Susceptibility of Mammalian (Hamster) Cell Culture to Infection with Herpesvirus of Turkeys

H. G. BEDIGIAN and MARTIN SEVOIAN

Department of Veterinary and Animal Sciences, University of Massachusetts, Amherst, Massachusetts 01002

Received for publication 21 March 1972

Herpesvirus of turkeys (HVT) infected and replicated in hamster kidney cells as evidenced by cytopathic effects, intranuclear inclusions, and by the presence of herpesvirus particles as seen by electron microscopy. Additional evidence for the presence of HVT in cell cultures was determined by the serum neutralization, complement fixation, and the fluorescent-antibody tests.

Type II (Marek's disease) leukemia, a lymphoproliferative disease of chickens, is reportedly caused by a highly cell-associated herpes-type virus (7). Although attempts have been made to infect mammalian cells, the disease has been considered to be specific to the avian species (1, 3). A herpesvirus isolated from turkeys (HVT) is moderately cell associated and antigenically related to the causative agent of type II leukemia (JM virus) (6), and reported by Okazaki (8) to offer protection against the development of lymphoid tumors. Purchase (9) investigated the susceptibility of HeLa cells and avian cell cultures from various species to HVT and concluded that all avian species examined were susceptible to HVT, whereas HeLa cultures were not. The purpose of this investigation was to investigate whether cells other than those of avian origin were susceptible.

MATERIALS AND METHODS

Virus. HVT (FC-126) was isolated by Witter et al. (11) and has been propagated on duck embryo fibroblast (DEF) monolayers for approximately six passages. A second strain of HVT (BS) was isolated from turkeys at the University of Massachusetts and was carried through nine passages on DEF. HVT-infected DEF cell cultures which showed nearly complete destruction of the monolayer were trypsinized (0.25% trypsin), collected in 1 ml of medium 199, and inoculated onto primary hamster kidney (HK) cell cultures. HVT-infected DEF cell cultures were also trypsinized and transferred to new plates, which were not seeded on to primary DEF, to determine how long the infected cultures remained viable and were able to maintain themselves.

Preparation of HK cell culture. Primary HK cell cultures were prepared from 1- to 6-week-old Syrian hamsters. Kidneys were removed, minced, washed in pH 7.2 phosphate-buffered saline (PBS) containing antibiotics (100 IU of penicillin, 0.2 mg of streptomycin, and 25 IU of mycostatin per ml), and trypsinized (0.25% trypsin) at room temperature until tubes could be seen under the microscope, and then the cells were collected (5). This was repeated until the minced kidney was digested and no more cells could be obtained. The cells were spun at 800 × g for 5 min, and the pellet was washed twice in PBS containing antibiotic as cited above. Finally, the pellet was suspended in medium 199 (Flow Laboratories) containing 10% tryptose phosphate broth, antibiotic as cited above, bicarbonate, and 10% fetal calf serum (FCS). The cell suspension was placed in disposable plastic Falcon petri dishes (60 by 15 mm) containing cover slips (11 by 22 mm) and placed at 37°C under 5% CO₂. When monolayers were formed, usually 4 to 5 days, the cells were inoculated with 20,000 plaque-forming units of HVT per ml and maintained in the above media with 2% FCS.

Fluorescent-antibody procedures. Antisera to HVT was prepared by inoculating HVT intravenously into 3-month-old New Zealand and Dutch Belted rabbits. Each rabbit received five to seven weekly injections of 10⁵ median, tissue culture-infective doses of HVT-DEF antigens which were frozen and thawed three times. Blood was withdrawn by cardiac puncture 10 days after the last inoculation, and the serum was collected. The serum was treated with saturated ammonium sulfate to precipitate the gamma globulin fraction (2). The globulin fraction was then conjugated with fluorescein isothiocyanate by the method of Cherry et al. (2). To reduce nonspecific fluorescence, the conjugate was absorbed three times with tissue powder prepared from FCS and 10-day-old duck embryos. Each adsorption was allowed to stand for 1 hr at 4°C. After each adsorption, the conjugate was centrifuged at 10,000 × g for 30 min, and the supernatant fraction
was collected. After the last adsorption, the conjugate was divided into 0.5-ml samples and stored at -70°C until use.

