Two-Hour Embedding Procedure for Intracellular Detection of Viruses by Electron Microscopy

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An embedding method requiring only 2 h to complete and giving excellent ultrastructural preservation has been used for the rapid detection of viruses in tissue cultures. The method has also been applied successfully to mammalian tissue. It provides a rapid technique for identifying viruses isolated in tissue cultures, for screening cultures for adventitious agents, and for examining tissue biopsies for viruses.

The standard diagnostic procedure used in most virus laboratories is to isolate a virus in tissue culture and then identify it either on the basis of the cytopathic effect or by serological tests involving complement fixation or virus neutralization. The final identification can often be guided or completely accomplished by electron microscopy (EM) (5). The simplest technique for EM identification is negative staining, which can be performed, in a matter of minutes, directly on the crude isolate (1). As a routine method of morphological identification, the negative staining technique is extremely practical. However, the procedure is actually destructive to many enveloped ribonucleic acid viruses, such as togaviruses and oncornaviruses. These and other viruses can readily be detected in thin sections of fixed and embedded cultures (2, 11), but because of the prolonged nature of most EM embedding procedures (1 to 2 days) this approach is rarely considered.

There have been several reports of rapid embedding methods for electron microscopy (3, 4, 6, 8, 9). The shortest of these has been described by Hayat and Giaquinta (7, 8), with the total processing time being reduced to approximately 3.25 h without loss of ultrastructural preservation.

In attempting to develop a rapid embedding method for use in virology, we have reduced the processing time even further, to 2 h. This procedure has been used routinely in our laboratory during the past 2 years for the rapid detection of viruses in tissue cultures. More recently, we have applied it with equal effectiveness to the electron microscopy examination of tissue biopsies.

MATERIALS AND METHODS

Cell cultures. The procedure used to prepare cell cultures for fixation and embedding was based on that described by N. Willis (Ph.D. thesis, Univ. of Toronto, Ontario, 1973). Cells from a single tube culture were scraped into the nutrient medium, transferred to a small conical-tipped centrifuge tube, and pelleted by light centrifugation (1,500 rpm for 3 min). The medium was drawn off, leaving only 2 to 3 drops in the tip of the tube to allow for the transfer of the cells to a flat waxed surface (e.g., Parafilm). A fine-bore glass tube, 1.3 by 75 mm, was touched to the drop so that the cell suspension was drawn in by capillary action. One end was sealed with plasticine, and the tube was then centrifuged in a hematocrit centrifuge for 3 min at top speed (12,500 rpm). The cells now formed a compact pellet immediately above the plasticine plug. The capillary tube was scored and broken at a distance of 6 to 7 mm above the cell pellet. The tube was then inverted, and a blunt wire (paper clip) that was slightly narrower than the bore of the tube was used to push against the plasticine, forcing the cell pellet into a vial of fixative. The cell pellet at this and subsequent stages remained tightly packed and could be transferred easily in the tip of a Pasteur pipette from one solution to another.

Animal tissue. Liver removed from a freshly killed, healthy mouse was used as an ultrastructural control, to provide an additional standard by which to compare the various procedures under study. Immediately after excision, the tissue was cut, in a drop of fixative, into pieces measuring approximately 1 mm³ and then transferred to a vial of fresh fixative.

Rapid embedding method. Two different fixation methods were investigated. In the combined fixation method, specimens were fixed for 30 min at 4°C in a freshly prepared mixture of 1 part 2.5% glutaraldehyde and 2 parts 1% osmium tetroxide in Millonig phosphate buffer (10); this was followed by three rinses of 1 min each in phosphate buffer. In the sequential fixation method, specimens were fixed first for 15 min at 4°C in 2.5% glutaraldehyde in phosphate buffer, rinsed three times in phosphate buffer, 1 min each, and then postfixied for 15 min at room temperature in 1% osmium tetroxide in phosphate buffer.

Fixed specimens were dehydrated through acetone as follows: 70% acetone, two changes in 3 min; absolute acetone, three changes in 5 min. After 10 min
in a 1:1 mixture of absolute acetone and Spurr embedding medium (12) and two changes of 100% Spurr medium, 5 min each, the specimen was placed in fresh Spurr medium in a BEEM capsule and heated to 95°C for 60 min to achieve polymerization of the embedding medium.

**Standard embedding method.** Specimens were fixed at 4°C for 1 h in 2.5% glutaraldehyde in phosphate buffer, rinsed three times in buffer for a total of 45 min, and then postfixed for 30 min at room temperature in 1% osmium tetroxide in phosphate buffer. Dehydration was accomplished with acetone, as follows: two changes of 50% acetone, 5 min each; 70% acetone, 5 min; 95% acetone, 5 min; three changes of absolute acetone, 5 min each. Plastic embedding in Spurr embedding medium was preceded by 30 min in a 1:1 mixture of absolute acetone and Spurr and 1 h in 100% Spurr. Specimens were finally transferred to fresh Spurr medium and placed at 70°C for 18 to 24 h.

