A HIGH-YIELD TECHNIQUE FOR PREPARING CELLS FIXED IN SUSPENSION FOR SCANNING ELECTRON MICROSCOPY

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Scanning electron microscopy (SEM), a useful technique for the study of cell surface architecture, has gained wide application in cell biology. The analysis of individual cells may have certain methodological limitations since it is required that the cells be attached to underlying surfaces which then serve as vehicles for SEM processing. In some studies (5, 9) cells have been settled live onto various substrates. Since unfixed cells may variably and unpredictably alter their shape and surface morphology when in contact with solid substrates (1, 4, 5, 10), it is often necessary to fix cells in suspension. For certain quantitative studies by SEM, it is also imperative that no major cell loss occurs, since this loss may be selective. Thus, it is desirable to develop a simple, reproducible, high-yield SEM preparative technique which itself does not substantially alter ultrastructural features. In the present investigation, poly-L-lysine-coated glass coverslips have been utilized as a substrate for processing human blood mononuclear leukocytes fixed in suspension for SEM. This technique is a modification of one initially described by Mazia et al. (6, 7). Nearly 100% of the cells initially placed on the coverslips were retained throughout the entire procedure.

MATERIALS AND METHODS

Human peripheral blood mononuclear leukocytes were obtained from four normal individuals. The freshly drawn blood was defibrinated with glass beads, placed in siliconized glass tubes, diluted volume per volume with RPMI 1640 culture medium, and processed by density gradient centrifugation according to Boyum (3). In our hands, this technique permits the collection of approximately 70% of the mononuclear leukocytes present in the whole blood. After centrifugation, the cells were washed three times and resuspended in 0.5 cc of medium. The population obtained consisted of leukocytes and residual erythrocytes. 95% of the leukocytes were lymphocytes and monocytes as seen on Giemsa-stained cytocentrifuge preparations. The viability as assessed by trypan blue dye exclusion was greater than 98%. The cells were fixed in suspension by adding at least 10 cc of 1% glutaraldehyde in Sorensen's buffer, pH 7.3 (Tousimis Research Corp., Rockville, Md.). After 24–48 h, the fixed cells were washed in Sorensen's buffer, and the final cell concentration was adjusted to 1.5×10^6 per cc. The entire procedure of cell isolation and fixation was carried out at room temperature.

Nickel locating grids (H-2 London 200 Finder Grid, Ernest F. Fullam, Inc., Schenectady, N. Y.) were affixed to one side of glass coverslips (no. 3 thickness, 6-mm diameter, Corning Glass Works, Science Products Div., Corning, N. Y.) with epoxy resin (Elmer's Epoxy,

Borden, Inc., New York.). The opposite side of the coverslips was then covered with a 25-μl drop of poly-L-lysine hydrobromine (mol. wt. 85,000, Sigma Chemical Co., St. Louis, Mo.; 1 mg/ml stock solution in phosphate-buffered saline, pH 7.2). After 1 h at room temperature, the coated coverslips were washed in distilled water and placed in 35-mm Petri dishes in a moist chamber. The coverslips were partially blotted with filter paper and a 20-μl drop of the cell suspension containing 3×10^5 cells was then placed on each coverslip. There was no loss of the cell suspension from the edges of the coverslip. Two to four coverslips were prepared for each of the four blood samples. The moist chamber containing the Petri dishes was stored at 4°C for approximately 14 h. After 14 h of unit gravity sedimentation, all cells were expected to have settled onto the poly-L-lysine-coated glass surface. Each of the coverslips was then observed under an inverted light microscope. By careful focusing, the examination of the 20-μl drop revealed that all of the cells had settled onto the surface of the poly-L-lysine-coated coverslips. Since the cells in a droplet do not settle uniformly onto a flat surface, the calculation of cell yields by the comparison of the theoretical to the observed cell density is not valid. Thus, we have elected to quantitate cell yields by monitoring the numbers of individual cells in premarked regions of the coverslip. Before SEM processing, three to five areas of each coverslip were photographed on Polaroid 52 film (Polaroid Corp., Cambridge, Mass.) at × 260 magnification, using the letters and numbers on the locating grid as reference points. The coverslips were subsequently dehydrated in a series of graded alcohols, placed in a metal holder (8), and critical point dried with CO_2 (2). The coverslips were coated with gold-palladium in a high vacuum evaporator on a rotating-tilting stage. After coating, the original fields were relocated and rephotographed with light microscopy. The cell counts were performed directly on the Polaroid photographs. An ETEC Autoscan (ETEC Corp., Hayward, Calif.) was subsequently used to examine the same samples for SEM at 20 kV and a 45° tilt.

