A Technique for Locating Rare Cells in the Circulation for Ultramicroscopy[1] (34510)

E. GAYNOR,[2] C. A. BOUVIER, AND J. R. CINTRON
(Introduced by T. H. Spaet)

Department of Hematology, Medical Division, Montefiore Hospital and Medical Center, Bronx, New York 10467

In the course of a study involving endothelial cells in the circulating blood of patients and experimental animals, it became necessary to locate these rare cells for ultramicroscopic examination. Although several investigators have demonstrated circulating endothelium using light microscopy (1–3), these cells have not yet been studied electron microscopically. This may be due to the extreme difficulty encountered in trying to locate such rare cells with routine ultramicroscopic embedding techniques. Endothelial cells, when present in the circulating blood, occur with an estimated frequency of less than 1 per 10^6 nucleated blood cells. The method to be described was developed to permit light microscopic scanning of peripheral blood cells embedded in Epon, in order to locate circulating endothelial cells for ultramicroscopic identification. Standard procedures for selecting specific cells for ultrastructural study using "thick" Epon sections provide only small areas for light microscopic scanning. This is impractical when the cell to be studied forms only a small proportion of the cell population, and difficult when the spatial orientation of the cells is random. Methods which permit light microscopic examination of large numbers of cells prepared for electron microscopy have been developed for tissue culture monolayers (4-6), but these are not applicable to heterogeneous cell suspensions.

The present report describes a simple technique of fixation and embedding suspended material in Epon polymerized in the shape of an ordinary glass slide. It has several advantages over other such techniques: (1) large volumes of suspended cells may be examined for the presence of rare cells; (2) cells may be observed and photographed under oil immersion and then sectioned and studied ultramicroscopically; (3) cells may be selected which are oriented in the most advantageous plane for thin sectioning.

Methods. Leukoconcentrates were prepared by the method of Herbeauval (8). Briefly, 5 ml of venous or arterial blood are collected through an 18-gauge needle and plastic tubing, into 10 ml of citrated buffered solution (8). The specimen is then hemolyzed with 2% saponin in 50% alcohol to remove the red cells and diluted with the buffer to deter further lysis of the nucleated cells. These cells are collected by centrifugation for 10 min at 340*g*. The supernatant fluid is decanted, an aliquot of cells is removed with a Pasteur pipette, smeared on glass slides, stained with Wright-Geimsa, and prescreened for the presence of endothelial cells.

The remainder of the cells are resuspended in 2 drops of 0.067 *M* phosphate buffer and prepared for electron microscopy. Fixation is accomplished by adding 2 vol of 5% glutaraldehyde in 0.067 *M* phosphate buffer, pH 7.4. After 1 hr the suspension is washed in phosphate buffer and postfixed in 1% phosphate-buffered OsO_4 for 30 min.

It is essential to keep the cells well dispersed during each step of fixation, dehydration, and embedding, in order to facilitate screening of the embedded suspension for individual cells. Therefore, after centrifugation

[1] Supported by Grant HE 05414-09 from the National Heart Institute, United States Public Health Service.

[2] Fellow supported by a grant from the National Leukemia Association.

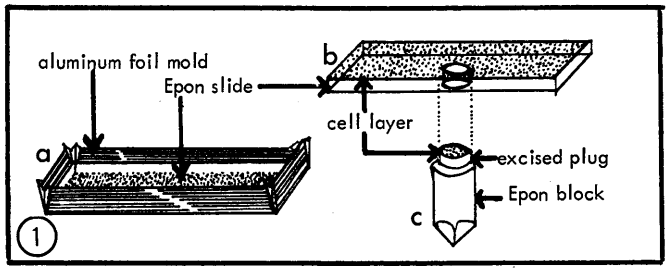

FIG. 1. Steps in specimen preparation. a. Aluminum foil mold, in which cell suspension in Epon is sedimented and polymerized. b. Areas selected by light microscopy for EM study are excised with a cork borer, and c. mounted on an old Epon block with fresh Epon.

at 1000*g* for 5 min for each solution change, the cells are resuspended, using an Adams Cyclo Mixer, in a drop or two of remaining supernatant fluid before addition of the next solution.

