Host-Cell Lysosomal Response to Two Strains of Herpes Simplex Virus¹

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A correlation has been made between the host lysosomal responses to and release of infectious virus from HEp-2 cells infected with two strains of herpes simplex virus (HSV). Supravital staining with acridine orange was used for morphological studies of macroplaque and microplaque HSV-infected cells. With the progression of infection, cells infected with either microplaque HSV or macroplaque HSV were observed to undergo different lysosomal and cytopathic changes, which could be correlated with increased accumulation of acid phosphatase and infectious virus in the extracellular fluid.

A direct temporal correlation between activation of lysosomes, genesis of cytopathic effects (CPE), and virus release has been demonstrated for several virus-host cell systems (3, 4, 6, 11). In general, the contention is that lysosomal activation leads to CPE in virulent lytic viral infections, whereas during moderate nonlytic infections lysosomes retain their integrity such that the host cell is not damaged.

Previous studies in this regard have simultaneously compared the host lysosomal response to such widely different agents as poliovirus and Newcastle disease and herpes simplex viruses (S. K. Dusing and D. A. Wolff, *Bacteriol. Proc.*, p. 187, 1969). Others have compared lytic and nonlytic infections, but simultaneously in different cell types (2). Such comparisons make conclusions concerning the differences in host-cell response and sequence of events difficult. By studying two strains of herpes simplex virus (HSV) which differ fundamentally in both the type and degree of host-cell cytopathology they induce, we have attempted to remove many of the variables involved in establishing a correlation between activation of lysosomes, type of CPE, and release of matured virus.

MATERIALS AND METHODS

Virus and cell cultures. Human epidermoid carcinoma no. 2 (HEp-2) cells were propagated in monolayer cultures in medium 199 supplemented with 10% bovine serum and antibiotics.

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HSV strains were originally obtained from Bernard Roizman (University of Chicago, Chicago, Ill.). The pertinent properties of the microplaque and macroplaque strains of HSV have been described elsewhere (5). The virus strains were assayed in HEp-2 cells under a liquid overlay of medium 199 containing 5% bovine serum and 0.25% human immune gamma globulin. The virus titer was expressed as polykaryocyte-forming units (PoFU/ml).

Microscopy and photography. A Leitz Orthomat microscope equipped for simultaneous phase-contrast fluorescence microscopy was used for the examination of all preparations. The light source consisted of a Leitz lamp housing fitted with an Osram mercury burner (HBO 200W) and a Leitz tungsten bulb (12 v, 60 w). The filters used and their positions in the system were: 4-mm BG38 red suppression filter, 2-mm BG12 ultraviolet transmitting filter between the light source and the specimen, and a K 530 absorbing filter between the specimen and ocular.

Photographs were taken with Anscochrome high-speed daylight film (ASA 500). Exposure times were dependent on the intensity of the fluorescence and were automatically determined by a built-in photoelectric cell.

Acid phosphatase determinations. Hep-2 cells were infected with 10 PoFU/cell of either macroplaque or microplaque HSV. At designated intervals postinfection (PI), 5-ml samples of supernatant culture fluid were taken for measurement of acid phosphatase released from 2×10^6 cells. A 1-ml amount of a buffer-substrate mixture (0.05 M citrate buffer, pH 4.8, containing 5.5×10^{-3} M *p*-nitro-phenyl-phosphate, sodium salt) and 0.2 ml of enzyme sample were mixed and incubated for 30 min at 37 C. The reaction was stopped by adding 10 ml of 0.02 N NaOH. Absorbance of the assay mixture was read against a blank at 405 nm.

Supravital staining with acridine orange (AO). The solutions of AO were prepared fresh daily and consisted of 10^{-3} M stock solution which was diluted with 199 growth medium to 10^{-5} M. Cover slip HEp-2 cell

cultures were removed from incubation at predetermined intervals, gently blotted, and mounted on several drops of the 199-AO medium.

Production curves of HSV strains. Cell-associated and extracellular virus production curves were determined for both strains of HSV. Confluent HEP-2 cell monolayer cultures were each inoculated with 0.1 PoFU/cell. After a 2-hr adsorption period, the inoculum was decanted and the monolayer was washed two times with prewarmed 199 growth medium. The monolayer was then overlaid with 5 ml of conditioned medium. At intervals PI, the supernatant fluids were decanted. The cultures were overlaid with 5 ml of 199 growth medium and then were subjected to three cycles of freeze-thawing; corresponding fluids were titrated for infectious virus.

RESULTS

Cytopathological changes monitored by supravital staining. HEP-2 cells were infected at an input of 1 to 5 PoFU/cell with either the macroplaque or microplaque strains of HSV and stained with AO. This specifically stains the acid phosphatase-positive multivesicular bodies of the Golgi-endoplasmic reticulum-lysosome complex, which fluoresce a brilliant yellow-orange when examined under the fluorescence microscope. Figure 1 shows a typical field of normal uninfected HEP-2 cells containing the fluorescent AO particles (AOP). The AOP are sometimes polar or perinuclear in location but heterogeneous with respect to size. Also seen with this vital stain are the pale yellow-green nucleoli.

