HAPTEN-SANDWICH LABELING

II. Immunospecific Attachment of Cell Surface Markers Suitable for Scanning Electron Microscopy

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ABSTRACT

A hapten-sandwich procedure has been used for immunospecific labeling of cell surface antigens with markers visible by scanning electron microscopy. Antihapten antibody was used to link hapten-modified tobacco mosaic virus, bushy stunt virus, or hemocyanin to hapten-modified human erythrocytes. The antihapten antibody bridge was also used to link the hapten-virus marker to hapten-modified antibodies against mammary tumor virus on mouse mammary tumor cells, or against immunoglobulin receptors on mouse splenic lymphocytes. In all cases, labeling was highly specific. With this technique, it is possible to (*a*) compare morphological features of cells bearing differing cell surface antigens, and (*b*) examine the arrangement of specific antigenic sites on a cell surface or their distribution relative to membrane structures such as microvilli.

With continuing improvements in resolving power and techniques, scanning electron microscopy (SEM) has become a powerful tool for examining cell surfaces (10, 17, 19, 20). In some instances, immunological or related methods (8) have been used to attach surface markers, including latex particles conjugated with antibody (12, 14, 15), for SEM studies. However, a general approach to labeling cell surface antigens with various suitable SEM markers has been lacking.

An ideal SEM marker should be recognizable at relatively low survey magnifications while displaying high-resolution site specificity. We report here the use of a hapten-sandwich method (9, 23) for attaching an SEM marker to antibodies specifically bound by cell surface antigens. The hapten-sandwich procedure we have employed involves this sequence: (*a*) hapten groups are azocoupled under mild conditions to an immunoglobulin (Ig) preparation with specificity against a cell surface antigen and, separately, to distinctive virus particles or other markers; (*b*) after cells are reacted with the hapten-modified Ig, they are treated with antihapten antibody and then labeled with the hapten-modified marker, as illustrated in Fig. 1.

As demonstrated in an earlier paper (23), this method can be applied readily with any anticell-surface antibody and permits highly specific recognition of a variety of antigens with fluorescent or transmission electron microscopy. In this study, we have used hapten-modified tobacco mosaic virus (TMV) as our principal SEM marker in conjunction with antibodies against antigens (or haptens) on mouse mammary tumor cells, human erythrocytes, and mouse lymphocytes. TMV has been used previously as a marker in transmission electron microscopy (2). Preliminary reports of our results have been presented (16, 18).

MATERIALS AND METHODS

Antisera and Purified Antibodies

Antibodies to the haptens *p*-azophenyl-β-D-lactoside (anti-lac), *o*-azophenyl-β-D-galactoside (anti-*o*-gal), and ε-*N*-2,4-dinitrophenyl lysine (anti-dnp) were isolated from pooled, high-titer antisera from rabbits hyperimmunized by standard procedures against hapten conjugates of keyhole limpet hemocyanin (KLH), prepared as previously described (6), and were purified by sepharose affinity chromatography (24).

We received two gifts of rabbit antisera against mouse mammary tumor virus (anti-MTV), prepared as previously described (3), from Drs. P. B. Blair and S. Nandi.

Normal rabbit Ig was purchased from Miles Laboratories, Inc. (Kankakee, Ill.). The preparations of rabbit anti-MTV and normal Ig were precipitated twice from 16% sodium sulfate and passed over DEAE-cellulose (Whatman no. DE-52) in 0.07 M phosphate, pH 6.3 (22).

Goat antimouse immunoglobulin (anti-MIg), purchased from Antibodies, Inc. (Davis, Calif.), was absorbed with mouse red blood cells and precipitated twice with 40% saturated ammonium sulfate.

Modification of Immunoglobulin and Markers

The lac-hapten conjugates of immunoglobulins (lac-anti-MTV, lac-normal rabbit Ig, and lac-anti-MIg) and of SEM markers (lac-TMV, lac-bushy stunt virus [lac-BSV], and lac-KLH) were prepared under standard reaction conditions as described elsewhere (23): 3 mg protein/ml, 4×10^{-4} M lac-diazonium reagent, in 0.2 M borate buffer, pH 8.5, overnight at 4°C. TMV, BSV, and KLH were treated for 2 h with 0.1% glutaraldehyde before hapten modification; this treatment prevents the disruption of large molecular-weight markers during hapten coupling (23). Modified marker preparations were passed over Agarose-15 sizing columns (Bio-Rad Laboratories, Richmond, Calif.) to remove low-molecular-weight material.

Fluorescent rabbit anti-lac antibody (Fl-anti-lac) was prepared and used according to described procedures (23).

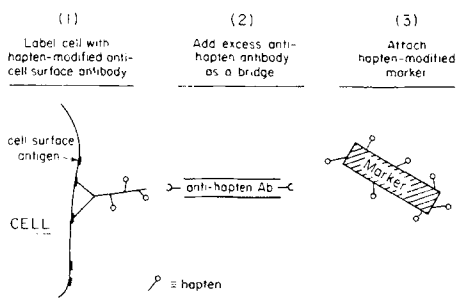

FIGURE 1 A schematic representation of the hapten-sandwich labeling method.

