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Application of Freeze-Drying Intact Cells to Studies of Murine Oncornavirus Morphogenesis

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Using a method for freeze-drying intact cells, uninfected and murine leukemia virus (MuLV)-infected $JLSV_9$ cell surfaces, as well as murine mammary tumor virus (MuMTV)-infected cell surfaces, were examined by electron microscopy. The 10-nm knobs of MuLV and the 5-nm spikes of MuMTV were clearly revealed on the surfaces of budding viruses and were also found dispersed over the cell surface. The MuLV knobs are randomly arranged on the virus surface, whereas the MuMTV spikes are much more ordered. Because freeze-fractured budding viral envelopes are devoid of intramembranous particles, the observed surface particles do not appear to be merely accentuated intramembranous particles. This technique should permit further analysis of the morphogenesis of viral envelopes without the need for externally applied labels.

Murine leukemia viruses (MuLV) and murine mammary tumor viruses (MuMTV) mature by budding through the cell surface. In this process, either a developing viral nucleocapsid (e.g., MuLV) or a preformed viral nucleocapsid (e.g., MuMTV) becomes enveloped by a portion of the host plasma membrane. During budding, this portion of the cell surface membrane becomes morphologically, biochemically, and antigenically differentiated from the rest of the membrane (3, 16, 17). How such localized topographical differentiation occurs within a presumably fluid membrane is an intriguing problem and may have profound physiological significance.

The major virus-specific component of the MuLV envelope is glycoprotein of molecular weight 69,000 to 71,000 and is thus designated gp69/71 or gp70 (1, 26, 33). This molecule possesses type-, species-, and interspecies-specific antigenicities, has hemagglutinating activity, and can elicit production of virus-neutralizing antibody (12), as well as immunize mice against MuLV-induced leukemia (13). The gp70 molecules have been visualized on the envelopes of purified MuLV by transmission electron microscopy of negatively stained and freeze-dried preparations and appear as spherical projections, or "knobs," about 10 nm in diameter (18, 36).

Similarly, the major virus-specific component of the MuMTV envelope appears to be glycoprotein of 52,000 to 55,000 molecular weight, designated gp52 (22, 24, 35). This molecule is associated with prominent projections, or "spikes," on the MuMTV surface, which are about 5 nm in diameter and have an interspike distance of about 8 nm (23).

Knobs and spikes presumably form on the virus envelope during budding, and they may be present on the generalized cell surface as well. If so, it might be possible to follow the morphogenesis of the virus envelope by directly visualizing the dynamic movement and distribution of these projections on the surfaces of virus-infected cells. This would eliminate the need for externally applied ligands, which are generally required to identify a specific molecule but which unavoidably perturb the native configuration of the labeled molecules (4, 6, 32).

We have developed a technique that permits this type of investigation (25). Freeze-drying of intact cells has enabled us to visualize knobs and spikes on viruses in the process of budding by revealing surface detail superior to that obtained with other techniques (2, 7, 10, 19, 31).

MATERIALS AND METHODS

Cells. Monolayer cultures of $JLSV_9$ mouse fibroblasts (V_9), derived from BALB/c-strain bone marrow, and Rauscher leukemia virus (RLV)-infected $JLSV_9$ cells (V_9-RLV) were provided by J. Gruber, The John L. Smith Memorial for Cancer Research, Pfizer & Co., Maywood, N.J. STU-Eveline cells, Friend MuLV-infected mouse fibroblasts adapted to growth in suspension culture (28), were obtained from W. Schäfer, Max-Planck-Institut für Virusforschung, Tübingen, W. Germany. A monolayer culture of MuMTV-producing cells, designated MuMT-73, was derived from a BALB/cfC3H mouse mammary tumor (N. Sarkar et al., manuscript in preparation) and was obtained from N. Sarkar, Sloan-Kettering Institute.

V_9 and V_9-RLV cells were cultured in RPMI 1640 medium supplemented with 8% fetal bovine serum, 100 U of penicillin per ml, and 100 μg of streptomycin sulfate per ml, in an atmosphere of 5% CO_2. MuMT-73 and STU-Eveline cells were grown in Dulbecco modified medium with penicillin and streptomycin and 15% fetal bovine serum.