The following schedule was used for embedding:

1. Wash with one change of distilled water.
2. Postfix with filtered saturated solution of uranyl acetate in 50% ethanol for 30 min.
3. Stain with 5-7 drops of Paragon 1301 in 5 ml of 50% ethanol for 15 min. This stain does not affect ultramicroscopic morphology (10).
4. Dehydrate by the following schedule: 50% ethanol for 10 min. 75% ethanol for 10 min. 90% ethanol for 10 min. 95% ethanol for 10 min. 100% ethanol-three changes, 15 min each. One part propylene oxide: One part Luft's Epon (9) 20 min. Pure Luft's Epon, two changes for 20 min each. During the steps containing Epon, it was necessary to centrifuge the specimens in a Clay Adams Sero-Fuge 5–10 min, in order to sediment the cells.
5. Resuspended cells in 1-2 ml of fresh Epon, pour into molds, cover, and leave undisturbed at room temperature overnight. Molds are made of household aluminum foil shaped over two standard glass slides (Fig. 1a).
6. After the suspension has settled to the bottom of the mold, the Epon is polymerized for 12 hr at 60°.
7. Add fresh Epon to a final height of 1–1.5 mm, and replace mold in 60° oven for 12 hr. This increased slide thickness permits use of a mechanical microscope stage and still allows ample light penetration for oil-immersion microphotography.
8. After cooling the Epon slide at room temperature, the aluminum foil is stripped off, and the slide is inverted and is examined with a light microscope. Areas chosen for ultramicroscopic study are scored with a fine needle, and the location of the cells to be studied relative to the score marks is "mapped."
9. Scored areas are excised with a cork borer, the disc is glued to an old Epon capsule with a drop of fresh Epon, and repolymerized at 60° for 12 hr (Fig. 1b, c).
10. Blocks are trimmed using the "maps" as a guide, and thin sections are cut parallel to the surface of the excised disc. Cells may be chosen in step 8 at sufficient depth to permit the microtomist to adjust the knife and section thickness before they are reached.
11. Sections are picked up on uncoated grids and stained with uranyl acetate and lead citrate.

Results and Discussion. This procedure has been used in our laboratory for the ultrastructural identification of circulating endothelial cells in patients with thrombotic diatheses (unpublished data), and in endotoxin-treated rabbits (7). For reasons noted above, attempts to locate these cells in Epon-embedded leukocyte buttons prepared by centrifugation or from buffy coats proved

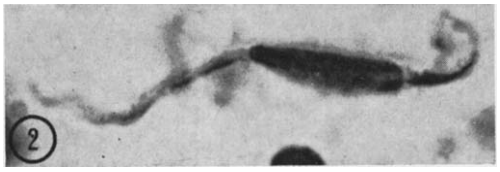

FIG. 2. Light micrograph of an Epon-embedded whole endothelial cell from the circulation of an endotoxin-treated rabbit. × 1300.

unfeasible. Using these techniques, no endothelial cells were ever identified in serial sections of known positive specimens. However, with the Epon slides, the sedimented cell suspension was easily scanned, and the elongated endothelial cells were readily distinguished from leukocytes. Figure 2 presents a light micrograph of an Epon-embedded endothelial cell from the circulation of an endotoxin-treated rabbit processed as described. Using both glass and Epon slide techniques, endothelial cells were never observed in the blood of normal rabbits.

Large numbers of red cells contaminating the cell suspension interfere with easy scanning, and must be removed. Of the various techniques for their removal, such as lysis, dextran sedimentation, or differential centrifugation, only the Herbeuval method employing saponin was satisfactory. However, this method alters ultrastructural detail in endothelial cells. Figure 3a shows the cytolysis and loss of some intracellular components produced by saponin in an endothelial cell scraped from rabbit aorta, added to normal rabbit blood and processed as above. In contrast, endothelial cells scraped from the rabbit aorta, and fixed in glutaraldehyde before addition to blood and saponization, show good preservation of ultrastructural morphology after Epon "slide" embedding (Fig. 3b). It is noteworthy that saponin treatment has

