

Thermoinactivation of Herpes Simplex Virus and Cytomegalovirus

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ABSTRACT

PLUMMER, GORDON (Baylor University College of Medicine, Houston, Tex.), AND BRIAN LEWIS. Thermoinactivation of herpes simplex virus and cytomegalovirus. *J. Bacteriol.* **89**:671-674. 1965.—The stability of herpes simplex virus and of human cytomegalovirus at 4, 22, 36, and 50 C was studied. A plateau or lag phase, during which no loss of viability was detected, was a constant feature of the inactivation curves of herpesvirus and cytomegalovirus at 36 and 22 C and of herpesvirus at 4 C. Unlike herpesvirus, cytomegalovirus was repeatedly found to be less stable at 4 C (and at 10 C) than at room temperature (22 C). Extracellular herpesvirus harvested from the supernatant culture fluid seemed to be more stable than intracellular virus obtained by sonic disintegration of the infected cells. Serum had a stabilizing effect at 36 C on both intracellular and extracellular virus.

The thermoinactivation of a number of animal viruses has been studied. Among these are foot-and-mouth disease virus (Bachrach et al., 1957), poliovirus (Youngner, 1957; Wallis and Melnick, 1963), and vaccinia virus (Kaplan, 1958). Particularly at the higher temperatures, a two-component curve of inactivation was frequently observed. The first portion was usually steeper than the later portion. At lower temperatures, a single straight line tended to replace the two components. Little work has been done on the thermoinactivation of herpesviruses. However, Kaplan (1957) and Hoggan and Roizman (1959) mentioned that the inactivation curve of herpes simplex at 37 C had a "plateau" or "lag phase" for the first few hours, during which little or no loss of viability occurred. The work on cytomegalovirus by Krugman and Goodheart (1964) showed no such plateau during inactivation.

In this paper, we report on studies of the thermoinactivation of two human members of the herpesvirus group—herpes simplex virus and cytomegalovirus.

MATERIALS AND METHODS

Viruses. The MS strain of herpes simplex virus was used; it was isolated from the central nervous system of a patient with multiple sclerosis, and was kindly made available by M. Gudnadottir of

the University of Iceland. It was somewhat different serologically from most other strains of simplex (Plummer, 1964). Two strains of cytomegalovirus were studied: strain C87, isolated by M. Benyesh-Melnick from a human kidney culture, and strain Davis, obtained from T. Weller; the two viruses are serologically similar (Plummer and Benyesh-Melnick, 1964).

Preparation and testing of the virus. Herpes simplex virus was grown in confluent 48-hr-old cultures of human lung fibroblasts (8th to 15th passage) in 16-oz flat bottles, each bottle being inoculated with 3 ml of a stock virus suspension with a titer of 10^6 plaque-forming units (PFU) per milliliter. After 0.5 hr of adsorption, the cell sheet was washed and covered with 30 ml of Eagle's medium containing 5% calf serum and 0.22% NaHCO_3 . The cultures were used after 24 hr when 90 to 100% of the cells showed cytopathic effect (CPE). Both this and the following procedures were standardized to make the results comparable.

With most cytomegalovirus strains, little infectious virus is found in the fluid of infected cultures, necessitating the infection of cultures by transfer of infected cells. In the present work, approximately 5×10^5 human lung fibroblasts infected with cytomegalovirus were mixed with 7×10^6 healthy human lung fibroblasts and seeded into a 16-oz bottle in 20 ml of Eagle's medium with 5% calf serum. The culture was used 3 days later when it showed 90 to 100% CPE.

Virus free in the culture fluid (extracellular virus) and virus associated with the cells (intracellular virus) were tested separately in the case of herpes simplex. Intracellular virus was prepared by sonic disintegration (in a Raytheon oscillator for 40 sec) of the trypsinized cells from one in-

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fected bottle culture, resuspended in 1 ml of tris-(hydroxymethyl)aminomethane (Tris) buffer. After centrifugal clarification, the supernatant fluid was diluted 1:5 in Tris and filtered to remove virus clumps (Millipore filters, 3μ and then 0.45μ). The filtrate was diluted 1:20 in Tris held at the appropriate temperature, making a final dilution of 1:100. Herpesvirus from the culture fluid was prepared by a similar filtration, followed by sonic treatment (to make it more comparable with intracellular virus) and a final dilution of 1:100 in Tris.