**Preparation of thin sections.** Embedded specimens prepared by either the rapid method or the standard method could be trimmed for sectioning within 15 min after removal from the oven. Thin sections were cut with both glass and diamond knives and were collected on uncoated 300 mesh copper specimen grids prior to staining with uranyl acetate and lead citrate (approximately 13 min). They were then ready for examination in the electron microscope.

**RESULTS**

Total processing time by the rapid embedding method requires 2 h as compared with 22 to 28 h by the standard method (Table 1). Preservation of ultrastructural detail, in cultures and in mouse liver, is excellent using the shorter method, both with combined and sequential fixation. However, there is a slight diminution of specimen detail, and sections do not stain as sharply as those prepared by the standard procedure (Fig. 1). Cultures infected with a variety of viruses have been processed by the rapid embedding method, and in all cases viruses have been readily detected and virus structure has been well preserved (Fig. 2).

In the rapid method it was important to keep the polymerization temperature at 95°C. Above that temperature the BEEM capsules showed a tendency to soften, resulting in misshapen blocks. Specimens prepared by both methods were ready for trimming within 15 min of removal from the oven. There was no obvious difference in cutting properties of embedded material processed by either method.

**DISCUSSION**

With the advent of viral chemotherapy, there is an increasing need for rapid methods of virus diagnosis. As antiviral drugs tend to be group specific in their action, the group identification provided by electron microscopy often may be adequate for determining appropriate chemotherapy. The negative staining technique is the method of choice for the rapid processing of virus specimens for electron microscopy (1, 2). By using this technique, undoubtedly a few fragile viruses are missed due to structural disruption by the stain itself. If the specimens are first fixed and embedded, however, the fragile viruses are well preserved and can easily be detected by electron microscopy in thin sections.

Because of the long processing times normally required to embed specimens, this approach has not generally been considered to be applicable to rapid virus diagnosis. But it is obvious that embedding procedures can be shortened by many hours without loss of cellular preservation (3, 4, 6, 8, 9), and in our experience the full procedure can be shortened to 2 h.

In the present report, two rapid methods are described: one uses a single fixative mixture of glutaraldehyde and osmium, and the other uses sequential fixation with the same reagents. The combined fixation procedure was tested in light of the findings of Trump and Bolger (13), who observed that most of the "undesirable effects" produced by the more conventional sequential fixation method could be minimized or avoided by using a combined fixative. We have not been able to detect any significant improvement by using a fixative mixture. From a practical point of view, the combined fixative is more inconvenient, as it is unstable and must be prepared immediately prior to use to avoid the formation of a precipitate.

Millonig phosphate buffer was chosen because of the ease of its preparation. Similar ultrastructural preservation was obtained with cacodylate or s-collidine buffers. Acetone was used as a dehydrating agent because of its rapidity of action, its nonreaction with osmium tetroxide, and its miscibility with epoxy resins (7).

Although en bloc staining with aqueous uranyl acetate has been recommended by Hayat (7,
FIG. 1. Thin sections of mouse liver fixed and embedded by different procedures. A, Rapid method with combined fixation. B, Rapid method with sequential fixation. C, Standard method. Bar equals 1 μm.

FIG. 2. Thin sections of virus-infected Vero cell cultures processed by rapid embedding method. A, Adenovirus infection; combined fixation. B, Herpes simplex virus infection; sequential fixation. Bar equals 1 μm. Insert shows higher magnification of two herpes virus particles. Bar equals 0.1 μm.
8), we found that this extra step did not significantly improve the final image. Consequently, staining was carried out only at the end of the procedure, on the freshly cut sections. We have used both Spurr medium and an Epon-Araldite mixture as embedding media, with equal success. Specimens are polymerized in preshaped BEEM capsules rather than in gelatin capsules, thereby significantly reducing the block trimming time.

The sequential rapid embedding method is now used routinely in our laboratory, in conjunction with the negative staining technique, to assist in the identification of viruses isolated in cell cultures and to screen cultures for adventitious agents, notably latent viruses and mycoplasma. We have recently found the same method to be completely applicable to the processing of tissue biopsies. It is in this area that a rapid embedding method has its greatest potential. By such a procedure it is now possible to prepare and examine by electron microscopy thin sections of biopsy specimens within less than 3 h, thereby providing a valuable tool in the diagnosis and treatment of virus infections.

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