Tubular Structures in Mixed Infection with Herpes Simplex Virus Type 1 and Type 2

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SUMMARY

The nature of tubular structures (TS) specifically found in cells infected with herpes simplex virus type 2 (HSV-2) was investigated by studying the appearance of TS in mixed infections with herpes simplex virus type 1 (HSV-1) and HSV-2 in Vero cells. Mixed infections with HSV-1 and HSV-2 resulted in a notable reduction in TS appearance. Accumulation of TS in cells infected with u.v.-irradiated HSV-2 was decreased by superinfection with HSV-1. The majority of progeny viruses obtained from mixed infection with HSV-1 and HSV-2 was neutralized by type-specific anti-HSV-2 serum, but not by type-specific anti-HSV-1 serum. Analysis of the genotype of the progeny revealed that the yield from mixed infection contained both HSV-1 and HSV-2 genotypes. These observations indicate that the majority of HSV-1 progeny of mixed infection is phenotypically mixed and make it possible to propose that TS are materials related to the type-specific antigen for HSV-2 which can be utilized by superinfection with HSV-1, producing phenotypically HSV-2 particles.

It is now generally accepted that tubular structures (TS), originally reported by Murphy et al. (1967), are specifically found in herpes simplex virus type 2 (HSV-2)-infected cells (Couch & Nahmias, 1969; Schwartz & Roizman, 1969; Aurelian et al. 1971; Young et al. 1977) and that this property is useful for the differentiation between herpes simplex virus type 1 (HSV-1) and HSV-2.

In previous papers it has been reported that the appearance of TS was observed as early as 6 h p.i., reached a maximum about 9 h p.i. and decreased thereafter, while infectivity reached a maximum 24 h p.i. (Oda & Mori, 1976; Iwasaka et al. 1979). It was also shown that TS appearance was one of the late events and that TS could be induced by a part of the total genome of HSV-2. In addition, the accumulation of TS in the nuclei when cells were infected with u.v.-irradiated HSV-2 was observed (Iwasaka et al. 1979). These observations suggested the possibility that TS are materials required for virus structural components or for the maturation of the virions. In the present study, superinfection with HSV-1 caused the disappearance of TS from HSV-2-infected cells and this phenomenon was utilized to analyse the nature of TS.

The procedures for tissue culture, virus propagation and titration of infectivity were described recently (Iwasaka et al. 1979). The virus strains used were strain KOS of HSV-1 and strain YS-4 of HSV-2 (Mori et al. 1973; Tasaki et al. 1975). Strain KOS forms syncytial plaques and strain YS-4 non-syncytial plaques.

Monolayer cultures of Vero cells were infected with the first virus (5 p.f.u./cell, unless otherwise indicated) and, after various incubation times, the cells were superinfected with the second virus (5 p.f.u./cell). For all experiments, one virus was of syncytial plaque morphology to allow ready identification in mixed virus yields. At the times indicated, cells were harvested for assay of infectivity and preparation for electron microscopy (EM).
Thin sections were examined in a JEM T8 electron microscope at a magnification of \( \times 13000 \). The frequency of TS in the infected nuclei is recorded as the percentage of nuclei containing TS relative to the total number of nuclei examined.

Type-specific antiserum was prepared by the method of Glorioso et al. (1978). Japanese white rabbits were injected with \( 3 \times 10^6 \) p.f.u. of HSV-1 or HSV-2 intraperitoneally on days 1, 8, 15 and 22, bled on day 32 and the sera separated. Two ml portions of sera were absorbed with \( 2 \times 10^6 \) lyophilized Vero cells infected with heterotypic virus. These sera had type-specific neutralizing activity. Anti-HSV-I and anti-HSV-2 titres of anti-HSV-1 serum after absorption were \( \times 48 \) and \(< \times 2\), respectively, and those of anti-HSV-2 after absorption were \(< \times 2 \) and \( \times 48 \), respectively.