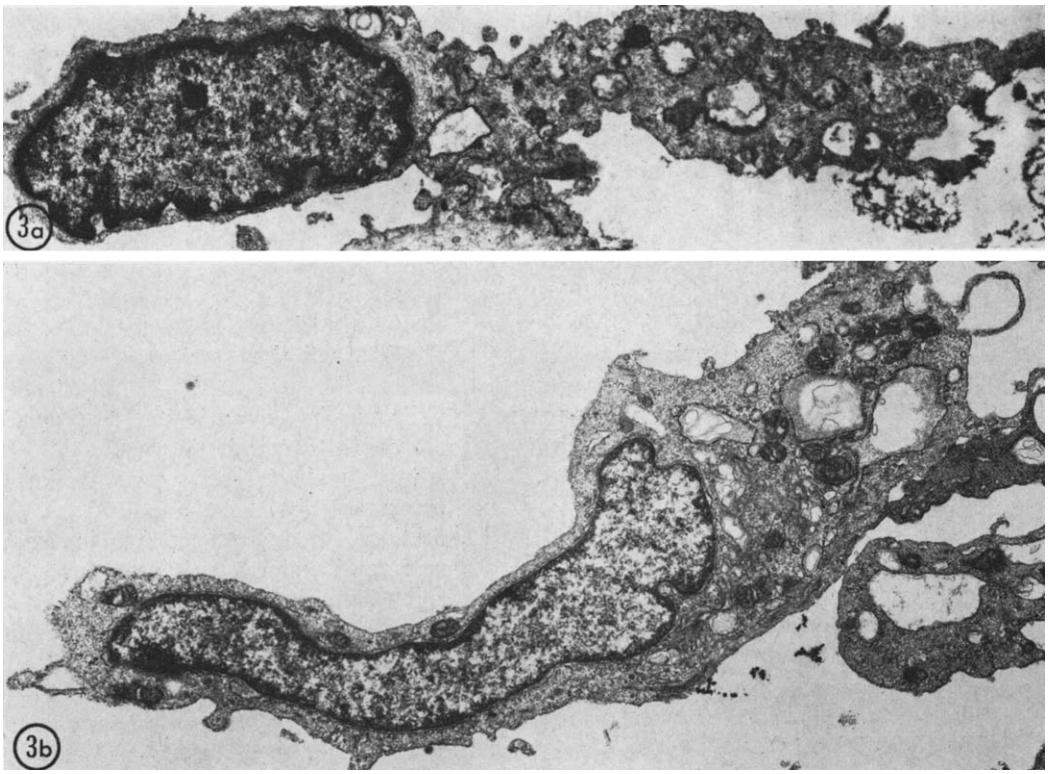

FIG. 3. a. Endothelial cell scraped from normal rabbit aorta, added to rabbit blood, and saponized prior to fixation. Note membrane lysis and slight loss of intracellular detail. × 8,000. b. Endothelial scrapings fixed in glutaraldehyde prior to addition to blood and saponization, and embedded as in a. × 9,980.

not altered morphology enough to prevent easy identification of the cells in Fig. 3a as endothelium. Moreover, some degree of damage is expected in mechanically traumatized cells as well as in disease-induced cell sloughs. Indeed, undamaged endothelium can probably be seen only after *in situ*-perfusion fixation of normal vessels.

The present study provides an embedding technique which is useful in the study of rare cells in heterogeneous cell suspensions. It permits their localization and identification by ordinary light microscopy, and subsequent ultramicroscopic examination. Application of the method has made possible the ultramicroscopic identification of circulating endothelial cells in human disease and in experimental animals.

Summary. A method has been developed for the isolation of rare cells in heterogeneous cell suspensions, for ultramicroscopic examination. Such cells cannot be located using conventional embedding screening techniques. The cells are embedded in Epon which is polymerized in the shape of an ordinary glass slide. Large numbers of suspended cells may be screened by light microscopy for the rare cells, which may then be sectioned for ultramicroscopy. The method has been used to locate and ultramicroscopically identify circulating endothelial cells in the blood of patients and experimental animals.

Although artifacts were induced by the techniques used for collecting and concentrating circulating endothelial cells, the embedding method itself did not cause further morphological alterations.

1. Bouvier, C. A., Maurice, P. A., and Roch, R., Oncologia **15**, 129 (1962).

2. Jalaitis, J., McGrew, E. A., McGrath, R. G., and Roberts, S. S., Acta Cytol. **12**, 439 (1968).

3. Bouvier, C. K., Maurice, P. A., and Roch, R., Bull. Acad. Suisse Sci. Med. **20**, 15 (1964).

4. Anderson, N. and Doane, F. W., Stain Technol. **42**, 169 (1967).

5. Brinkley, B. R., Murphy, P., and Richardson, C., J. Cell Biol. **35**, 279 (1967).

6. Egeberg, J., Stain Technol. **40**, 343 (1965).

7. Gaynor, E., Bouvier, C. A., and Spaet, T. H., Clin. Res. **16**, 535 (1968).

8. Herbeuval, R. and Herbeuval, H., C. R. Acad. Sci. Paris **250**, 3070 (1960).

9. Luft, J. H., J. Biophys. Biochem. Cytol. **9**, 409 (1961).

10. Spurlock, B. O., Skinner, M. S., and Kattine, A. A., Amer. J. Clin. Pathol. **46**, 252 (1966).

Received Oct. 6, 1969. P.S.E.B.M., 1970, Vol. 133.