When HVT-infected HK cell cultures began to show cytopathic effects (CPE) characteristic of herpes-type infection, cover slips were removed, fixed in acetone for 2 min, and examined by using the direct fluorescent-antibody (FA) method as described by Spencer et al. (10) with a Leitz-Ortholux (FA) microscope with a 150-w high-pressure xenon bulb with ultraviolet filters BG 38, BG 12, and K 530. For determination of the specificity of the conjugate, cover slips from control HK cell cultures, HVT-infected HK cell cultures treated first with unlabelled HVT antiserum, DEF cultures, and HVT-infected DEF cultures were treated with conjugate and examined.

Cytopathic changes. When cytopathic areas were visible on HVT-infected HK cell cultures, cover slips were removed, fixed in Bouins fixative, and stained with hematoxylin and eosin (HE).

Serum neutralization (SN) test. Cell-free HVT was prepared from cell-virus pools which were frozen and thawed twice in medium 199 containing 10% dimethyl sulfoxide and 10% FCS, and passed through membrane filters (Millipore Corp.) with a pore diameter of 0.45 nm. The serum was first diluted 1:5 in PBS and then into twofold dilutions. The various serum dilutions were mixed with an equal volume of filtered virus, allowed to stand at room temperature for 30 min, and then placed on DEF cultures. An agar overlay was applied after 4 days. The neutralizing antibody titer was expressed as the highest serum dilution which caused a 50% reduction in plaque count (Table 1).

Complement fixation test. The complement fixation (CF) test as described by Hamdy and Sevoian (4) was used to quantitate CF antigens in HVT-infected HK cell cultures.

Electron microscopy. Preparations of fourth-passage HVT-infected and control HK cell cultures were scraped from petri dishes with a rubber policeman and fixed in 1% glutaraldehyde, followed by washing in 0.1 M cacodylate buffer and further fixation in 1% osmium tetroxide. The preparations were then dehydrated in graded alcohols, infiltrated, and embedded in Epon (Ernest Fullman, Inc., New York). After polymerization for 24 hr at 61°C, the blocks were sectioned with a Porter Blaum-11 microtome. Photomicrographs were taken with a Phillips 200 electron microscope.

RESULTS

CPE. On first passage, HK cell cultures inoculated with HVT did not show any CPE for the first 7 days postinoculation, but after that time the cell cultures began to show rounding, clumping, and retraction of cells.

Twelve days after infection, the infected HK cell cultures of the first passage were trypsinized and collected in 2 ml of medium 199. A portion (0.5 ml) of this culture was placed on DEF to determine whether the virus was still active, indicated by the characteristic CPE on fibroblasts as described by Witter (12). The remainder (1.5 ml) of the infected HK cell cultures was transferred to new primary HK monolayers. The same procedure was followed for noninfected control HK cell cultures. After 4 days of the second passage, CPE characteristic of herpes-type infection was seen on DEF cultures, whereas CPE was not observed until 7 days on HK cell cultures. No CPE was observed on normal control HK cell cultures or DEF. The infected HK cells were transferred again to newly prepared HK monolayers and DEF; CPE was observed in third-passage infected HK and DEF cultures after 4 days (Fig. 1), but not in the parallel control cultures. These cultures have been passed through seven passages of HK cells with continued CPE characteristic of HVT infection. Similarly, the BS strain of HVT exhibited CPE on HK cell cultures and has been carried through six passages. Again, uninoculated control HK cell

| Passage | Primary plaques per plate on hamster kidney* | FA | SN | CF | VI on DEF primary plaques per plate† | Control HK‡ |
|---------|---------------------------------------------|----|----|----|-------------------------------------|-----------|
| FC-125 1* | 0 | ND | ND | ND | 20 | – |
| FC-126 2* | 10 | + | ND | ND | 50 | – |
| FC-125 3* | 55 | + | 1:40 | ND | 60 | – |
| FC-126 4* | 55 | + | 1:40 | ND | 60 | – |
| FC-126 5* | 60 | + | ND | ND | 45 | – |
| FC-126 6* | 80 | + | ND | ND | 90 | – |
| BS 1* | 0 | ND | ND | ND | 10 | – |
| BS 2* | 7 | + | ND | ND | 25 | – |
| BS 3* | 15 | + | 1:40 | ND | 30 | – |
| BS 4* | 20 | + | ND | ND | 30 | – |
| BS 5* | 40 | + | ND | ND | 35 | – |