To determine the phenotype of progeny virus produced in mixed infections with HSV-I and HSV-2, the samples from mixed infection and control single infections at 24 h p.i. were treated with type-specific anti-HSV-1 serum, with type-specific anti-HSV-2 serum or with pre-immune control serum. Samples from mixed infection and from control single infections of known titre were diluted in Eagle's minimal essential medium containing 2% calf serum (MEM-2) to approximately the same infectivity. To each virus sample in 0.5 ml, 0.5 ml of a 1:8 dilution of type-specific antiserum or preimmune serum and 0.5 ml of a 1:10 dilution of fresh guinea-pig serum were added. After incubation at 37°C for 1 h, the samples were assayed for infectivity. To investigate the genotype of progeny from mixed infection, the diluted harvest from mixed infection was plated on Vero cells in 35 mm Petri dishes to produce approx. 5 plaques/dish. Well-isolated plaques were picked and suspended in 1 ml of MEM-2 and suspensions were inoculated directly into Vero cell cultures in bottles. At 24 h p.i., cells were scraped from the glass and pelleted by centrifugation. The pellets were used for assessment of TS in EM study, while the supernatants were used for neutralization tests to determine the virus type. To each virus sample in 0.3 ml, 0.3 ml of a 1:16 dilution of type-specific antiserum or preimmune serum and 0.3 ml of a 1:10 dilution of fresh guinea-pig serum were added. After incubation at 37°C for 1 h, the samples were assayed for infectivity by plaque counts on Vero cells.

In the first experiment, Vero cells were first infected with HSV-2 or HSV-1 or, in the case of control cultures, mock-infected. Simultaneously or 1 h after the initial infection, the cells were superinfected with heterotypic HSV. The time course of TS appearance was quantified by EM and the yield of HSV-1 and HSV-2 was calculated from the numbers of syncytial or non-syncytial plaques. Neither prior infection nor superinfection with HSV-1 had an appreciable effect on the yield of HSV-2. Conversely, both prior infection and superinfection with HSV-2 greatly inhibited the replication of HSV-1, especially when HSV-2 was the first virus. TS appearance reached a maximum (30%) 9 h p.i. and decreased to 8% by 12 h in standard infection with HVS-2. In all the mixed infections, namely in prior infection with HSV-1, in prior infection with HSV-2 and in simultaneous infection with both types, the percentage of the appearance of TS in the nuclei was less than 5% 9 h p.i. and was 0% at 12 h. Mixed infections seem to utilize TS rather than to inhibit TS production, because yields of HSV-2 were not affected by mixed infections.

On the basis of our previous observation that TS accumulated in the nuclei when cells were infected with u.v.-irradiated HSV-2 (Iwasaka et al. 1979), the question arose as to whether superinfected HSV-1 would modify the appearance of TS produced in u.v.-irradiated HSV-2-infected cells. To answer this question Vero cells were first infected with u.v.-irradiated HSV-2 and superinfected with HSV-1 after 2, 4, 6 and 8 h. Infected cultures were harvested at the times indicated in Fig. 1 for infectivity assays and EM preparations. As shown in Fig. 1(b) the appearance of TS was reduced by superinfection with HSV-1. The closer the interval between the first and the second infection, the more marked was the reduction in TS. After 12 h p.i. with HSV-2, TS appeared in 26, 11, 4 and
Fig. 1. (a) Virus replication and (b) TS appearance in mixed and single infections of u.v.-irradiated HSV-2 (YS-4) as the initial virus and HSV-1 (KOS) as the second virus. Monolayer cultures of Vero cells were infected with u.v.-irradiated HSV-2 at 0-05 p.f.u./cell (5 p.f.u./cell equivalent before u.v. treatment); the cells were then infected with HSV-1 at 5 p.f.u./cell at 2, 4, 6 and 8 h p.i. with u.v.-irradiated HSV-2 or in the case of control cultures, mock-infected. At the times indicated, the cultures were harvested and the infectivity of HSV-1 and HSV-2 was determined on the basis of plaque morphology, while TS appearance was quantified under the electron microscope. The quantity of TS was indicated as the percentage of nuclei containing the structures. ○, U.v.-irradiated HSV-2 single infection; ●, HSV-1 single infection and mixed infections with u.v.-irradiated HSV-2 at 0 h and HSV-1 at: ●, 2 h; △, 4 h; ▲, 6 h; and □, 8 h p.i. The solid lines indicate the replication of HSV-2 and the broken lines HSV-1.

0% of cells superinfected with HSV-1 at 9, 6, 4 and 2 h p.i., respectively, while they appeared in 33% of the cells infected with u.v.-irradiated HSV-2 only.

