C for 1 h, the infected monolayers were washed twice with MEM, overlaid with maintenance medium containing 2% methylcellulose and incubated in a humidified atmosphere of 5% CO₂. After 72 h of incubation, 0.025% neutral red solution dissolved in PBS was added. Plaque counts were made 12 h later.

Quantification of tubular structures. Thin sections were examined in a JEM T8 electron microscope at a magnification of ×13,000. Frequencies of tubular structures in the infected nuclei were expressed as the percentage of nuclei containing tubular structures relative to the total number of nuclei examined, i.e.

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\% \text{ nuclei containing tubular structures} = \frac{\text{no. of nuclei containing tubular structures}}{\text{total no. of nuclei examined}} \times 100.
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All of the three available blocks for each test group were examined. Random counting was made in order to avoid bias in the selection of fields, and care was taken so that similar areas of adjacent sections were not examined. Finally, at least 200 nuclei were observed for quantification.

Quantification of virus particles in the nucleus. Thin sections were examined in the same conditions as described above. Quantification of virus particles of all capsid types in the nucleus was expressed as the relative number of virus particles per infected nucleus:

− = 0; 1+ = 1 to 20; 2+ = 21 to 40; 3+ = 41 to 60; 4+ = 61 to 80. At least 100 nuclei were examined for this measurement.

RESULTS

Effects of antimetabolites on the synthesis of infectious virus and the appearance of tubular structures in Vero cells

Cytosine arabinoside (Ara-C)

Figure 1 shows the effects of varying concentrations of Ara-C on the appearance of tubular structures relative to the synthesis of infectious virus. The production of infectious virus was markedly reduced by Ara-C in proportion to its concentration. At a drug concentration of 60 μg/ml, both the synthesis of infectious virus and the appearance of tubular structures were inhibited by more than 99%. At drug concentrations of 0.3 and 1.0 μg/ml, however, the percentage of nuclei containing tubular structures was even higher than that in untreated cultures at 9 h p.i. Moreover, it continued to increase until 24 h p.i., whereas a marked decrease was observed in untreated cultures. Since at drug concentrations of 10 and 30 μg/ml suppression of both multiplication of HSV-2 and production of tubular structures were in proportion to the drug concentrations, curves at these two concentrations were omitted from the figure. Toxicity of Ara-C at the concentration of 60 μg/ml was minimal as estimated by morphology of Vero cells.
Fig. 1. The effects of varying concentrations of Ara-C on (a) the synthesis of infectious virus and (b) the appearance of tubular structures in HSV-2-infected Vero cells. Vero cells were infected with HSV-2 at a multiplicity of 7, and after incubation for varying times, they were harvested from the preparation of thin sections and plaque assays for infectivity. Quantification of tubular structures was indicated as the percentage of the appearance in the nuclei under electron microscope. O—O, HSV-2-infected cells without Ara-C; △—△, HSV-2-infected cells treated with 0·3 µg/ml of Ara-C; ●—●, HSV-2-infected cells treated with 1·0 µg/ml of Ara-C; ▲—▲, HSV-2-infected cells treated with 3·0 µg/ml of Ara-C; ■—■, HSV-2-infected cells treated with 60 µg/ml of Ara-C.

**Cycloheximide (CH)**

The effects of CH are shown in Fig. 2. Both the synthesis of infectious virus and the number of nuclei containing tubular structures were markedly reduced in proportion to the concentration of CH. At a concentration of 1·0 µg/ml, the drug almost completely inhibited both the synthesis of infectious virus and the appearance of tubular structures.

**Effect of Ara-C and CH on the appearance of virus-specific surface antigens, tubular structures and virus capsids in HSV-2-infected cells**

Monolayer cultures of Vero cells were infected with HSV-2 at a m.o.i. of 7 p.f.u./cell. After 1 h adsorption, medium containing either 60 µg/ml Ara-C or 10 µg/ml CH or medium without drugs was added. At different times p.i., the infectivity of the virus was measured by plaque assay; the appearance of virus-specific surface antigens was detected by immunofluorescent antibody and the tubular structures and all species of virus capsids in the nuclei were examined under the electron microscope.
As shown in Fig. 3, the appearance of tubular structures and virus capsids in the nucleus was almost completely inhibited in the presence of 60 μg/ml Ara-C, whereas virus-specific surface antigens were detected in about 60% of the infected cells at 9 h p.i. and 80% at 12 h p.i. On the other hand, 10 μg/ml of CH almost completely blocked both the appearance of the tubular structures and virus capsids in the nucleus and the induction of virus-specific surface antigens.

Effects of u.v. irradiation of HSV-2 on the capacity to produce surface antigens, tubular structures and infectious centres

To investigate the comparative genome size required for the production of surface antigens, tubular structures and infectious centres, the effect of u.v.-inactivation of HSV-2 on these processes was studied.

