A SIMPLE, RAPID METHOD FOR PREPARATION OF VIRUS ISOLATES FROM CELL CULTURE FOR ELECTRON MICROSCOPY

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SUMMARY: A simple procedure for the rapid preparation of virus isolates from cell culture for negative-contrast electron microscopy was devised. Using only conventional centrifugation steps (i.e. without ultracentrifugation), the procedure produced consistent, fine-quality preparations of a variety of virus types differing in size/shape and buoyant density.

Key words: virus isolates; cells, cultured; electron microscopy; centrifugation.

I. INTRODUCTION

Negative-contrast electron microscopy (NCEM) is a simple and efficacious means of visualizing viruses present in cell culture supernatants and in clinical specimens of various kinds (1,2,7,12,21). Several NCEM approaches for improved results involving concentration of samples by centrifugation have been described (7,8,13,17,22,23). Conventional methods have utilized preparatory low-speed centrifugation (clarification) to remove unwanted debris, followed by ultracentrifugation to pellet virus particles (6–8,11,21,23).

We report here a simple and rapid procedure for the preparation of virus particles from cell culture material for NCEM that eliminates the need for an ultracentrifugation step. This technique is used routinely in our laboratory and has proven to be extremely useful for the rapid detection of viruses present in small quantities of cell culture material.

II. MATERIALS

A. Cell culture

Minimum essential medium Eagle, with Earle's balanced salt solution without L-glutamine, No. 12-125Y, M.A. Bioproducts¹
HyCloneTM fetal bovine serum, Lot no. 100418, HyClone Laboratories²
L-Glutamine (100×), No. 320-5030, GIBCO³
Penicillin-streptomycin solution, No. 600-5070³
Gentamycin sulfate, No. G-7632, Sigma⁴
Trypsin-EDTA (1X), No. 610-5300³
Culture tubes, polystyrene, 16 × 125 mm, No. 25200, Corning⁵

Shel-lab incubator, CO₂, dual-chamber, No. 350, Sheldon⁶
Roller drum, No. 1240, Lab-line⁷

B. Centrifugation

Freezer, biological, to –90° C, No. 8326, Forma⁸
Vortex mixer, No. 1290⁷
Conventional centrifuge, No. UV, IEC⁹
Micro tubes, polypropylene, 43 × 10 mm, No. 72.692, Sarstedt¹⁰
Bench-top centrifuge, CENTRA^R-4, No. 2382⁹, equipped with model IEC 817 fixed-angle rotor⁹

C. Electron microscopy

MILLI-Q^R Water Purification System, Millipore¹¹
Parafilm^R, American Can¹²
Grids, 300C (300 mesh, copper), Ted Pella¹³
Formvar solution (0.5%), No. 01582, Electron Microscopy Sciences¹⁴
Phosphotungstic acid (1.5%, pH 7.0), No. 19403¹³
Folded filter paper, 18.5 cm, No. 12, Whatman¹⁵
Germicidal lamp, No. G30T8, GTE Sylvania¹⁶
Transmission electron microscope, No. EM 300, Philips¹⁷

III. PROCEDURE

1. Passage clinical materials into culture tubes containing appropriate cell monolayers and 1.5 ml cell culture medium.
2. Incubate tubes at 37° C on a roller drum (0.33 rpm) until 4+ cytopathic effect is observed.
3. Harvest cultures by one or more freeze-thaw cycles (–70° C).
4. Vortex cultures and clarify at 850 × g for 10 min (room temperature) in a conventional centrifuge.
5. Transfer supernatants to polypropylene micro tubes

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- and centrifuge at $2500 \times g$ for 10 min (room temperature) in a CENTRA^R-4 bench-top centrifuge equipped with a model IEC 817 fixed-angle rotor.
6. Transfer supernatants to fresh micro tubes and respin at $8850 \times g$ for 20 min (room temperature) in the CENTRA^R-4.
 7. Resuspend pellets in 10 μ l sterile-filtered distilled water.
 8. Transfer one drop of each sample to Parafilm^R.
 9. Float individual Formvar-coated copper grids for 2 min on each drop, then blot dry with filter paper.
 10. Touch each grid to a drop of sterile-filtered distilled water, then blot dry with filter paper.
 11. Float each grid for 1 min on a drop of 1.5% phosphotungstic acid (pH 7.0), then remove excess stain with filter paper.
 12. Leave all grids under ultraviolet light for at least 15 to 20 min.