RESULTS

Processing cells fixed in suspension on poly-L-lysine-coated glass coverslips to which locator grids were attached permitted the monitoring of cell yields at various steps in the preparation of the samples for SEM. In Table I, the total number of cells present in several fields of the coverslips before ethanol dehydration, critical point drying, and metal coating (starting population) is compared to the number present in the same fields at the end of processing (final population). In each of four samples, nearly 100% of the cells were retained on the coverslips throughout the entire procedure. Since there was no loss of fluid from the cell suspension droplets applied to the coverslips and since there was no significant difference between the numbers of settled and attached cells, it is unlikely that substantial selective cell loss due to differences in cell surface charge could have occurred.

Fig. 1 demonstrates an area of one of the coverslips after cell settling before (Fig. 1 *a*) and after (Fig. 1 *b*) SEM processing. As can be observed on a cell per cell basis, virtually all of the mononuclear cells present initially (Fig. 1 *a*) were retained for final viewing in the SEM (Fig. 1 *b*). Fig. 2 is a low power SEM micrograph of one of the samples. The individual cells are distributed in a monolayer over the flat, uniformly dark surface of the glass coverslip. Fig. 3 illustrates the spherical shape of the cells and their detailed surface architecture with multiple microvilli or ruffles.

DISCUSSION

Many studies of the surface morphology of individual cells have been performed on populations which have been cultured, settled, or aspirated onto various substrates before fixation (5, 9). It has been well documented (1, 4, 5, 10) that cell surface architecture can change rapidly and dramatically when live cells come into contact with underlying surfaces for even brief periods of time. Furthermore, when an attempt is made to attach live cells in suspension to solid substrates, selective losses may occur in populations of cells with different adherent capacities. It is thus advantageous to process cells fixed in suspension provided no substantial cell loss occurs.

With few exceptions (1, 10), previous investigators have failed to address themselves to the fundamental problems of cell yields, potentially

TABLE I
Total Cell Yields

Sample	Starting population*	Final population‡	Yield ± SD
1	479	462	96.7 ± 1.4
2	1013	991	97.8 ± 1.4
3	1456	1466	98.8 ± 1.0
4	1608	1583	98.5 ± 2.7

* Cells attached to coverslips after completion of cell settling. The numbers were obtained by counting several photographed fields on different coverslips in each sample.

‡ Cells remaining attached to coverslips after alcohol dehydration, critical point drying, and metal coating. The numbers were obtained by relocating and counting identical fields as in starting population.

selective losses, and alterations in surface architecture induced by SEM preparative techniques. When populations are heterogeneous or when quantitative correlation between SEM and other morphological or functional parameters is required, it is essential to avoid uncontrolled cell surface changes during processing as well as to retain most of the cell population for analysis.

Preliminary observations have suggested that poly-L-lysine, a synthetic polypeptide, may be utilized as a substrate to attach cells and cell fragments to different surfaces for processing for SEM (6, 7). We report here a simple, reproducible procedure for processing cells fixed in suspension on poly-L-lysine-coated glass coverslips. Unlike previous procedures, this technique permits the retention of almost 100% of fixed cells despite the multiple manipulations involved in the SEM preparatory steps. In addition to the high cell yields and the preservation of cell surface architecture, this technique requires only a small number of cells and permits combined light and SEM studies to be performed sequentially on the same individual cells. Furthermore, the flat glass coverslip provides a dark, uniform background which improves the quality of photomicrography. The technique can be widely applied to many types of cells in suspension regardless of their inherent adherent properties.

We anticipate that this technique will both simplify the difficult task of processing cells in suspension for SEM and enhance the quality of the information that can be obtained from such studies.

