Use of Japanese Quail Embryo Fibroblast Cells for Propagation and Assay of Turkey Herpesvirus FC-126

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Japanese quail (Coturnix coturnix japonica) fibroblast cell culture monolayers were found to provide a very satisfactory system in which to propagate and assay turkey herpesvirus FC-126, which is used for production of Marek's disease vaccine. Japanese quail cells were more sensitive than duck cells and of approximately equal sensitivity to chicken cells. Foci of infection developed rapidly and uniformly, were of larger size, and were more easily discernible in quail cells than in chicken cells.

The use of a group B turkey herpesvirus (HVT) as a vaccine for protection of chickens against Marek's disease (MD) has proved to be highly successful (6, 10). A majority of the commercial poultry flocks raised in this country now receive HVT vaccine as a routine part of the total disease control program. Quality control in HVT vaccine production laboratories as well as surveillance activities by vaccine regulatory agencies necessitate accurate assays of viral content. Basic investigations in the research laboratory also require virus propagation and accurate quantitating systems.

Even though the cultured cells of a number of avian species have been shown to be susceptible to HVT (9), chicken embryo fibroblast (CEF) or duck embryo fibroblast (DEF) monolayers are most often employed for commercial propagation and laboratory study. The susceptibility of Japanese quail embryo fibroblast (JQEF) cells to HVT has been reported by Ono et al. (7) and Purchase et al. (9). After considerable adaptation, Onoda et al. (8) were able to successfully propagate Biken C strain MD virus of chickens in JQEF cells. The purpose of this study was to evaluate the quantitative and qualitative efficiency of JQEF cells for laboratory propagation and assay of HVT.

**MATERIALS AND METHODS**

**Cell cultures.** Fertile chicken eggs, duck eggs, and Japanese quail eggs were obtained from SPAFAS, Inc., Norwich, Conn., Maag and Easterbrooks, Inc., Raleigh, N.C., and North Carolina State University Department of Poultry Science, respectively. Embryos near the mid-point of their respective incubation periods (i.e., 14-day duck, 11-day chicken, and 8-day quail embryos) were used. Embryos were removed aseptically from the embryonated eggs, and primary CEF, DEF, and JQEF cells were prepared essentially as described by Witter et al. (12). Approximately \( 8 \times 10^4 \) cells in 4 ml of culture medium were seeded into plastic culture dishes (15 by 60 mm) (Falcon Plastics, Oxnard, Calif.). Complete monolayers were formed within 24 h.

**Culture medium.** Culture medium consisted of 0.25% lactalbumin hydrolysate, 0.1% yeast autolysate, and 10% tryptose phosphate broth, in Earle balanced salt solution. Penicillin, 500 U; streptomycin, 125 \( \mu g \); and mycostatin 50 \( \mu g \) (E. R. Squibb and Sons, New York, N.Y.). per ml were added, and the medium was adjusted to pH 7.2 by addition of sodium bicarbonate solution. Growth medium contained 5% fetal bovine serum (FBS), and maintenance medium contained 0.5% FBS.

**Viruses.** Serials (22) of MD vaccine were obtained from 3 commercial manufacturers: Agri-Bio Corp., Ithaca, N.Y.; Maag and Easterbrooks, Inc., Raleigh, N.C.; and Salsbury's Laboratories, Charles City, Iowa. Viruses contained in all commercial vaccines originated from HVT FC-126 isolated and described by Witter et al. (13).

**Titers of commercial MD vaccine.** The 22 serials of commercial MD vaccine were assayed for viral content on JQEF and CEF monolayers essentially as described by Caine et al. (1). Foci of infection were enumerated on the 4th day after inoculation.