13. Examine grids with an electron microscope at an accelerating voltage of 80 kV.

IV. DISCUSSION

Viruses of infected cell cultures identified by NCEM have included members both of DNA (Adenoviridae, Herpesviridae, Poxviridae) and RNA (Picornaviridae, Caliciviridae, Coronaviridae, Reoviridae, Paramyxoviridae) virus families (Fig. 1). This technique has permitted detection of a wide variety of viral agents differing in size/shape and buoyant density. In our experience it has shown itself to be an especially reliable procedure for the rapid morphological identification of new virus isolates from cell culture. Only small volumes of material are required; with the development of cytopathic effect in a single culture tube, enough material is provided not only to passage the isolate onto

FIG. 1. Representative examples of selected virus families, as revealed by NCEM. A, Adenoviridae (No. WADDL 82-5536-12), bovine, from bovine turbinate cell culture; B, Herpesviridae (No. M255T), bovine, from calf kidney cell culture; C, Poxviridae ("Pandora"), cetacean, from cutaneous lesion; D, Picornaviridae (No. OSU 475R), bovine, from calf kidney cell culture; E, Caliciviridae (No. SDZ 041), cetacean, from Vero cell culture; F, Coronaviridae (No. M254T), bovine, from calf kidney cell culture; G, Reoviridae (No. OSU 568R), bovine, from bovine turbinate cell culture; H, Paramyxoviridae (No. M14CN), bovine, from calf kidney cell culture; I, Paramyxoviridae (No. OSU 463CN), bovine, from Vero cell culture, illustrating typical "herringbone" nucleocapsid. Bars = 100 nm. A through H, $\times 108\,571$; I, $\times 81\,905$. Isolate A prepared from material kindly provided by Dr. J. F. Evermann, Washington Animal Disease Diagnostic Laboratory, Washington State University, Pullman, Washington.

fresh monolayers but also to examine it by NCEM at the same time. In many cases we used this technique in conjunction with immune electron microscopy for preliminary serologic identification of isolates. It is useful for the identification both of cytopathic and non-cytopathic isolates, and we have obtained successful results by direct examination of clinical specimens, including feces and skin scrapings (Fig. 1 C). The success of the procedure lies in part in the intermediate centrifugation step ($2500 \times g$ for 10 min), which effectively cleans the preparation by removing most of the nonviral, particulate (cellular) debris remaining after preliminary clarification. Of corresponding significance is the final, moderate-speed centrifugation step ($8850 \times g$ for 20 min) which we have found to be most effective for pelleting many viruses and a desirable alternative to ultracentrifugation. The resulting pellets have produced clear, even spreading of virus particles on grids, with minimal amounts of unwanted virion aggregation ("clumping"), background staining, and subcellular debris (a frequent contaminant of ultracentrifuged preparations). Importantly, the ultrastructural integrity of most virus particles has been well preserved.

The major advantages of NCEM that have been cited traditionally are its speed, simplicity, and capability of identifying viruses on a morphological basis without the need for intermediate, specific reagents (1,2,6,7,12). Its major disadvantage is a relative insensitivity when compared to certain other techniques, such as enzyme immunoassay, radioimmunoassay, or virus isolation (1,2,22). Because of this, various preparatory procedures for concentrating virions from sample material have been described. These have included ultracentrifugation (3,7,8,19,20), pseudoreplication (10,16), density gradient centrifugation (4,9), immune electron microscopy (1,6,14,19,21,23), ammonium sulfate precipitation (5), and polyacrylamide hydrogel absorption (22). Some have concentrated viruses by simple low-speed centrifugation (13,17), although the validity of this type of procedure has been questioned (18). Of all these methods, ultracentrifugation has received without doubt the widest acclaim and acceptance for rapid viral diagnosis (1,6–8, 11,17). However, ultracentrifugation is not without its own disadvantages (sedimentation time, disruption of virion morphology, sedimentation of subcellular debris, requirement for ready access to an ultracentrifuge) (9,15,20,22). With the procedure described in this report, the need for a relatively prolonged (1 or 2 h) ultracentrifugation step has been eliminated, decreasing sample manipulation and resulting in even greater simplification of the process. Importantly, the time required for the preparation of samples has been reduced considerably. Search time for the location and identification of isolates with the electron microscope has been minimized (usually averaging less than 1 min, frequently much less) by the fine, even quality of the

final preparations. Although some degree of virion loss is inevitable in the two lower-speed centrifugation steps, this had had little effect on our ability to rapidly locate and identify isolates from cell culture supernatants. This procedure will probably prove useful for the detection and identification of a broad range of virus isolates in addition to those described in the present communication.

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