SUMMARY

Human leukocytes fixed in suspension were allowed to settle onto poly-L-lysine-coated glass coverslips and prepared for observation with the scanning electron microscope (SEM). The coverslips were dehydrated in ethanol, critical point dried with CO_2, and coated with gold-palladium. With the aid of a locator grid, several fields were photographed with light microscopy after the cells

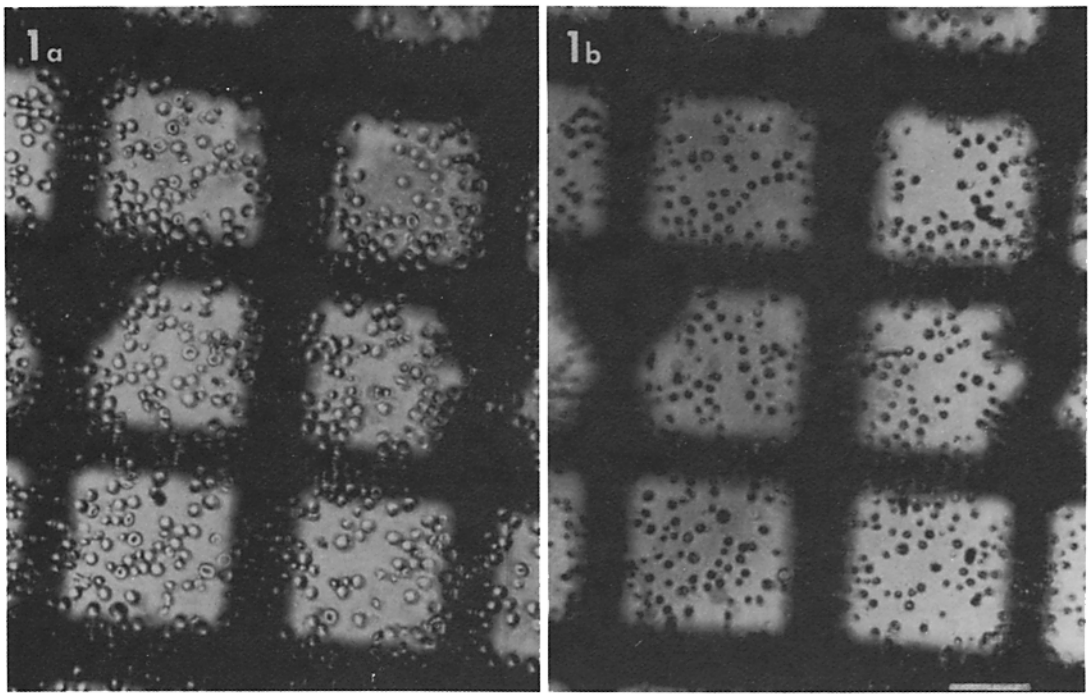

FIGURE 1 (*a*) Cells fixed in suspension and subsequently attached to a poly-L-lysine-coated coverslip (starting population). (*b*) The same area as seen in Fig. 1 *a* after completion of alcohol dehydration, critical point drying, and metal coating (final population). Virtually all cells have been retained on the glass surface. The frames and indicating letters are not in focus because the locator grid is attached to the undersurface of the coverslip. (Bar 50 μm, × 215.)