Cell Preparations

To evaluate our labeling procedures, we used human red blood cells (HRBC) to which haptens were randomly attached in covalent linkage by reaction with a diazonium reagent. HRBC from a healthy donor were reacted with 2×10^{-3} M lac-diazonium reagent at pH 7.5, overnight at 4°C, as previously described (21). The hapten-modified cells were washed four times at 4°C in Veronal-buffered saline, pH 7.2, containing 0.1% bovine serum albumin (VBS-BSA) and were used immediately for SEM labeling experiments.

7–10-day primary cultures of cells dissociated from spontaneous mammary carcinomas in female C3H/Crgl and BALB/cfC3H mice, infected with the mammary tumor virus (MTV), were used for labeling of MTV antigens on cell surfaces; culture methods were described in detail elsewhere (7).

Spleen lymphocytes were obtained from BALB/c mice and prepared by the same procedures as before (23).

Labeling of Cells for SEM

LAC-HRBC: 4×10^7 lac-HRBC were washed twice in VBS-BSA and incubated for 15 min at 0°C in 0.2 ml of anti-lac (1 mg/ml). Here, as in each cell preparation, a saturating amount of anti-lac was used to maximize labeling efficiency and minimize cross-linking. The cells were washed twice again with VBS-BSA-azide, and incubated as above in 0.2 ml lac-TMV. After two washes in VBS-azide, the cells were fixed in 5 ml 1% glutaraldehyde in 0.1 M Na phosphate buffer, pH 7.2 (PB) for 10 min at room temperature. After one wash in PB, the cells were postfixed in 1% OsO_4 in PB for 15 min at room temperature.

MAMMARY TUMOR CELLS: The tumor cells on cover slips were washed three times with culture medium (37°C) and then fixed in 5% Formalin or 2% glutaraldehyde in culture medium at room temperature for 30 min. (Glutaraldehyde fixation retains the labeling specificity but eliminates surface etching observed with Formalin.) The cells were washed three times in PB. Each cover slip was incubated successively for 30 min at room temperature with 0.2 ml lac-anti-MTV (0.6–0.006 mg/ml), anti-lac (0.3 mg/ml), and an excess of lac-TMV (determined empirically for each preparation of the marker); buffer washes separated the incubations. At this stage the labeled cells on cover slips were loaded onto springs (10 cover slips per spring) to facilitate subsequent handling of many cover slips together. The cover slips were fixed (postincubation) in 2% glutaraldehyde in 0.1 M cacodylate, pH 7.3, at 20°C for 30 min and subsequently washed two times in a large volume of 0.1 M cacodylate buffer.

SPLEEN LYMPHOCYTES: 1×10^7 mouse spleen lymphocytes in VBS-BSA were incubated for 15 min at 0°C with 0.1 ml lac-anti-MIg (3 mg/ml. Washes and subsequent sequential additions of the anti-lac antibody (0.2 ml) and the lac-TMV (0.1 ml) were per-

formed as described above for labeling lac-HRBC. Lymphocytes were fixed for 10 min at room temperature in 1% glutaraldehyde in PB, either in suspension or after adsorption onto glass cover slips. In the latter procedure, cells were allowed to settle onto cover slips for 1 h at room temperature. Cover slips were loaded onto springs for dehydration.

Preparation of Cells for SEM

After fixing, suspensions of labeled lac-HRBC or mouse spleen cells were washed and resuspended in 0.1–1 ml PB before dehydration. A dehydration chamber is formed from three (14 mm OD, 6 mm ID) brass washers, two gold-coated Nucleopore (VWR Scientific, San Francisco, Calif.) filters (13-mm diameter, 0.6-μm pore; 20–40 nm of gold is evaporated onto the shiny side of the filters to provide a conductive substrate), two paper washers (fashioned from the paper disks separating Nucleopore filters, with a 6-mm hole made with a standard paper punch), and a bent 22-mm paper clip. Fig. 2 shows the chamber before and after cell loading. The entire assembly, with the loaded cells, is held together with the appropriately bent paper clip.

The chamber assembly and spring-loaded cover slips of fixed tumor cells or lymphocytes were transferred through increasing concentrations of ethanol (20, 50, 70, 80, 90, 95, 100, and 100% ethanol) and Freon-113 (Matheson Gas Products, Newark, Calif.) (25/75, 50/50, 75/25, 100/0, and 100/0 Freon in ethanol, vol/vol) at 5 min/step. The chambers and cover slips were then critical-point dried in Freon-13 (Matheson) according to the procedure of Cohen et al. (5). Individual Nucleopore filters and cover slips were mounted on 13-mm sheet metal disks with carbon conductive cement (Coates and Welter Instruments, Sunnyvale, Calif.) and were then placed on a rotating or a tilt-rotating stage and coated with about 75 Å of platinum. The filters and cover slips were examined in a Cambridge Stereoscan Mark IIa, or a Coates and Welter Cwikscan 100-4 field emission scanning electron microscope.

Preparation of Cells for Transmission Electron Microscopy

Tumor cells grown on 35-mm Falcon plastic petri dishes were prepared through ethanol dehydration in the same way as cells on cover slips. The petri dish cultures were then embedded *in situ* in Epon 812. Sections were cut perpendicular to the cell layer, stained with uranyl acetate and lead citrate, and examined in an RCA EMU3F microscope or a Siemens Elmiskop 102.