With cytomegalovirus only intracellular virus was tested, because with these strains the culture fluid usually contains insufficient virus to make testing it practical.

Virus assay. Infectious virus was assayed by the plaque technique with petri-dish cultures of human lung fibroblasts overlaid with Eagle's medium containing 5% calf serum, 0.22% NaHCO_3 , and 2% Methocel. Herpes simplex tests were read after 3 days and cytomegalovirus tests after 14 to 18 days (Plummer and Benyesh-Melnick, 1964).

Electron microscopy. Particle counts were kindly carried out by K. O. Smith with methods already described (Smith and Melnick, 1962). Naked and enveloped virions were enumerated (Smith, 1964).

RESULTS

Stability of herpesvirus at 4, 22, and 36 C. The stability at 36 C of extracellular and intracellular virus is shown in Fig. 1a. There is a plateau in each case, but with the extracellular virus the plateau is less sharply defined and the subsequent drop in titer is less precipitous. At 22 and 4 C, the plateaus became longer and the subsequent falls were less steep, but the marked difference in stability between the intracellular and extracellular virus still remained (Fig. 1b and 1c).

Electron microscopy of the intracellular virus and the extracellular virus prior to filtration showed ratios of nonenveloped to enveloped particles as 4:1 and 1:3, respectively. The greater

stability of the extracellular virus may reflect the "protective" nature of the envelope. Nevertheless, a further point to be considered was the possibility that the difference in stability between the intracellular and extracellular virus was due to the presence (even though diluted 1:100 in Tris) of the 1% Eagle's medium, 0.05% calf serum, or the 1% of the cell contents. It was thought that in the latter case deoxyribonuclease from the disrupted cells might account for the seemingly greater instability of the intracellular virus. However, when either 1% sterile medium or 1% cell extract, made by sonic disintegration of healthy human lung cells, was added to the intracellular or extracellular virus, relatively little change in their stability was observed.

Experiments were done to determine whether the changes leading to the ending of the plateau were readily reversible by lowering the temperature. A suspension of intracellular virus was heated at 36 C, and after 4 hr (i.e., about 2 hr after the ending of the plateau) a sample was removed and cooled to 4 C for 1 hr and then reincubated at 36 C and at 22 C. The original 36 C curve was also continued. The fresh curve at 36 C showed no plateau and was similar to the remaining portion of the original curve. No plateau was observed in the curve at 22 C, but the curve was similar to the straight line, post-plateau portion of the curve in Fig. 1b. Therefore, once the plateau has been passed at 36 C it is not, after cooling, encountered again at 36 C or at 22 C.

The addition of 5% calf serum to the Tris medium at 36 C had a considerable stabilizing influence (Fig. 2a and 2b). It became very difficult to see where the plateau ended, particularly with the extracellular virus.

Stability of cytomegalovirus at 4, 10, 22, and 36 C. The stability of the intracellular virus of C87 and Davis strains was similar. At 36 C, the

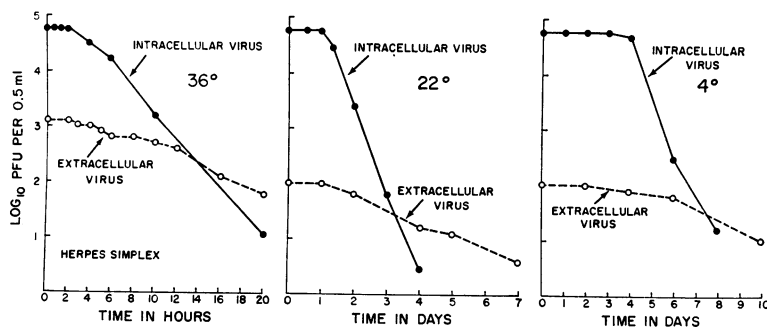


FIG. 1. Thermostability of intracellular and extracellular herpesvirus, (a) at 36 C; (b) at 22 C; and (c) at 4 C.

