IN VITRO METHODS FOR ASSAY OF TURKEY HERPESVIRUS [a]

INTRODUCTION

The discovery that an apparently nonpathogenic group B herpesvirus from turkeys (HVT) is antigenically related to Marek's disease virus (MDV) and can be employed as a vaccine for the protection of chickens against Marek's disease (MD) has led to widespread distribution of the virus. Many laboratories, large and small, are presently involved in the propagation of HVT for use as a vaccine. Most workers have been concerned with virus in its cell-associated form; however, recent methods for the production of high-titered, cell-free preparations have encouraged investigations with this form of virus also. Regardless of whether cellular or cell-free virus preparations are employed for vaccine, a most important consideration is the titer since there are minimum standards for vaccine dosage. Thus, the method of virus assay is important in order to provide uniformity within a laboratory and also among laboratories.

In 1970, the Leukosis Committee of the American Association of Avian Pathologists held a Workshop Conference and subsequently prepared a report dealing with methods in Marek's disease research. Unfortunately, there was little information at that time about special requirements, optimum conditions, or specific

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[a] A report by a subcommittee of the Leukosis Committee of the American Association of Avian Pathologists.

C. Garrido
W. Okazaki
I. V. Patrascu
B. W. Calnek, Chairman

problems encountered in in vitro assay of HVT. The purpose of this report is to present that kind of information. The report is divided into 2 sections, one dealing with cell-associated virus, the other with cell-free virus. In each case, suitable methods are described without attempting to provide an exhaustive list of possible alternatives.

CELL-FREE VIRUS

Various aspects of the in vitro assay system for cell-free virus are discussed without consideration of whether the preparation had been freshly prepared, stored frozen, or stored in the lyophilized state. The methods described should apply to all preparations.

1. Cell type, passage, and genetic strain. Suitable cell types for assay cultures include chicken embryo fibroblasts (CEF), derived from 9- to 11-day-old embryos, chicken embryo kidney cells (CEK), from 19- or 20-day-old embryos, and chicken kidney (CK), from 2- to 6-week-old chickens free of avian pathogens. CEF derived from a variety of genetic strains of embryos have been equally satisfactory. Foci in kidney cells generally are observable a little earlier and are morphologically more distinctive than those in CEF; however, the cell types are of equal sensitivity. Primary, secondary, or tertiary cultures of CEF have been found to be of about the same sensitivity in some cases; in others, primary cultures appeared to be up to 3-6 times as sensitive as secondary cultures. For uniformity, primary cultures are recommended. Titers in cultures of duck

embryo fibroblasts (DEF) may be only one-fourth those in other cell types.

2. Number of cells seeded per culture (60 mm Petri dish) . There should be enough cells seeded to provide a complete, or nearly complete monolayer at the time of inoculation or within 18-24 hours after inoculation. For primary CEF plated in 60 mm plastic Petri dishes, about 7.5×10^6 cells in 5 ml medium is optimal. With subcultured CEF, about 1.0×10^6 is suitable and for kidney cells, about 3.0 to 3.5×10^6 cells per 5 ml should be plated.

3. Culture age. The age of the assay culture at the time of inoculation is quite important in the case of cell-free virus ; in this respect the optimum assay conditions appear to be different from those for cell-associated virus. Inoculation of cells while in suspension before plating is inferior to inoculation of a monolayer, and cultures inoculated at 48 or more hours after plating usually are less sensitive than those inoculated after 24 hours of incubation. CEF attach and spread rapidly; 3-hour cultures have been found as sensitive as 24-hour cultures. CK cultures, on the other hand, attach and spread slowly. Even after 24 hours of incubation (the optimum time for inoculation of CK cultures), cell clumps have not fully spread and the monolayer often appears only one-half or two-thirds complete. By the next day, the CK monolayer should be complete.

4. Culture medium and incubation. A satisfactory medium consists of Medium 199 supplemented with 10% tryptose phosphate broth and bovine fetal serum (BFS). The level of BFS is usually 5% for CK cells and 2% for CEF during the first 24 hours (preinoculation) and is reduced to 1% or 0.5% (depending on

how heavy the monolayer is) after inoculation. An agar-overlay medium (same formulation but with about 0.8% agar) may be applied at 24 hours post-inoculation. Incubation should be at 38-39 C.

5. Virus diluent and inoculation procedure. Cell-free HVT which is to be inoculated immediately after dilution may be diluted in any of several solutions (PBS, tissue culture medium, SPGA stabilizer). If the inoculum is to be held for any period of time, especially at 4 C, it is important that the SPGA stabilizer be employed since cell-free virus is rapidly inactivated when suspended in PBS or tissue culture medium and held at 4 C. SPGA stabilizer contains the following: 0.218 M sucrose, 0.0038 M monopotassium phosphate, 0.0072 M dipotassium phosphate, 0.0049 M monosodium glutamate and 1% bovine albumin powder.