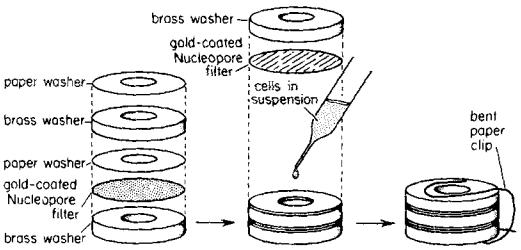

FIGURE 2 Chamber for processing cell suspensions for SEM. The cell suspensions are placed in contact with the gold-coated side of each Nucleopore filter.

RESULTS

Labeling of Hapten-Modified Erythrocytes

Studies to determine the specificity of coupling of lac-modified SEM markers by anti-lac antibody were performed on lac-HRBC and unmodified HRBC. When lac-HRBC were reacted with anti-lac antibody followed by lac-TMV, all cell surfaces were shown by SEM to be labeled extensively (Fig. 3 *a*). Some of the lac-TMV markers seen in Fig. 3 *a* lie flat along the cell surface; others stand out from the membrane and are readily seen, even at low magnification, as bright rods against a darker background. Considerable agglutination of lac-HRBC occurs after treatment with anti-lac antibody. When lac-HRBC were treated with anti-dnp instead of anti-lac, subsequent exposure to lac-TMV resulted in no labeling. When anti-*o*-gal (antibody to a chemically related hapten) was substituted for anti-lac, cross-reaction was indicated by some agglutination of lac-HRBC, but after treatment with lac-TMV and the usual washing procedures, the cells were unlabeled (Fig. 3 *b*).

Lac-HRBC, treated with anti-lac, were also labeled with lac-KLH (Fig. 3 *c*). Unmodified HRBC were never labeled after treatment with anti-lac followed by lac-TMV or lac-KLH.

Most recently, we have labeled lac-HRBC with lac-BSV. Fig. 4 shows a cell preparation in which lac-TMV and lac-BSV were added simultaneously in the final step of the labeling procedure—that is, after treatment of lac-HRBC with anti-lac. The two labels are readily distinguishable on the cell surface.

Distribution of Mammary Tumor Virus Antigen on Mouse Mammary Tumor Cells

Mouse mammary tumor cells were treated in sequence with lac-anti-MTV, anti-lac, and lac-TMV. Fig. 5 *a* shows part of the surface of a typical labeled cell: lac-TMV is attached to a 100-nm sphere (the size of an MTV virion) and to microvilli. The marker was almost always attached by one end to the cell surface and was visible at relatively low magnifications as protruding bright rods (Fig. 5 *b*).