**Growth profile of HVT in QEF, CEF, and DEF.** Serial 10-fold dilutions of cells infected with HVT through \( 10^{-4} \) were prepared in growth medium. Twenty-four-hour CEF and JQEF monolayers were inoculated with 0.5 ml of each dilution as described by Caine et al. (1). One culture dish each of CEF and JQEF which had been inoculated with the \( 10^{-1} \) dilution were removed after 24, 48, 72, and 96 h of incubation. Infected cell monolayers were removed by trypsinization, and serial 10-fold dilutions were prepared. Virus content was assayed by inoculation onto...
primary 24-h CEF and JQEF monolayers as described above. In a second trial, the growth profile of HVT was compared in DEF and JQEF monolayers. The procedure was the same as described above except that subtitrations were continued for 5 days rather than 4.

Propagation of HVT in CEF and JQEF monolayers in roller bottles. Methods used for propagation of cells and HVT in roller bottles were essentially as described by Lee (3). Four bottles each of CEF and JQEF monolayers were prepared in small prototype bottles approximately 3.5 cm in diameter by 10.0 cm in length. The bottles were rotated by a multi-purpose rotator (Scientific Industries, Inc., Springfield, Mass.) at 0.2 rpm in an incubator maintained at 37 C. Each roller bottle was seeded with 6 ml of culture medium containing approximately 3 x 10^4 cells per ml. After 24 h of incubation, the monolayers were 80% confluent. Growth medium was removed, and each bottle was inoculated with 6 ml of an infected cell suspension which contained approximately 2 x 10^6 focus-forming units (FFU) of HVT. The inoculum was removed after 24 h and replaced with maintenance medium. Infected cell monolayers were removed by trypsinization 72 h after inoculation, and viral content was assayed as previously described (1).

Primary isolation of HVT in JQEF cells. Primary isolation of HVT from a commercial turkey flock was attempted by inoculating 0.2 ml of freshly drawn whole blood (collected in 20 U of heparin per ml) onto JQEF monolayers, and by co-cultivating a mixture of primary adult turkey kidney cells and JQEF cells.

RESULTS

Cytopathology in JQEF cells. Enlarged refractile cells were often visible 24 h postinoculation (PI) in JQEF cells infected with HVT (Fig. 1). At 48 h PI, foci of infection consisted of groups of two or more enlarged cells which were either rounded or angular in appearance (Fig. 2). Foci became quite large by 72 h PI (Fig. 3) and often covered the entire microscopic field at 96 h PI (Fig. 4). As previously noted by Purchase et al. (9), individual infected cells were considerably larger in JQEF monolayer cells than in CEF cells. Typical foci of infected cells were also much larger at 4 days PI in JQEF than in CEF cells, and were equal in size or slightly larger than foci seen in DEF cells (Fig. 4-6).

Titers of commercial MD vaccine. Titers of 15 commercial MD vaccine serials which were
TABLE 1. Comparison of commercial MD vaccine titers in Japanese quail embryo fibroblast and chicken embryo fibroblast cells

| Commercial MD vaccine serial | Titer in JQEF cells* | Titer in CEF cells* |
|-----------------------------|----------------------|---------------------|
| 1                           | 3.74                 | 3.88                |
| 2                           | 4.04                 | 3.93                |
| 3                           | 3.79                 | 4.08                |
| 4                           | 3.77                 | 4.08                |
| 5                           | 3.90                 | 3.79                |
| 6                           | 3.79                 | 3.88                |
| 7                           | 3.83                 | 4.04                |
| 8                           | 4.04                 | 4.04                |
| 9                           | 4.04                 | 3.88                |
| 10                          | 4.15                 | 4.15                |
| 11                          | 3.95                 | 3.81                |
| 12                          | 4.15                 | 4.11                |
| 13                          | 3.85                 | 3.78                |
| 14                          | 4.45                 | 4.40                |
| 15                          | 4.56                 | 4.41                |

* Mean and standard deviation, 4.00 ± 0.23.

