Growth Characteristics of a Herpesvirus of Turkeys

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The herpesvirus of turkeys remains associated with cells attached to the culture vessel throughout its growth cycle. The level of infectious centers is essentially constant between 24 and 48 hr when the culture medium is unchanged. Thereafter, the number of infectious centers decreases. The cessation of virus synthesis is not due to insufficient numbers of uninfected cells or interferon production. Herpesvirus of turkeys does not replicate at 22 C and is similar to other herpesviruses in its sensitivity to interferon. The results define conditions to be considered in the preparation of infected cell suspensions commonly used as a vaccine against Marek's disease virus.

A type B herpesvirus was isolated from turkeys by Witter and co-workers in 1970 (10). Cells infected with this virus were shown to protect chickens from infection with Marek's disease herpesvirus (7). This represents an example of immunization against herpetic and malignant disease by employing an attenuated virus. For this reason, the turkey herpesvirus may be a good model for studies on the protection of humans against similar type herpes infections.

The herpesvirus of turkeys (HVT) is extremely cell associated, in agreement with data on other type B herpesviruses (6). Because of this, infected cell suspensions are used to vaccinate susceptible chickens throughout this country. We have reported previously on studies dealing with basic aspects of the propagation and assay of HVT (1). We present data here on quantitative aspects of HVT infection of chick cells.

MATERIALS AND METHODS

Virus and cells. All experiments were performed with HVT, strain FC-126, in secondary chick fibroblasts. Petri dish cultures (60 mm) containing 4 x 10⁴ to 5 x 10⁴ cells were inoculated with infected cell suspensions as described in the Results section. The number of free or attached infectious centers was determined by plaque assay as previously described (1). Attached cells were removed from surfaces after a brief trypsin (0.25%, w/v) treatment and were diluted in Eagle minimum essential medium (MEM) for assay. The term "plaque-forming unit" (PFU) in the text refers to a focus initiated by a single infected cell.

Interferon production and assay. Interferon used for determining the sensitivity of HVT was prepared by infection of secondary chick fibroblasts with Chikungunya virus at 0.1 PFU/cell (2). Residual virus and much of the contaminating protein were precipitated with perchloric acid (5) followed by dialysis against several changes of Earle salt solution (pH 7.2). The inhibitor displayed the properties common to previously described interferons, i.e., pH stability, trypsin sensitivity, cell species specificity, and lack of viral specificity. Interferon was assayed by a plaque reduction method (9) employing a vaccinia virus (IHD strain) challenge. Cell cultures were treated with dilutions of the interferon (4.0 ml/culture) for 18 hr at 37 C. The fluids were then removed, and each dish was challenged with 100 to 200 PFU of vaccinia virus in a volume of 0.2 ml. The challenge virus was adsorbed for 60 min and replaced with MEM. Plaques were counted 36 to 48 hr later after staining with crystal violet. Mock interferon, prepared from uninfected chick cells, was used as a control in all assays.

RESULTS

Infectious centers. The number of attached infectious centers as a function of time at 22 or 37 C is presented in Fig. 1. The inoculum in this experiment is indicated on the ordinate (0 time) and represents an input multiplicity of 0.025 infected cell per each uninfected cell. We have shown previously that 90% of the infected cells attach to the uninfected monolayer within 2 hr after addition (1). In this experiment, the lack of efficient virus replication at 22 C was evident, whereas at 37 C there was a 10-fold increase in the number of attached infectious centers at 24 hr postinfection. The rate of
The progression of infection at earlier time periods is shown in Fig. 4. In this experiment, the number of attached infectious centers was again determined. An initial doubling in the number of infected cells occurred due to the transfer of infectivity from the adsorbed inoculum to the cells of the monolayer. This period was followed by a lag of about 6 hr with subsequent dissemination of virus to other cells in the culture. The rate of virus transfer to uninfected cells decreased between 12 and 24 hr.

The influence of inoculum size on the increase in infected cells is presented in Fig. 5. The increase in attached infectious centers at 24 hr postinfection as a function of different inocula in several experiments is illustrated. At an inoculum of 10^5 infected cells per culture (multiplicity of infection = 0.02), a 12-fold increase in infectious centers was obtained; at larger inocula the increase was consistently less. At multiplicities less than 0.02 there

production of infected cells decreased sharply between 24 and 48 hr even though in this particular experiment 60 to 70% of the cells in the culture remained noninfectious. We consistently found a cessation of virus synthesis at 24 hr when the input multiplicity exceeded 0.02 PFU/cell. Frequently, the number of attached infectious centers actually decreased between 24 and 48 hr. This is especially true if the medium is changed at daily intervals (1).