Frequently, adjacent cells could be found with

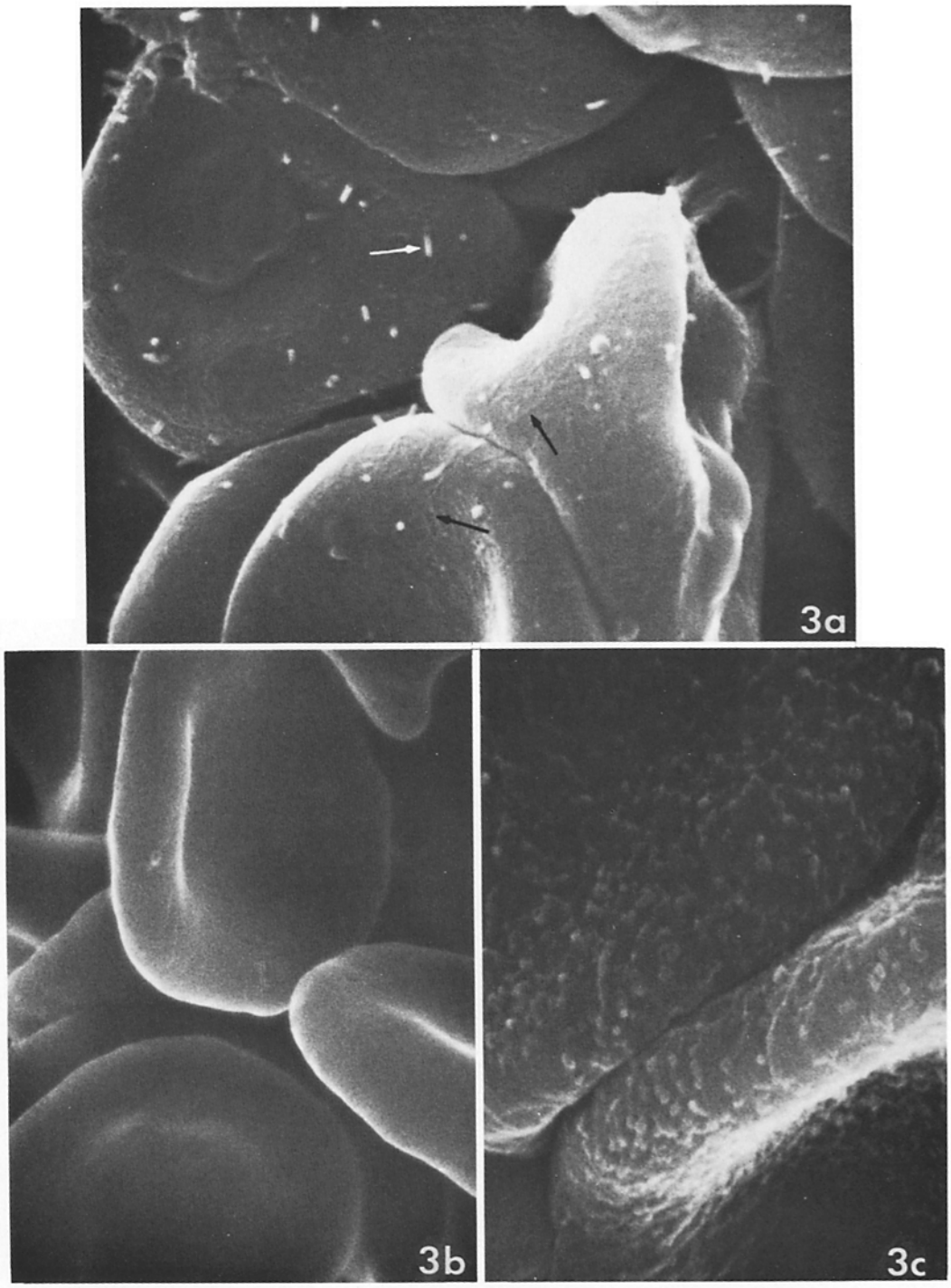

FIGURE 3 SEM micrographs of lac-HRBC. (*a*) Cells after treatment with anti-lac and lac-TMV. A white arrow indicates representative lac-TMV extending from the cell surface; black arrows indicate lac-TMV lying parallel to the cell surface. Compare with control, Fig. 3 *b*. (*b*) Cells after treatment with anti-*o*-gal and lac-TMV. (*c*) Cells after treatment with anti-lac and lac-KLH. (*a*) × 16,000 at 30 keV, (*b*) × 10,000 at 20 keV, (*c*) × 23,000 at 16 keV.

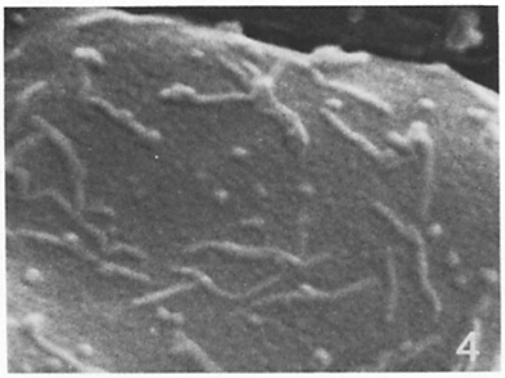

FIGURE 4 SEM micrograph of lac-HRBC labeled with lac-TMV (rods 25-nm diam) and lac-BSV (spheres, 30-nm diam). × 50,000.

similar densities of microvilli but with great differences in the degree of TMV labeling. Figs. 5 *b*, 5 *c*, and 5 *d* illustrate such contrasting cell surfaces. Some heavily labeled cells had more marker attached than seemed reasonably attributable to the presence of underlying virus (Fig. 5 *b*); this suggests that viral antigen can occur on the cell membrane in the absence of visible virus in the underlying cytoplasm. Similar findings have been reported recently for MTV (4) and for murine leukemia virus (1).

Heavily labeled cells were found in cell preparations incubated with 0.06 mg/ml lac-anti-MTV reagent. When lac-modified normal rabbit Ig (i.e., Ig from a rabbit not immunized against MTV) at a tenfold higher concentration was substituted for lac-anti-MTV, the label was very rare on tumor cell surfaces, although large areas of the culture were scanned. When tumor cells were treated first with unmodified anti-MTV (0.5 mg/ml), the usual labeling sequence of lac-anti-MTV, anti-lac, and lac-TMV resulted in no labeling; in contrast, a similar pretreatment with lac-normal rabbit Ig produced no detectable inhibition of labeling.

Fig. 6 is a transmission electron micrograph of a mammary tumor cell labeled with the lac-anti-MTV hapten-sandwich procedure. The lac-TMV marker appears on most discernible MTV particles (exceptions may be accounted for by plane of section).

Labeling of Ig-Bearing Mouse Lymphocytes

Mouse splenic lymphocytes were treated with lac-anti-MIg, anti-lac, and lac-TMV. From 40 to 60% of the lymphocytes were labeled (Fig. 7 *a*), some heavily and some moderately, while no label appeared on the remaining cells (Figs. 7 *b* and 7 *c*). A similar ratio of labeled to unlabeled splenic lymphocytes was observed with fluorescence, using Fl-anti-lac, in this and the previous study (23). Labeled cells were indistinguishable morphologically from other cells devoid of label. The cell shown in Fig. 7 *a* is typical of most labeled cells prepared in suspension: round, with an undulating surface and few microvillous structures, which do not exhibit the lac-TMV marker. The cell shown in Fig. 7 *b* is very similar in appearance, yet is altogether unlabeled. When lymphocytes were labeled in suspension, only a small percentage (< 10%) had numerous microvilli, and the lac-TMV marker for Ig determinants was not detected on these cells (Fig. 7 *c*).

With the procedure of adsorption to glass cover slips, the fraction of lymphocytes with many microvilli increased considerably. Under these conditions, the marker for Ig was detectable on some cells of both types (with or without numerous microvilli). Fig. 8 shows labeled and unlabeled cells prepared using the cover slip procedure.

