

# Electron Microscope Methods for the Identification of Adenoviruses Isolated in Micro Tissue Cultures<sup>1</sup>

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Received for publication 4 August 1971

A tissue culture micromethod is described for adenovirus isolation and preparation for presumptive identification by electron microscopy. These procedures are easier, more economical, and faster than conventional methods. The micro techniques make it more feasible to utilize direct visualization of virus in infected cells as an adjunctive diagnostic and research tool.

Micromethods for cell cultivation in disposable plastic plates have been shown to be reliable, rapid, and economical techniques for the sero-diagnosis of viral infections (3, 4). Recent investigations have demonstrated that these methods also can be used for the isolation of various respiratory disease viruses, particularly, the adenoviruses (E. J. Sullivan, manuscript in preparation). In addition to the technical advantages mentioned above, the recognition of viral cytopathic effect (CPE) is facilitated by the micro technique. It was hypothesized that if electron microscopy could be applied to the specimen, at the time CPE was first evident, then direct visualization of viral morphology might be obtained. This procedure would further decrease the time required for identification of a viral isolate.

In 1968 Rosenbaum et al. reported a simplified method for electron microscopy of tissue culture cells by employing the micromethod (2). Modifications of these techniques were used in the present investigation of virus-infected cells. The results obtained verified the usefulness of the method as a practical and economical tool for improving the identification of viral agents.

## MATERIALS AND METHODS

The procedures for the cultivation, inoculation, and preparation of infected tissue cultures for electron microscopy are diagrammed in Fig. 1.

<sup>1</sup> This investigation was done in connection with research project MF12.524.009-4019AF61 of the Bureau of Medicine and Surgery, Navy Department, Washington, D.C.

The technique for cultivation of cells in microplates has been previously described (4). For virus isolation, HeLa cells were added to each well of a microplate at a concentration of 7,500 cells per 0.025 ml of growth medium (Eagle's minimum essential medium plus 10% fetal calf serum). Then one drop (0.025 ml) of throat-swab specimen obtained from a patient with acute respiratory disease was placed in each of eight wells containing the seed cells. Cells and inoculum were mixed by shaking and incubated in a humidified CO<sub>2</sub> incubator (2% CO<sub>2</sub> in air) at 34 C. When cultures showed CPE involving approximately 25% of the cell monolayer, the media in wells

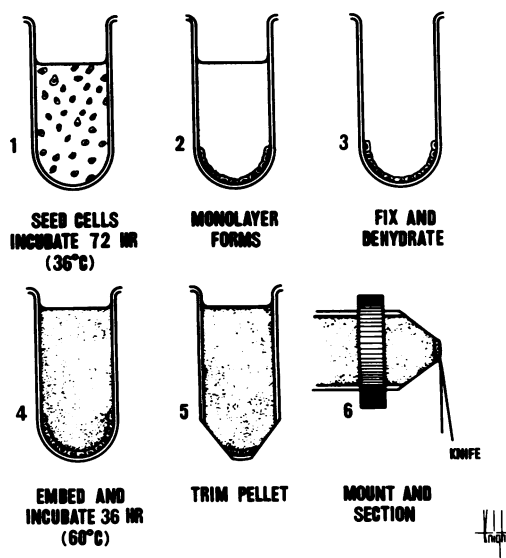


FIG. 1. Preparation of microplate cell cultures for embedding and ultrasectioning.