The level of cell-free virus in the supernatant fluid at 24 hr was 36 PFU/ml in this experiment. This value is particularly unimpressive when one considers that there were 10^8 infectious centers in the culture at this time. This illustrates the cell-associated nature of HVT.

A comparison between attached and free infectious centers is presented in Fig. 2. The number of attached infectious centers followed the pattern of the first experiment. The number of infected cells in the supernatant fluid was low and never exceeded 10^9/ml at maximum. The decrease in attached PFU was accompanied by a decrease in free PFU. The number of total free cells and total free infectious centers from the same experiment is illustrated in Fig. 3. The low infectivity of free cells is apparent (broken line). At 24 and 48 hr postinfection only 1 out of every 100 cells was infectious. The ratio of noninfectious to infectious cells increased to over 1,000 to 1 at 72 hr when cellular degeneration was advanced.
GROWTH OF TURKEY HERPESVIRUS

**Interferon production.** Interferon has been shown to limit the spread of certain viruses both in vivo and in vitro (8). Since increases in the number of HVT infectious centers are not evident after the first day of infection, the possibility was considered that interferon was produced. Culture fluids were tested for interferon at various times postinfection with different HVT inocula. Interferon activity was detected when low dilutions of the fluids were tested. The level of plaque inhibition at a 1:2 dilution varied from 40 to 60% when infected culture fluids were compared to uninfected control medium. In one experiment the culture fluid inhibited plaque formation by 80% at a 1:2 dilution. These are low levels of activity and are similar to those found in herpes simplex virus-infected cells (3). It is also important to note that these interferon preparations were assayed against vaccinia virus. The sensitivity of HVT to interferon (prepared as described in Materials and Methods) is compared with vaccinia virus in Fig. 6. It is apparent that vaccinia virus is about six times more sensitive to chick interferon than HVT. The low sensitivity of herpesviruses has been reported by others (4). When HVT-infected cells, rather than free virus, were employed as a challenge in the

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**Fig. 4.** Production of HVT infectious centers at 37 C.

**Fig. 5.** Effect of inoculum size on the production of infectious centers. The inoculum refers to the number of infectious centers added to each culture of 5 x 10⁴ cells. The fold increase refers to the number of infectious centers at 24 hr postinfection divided by the inoculum.

was a two to threefold stimulation of the relative number of infectious centers. Thus, inocula greater than 10⁴ infected cells per culture (multiplicity = 0.002) resulted in a lower relative increase in attached infectious centers. The low increase at the highest inoculum was not due to insufficient numbers of uninfected cells since no more than 50% of the cells in the culture were infectious at the highest inoculum employed.
addition, this suggests that significantly supernatant vessel. The inference of infected cells approximates the situation in an infected culture more closely than the use of cell-free virus because virus is spread primarily through cell-to-cell contact. These data indicate that low levels of interferon are produced in HVT-infected cultures but are probably not sufficient to suppress the spread of HVT in vitro.

DISCUSSION

These experiments have demonstrated that HVT remains associated with cells which, for the most part, are attached to the culture vessel. The number of infected cells in the supernatant fluid is low. On a practical level, this suggests that the source of cells used for vaccination purposes should be the attached infectious centers.

The level of infected cells does not increase significantly between 24 and 48 hr postinfection when the input multiplicity exceeds 0.02. In addition, cytopathology is not prominent when maximum numbers of infected cells are present. It appears that there is an overall decrease in the number of infectious centers with progressive cellular degeneration.

The transfer of infectivity is more efficient at low inocula. It is possible that the addition of large inocula is accompanied by the simultaneous addition of defective HVT particles which interfere with virus replication. It is also conceivable that the production of defective particles is a factor limiting the spread of HVT in vitro. Interferon does not appear to play a role in suppressing HVT spread in infected cultures for two reasons: (i) little interferon is produced by HVT-infected cells, and (ii) HVT is relatively insensitive to interferon action. We are attempting to detect the presence of defective virions and their role, if any, in the replication of HVT.

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