DISCUSSION

These results demonstrate that hapten-sandwich labeling can be a general and versatile procedure for immunospecific attachment of cell surface markers suitable for SEM. We have concentrated here on establishing the specificity of the method, examining labeled surfaces on three different kinds of cells: human erythrocytes, mouse mammary tumor cells, and mouse lymphocytes.

Erythrocytes to which the lac hapten was azo-coupled (lac-HRBC) were readily labeled with anti-lac antibody followed by lac-TMV, lac-KLH, or lac-BSV (Figs. 3 *a*, 3 *c*, and 4); unmodified HRBC were unlabeled. Labeling of lac-HRBC required the correct (anti-lac) anti-hapten antibody, since anti-dnp and even weakly cross-reactive anti-*o*-gal (Fig. 3 *b*) did not attach the lac-TMV marker.

Lac-anti-MTV antibody, followed by anti-lac and then lac-TMV, labeled mouse mammary tumor cells (Fig. 5 *a*). Labeling could be prevented by prior treatment of the cells with unmodified anti-MTV antibody. No significant labeling was evident when lac-normal rabbit Ig was substituted for lac-anti-MTV in the regular labeling sequence. The SEM results with hapten-sandwich labeling of MTV were consistent with those observed with transmission electron microscopy of thin sections of the labeled mouse mammary tumor cells (Fig.

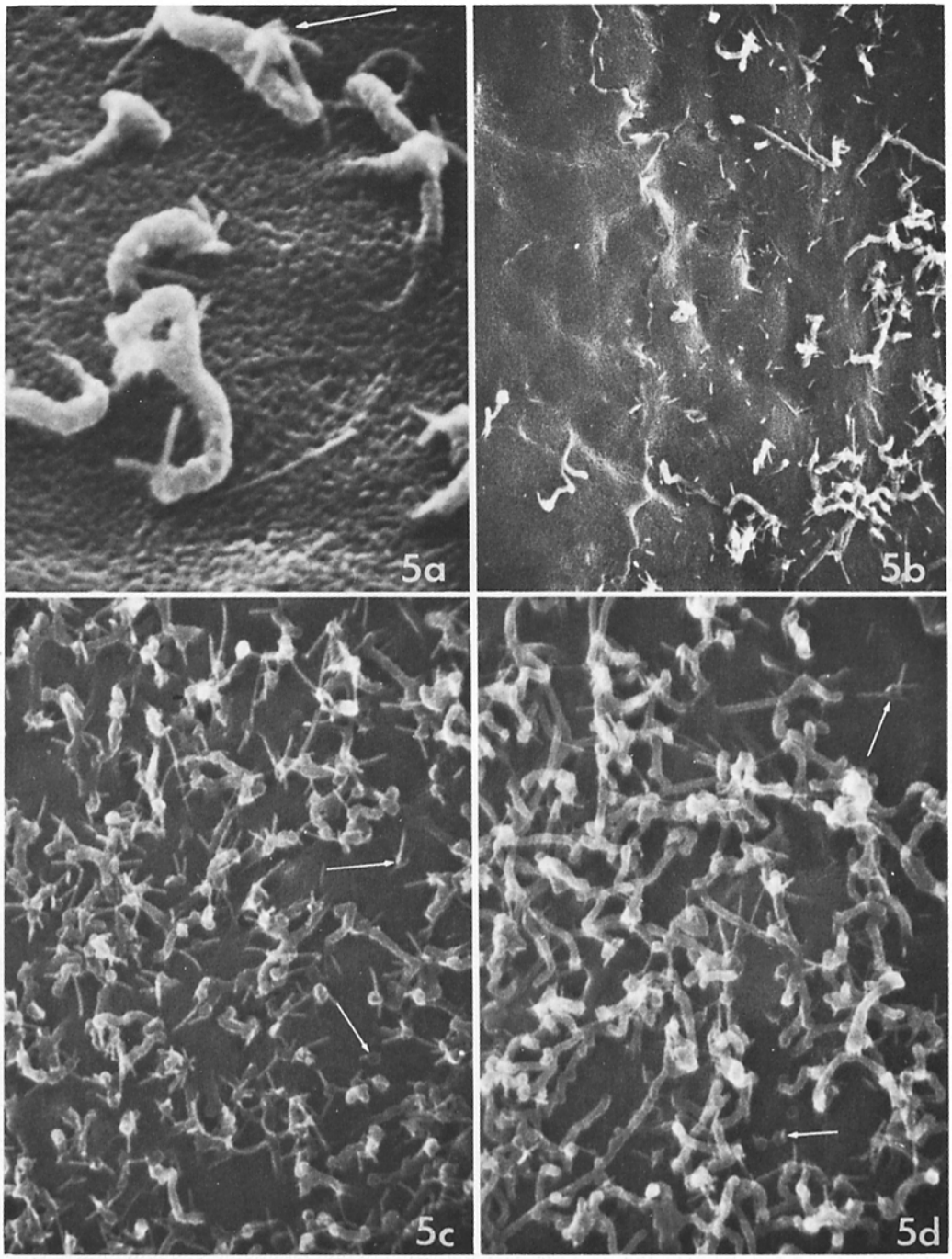

FIGURE 5 SEM micrographs of cultured mouse mammary tumor cells treated as shown in Fig. 1. (*a*) TMV is attached to microvilli and to a 100-nm particle (arrow) the size of an MTV virion. This preparation was Formalin fixed; note the etched cell surface. (*b*) Two cells and the border between them. The right cell surface is heavily labeled with TMV (slender bright rods) both on microvilli and elsewhere, whereas the left cell is virtually unlabeled. (*c*) Heavily labeled and (*d*) lightly labeled patches of microvilli on different tumor cell surfaces. Arrows indicate labeled virion-like spheres on both cells. (*a*) × 40,000 at 16 keV, (*b*) × 7,000, (*c*) × 13,000, (*d*) × 13,000 at 30 keV.