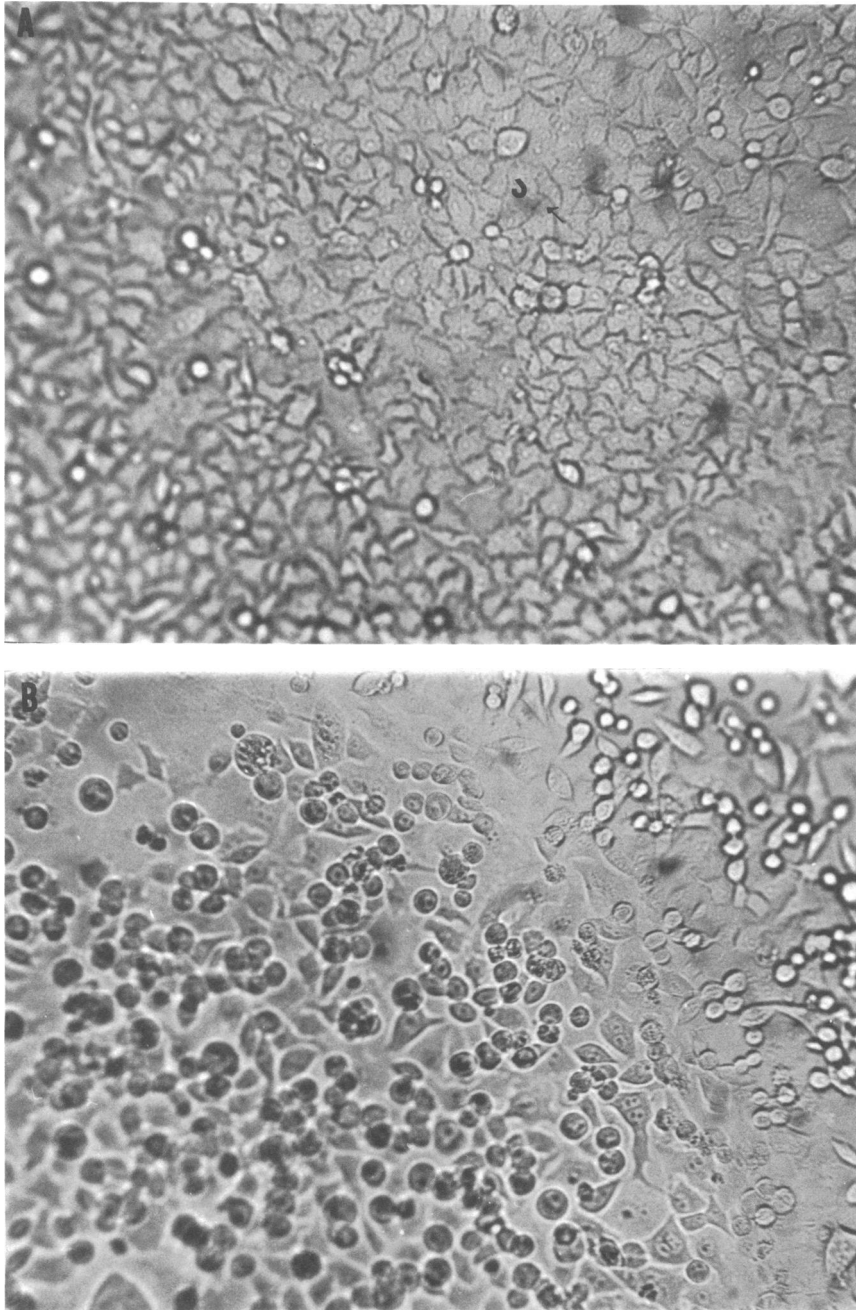


FIG. 2. Appearance of uninoculated control (A) and infected HeLa cells exhibiting viral cytopathology (B).  $\times 300$ .

were removed and the residual cells were gently washed three times with 0.1 M phosphate-buffered saline (PBS). The cells were fixed for 30 min with a 2.7% solution of osmium tetroxide in PBS. The fixed cells were then quickly washed with PBS to remove excess fixative. This step was followed by cell dehydration through graded concentrations of ethyl alcohol (50, 75, and 95%), allowing 3 to 5 min in each

bath. Final dehydration was accomplished by two 10-min changes in absolute ethyl alcohol.

Neither propylene oxide nor acetone could be used either as a transitional solvent or for infiltration, since these chemicals would have dissolved the plastic wells. Therefore, after the absolute alcohol was removed, the cells were immediately embedded in Epon 812 by the method of Luft (1) using three

parts of Epon A to seven parts of Epon B. The resin was polymerized at 60 C for 36 hr.

After polymerization, a plastic well containing the embedded cells was selected and cut away from the microplate with a razor blade. An LKB Pyramitome was used to section through the plastic until the interface between the plastic and the resin was reached. The block was then transferred to an LKB Ultratome III where thin sections for electron microscopy were prepared with a DuPont diamond knife.

The sections were mounted unsupported on a 300-mesh grid and counterstained with uranyl acetate and lead citrate. The tissue was examined and photographed with an RCA EMU-3G electron microscope at 50 kv.

### RESULTS AND DISCUSSION

The appearance of uninoculated cell control and cultures with evidence of CPE are shown in Fig. 2. Although specific viral cytopathology can be recognized, the causative agent usually cannot be definitely ascertained until virus typing tests are performed.

Figure 3 is an electron micrograph of a thin section prepared from a replicate culture inoculated with the same specimen. Intranuclear inclusions with crystalline packing typical of adenovirus can be seen. Also the characteristic morphology of the adenovirus particle is readily apparent. This will suffice to identify the viral group of the infecting agent, but serological tests still must be done to establish its type specificity. Nevertheless, the time for presumptive diagnosis of the etiological agent may be decreased from several weeks to a few days by eliminating the need to apply physicochemical procedures where tissue cytopathology is uncertain or misleading.