* Abbreviations: ND, not done; CF, complement fixation; SN, serum-neutralization titer of HVT antiserum against HVT from HVT-infected HK cell cultures; FA, fluorescent antibody.
† Inoculum: 1.5 ml/culture.
‡ VI, Isolation of FC-126 and BS strains from infected hamster kidney cultures transferred on to DEF to see whether the viral agents are still active and to demonstrate differences, if any, from characteristic HVT infection on DEF. Inoculum: 0.5 ml/culture.

‡ HK controls were overlaid onto new primary cultures with each passage of infected HK cultures.
cultures and DEF cells had no CPE. The HVT-infected DEF cell cultures which were replated onto new plates, which had not been previously seeded with DEF, showed only a few cells which had settled and remained viable. Upon third passage no seeding of either HVT-infected or noninfected DEF cells was observed.

Cover slips from infected HK cultures stained with HE showed type A intranuclear inclusions (Fig. 2), whereas no inclusions were seen in noninfected HK control cultures (Fig. 3). Upon fluorescent microscope examination, infected HK cell cultures (stained with conjugate by using the direct method) showed intranuclear fluorescence (Fig. 4). Cytoplasmic fluorescence was also observed and appeared somewhat granular. HVT-infected DEF also

---

Fig. 1. Cytopathic effect of HVT on third passage hamster kidney cell culture 5 days after inoculation. Notice rounded refractile cells. Unstained; x6.

Fig. 2. Fourth passage hamster kidney infected with HVT 5 days after infection. Note the many intranuclear inclusion bodies (arrows). HE stained; x97.
showed nuclear and cytoplasmic fluorescence, whereas noninfected HK and DEF cell cultures, and HVT-infected HK cell cultures treated with unconjugated HVT antiserum before staining, were all negative.

**CF and SN.** CF antigens were demonstrated in both DEF- and HK-infected cultures by using antisera prepared in rabbits. Specific CF antigen from fourth passage HVT-HK cell cultures was demonstrated above a 1:32 dilution. Control antigens (normal DEF and HK cell cultures) did not fix complement with anti-HVT rabbit antiserum, nor did the normal control rabbit serum when tested with HVT-infected HK and DEF cell cultures. The CF test was considered positive when complement-fixing activity was above 1:4.

The SN test demonstrated that HVT grown on HK cell cultures was neutralized by HVT antiserum prepared in rabbits. A serum dilution of 1:40 gave a 50% reduction in plaque counts in third- and fourth-passaged HVT-HK (FC-126) cell cultures as well as in third passage HVT-HK cell cultures with the BS strain (Table 1). The 1:40 SN titer was comparable to the SN titer of HVT-infected DEF cultures.

**Electron microscopy.** Electron microscope examination of fourth passage HVT-infected HK cell cultures showed herpesvirus-type particles (Fig. 5), whereas viral particles were not

---

**Fig. 3.** Control hamster kidney. HE stained; ×97.

**Fig. 4.** Fourth passage HVT-infected kidney cell culture stained by the direct fluorescent-antibody method. Note nuclear fluorescence. ×400.
observed in control HK cell cultures. The viral particles appeared as hexagonal-shaped capsids, some containing nucleoids. Electron-lucent crosses, characteristic of HVT infection (7) were also observed. Because of vast cell destruction, it is difficult to ascertain whether the viral particles are located in the nucleus or cytoplasm, or both.