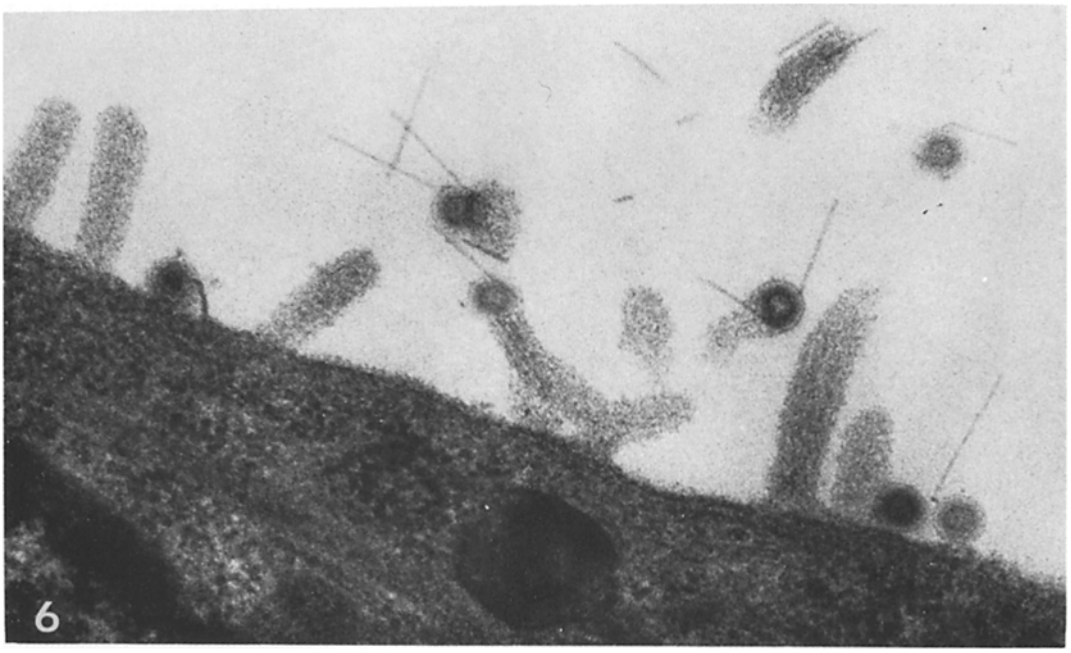

FIGURE 6 Transmission electron micrograph of cultured mouse mammary cell labeled as shown in Fig. 3; a thick section was used to increase the length of TMV label included in the section. Budding MTV particles on sectioned microvilli or on the cell surface are visible as dense rings surrounded by membrane derived from the plasma membrane. × 60,000.

6). Transmission electron microscopy revealed that identifiable MTV particles were the main sites of attachment of lac-TMV at the cell surface. SEM, on the other hand, revealed features of the topography of the labeled surfaces, especially the distribution of the MTV antigen relative to the microvilli that are present on many mammary tumor cells: specific label did not appear on cells without microvilli; label was most often found directly attached to microvilli (Fig. 5 *c*); however, some cells with dense clusters of microvilli were free of label (Fig. 5 *d*).

The proportion of mouse spleen lymphocytes labeled for surface immunoglobulin (Fig. 7 *a*) was about the same for SEM and for fluorescent microscopy. Although transmission electron micrographs of lymphocytes labeled with lac-anti-MIg, anti-lac, and lac-ferritin have shown Ig at the base of microvilli (23), these initial SEM studies with lymphocytes prepared in suspension do not exhibit the Ig marker (lac-TMV) on extended microvilli (Figs. 7 *a* and 7 *b*). There have been reports of SEM studies which suggested that B lympthocytes from human peripheral blood have multiple, long microvilli (10, 19). Such interpretations, however, have not been based on specific labeling of cell surface Ig with SEM markers. A major difficulty in assessing microvilli and their possible relationship to the distribution of receptors on lymphocyte surfaces is that these structures appear to depend in number and appearance on a variety of factors, including external environment (11). When cells were labeled and prepared for SEM in suspension, less than 10% of the mouse lymphocytes exhibited numerous microvilli; when adsorbed on glass before fixing, the number of cells with numerous microvilli greatly increased, as noted for rabbit lymphocytes (13), and included some Ig-bearing cells.

The specificity and usefulness of the hapten-sandwich method for SEM studies is well illustrated by the fact that apparently similar cells, or adjacent surface areas, within a given cell population can be sharply distinguished at moderate magnifications as heavily or lightly labeled or unlabeled (Figs. 5 *b*, 5 *c*, and 5 *d*). The same kind of uneven distribution is routinely observed in preparations labeled for fluorescent microscopy.