Figure 4 shows the u.v.-sensitivities of HSV-2 to induce surface antigens or tubular structures as measured at 9 h p.i. when production reached a maximum in the cells infected with non-irradiated virus. The u.v.-sensitivity of HSV-2 to form infectious centres is also shown in Fig. 4. Infectivity was found to be very sensitive to u.v. irradiation. The capacity of HSV-2 to induce the production of tubular structures was inactivated at a slower rate than infectivity, but at a faster rate than the capacity to induce surface antigens.
Fig. 3. Time course of (a) virus multiplication, (b) appearance of virus specific surface antigens, (c) tubular structures and (d) virus capsids in HSV-2-infected Vero cells. Monolayer cultures of Vero cells were infected with HSV-2 at a multiplicity of 7. After 1 h adsorption, media containing 60 μg/ml of Ara-C or 10 μg/ml of CH or without drugs was added to Vero cell cultures. At different times after infection, the infectivity, the appearance of tubular structures and virus capsids in the nucleus and the appearance of virus specific surface antigens in infected cells were quantified. Quantification was as described in the Methods. ○—○, HSV-2-infected cells without drug; △—△, HSV-2-infected cells with Ara-C; ●—●, HSV-2-infected cells with CH. The relative number of virus particles per infected nucleus was established as follows: − = 0; 1+ = 1 to 20; 2+ = 21 to 40; 3+ = 41 to 60; 4+ = 61 to 80.

Figure 5 shows the time course of the production of infectious virus and the appearance of tubular structures in Vero cells infected with either non-irradiated or u.v.-irradiated HSV-2. Monolayer Vero cells were infected with non-irradiated HSV-2 at a m.o.i. of 4 p.f.u./cell or with the same vol. of HSV-2 after u.v.-irradiation. The percentage of the appearance of tubular structures in the nuclei 9 h p.i. with the non-irradiated and u.v.-irradiated (2400 erg/mm²) virus was 48 and 11 %, respectively. The latter value is high compared to the actual m.o.i. (m.o.i. = 0.05 p.f.u./cell) and is even higher at 12 h p.i. Similar results were obtained when the virus was irradiated with a dose of 4800 erg/mm² (actual m.o.i. = 0.01 p.f.u./cell). These results again suggest that the virus genome required for the induction of tubular structures is much smaller than a whole virus genome. Fig. 6 shows the accumulated tubular structures in the nuclei of Vero cells 12 h p.i. with u.v.-irradiated (2400 erg/mm²) HSV-2.
Fig. 4. U.v.-inactivation curves of HSV-2. Residual infectivity (○—○) was measured by plaque assay as described in the Methods. After infection of Vero cells with non-irradiated or u.v.-irradiated HSV-2 at a multiplicity of 4 (prior to irradiation), the capacity to induce tubular structures in the nucleus (△—△) and virus specific surface antigens (●—●) were measured at 9 h p.i., when production of these reached a maximum in the cells infected with non-irradiated virus. The u.v.-inactivation rates were calculated as the percentage of activity relative to that of non-irradiated virus.

In these experiments, however, we should consider the possibility that the second cycle of infection with progeny virus might take place within 12 h p.i., because it takes about 5 h for virus particles to be recognized under the electron microscope and about 6 h to recognize tubular structures. If this is the case, the phenomenon we have observed might reflect a mixture of the first and the second cycles of infection. Therefore, in order to clarify this point, further experiments were performed, in which we compared the ability of u.v.-irradiated virus to induce tubular structures and infectious centres with that of non-irradiated virus diluted to the same infectivity titres as u.v.-irradiated virus. As clearly shown in Fig. 5, u.v.-irradiated virus has a much higher ability to induce tubular structures than non-irradiated virus with comparable infectivity. These results also strongly suggest that expression of only a part of the genome of HSV-2 can induce tubular structures in infected Vero cells.

DISCUSSION

Results obtained from our experiments are summarized as follows:

1. Considering the evidence of inhibition by Ara-C and CH, the appearance of tubular structures seemed to be one of the late events of virus replication.