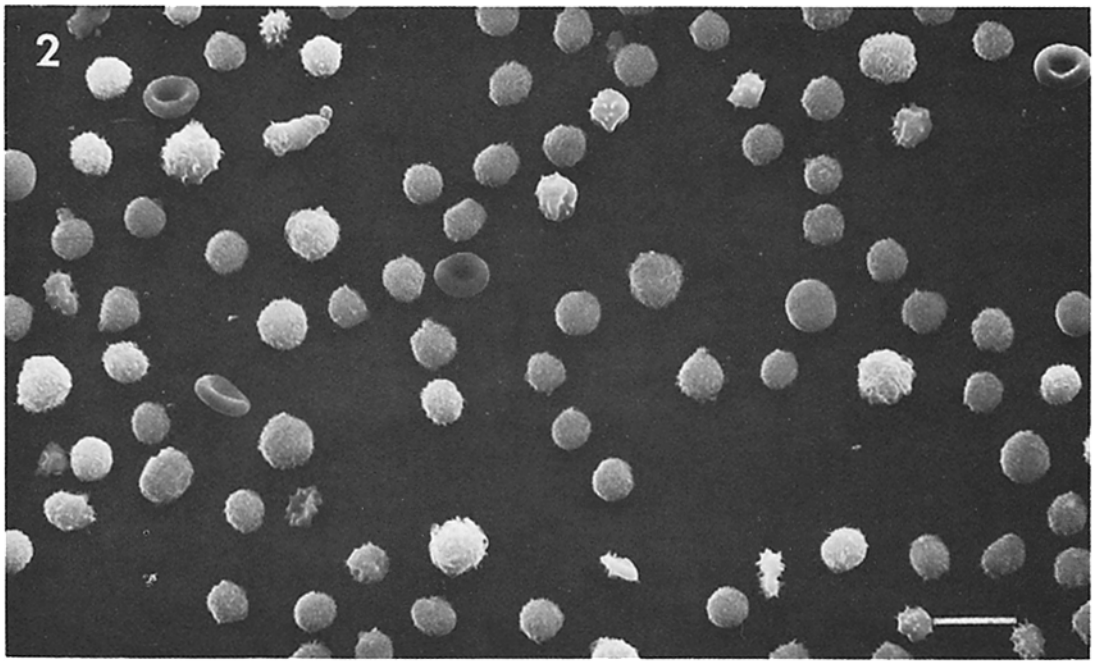

FIGURE 2 Low-power SEM view of human blood mononuclear leukocytes fixed in suspension and attached to a poly-L-lysine-coated coverslip. The distinct individual cells preserve their shape and stand out against the dark uniform background of the flat glass. (Bar 10 μm, × 2,110.)

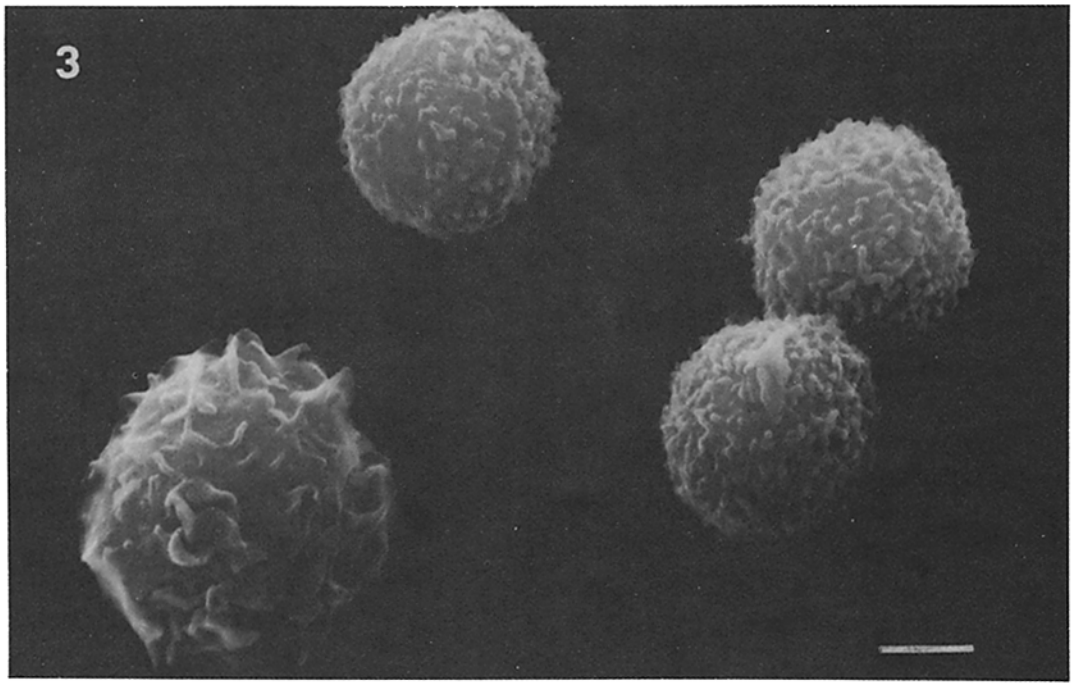

FIGURE 3 Higher magnification of adherent cells. The large cell at the left, probably a monocyte, exhibits numerous, broad-based ruffles. The other three cells display multiple short microvilli. (Bar 2 μm, × 6,240.)

had settled onto the poly-L-lysine-coated coverslips and again after completion of the processing before SEM observation. Quantitative comparison of the number of cells present after settling with the number retained for final viewing with the SEM revealed a cell yield approaching 100%. This simple, reproducible, high-yield technique for processing cells fixed in suspension for SEM prevents changes in surface architecture induced by collecting live cells onto various substrates before fixation and also avoids potentially selective cell losses. Such a technique should allow quantitative correlations between SEM and other morphological and functional parameters.

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