In principle, it should be possible to identify more than one antigen at a time (23), utilizing a variety of hapten-modified viruses or bacteriophages with distinctive size and shape. Preliminary

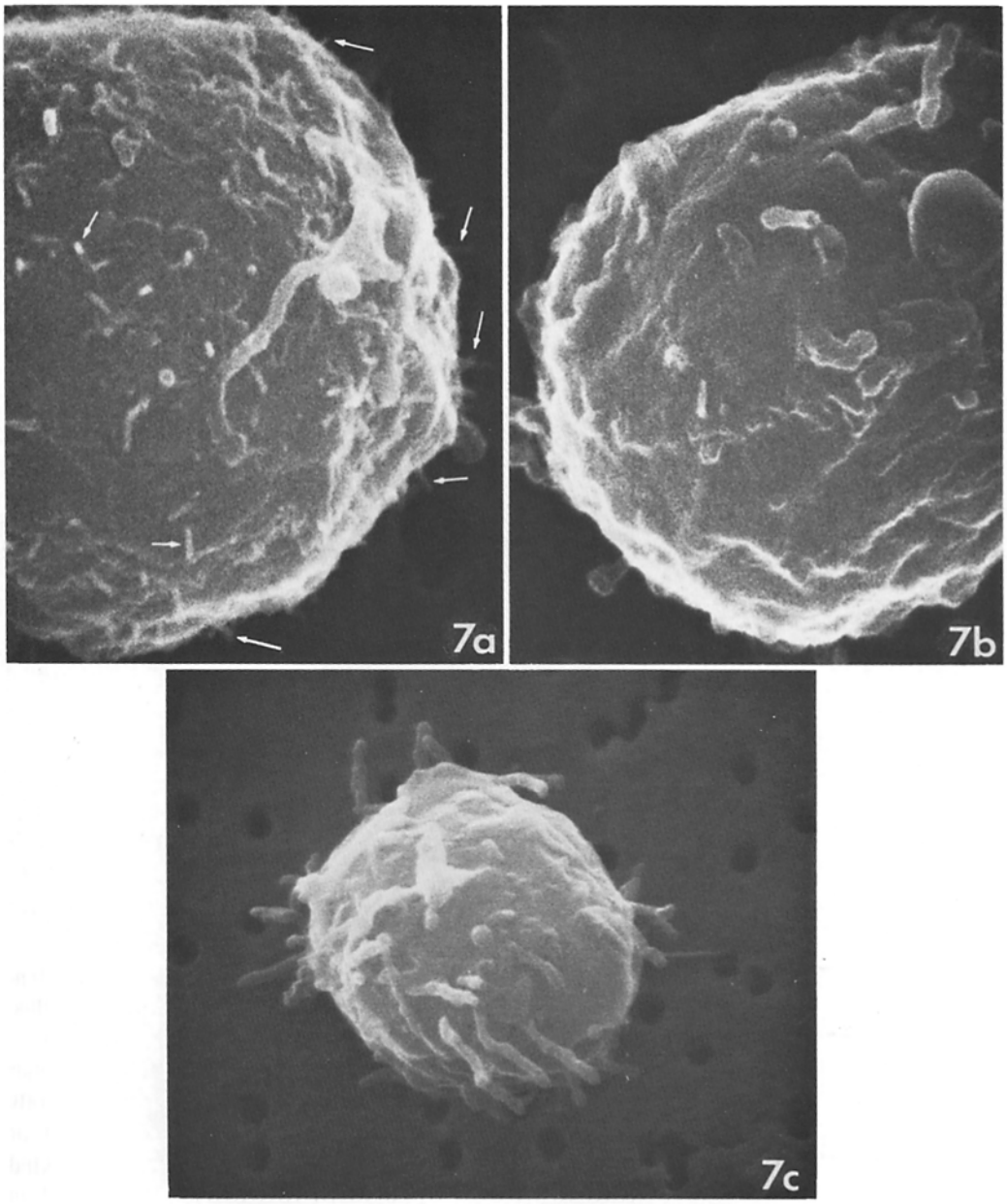

FIGURE 7 SEM micrographs of mouse spleen lymphocytes, all from the same preparation, treated with lac-anti-MIg, anti-lac, and lac-TMV. (*a*) Labeled cell with TMV attached to exposed cell surface and periphery (arrows). (*b*) Unlabeled cell. Occasional surface features that might be mistaken for label were determined at higher magnification not to be TMV. (*c*) Unlabeled cell with numerous microvilli. (*a*) × 22,000, (*b*) × 22,000, (*c*) × 15,000 at 30 keV.

labeling experiments using lac-TMV and lac-BSV simultaneously show that this is indeed feasible (Fig. 4). In practice, discrimination between two or more antigens on the same cell surface may hinge on consideration of at least two factors: (*a*) the nature of the cell itself—e.g., it is much easier to distinguish markers on an erythrocyte than on the turbulent surfaces of most lymphocytes; and (*b*)