The incorporation of a chelator (EDTA) in the inoculum diluent does not improve the virus titer as it does with MDV and is not recommended. Variation of inoculum pH from 6.0 to 8.5 is apparently without effect on virus titer.

Virus adsorption is enhanced if the inoculum is applied to a drained monolayer. The inoculum volume should be small; 0.05 ml or less per 60 mm Petri dish culture is optimal. While virus adsorption in cultures incubated at 38-39 C is not complete until between 1 and 2 hours and penetration is not completed until about 3 hours postinoculation, 5 ml of medium should be added at 30 minutes postinoculation (without removal of the inoculum) to avoid deterioration of the culture.

6. Recommended procedure. (1) 24-hour primary CK cultures or primary CEF culture are drained and inoculated with 0.05 ml (per 60 mm Petri dish culture) of appropriate $\log_{10}$ dilutions of cell-free HVT. Inoculum dilutions should be made in SPGA or other suitable stabilizing diluent and should be pipetted vigorously (or briefly sonicated) to disperse virus clumps. (2) Cultures are incubated at 38-39 C for 30 minutes. (3) Five ml of culture medium are added to each culture and incubation continued. A medium change may be made at 24 hours postinoculation and then at 2- to 3-day intervals. Alternatively, an agar overlay may be applied at 24 hours with a "feed agar" layer applied after an additional 2 or 3 days. (4) Focal lesions appear after 2-3 days of incubation but final counts should not be made until 5 days postinoculation.

CELL-ASSOCIATED VIRUS

1. Cell type passage and genetic strain. Primary or secondary CEF cultures from several genetic lines of chickens are equally sensitive for assay of cell-associated HVT. Titers obtained on DEF cultures, however, have been found to be only one-third to one-half of the titers obtained on CEF cultures. In some instances CEF assay titers have been as high as 20 times that obtained on DEF. Chick kidney cells appear to be as sensitive to cell-associated HVT infection as CEF cultures.

2. Number of cells seeded per culture (60 mm Petri dish). The number of cells seeded per plate will definitely influence the assay results. A complete

monolayer is essential for expressing the full potential of a given inoculum when 24 hour cultures are used for the assay. To achieve this, at least 1.0×10^6 secondary CEF cells or 4.0×10^6 primary CEF cells are required per 60 mm plate on initial seeding. If a lesser number of cells are seeded and the monolayer is not completely formed at the time of infection, the titers obtained will be significantly lower. If the infection is made at the time of seeding (in suspension) at least 1.5×10^6 secondary CEF cells are required for the full expression of the plaque-forming potential in a given inoculum.

3. Culture age. The age of the culture (up to 48 hours) does not appear to be of great importance in the assay of cell-associated HVT. Equivalent titers have been obtained using 0-, 12-, and 48-hour secondary CEF cultures and 24-, 48-, and 72-hour primary CK cultures. It should be noted, however, that when titrations are made with CEF cells inoculated while in suspension (0 hours) or on 12-hour CEF monolayers, slightly greater numbers of cells are required to obtain the maximum plaque-inducing potential from the inoculum as described in the previous section.

4. Culture media and incubation. The generally acceptable cell culture media, which include: Medium 199, Eagles' Basal Medium (BME), or a mixture of F10 and Medium 199, each supplemented with 10% tryptose phosphate broth and 2% bovine fetal calf serum (BFS) or 4% calf serum (CS) appear to be comparable when titrating cell-associated HVT on CEF or DEF cells. The conditions of incubation most widely accepted are a humidified atmosphere of 4-5% CO_2

in air at temperatures ranging from 37.5° C to 39° C.

5. Inoculum diluent and inoculation procedure. Phosphate buffered saline or cell culture medium with 4% CS is a far superior diluting fluid than is PBS alone. Experiments have shown that the inactivation rate of cell-associated HVT held at room temperature (25° C) is significantly greater when suspended in PBS than when suspended in PBS with 4% CS or complete culture medium.

6. Recommended procedure. A suitable procedure for titrating cell-associated HVT is as follows: (1) After a medium change, fully formed 24-hour secondary CEF monolayers, initially seeded with $1.2\text{-}1.5 \times 10^6$ cells are inoculated with 0.5 ml of the appropriate $\log_{10}$ dilutions of cell-associated HVT, Unlike the situation with cell-free HVT, the inoculum volume of cell-associated HVT does not appear to have any effect on the resultant titer calculations, since most if not all of the infected cells settle to the monolayer to induce a focal area of CPE. (2) The cultures are then gently but thoroughly shaken to allow the infected cells to be evenly suspended and distributed in the fluid layer. (3) The cultures are returned to the incubator (38° C) and the infection and resultant cytopathic effect (CPE) allowed to progress with media changes made at 48-hour intervals.

Agar overlay may be applied at 24 hours, if desired. Additional agar medium is added after 2 or 3 days. Focal areas of cytopathic alteration are enumerated on the 4th or 5th day after infection. If enumeration is made after the 5th day,

secondary plaques will be found and therefore may confound the titration.