Preparation of Herpes Simplex Virus of High Titer

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In nonpermissive DK cells abortively infected with the MPdk⁻ strain of herpes simplex virus, viral deoxyribonucleic acid (DNA) and protein are produced only after infection with ≥ 100 plaque-forming units (PFU)/cell (L. Aurelian and B. Roizman, *J. Mol. Biol.* **11**:539, 1965). To study the nature and products of macromolecular synthesis in abortively infected cells, it was necessary to develop methods for the production of virus preparations of high potency. The objective of this paper is twofold: (i) to document several factors affecting virus yield, and (ii) to describe the procedure used for large-scale production of virus.

Previous papers from this and other laboratories have shown that the optimal temperature for the multiplication of most virus strains is at or near 34°C. (A. E. Farnham and A. A. Newton, *Virology* **7**:449, 1959; M. D. Hoggan and B. Roizman, *Virology* **8**:508, 1959; K. O. Smith, *J. Bacteriol.* **86**:99, 1963). The exceptions are mutants specially selected for their ability to grow at high temperature [A. B. Germanov and M. I. Sokolov, *Prob. Virol. (USSR)* **12**:182, 1967] and host range mutants such as the MPdk⁺sp virus selected for capacity to grow in DK cells at 37°C (B. Roizman, *Virology* **27**:113, 1965). Incubation at 32 to 34°C is particularly advantageous, because at these temperatures infectious virus accumulates inside the cells and is not released efficiently into the extracellular fluid (M. D. Hoggan and B. Roizman, *Virology* **8**:508, 1959). It has also been shown previously (B. Roizman, *Proc. Soc. Exptl. Biol. Med.* **119**:1021, 1965) that the pH of the extracellular fluid affects virus yield. The optimal pH for the MPdk⁻ mutant is 6.8. The optimal pH for the MPdk⁺sp in both HEp-2 and DK cells is at or near 7.3.

In recent studies on the nutritional and physiological requirements for maximal yields of MPdk⁻ virus strain in HEp-2 cells and of MPdk⁺sp mutant in DK cells, it was found that maintenance media for infected cells consisting of mixture 199 with 1 to 5% calf serum are superior to media consisting of 1 to 10% calf serum, salts, amino acids, and vitamins once or twice the concentration recommended by H. Eagle (*Science* **130**:432, 1959). The factor

present in mixture 199 and responsible for the 4- to 10-fold higher yields is unknown. It was also found that cells grown and maintained in monolayer cultures yield more virus than those suspended after infection. In the experiments summarized in Fig. 1, HEp-2 cells in replicate monolayer cultures were exposed to 50 PFU of MPdk⁻ virus per cell for 2 hr at 37°C. At that time, the cells in one culture were suspended in 10 ml of medium and placed in a siliconized 125-ml Erlenmeyer flask. The fluid in each of the remaining cultures was aspirated and replaced with 10 ml of fresh medium. The Erlenmeyer flask and the monolayer cultures were placed at 34°C on a rotary shaker (80 rev/min). At intervals after infection, samples consisting of 0.5 ml from the Erlenmeyer flask and one infected monolayer culture were removed from the incubator for assay. As shown in Fig. 1, HEp-2 cells adhering to glass produced approximately 10-fold more virus than those suspended after infection. It is interesting to note that DK cells suspended after infection with MPdk⁺sp virus yielded 50-fold less virus than those maintained in monolayer cultures. Lastly, young, barely confluent monolayer cultures of rapidly growing cells yield more virus than multilayered cultures. The difference in MPdk⁻ virus yield between infected monolayered and multilayered HEp-2 cell cultures is two- to threefold. As shown in Table 1, considerably greater differences in MPdk⁺sp virus yield were obtained from 1- and 4-day-old cultures of DK cells. In this experiment, replicate monolayer cultures of DK cells were infected at daily intervals with a multiplicity adjusted to yield 10 PFU/cell. The cells were harvested after 19 hr of incubation at 37°C and assayed for virus content. At optimal conditions, the yield of MPdk⁺sp from 1-day-old cultures of DK cells was approximately 20 PFU/cell, i.e., only 5 to 10% of the yield of MPdk⁻ virus from infected HEp-2 cells. DK cells infected 4 days after seeding yielded less than 1 PFU/cell. The DK cell cultures were barely confluent on the 2nd day, but consisted of several layers of cells 4 days after seeding.

The procedure for the preparation of large quantities of virus follows that of W. House and P. Wilby (*Lab. Pract.* **14**:594, 1963) with

FIG. 1. Multiplication of MPdk⁻ strain of herpes simplex virus in HEp-2 cells maintained in monolayer cultures and in cells suspended after infection.

several modifications as follows. (i) HEp-2 cells are seeded in cylindrical bottles approximately 10 cm in diameter and 40 cm long. The bottles are then rolled in an apparatus (constructed by Bellco Glass Co., Vineland, N.J.) capable of rolling 28 bottles simultaneously. For uniform attachment and rapid growth, the bottles are seeded with 10^8 cells dispersed in 200 ml of prewarmed medium consisting of 10% calf serum, Earle's saline, and twice the amounts of amino acids and vitamins recommended by H. Eagle (Science **130**:432, 1959). The bottles are rolled at 0.3 to 0.5 rev/min at 37 C. The cell yield per bottle 3 to 4 days after seeding ranges

TABLE 1. Relation between the age and density of the DK cell culture and the yield of MPdk⁺ sp virus

Age of culture (days)	No. of cells	Virus yield	PFU/cell
1	1.1×10^6	2.1×10^7	19.1
2	2.3×10^6	3.5×10^7	15.2
3	4.1×10^6	1.1×10^7	2.6
4	6.8×10^6	0.6×10^7	0.9

between 5×10^8 and 7×10^8 cells. (ii) Three days after the seeding of roller bottles, monolayer cultures containing 4×10^7 cells per 32-oz (907 ml) prescription bottle are exposed to 10 PFU of virus per cell, and then incubated at 34 C for 18 hr. At that time, the infected cells are suspended in 100 ml of maintenance medium consisting of 1% calf serum in mixture 199 and buffered with CO₂ at pH 7.0. The growth medium in the roller bottle is replaced with the suspension of infected cells, and the culture is then rolled at 34 C. (iii) After 20 to 24 hr of incubation at 34 C, the infected cells are shaken off the glass into the medium and pelleted by centrifugation at $1,000 \times g$ for 10 min. Two techniques have been used to extract the virus from infected cells. The first is to suspend the infected cells in 3 to 10 volumes of medium and to freeze-thaw them three times and then apply brief sonic treatment. The second procedure is to suspend the cells in 5 volumes of distilled water. The swollen cells are then lysed by 20 strokes with a tight Dounce homogenizer. The yield of virus per roller bottle (5×10^8 cells) is approximately 5×10^{10} to 8×10^{10} PFU in 10 ml or less of fluid.

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