DISCUSSION

The data indicate that HK cells are susceptible to infection with two strains of HVT. The evidence includes both morphological and immunological examinations. Morphological observations included the presence of highly refractile rounded cells, the demonstration of type A intranuclear inclusions in epithelial cells of infected areas, and the demonstration of herpes-type virus particles in HVT-infected HK cell cultures by electron microscopy. Further evidence for HVT infection was given by the demonstration of HVT virus-specific CF and immunofluorescent antigens in HVT-infected HK cell cultures. Moreover, specific anti-HVT-antisera could neutralize the infectivity of HVT-infected HK cell cultures. These results are in agreement with A. Y. Elliot, University of Minnesota (personal communication), who has also concomitantly succeeded in infecting HK cell cultures with HVT.

It is improbable that the morphological and immunological changes of HVT-inoculated HK cell cultures was due to residual HVT infectivity from DEF as the plaque count increased with serial passage in HK cell cultures, indicating the development of a higher virus titer. Also HVT infection is known to be of the lytic form, and it would not be expected that the HVT-infected DEF inoculum would last through several passages. No attempts were made to investigate the absolute amounts of virus synthesized, for the primary purpose of this investigation was to see whether HVT could infect the HK cell cultures. No CPE was observed in noninfected control cultures which had been similarly passed in parallel onto primary HK cells.

It is most unlikely that the morphological and immunological changes observed in HVT-infected HK cultures were due to a latent virus of hamsters since control uninoculated HK cultures passed in the same manner as infected cultures did not show CPE or the presence of virus particles upon electron microscope examination. The viral particles observed in HVT-infected HK cell cultures were identical to those described by Nazerian (6) for HVT.

ACKNOWLEDGMENTS

Thanks are given to Farouk Hamdy for the electron micrographs and to Patricia Rust for her technical assistance.

This research was supported by Public Health Service grant CA-13272-01-VR from the National Cancer Institute.

LITERATURE CITED

1. Calnek, B. W., S. H. Madin, and A. J. Kniazeff. 1969.
Susceptibility of cultured mammalian cells to infection with a herpesvirus from Marek's disease and T-virus from reticuloendotheliosis of chickens. Amer. J. Vet. Res. 30:1403-1412.
2. Cherry, W. B., M. Goldman, and T. R. Carski. 1960. Fluorescent antibody techniques in the diagnosis of communicable disease. Publication no. 729. U.S. Dept. Public Health Service, Department of Health, Education, and Welfare, Washington, D.C.
3. Esposito, J., P. D. Lukert, and C. S. Eidson. 1972. Marek's disease herpesvirus: growth and detection in vivo and in vitro. Amer. J. Vet. Res. 33:171-175.
4. Hamdy, F., and M. Sevoian. 1970. Complement fixation reaction for the diagnosis of type II avian (Marek's) leukemia. Appl. Microbiol. 20:356-361.
5. Madin, S. H., B. S. Andriese, and N. B. Darby. 1957. The in vitro cultivation of tissues of domestic and laboratory animals. Amer. J. Vet. Res. 18:932-941.
6. Nazerian, K., L. F. Lee, R. L. Witter, and B. R. Burmester. 1971. Ultrastructural studies of a herpesvirus of turkeys antigenically related to Marek's disease virus. Virology 43:442.
7. Nazerian, K., J. J. Solomon, R. L. Witter, and B. R. Burmester. 1968. Studies on the etiology of Marek's disease. II. Findings of herpesvirus in cell culture. Proc. Soc. Expt. Biol. Med. 127:177-182.
8. Okazaki, W., H. G. Purchase, and B. R. Burmester. 1970. Protection against Marek's disease by vaccination with a herpesvirus of turkeys. Avian Dis. 14:413.
9. Purchase, H. G., B. R. Burmester, and C. H. Cunningham. 1971. Response of cell cultures from various avian species to Marek's disease virus and herpesvirus of turkeys. Amer. J. Vet. Res. 32:1811-1823.
10. Spencer, J. L., and B. W. Calnek. 1970. Marek's disease: application of immunofluorescence for detection of antigen and antibody. Amer. J. Vet. Res. 31:345-358.
11. Witter, R. L., K. Nazerian, H. G. Purchase, and G. H. Burpeyne. 1970. Isolation from turkeys of a cell associated herpesvirus antigenically related to Marek's disease virus. Amer. J. Vet. Res. 31:525-538.