2. The u.v.-irradiation studies showed that only a part of the total genome of HSV-2 was involved in the formation of tubular structures.
Fig. 5. Time course of the synthesis of (a) infectious virus and the appearance of (b) tubular structures in the nucleus of Vero cell. The cells were infected with non-irradiated or two kinds of u.v.-irradiated HSV-2 (u.v.-dose 2400 and 4800 erg/mm², respectively) at a multiplicity of 4 (prior to irradiation). At the same time, monolayer cells were infected with two groups of non-irradiated virus diluted to the same infectivity titre as the u.v.-irradiated virus. After varying incubation times, cells were harvested for the quantification of infectivity and the appearance of tubular structures in the nucleus. ○—○, Vero cells infected with non-irradiated HSV-2; △—△, Vero cells infected with HSV-2 irradiated with a u.v. dose of 2400 erg/mm²; •—•, Vero cells infected with HSV-2 irradiated with a u.v. dose of 4800 erg/mm²; ○—○, Vero cells infected with non-irradiated HSV-2 diluted to the same infectivity titre as HSV-2 irradiated with a 2400 erg/mm²; ○—○, Vero cells infected with non-irradiated HSV-2 diluted to the same infectivity titre as HSV-2 irradiated with a u.v. dose of 4800 erg/mm².

(3) Accumulation of tubular structures in the infected nuclei was observed when infected cells were treated with Ara-C at appropriate concentrations and when cells were infected with u.v.-irradiated virus.

Inhibitors of DNA synthesis such as Ara-C (Buthala, 1964; Falke et al. 1972; Ito & Barron, 1972; Nutter & Rapp, 1973), prevent the synthesis of late virus protein, and inhibitors of protein synthesis such as CH (Haff, 1964; Cooper et al. 1967; Peterknecht et al. 1968; Ito & Barron, 1972), prevent the synthesis of both early and late virus proteins. As clearly shown in the experiment using 60 µg/ml of Ara-C, virus specific surface antigens (O'Dea & Dineen, 1957; Nahmias et al. 1971; Falke et al. 1972; Ito & Barron, 1972), which are considered to be early proteins, were detected by the indirect fluorescent antibody technique. About 60% of the cells were positively stained at a concentration of 60 µg/ml Ara-C at 9 h p.i.,
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Fig. 6. Vero cells, 12 h p.i. with HSV-2 irradiated with a u.v. dose of 2400 erg/mm$^2$. Accumulated tubular structures, viewed in longitudinal section or cross-section, and virus particles are present in the nuclei.

while the appearance of tubular structures and virus particles was almost completely inhibited under the same conditions. On the other hand, the experiment using CH revealed that both the induction of virus specific surface antigens and the production of tubular structures and virus particles were almost completely inhibited in the presence of 10 μg/ml CH. All these observations suggest that the appearance of tubular structures is one of the late events.

In the next series of experiments, we compared the reduction of infectivity, the capacity to induce virus-specific surface antigens and capacity to form tubular structures using HSV-2 exposed to u.v. light for varying times. Results show that infectivity was very sensitive to u.v.-inactivation; the capacity to induce surface antigens or tubular structures was less sensitive than infectivity. Furthermore, the results obtained from these experiments, as shown in Fig. 5, indicate that when the cells were inoculated with u.v.-irradiated virus, tubular structures appeared in more cells than could be predicted from the residual infectivity. This observation confirmed the idea that a virus which has lost its infectivity by u.v.-irradiation still has the capacity to induce tubular structures. In other words, only a part of the whole genome of HSV-2 is involved in induction of tubular structures in the nuclei of infected Vero cells.

Another interesting phenomenon is the delayed appearance and accumulation of tubular structures in the nuclei of the cells infected with u.v.-irradiated HSV-2. The mechanism of
this phenomenon is not clear, but the delayed appearance might be caused by the time required for the repair process of damaged virus DNA, or, for example, by multiplicity reactivation occurring. On the other hand, gradual accumulation of tubular structures could be caused by their not being required in the assembly of virus particles, although it has not yet been confirmed whether tubular structures are virus structural subunits.

Accumulation of tubular structures was also observed when infected cells were treated with certain concentrations (0.3 and 1.0 μg/ml) of Ara-C. In pseudorabies virus-infected cells, Kamiya et al. (1965) observed a remnant of 3 to 5% virus DNA synthesis even when high doses of Ara-C had been applied. They concluded that Ara-C would be unable to stop virus DNA synthesis completely and that some molecules of newly synthesized virus DNA would be responsible for the production of some virus proteins. In addition to this report, Falke et al. (1972) observed under the electron microscope that 1.5 μg/ml of Ara-C allowed the synthesis of capsids and envelopes of HSV, although the incorporation of 3H-thymidine into DNA under the same concentration of Ara-C was almost completely blocked. On the basis of these reports, it is tempting to suppose that certain concentrations (0.3 and 1.0 μg/ml) of Ara-C cannot completely stop the synthesis of progeny DNA, and enough is made to allow synthesis of virus proteins but not enough to assemble into complete virions. Consequently, tubular structures could accumulate in the nucleus under these conditions. However, immuno-electron microscopy would be required to clarify the relationship between the tubular structures and virions. Finally, temperature-sensitive mutants of HSV-2 as studied by Atkinson et al. (1978) should give useful clues on the function and nature of the tubular structures in HSV-2 infected cells.

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