It is recognized that not all groups of viruses produce distinctive cellular inclusions, but tentative diagnoses may be deduced from the morphological appearance of the virus particle and its site of cellular localization. Application of ferritin antibody techniques in conjunction with the method described may aid in obscure cases.

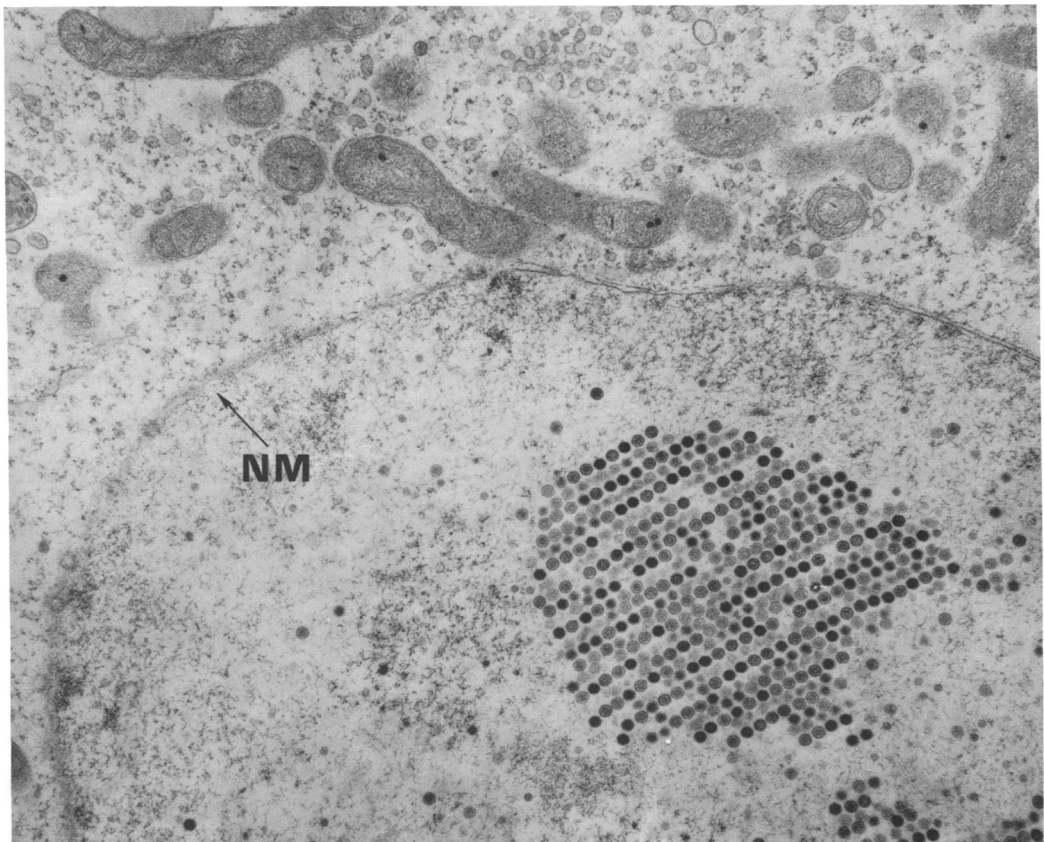


FIG. 3. Electron micrograph of a HeLa cell showing intranuclear adenovirus particles in crystalline array. (NM, nuclear membrane;  $\times 39,500$ ).

These micro techniques would be particularly useful for the identification of coronaviruses or other viruses which produce little or no discrete cytopathology. Organ cultures in microplates could be inoculated with such suspected viruses and later thin sections could be prepared *in situ* and examined for the presence of the coronavirus particles. It is conceivable that viral agents, heretofore unrecognized, may be detected by employing these techniques.

#### LITERATURE CITED

1. Luft, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9**:409-414.
2. Rosenbaum, M. J., A. M. Earle, A. L. Chapman, and E. J. Sullivan. 1968. Simplified procedure for *in situ* embedding cell monolayers for electron microscopy. *Lab Pract.* **17**:713-714.
3. Rosenbaum, M. J., E. J. Sullivan, and E. A. Edwards. 1970. Micromethods for respiratory virus seroepidemiology. *Health Lab. Sci.* **7**:43-52.
4. Sullivan, E. J., and M. J. Rosenbaum. 1967. Methods for preparing tissue culture in disposable plastic plates and their use in virology. *Amer. J. Epidemiol.* **85**:424-437.