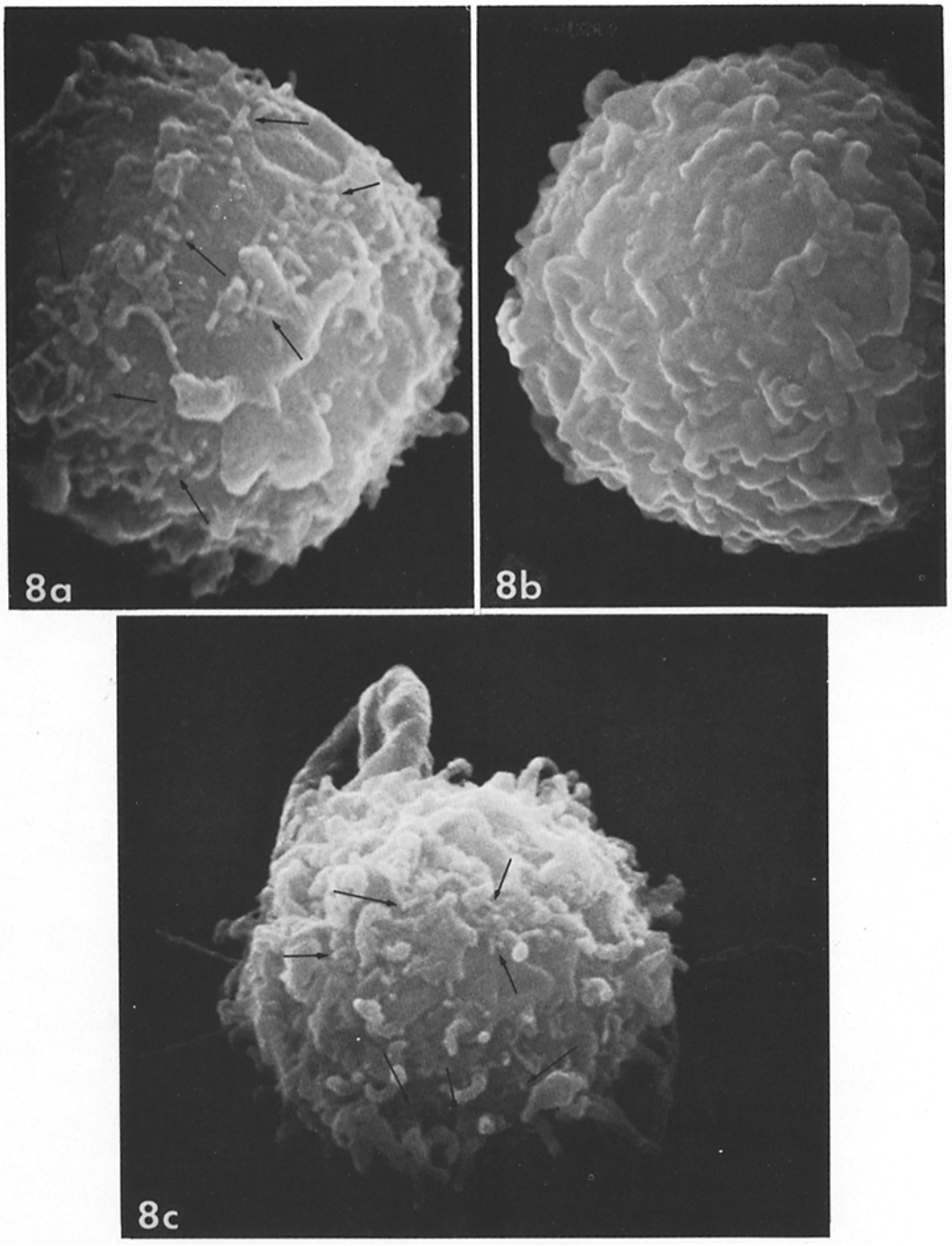

FIGURE 8 SEM micrographs of mouse spleen lymphocytes, all from the same preparation, treated with lac-anti-MIg, anti-lac, and lac-TMV, and allowed to settle onto glass cover slips before fixing. (*a*) Labeled cell: label is obviously in a patchy distribution (arrows). (*b*) Unlabeled cell with numerous microvilli. (*c*) Labeled (arrows) cell with numerous microvilli. (*a*) × 28,000, (*b*) × 25,000, (*c*) × 17,000 at 16 keV.

the size and distinctive form of each of the markers—e.g., TMV may in some cases prove too long, especially when it lies horizontally on a surface, to be useful in defining clearly the relative positions of different antigens. Precise localization of surface antigens is limited in any labeling technique that utilizes multivalent complexes as markers, and even more by the fact that SEM requires markers that are both large and recognizable enough to be distinguished from features intrinsic to the cell surface.

As shown in an earlier paper (23), large, multiple-subunit markers can be stabilized without appreciable aggregation by a limited cross-linking procedure, so that they will not dissociate when modified with hapten (cf. 9). This should make available a sizable number and variety of SEM markers that can be linked by anti-hapten antibody to hapten-coupled antibodies, mitogens, and, possibly, hormones specific for cell surface receptors. The versatility of the hapten-sandwich method is enhanced by the fact that a large variety of antibodies against azophenyl haptens can be prepared and purified easily. The simple azocoupling procedure permits the addition of amplifying hapten groups to antibodies directed against cell surface antigens with a high degree of retention of binding activity (23). Thus, the immunoglobulin fraction of any specific antiserum can be used conveniently and economically in an indirect (sandwich) labeling scheme. This method, unlike others applied to attachment of an SEM marker (15), makes it possible to label cells with alloantisera, which cannot be amplified by antibodies made in another species.

In conclusion, the utilization of new markers and improved immunospecific labeling techniques in conjunction with SEM makes it feasible (*a*) to identify particular cells in a heterogeneous population and (*b*) to determine the gross distribution of selected components relative to the topography of the cell surface.

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