In the present study, plaque morphology was used as a basis for differentiating between HSV-1 and HSV-2 progeny in the yields of mixed infections. To assess the validity of this method, progeny virus obtained from mixed infection 24 h p.i. was subjected to phenotypic and genotypic analysis. For this purpose we used antiserum to HSV-1 or HSV-2 absorbed with cells infected with heterotypic HSV. The 24 h yield from a mixed infection of HSV-2 at 0 h and of HSV-1 at 1 h p.i. was investigated along with the yield from HSV-1 or HSV-2 single infection. Appropriately diluted samples from each yield were tested with type-specific antisera for neutralization of infectivity. The results are shown in Table 1. Antiserum specific to HSV-1 reduced HSV-1 progeny from a single infection by more than 99-9% but failed to neutralize HSV-2 progeny from a single infec-
Table 1. Neutralization of progeny from yield of mixed infection with HSV-1 (KOS) and HSV-2 (YS-4)

<table>
<thead>
<tr>
<th>Infection conditions</th>
<th>Plaque form</th>
<th>Infectivity after neutralization (p.f.u./ml)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Preimmune serum</td>
<td>Anti-HSV-1- specific serum</td>
</tr>
<tr>
<td>HSV-1 alone</td>
<td>S*</td>
<td>$1.5 \times 10^4$</td>
<td>$1.0 \times 10^4$</td>
</tr>
<tr>
<td>HSV-2 alone</td>
<td>NS†</td>
<td>$2.9 \times 10^4$</td>
<td>$5.9 \times 10^4$</td>
</tr>
<tr>
<td>HSV-2 at 0 h p.i.</td>
<td>S</td>
<td>$9.0 \times 10^5$</td>
<td>$9.5 \times 10^5$</td>
</tr>
<tr>
<td>HSV-1 at 1 h p.i.</td>
<td>NS</td>
<td>$4.3 \times 10^4$</td>
<td>$5.6 \times 10^4$</td>
</tr>
</tbody>
</table>

* Plaques of syncytial morphology.
† Plaques of non-syncytial morphology.

neutralization or either syncytial or non-syncytial plaque-forming progeny from mixed infection. Type-specific antiserum to HSV-2 reduced HSV-2 from a single infection by more than 99.9% and both syncytial and non-syncytial progeny from mixed infection by more than 99%, while it failed to neutralize HSV-1 progeny from a single infection. These observations indicate that the majority of progeny viruses obtained from the mixed infection were antigenically HSV-2 irrespective of their plaque morphology.

To clarify the nature of the progeny obtained from mixed infection, 15 syncytial and five non-syncytial plaques were isolated and propagated in Vero cells. The isolates were investigated for their ability to produce TS in the nuclei and their antigenicity related to neutralization. None of the 15 syncytial plaques produced TS in the nuclei of infected cells, while all of the five non-syncytial plaques did. All of the progeny from five syncytial plaque isolates were neutralized by HSV-1-specific antiserum but not by HSV-2-specific antiserum. These observations indicate that in mixed infection, the majority of genotypically HSV-1 progeny have HSV-2 antigens related to neutralization and confirm that identification of virus genotype with the use of plaque morphology is a reliable method.

In the present experiments we observed that the appearance of TS was markedly reduced in mixed infection with HSV-1 and HSV-2 and, moreover, that TS accumulating in the nuclei after u.v.-irradiated HSV-2 infection decreased after superinfection with HSV-1. In addition to this phenomenon, our results showed that the majority of the progeny viruses of mixed infection had HSV-2 antigens related to neutralization but had little HSV-1 antigen in spite of the fact that some of them produced syncytial plaques and were genotypically HSV-1. Similar results were also reported by Purifoy & Powell (1977) in their progeny analysis of mixed infection. In their experiments the majority of HSV-1 progeny was phenotypically mixed, containing HSV-2 glycoproteins in the envelope, but nothing was mentioned about the TS production. We previously observed that the appearance of TS reached a maximum 9 h p.i. in Vero cells and that TS accumulated in the nuclei under the inhibitory conditions of virus maturation, such as when cells were infected with u.v.-irradiated HSV-2 or when infected cells were treated with some DNA inhibitors (Iwasaka et al. 1979; Oda et al. 1979). In addition, we observed that unusual membranous structures appeared frequently in the nuclei in parallel with the disappearance of TS when HSV-2-infected cells were treated with inhibitors of glycoprotein synthesis, such as 2-deoxy-D-glucose and cytochalasin B (Iwasaka et al. 1978). From these observations it is conceivable that TS are HSV-2-specific glycoprotein and are utilized for the HSV-1 envelope in mixed infection. Although the exact nature of TS in HSV-2-infected cells is still obscure, long tubular structures found in the nuclei of KB cells infected with adenovirus type 2 and 4 are reported to be made up, at least in part, by adenovirus structural proteins (Dunker & Brown, 1979).
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REFERENCES


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