** Mean and standard deviation, 4.02 ± 0.20.

† Log_{10} FFU per ml of vaccine when diluted for use according to manufacturer's instructions.

evaluated in both CEF and JQEF are presented in Table 1. Titers of these serials in CEF ranged from a low of 3.81 to a high of 4.41 (log_{10}), whereas titers of the same serials were 3.74 to 4.56 in JQEF. Average titers for the 15 vaccine serials were 4.02 in CEF and 4.00 in JQEF. CEF monolayers failed to provide a satisfactory assay system for seven serials due to erratic drops in titer between 10-fold dilutions. However, uniform titrations of all 22 serials were achieved in JQEF cells. All serials of commercial vaccine contained 1,000 FFU or more per 0.2-ml chicken dose when diluted according to manufacturer's instructions.

Growth profile of HVT in JQEF, CEF, and DEF. Growth profiles of HVT in JQEF and CEF are compared in Fig. 7 and 8. Virus yields were similar in JEF and CEF, with maximum virus titers present at 48 to 72 h PI. A 10-fold or greater decrease in virus titer had occurred by 96 h PI. Daily assays for virus in JQEF or CEF produced similar results, whether the inoculum originated from JQEF or CEF.

Growth profiles of HVT in JQEF and DEF are compared in Fig. 9 and 10. Even though the inoculum for JQEF and DEF was identical, there appeared to be an approximate 1 log_{10}
FIG. 11. Thin section of JQEF-inoculated HVT. Viral capsids (VP) containing cores of varying morphology may be seen within the nucleus (N). ×32,250.

FIG. 12. Intranuclear and intracytoplasmic HVT virus particles (VP) may be seen in the thin sections of a JQEF cell. Virus particles with cross-shaped cores may be seen within the nucleus. Note the 30-nm particles (single arrow) within the nucleoplasm. ×24,250.
difference between zero-time values when the infected cells were titered in the two cell systems. Maximum virus yields were obtained in both JQEF and DEF 72 h PI. The HVT level in JQEF had dropped drastically by 96 h PI; however, the virus present in infected DEF remained at a relatively high level for at least 120 h. Daily titrations of HVT in JQEF were always higher than in DEF by a factor of 5 to 21 times.

**Propagation of HVT in JQEF and CEF monolayers in roller bottles.** Survival of JQEF monolayers in roller bottles was satisfactory. Inoculation of $2.0 \times 10^4$ FFU or HVT onto 24-h JQEF cell cultures resulted in a virus yield of $1.2 \times 10^7$ FFU per bottle on the 3rd day PI. Identical virus yields were observed in roller bottle cultures of CEF cells which received the same inoculum.

**Primary isolation of HVT in JQEF cells.** Inoculation of 24-h primary JQEF monolayers with turkey blood and co-cultivation of primary adult kidney cells with JQEF cells resulted in the development of foci of infection typical of HVT within 5 to 7 days. One isolate was subsequently passed 22 times in JQEF cells, achieving titers as high as $6.7 \times 10^6$ FFU per 60-mm culture dish.

**Electron microscopy.** Electron microscopy studies of JQEF cells infected with HVT demonstrated intranuclear and intracytoplasmic herpesvirus particles (Fig. 11, 12). The virus particles measured approximately 90 to 110 nm in diameter and contained nucleoids of varying size and electron density. Particles with cross-shaped capsids similar to those reported by Nazerian et al. (4), Okada et al. (5), and Colwell et al. (2) were frequently encountered (Fig. 12). Electron-dense particles approximately 30 nm in diameter assumed to be core components (5) were observed within the nucleoplasm (Fig. 12).