At 8 hr PI, cells infected with microplaque HSV were observed to be rounded with a coalescence of AOP. Nucleopathic effect (NPE) was also observed at this time, typified by a breakdown of nucleoli and condensation of chromatin as commonly described by other investigators (7). The macroplaque HSV-infected cells at 8 hr PI showed no rounding or NPE and only slight coalescence of AOP.

FIG. 1. Uninfected HEP-2 cells stained with acridine orange. $\times 400$.

At 16 hr PI, microplaque HSV-infected cells showed extensive NPE, clumping, and loss of AOP. Similar cells infected with the macroplaque strain showed retention of AOP and polykaryocyte formation (Fig. 2).

After 32 hr PI, the microplaque HSV-infected cells showed complete absence of AOP, whereas macroplaque HSV-infected cells still retained AOP while undergoing extensive NPE and syncytium formation.

The time course and degree of these changes representing one of five such experiments have been quantitated as shown in Fig. 3. Loss of AOP and development of NPE are shown for both strains of HSV. The microplaque HSV-

FIG. 2. HEP-2 cells infected for 16 hr with microplaque herpes simplex virus (left) and macroplaque herpes simplex virus (right); stained with acridine orange. $\times 400$.

FIG. 3. Loss of acridine orange particles (AOP) and development of nucleopathic effect in microplaque (μ P) and macroplaque (MP) herpes simplex virus-infected HEP-2 cells.

infected cells began to lose AOP at about 8 hr, whereas macroplaque HSV-infected cells, although they lost some AOP, appeared to retain a significant percentage up to 32 hr PI. Correlated with this loss of AOP was the progression of infection and spread of NPE. Nuclear changes appeared earlier with microplaque HSV infection but spread more slowly than with macroplaque HSV.

Such changes suggest differences in the mode of virus spread; the macroplaque strain rapidly spreads virus via recruitment of neighboring nuclei, whereas with the microplaque strain spread may be cell to cell through the extracellular environment.

Release of acid phosphatase from macroplaque or microplaque HSV-infected cells. Parallel evidence supporting the notion that the loss of AOP is indeed a reflection of lysosomal activation is the concurrent appearance of acid phosphatase extracellularly in the medium of infected cells (Fig. 4). It is apparent that the supernatant fluids from microplaque HSV-infected cultures exhibited greater acid phosphatase activity than corresponding supernatant fluids from macroplaque HSV-infected cultures. This increased acid phosphatase activity in the microplaque HSV-infected culture commenced at the same time PI as the loss of AOP (illustrated in Fig. 3). Such a correlation between loss of AOP and activation of lysosomes has been well documented (1).

Virus production curves of macroplaque HSV and microplaque HSV. A very important part of the dynamic change in AOP is its relationship to HSV release. The comparative growth curves of these two strains are shown on Fig. 5. The two strains exhibited only subtle differences in cell-

associated infectious virus, but differed markedly in the amount of infectious virus released into the extracellular fluids.

DISCUSSION

Examination of host-cell cytopathology as monitored by supravital staining of AOP has revealed differences in the host-cell response to two strains of HSV which can be correlated with the amount of infectious virus released.

It is apparent from Fig. 5 that macroplaque HSV-infected cells, which retain AOP, release virus more efficiently than microplaque HSV-infected cells. Since AOP have been defined as the normally present acid phosphatase-positive multivesicular bodies (8) or "lysosomes," this observation suggests that disposition of lysosomes may be directly involved.

We interpret this to be a consequence of the fact that macroplaque HSV-infected cells have maintained cell integrity during the period 16 to 24 hr PI [Note persistence of AOP in macroplaque HSV-infected cells (Fig. 2).] With the retention of cell integrity, macroplaque HSV development and release can proceed longer with the result that a greater amount of virus is released. In microplaque HSV-infected cells during the period from 16 to 24 hr PI, cell integrity was destroyed, as evidenced by an increased loss of AOP (Fig. 3), with the result that a low ratio of extracellular or released virus as compared to the total virus was produced.

FIG. 4. Release of acid phosphatase from microplaque (μ P) and macroplaque (MP) herpes simplex virus-infected cells.

FIG. 5. Cell-associated and extracellular virus produced during microplaque (μ P) and macroplaque (MP) herpes simplex virus infection of HEp-2 cells.

The recent electron microscopic observations of Schwartz and Roizman (10), which revealed the existence of a network of tubules in infected cells involved in virus egress, corroborate this conclusion. If the cytoplasm of the infected cell is a hostile environment for mature enveloped virions, loss of cell integrity due to lysosome activation would also destroy this means for externalization of the virus. It is also possible that these tubules arise from the multivesicular bodies of the cell.

Allison and Black (2) described a pattern in polykaryocytes induced by herpes simplex in 3T3 mouse cells in which the lysosomes, as monitored by aminoacridine staining, were disrupted. A significant number of lysosomal clusters (AOP) were, however, retained in infected cells. The pattern we have observed with macroplaque HSV-infected polykaryocytes is similar. The AOP are no longer distributed throughout the cytoplasm but align themselves amongst the centrally located nuclei of the syncytium. They are, nonetheless, retained.

Although conclusions about the significance of these observations must be tempered by a current lack of details concerning the nature of vital staining with AO, it is apparent that the demonstrated differences in host AOP response are directly related to some basic biological event distinguishing these two strains of HSV. This event may be the same one which relates to differences in cytoplasmic membrane changes in cells infected with these two strains (9).

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