NOTES

Preparation of Infectious Cell-Free Herpes-Type Virus Associated with Marek's Disease

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Cell-free transmission of the herpes-type virus of Marek's disease was obtained by demineralized water treatment of infected chick embryo fibroblasts.

A large body of experimental and epidemiological evidence has associated a herpes-type virus (HTV) with Marek's disease (MD) of chickens (3, 12). However, most attempts to obtain cell-free transmission of HTV have been unsuccessful (2, 4). The necessity for using whole, living cells for virus transfer can produce confusion in the interpretation of tissue culture changes, complicates the interpretation of serologic tests, and makes it difficult or impossible to eliminate the other viruses which are present in at least three strains of Marek's disease (5). Thus, clarification of the role of HTV in the etiology of MD is greatly hampered by the inability to obtain cell-free infectious virus.

We found that we can routinely obtain infectious cell-free preparations of HTV from tissue cultures infected with either the GA or JM strain of MD by extracting the cells in demineralized water.

Whole blood from inoculated chickens was used to initiate tissue culture passage lines of the GA and JM strain viruses. Primary chick embryo fibroblasts (CEF) from 10- to 12-day-old embryonated hens' eggs from a closed, inbred, resistance-inducing factor-free flock maintained at the NIH Animal Production Center were used. Serial passages were carried out by standard procedures by using the fresh cells suspended in culture fluid. HTV infectivity titers were 10^5 to 10^6 TCID₅₀/0.5 ml, as measured by production of cytopathic effects (CPE) in either CEF or chick kidney (CK) cultures. The CPE consisted of foci of ballooning round cells or syncytia of various sizes. As found by other investigators (3, 11), 450-nm filtrates of these HTV-infected cell suspensions failed to produce HTV CPE in CEF in a 28-day observation period. Three serial blind passages of

viable cells inoculated with such filtrates into 1-day-old CEF monolayers also failed to reveal HTV CPE. Chick syncytial virus (CSV, reference 5) was demonstrated in both MD strains, but was removed by incubation of infected CEF with specific CSV antiserum prepared in rabbits and by further passage of the infected cells under specific CSV antiserum through three to five serial passages. Avian leukosis virus [detected by the complement fixation for avian leukosis (COFAL) test (11)] also was present in the GA strain of MD in the early CEF passages, but incubation and passage of infected cultures under chicken serum containing group A and group B avian leukosis neutralizing antibodies reduced the level of COFAL-positive virus present in the cultures.

To obtain cell-free infectious HTV, infected CEF monolayers, containing only HTV or HTV contaminated with only minimal amounts of CSV or COFAL-positive virus, were allowed to incubate until over 70% HTV CPE was observed. The infected CEF monolayers were washed two to three times with demineralized water, and 5 ml of water was placed on the monolayers for 5 min. The lysed cells were then suspended in the water supernatant fraction. The extracts regularly titered 10^4 to 10^5 TCID₅₀/0.5 ml. Dilutions for infectivity titrations also were made in water. When 450-nm Millipore filtrates of the lysed CEF cell suspensions prepared from GA or JM strains of MD were tested in CEF, HTV CPE was observed in 10 to 12 days, and HTV titers of the filtrates were 10^2 to 10^3 TCID₅₀/0.5 ml. Serial passages of HTV could be made with water-treated CEF filtrates.

HTV CPE was not demonstrated in primary

FIG. 1. A section through a nucleus of a cell within a focus of "transformed" cells showing immature particles (I), mature particles (M), mature particles with an additional outer layer (arrows), and large filamentous structures (LF). Magnification, $\times 30,000$.

human embryonic kidney culture inoculated with these filtrates. Further, complement-fixing antigens prepared from the HTV-infected CEF did not fix complement in the presence of human

serum containing herpes simplex antibodies. These findings indicated that the HTV observed was not due to a herpesvirus contaminant of human origin.

FIG. 2. Multiple virus-related particles in JM-infected cell. (a) Immature particles (I) with an outer capsid (100 nm in diameter) and an inner shell-like core (65 nm in diameter). (b) Mature particles (M) consist of outer capsid and dense cores. (c) Two immature particles with capsid, shell core, and small densely staining central region. (d) Mature and immature particles with irregular small filaments (SF) about 30 to 35 nm in diameter and shell-like structures 50 to 60 nm in diameter which resemble cores of immature particles (arrow). (e) Empty capsids (E) and immature particles (I). (f) An empty capsid in the cytoplasm (arrow). All other particles are intranuclear. (g, h) Large filaments (LF) apparently consisting of small filaments with an outer cylindrical shell 70 to 75 nm in diameter. (i) A mature particle with a second membrane or capsid layer partially enclosed in a third layer (arrow). (j, k, l) Mature particles with a second outer layer present a variable appearance of their cores. Magnifications (a-l), $\times 45,000$.

Electron microscopy confirmation that the CPE observed was due to HTV was obtained by fixing infected cell monolayers with glutaraldehyde, postfixing in 1% osmium tetroxide, and embedding in situ in Epon-Araldite. Sections of isolated foci were cut with a diamond knife, doubly stained with uranyl acetate and lead citrate, and examined in an RCA EMU-3E electron microscope.

The embedded foci observed by electron microscopy were similar to those observed in unfixed cells. As many as six nuclei were observed in syncytia, but cells in which a single nucleus was sectioned were also encountered. Essentially all of the nuclei contained herpes-like viruses about 100 nm in diameter (Fig. 1). These particles are indistinguishable from those previously observed in CK infected with blood of chickens inoculated with JM strain or other strains of MD (1, 9). Very similar virus forms were observed associated with two other tumors, the Lucké frog tumor (7) and tissue cultures derived from Burkitt's lymphoma (6). As seen with the Lucké frog tumor, Burkitt's lymphoma, and herpes simplex virus (10), multiple virus-related particles were observed primarily in the nuclei of JM-infected cells (Fig. 2). No evidence of herpesvirus was found in cells in the same culture dishes from areas other than the foci of altered cells. Leukosis-like viruses were not found in any cells.

Other workers (2, 12) explained the requirement for viable cells infected with HTV for the production of CPE in tissue culture or MD in chickens on the relative instability of the virus. It is possible that the tissue culture medium from HTV-infected cells or disrupted tumor cell extracts may contain enough toxic material to destroy the viral envelope and account for the relative instability of the virus. S. Nii et al. (10) postulated that an uncoating enzyme capable of digesting the envelope of herpesvirus at the moment of entry into uninfected cells may diffuse into tissue culture medium in sufficient quantity to digest the viral envelope of newly released virus particles and render the virus noninfectious. Removing the tissue culture medium and washing the cell monolayer with water would reduce the amount of any toxic substance. Further, lysing the cells by hypotonic shock would release intracellular enveloped virus into the demineralized

water. Historically, water has been used for many years as the suspending medium of choice for herpesvirus (8). It would be of interest to use this technique with other closely cell-associated herpesviruses, particularly the herpesvirus associated with Burkitt's lymphoma.

Obtaining infectious cell-free HTV now allows conventional virus neutralization tests to be performed and, further, permits inoculation of animals with only HTV to determine its ability to produce lympho-proliferative disease in chickens.

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