**DISCUSSION**

The rapid development and large size of foci of infection produced by HVT in JQEF cells afforded definite advantages in that counts could be made easily and more rapidly than virus titrations utilizing CEF cells. JQEF cells were uniformly sensitive to HVT whether the virus originated from vaccines propagated on CEF or DEF, or from initial field isolations. Satisfactory titrations of HVT were achieved in 33 separate preparations of JQEF cells, whereas unsatisfactory titration was not experienced in any JQEF cell preparations. Erratic titration results were observed in 7 of 27 preparations of CEF cells. Infection producing extensive cytopathic effects in CEF cells was always achieved after massive inoculation with HVT-infected cells. However, lighter inoculation of 10-fold dilutions often produced fewer than the expected number of foci of infection for a given dilution, or none at all. The underlying cause of the non-uniform sensitivity of CEF cells is not known, but a similar phenomenon has been observed in other laboratories.

Size of the foci of infection and ease of enumeration in virus titrations were approximately equal for JQEF cells and DEF cells. However, DEF cells were shown in individual experiments to be 5 to 21 times less sensitive than JQEF for HVT virus assay. This lack of sensitivity was overcome also by massive inoculation since DEF cells inoculated with high levels of HVT-infected cells produced virus yields equivalent to those produced in JQEF cells.

Unsurpassed sensitivity, uniform susceptibility, and ease of enumeration of foci of infection indicate that JQEF can provide a highly satisfactory cell system for titration of HVT virus. Successful propagation of HVT in JQEF monolayers in roller bottles also suggests their potential value for commercial vaccine production. The small size and resulting low cell yield per quail embryo would be partially off-set by the economy of maintaining a Japanese quail breeding flock.

**LITERATURE CITED**

1. Calnek, B. W., C. Garrido, W. Okazaki, and I. V. Patrascu. 1972. In vitro methods for assay of turkey herpesvirus. Avian Dis. 16:52-56.
2. Colwell, W. M., C. F. Simpson, L. E. Williams, Jr., and D. F. Forrester. 1973. Isolation of a herpesvirus from wild turkeys in Florida. Avian Dis. 17:1-11.
3. Lee, L. F. 1971. Large-scale production of Marek’s disease virus. Avian Dis. 15:565-571.
4. 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 43:442-452.
5. Okada, R., Y. Fujimoto, T. Mikami, and K. Yonehara. 1972. The fine structure of Marek’s disease virus and herpesvirus of turkeys in cell culture. Jap. J. Vet. Res. 20:57-68.
6. Okazaki, W., H. G. Purchase, and B. R. Burmester. 1970. Protection against Marek’s disease by vaccination with a herpesvirus of turkeys. Avian Dis. 14:413-429.
7. Ono, K., K. Koyama, T. Doi, T. Ishikawa, T. Konobe, M. Naito, and S. Kato. 1971. Studies on the herpes type virus of turkeys isolated. Proc. 72nd Meet. Jap. Vet. Sci. Ass., Tottori, Japan.
8. Onoda, T., K. Ono, T. Konobe, M. Naito, Y. Mori, and S. Kato. 1970. Propagation of a herpes type virus isolated from chickens with Marek’s disease in Japanese quail embryo fibroblasts. Biken J. 13:219-228.
9. Purchase, H. G., B. R. Burmester, and C. H. Cunningham. 1971. Responses of cell cultures from various avian species to Marek’s disease virus and herpesvirus of turkeys. Amer. J. Vet. Res. 32:1811-1823.
10. Purchase, H. G., W. Okazaki, and B. R. Burmester. 1972. Long-term field trials with the herpesvirus of turkey vaccine against Marek's disease. Avian Dis. 16:57-70.
11. Simmons, D. G., W. M. Colwell, K. E. Muse, and C. E. Brewer. 1972. Isolation and characterization of an enteric reovirus causing high mortality in turkey pouls. Avian Dis. 16:1094-1102.
12. Witter, R. L., G. H. Burgoyne, and J. J. Solomon. 1968. Preliminary studies on cells infected with Marek's disease agent. Avian Dis. 12:169-185.
13. Witter, R. L., K. Nazerian, H. G. Purchase, and G. H. Burgoyne. 1970. Isolation from turkeys of a cell-associated herpesvirus antigenically related to Marek's disease virus. Amer. J. Vet. Res. 31:525-538.