curve of inactivation (Fig. 3) was similar in shape to that of herpes simplex virus, with a plateau of 1 to 2 hr. At 22 C (Fig. 4), the inactivation was also similar to that of herpesvirus, but at 4 C (and 10 C) a peculiar phenomenon was observed: a greater loss of viability occurred than at 22 C (Fig. 4). This apparent rapid loss

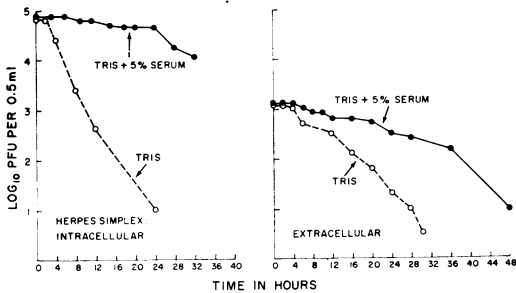


FIG. 2. Stabilizing action of 5% calf serum on herpesvirus at 36 C: on (a) intracellular virus and (b) extracellular virus.

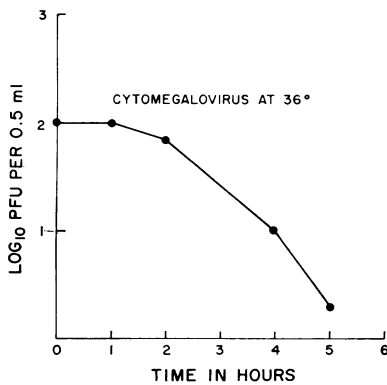


FIG. 3. Stability of cytomegalovirus at 36 C.

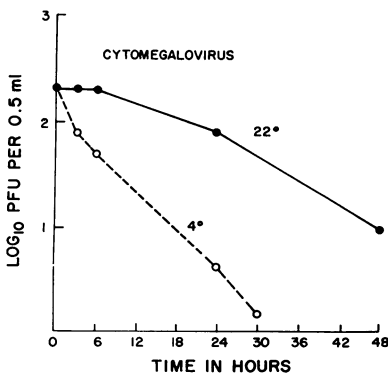


FIG. 4. Stability of cytomegalovirus at 22 and 4 C.

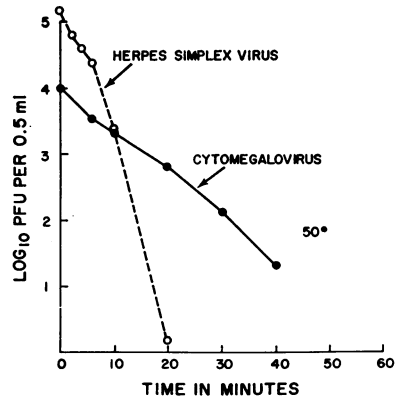


FIG. 5. Stability of herpes simplex virus and of cytomegalovirus at 50 C.

of viability at 4 C was repeatedly observed. No change (such as clumping or drop in particle count) was demonstrable by electron microscopy during the loss of viability at 4 C.

The stability of herpesvirus and cytomegalovirus at 50 C is shown in Fig. 5. The data indicate that herpesvirus was inactivated more rapidly than cytomegalovirus.

DISCUSSION

The plateau or lag phase was a constant feature of the inactivation curves of herpes simplex virus and of cytomegalovirus at 36 and 22 C, and of herpes simplex virus at 4 C. Its presence was particularly striking in the curves obtained with intracellular virus, and its duration was extended by lowering the temperature.

Extracellular herpesvirus was inactivated less rapidly than was intracellular virus. Two explanations are possible: (i) the two forms of virus have different properties; (ii) the suspensions of intracellular virus, even though diluted 1:100 in Tris, contained enzymes (such as deoxyribonuclease) responsible for the virus inactivation in greater quantities than did suspensions of extracellular virus. Addition of cell extract to the extracellular virus, however, indicated that the latter explanation alone is not correct and that the two forms of viruses differ in themselves. Perhaps naked capsids are infectious, but are less stable than their enveloped counterparts.

The phenomenon of the greater loss of detectable viable cytomegalovirus at 4 and 10 C than at 22 C is most odd. No reasons were found or can be suggested by us. It brings to mind the finding of Krugman and Goodheart (1964) that their strain of cytomegalovirus had a shorter half-life at 15 C than at 23 C.

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