Virus. RLV was purified from the medium of 2-day-old cultures of V_9-RLV cells. After short periods of centrifugation at low speed (3,500 × *g* for 5 min) and high speed (13,000 × *g* for 20 min) to remove cells and cell debris, virus was pelleted by centrifugation at 13,000 × *g* for 2.5 h in a Sorvall GS-3 rotor (DuPont, Wilmington, Del.). Virus was resuspended in 0.05 M aqueous sodium citrate and stored at 4°C.

Freeze-drying. Cell monolayers grown on 0.5-inch (ca. 1.2-cm)-diameter glass cover slips were rinsed with Earle balanced salt solution and fixed for 1 h at room temperature with either 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, followed by 1% cacodylate-buffered osmium tetroxide (OsO_4), or with 1% cocodylate-buffered OsO_4 alone. The pH of each solution was adjusted to 7.2, and the osmolarity was adjusted to 290 to 320 mOsm, approximately the same as the culture medium.

After fixation, cover slips with adherent cells were rinsed for 30 min with at least five changes of distilled water, drained of excess water, and rapidly immersed in liquid nitrogen (−190°C) or in liquid Freon 22 (−150°C) and then transferred to liquid nitrogen. The cover slips were then placed on the precooled (−150°C) specimen table of a Balzers 360 M freeze-etching apparatus (Balzers High Vacuum, Liechtenstein) and held firmly in place by a screw-on retaining ring. At a vacuum of 10^{-6} Torr, the temperature of the specimen table was raised to −82°C, a precooled knife (−150°C) was placed over the cover slip, and sublimation of ice was allowed to proceed for 15 to 25 min. To reduce condensation of contamination onto the specimen (in the form of fibrillar networks) during the initial stage of evacuation, a liquid nitrogen-cooled "cap" was placed over the specimen until the proper vacuum was attained. An effective cap was made from a Balzers single-specimen retaining ring by sealing the top. Under vacuum, the cap was knocked off with the knife assembly. Platinum-carbon replicas were made by evaporating platinum at a 45° angle and were strengthened with evaporated carbon. The replicas were removed from the glass rapidly with hydrofluoric acid (50% reagent grade; Fisher Scientific Co., Fair Lawn, N.J.) at room temperature and were cleaned in progressively increasing concentrations of sodium hypochlorite before being rinsed with distilled water and mounted on Formvar-coated 75-mesh or freeze-fracture electron microscope grids (Pella Co., Tustin, Calif.). Replicas were viewed with a JEM 100B electron microscope, and micrographs were printed with white shadows directed upwards.

Purified virus was adsorbed onto glow-discharged, Formvar- and carbon-coated grids. After rinsing with 0.05 M ammonium acetate, grids were plunged into liquid nitrogen and freeze-dried as described above. The specimen table was warmed to room temperature before venting the bell jar.

Negative staining. Purified virus was adsorbed onto grids prepared as above. The grids were rinsed briefly with distilled water and then immersed in 1% aqueous uranyl acetate for 15 to 30 s.

Freeze-fracture. For freeze-fracturing, STU-Eveline cells were rinsed with Earle balanced salt solution and fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer for 1 h at room temperature. After several rinses with cacodylate buffer, glycerol was added dropwise to a final concentration of 25%. Cells were pelleted, and thick suspensions were applied to gold specimen holders, frozen in liquid Freon 22, and stored in liquid nitrogen. Cells were freeze-fractured at −115°C, and replicas were cleaned with sodium hypochlorite and mounted on Formvar-coated grids.

Thin sectioning. Cell monolayers growing in plastic petri dishes were rinsed with Earle balanced salt solution and fixed with either 1% cacodylate-buffered OsO_4 or 1% cacodylate-buffered glutaraldehyde followed by 1% cacodylate-buffered OsO_4; each fixation was for 1 h at room temperature. Monolayers were immersed in 0.5% aqueous uranyl acetate overnight at 4°C, dehydrated with ethanol, and embedded in Epon. Thin sections were stained with 5% methanolic uranyl acetate and with lead citrate.

RESULTS

The described method of freeze-drying monolayer cultures permits visualization of entire exposed surfaces of a large number of cells in a manner similar to scanning electron microscopy (Fig. 1), but with considerably greater resolution. At higher magnifications no indication of budding virus could be detected on the V_9 cells (Fig. 2). However, the surfaces of similarly prepared V_9-RLV cells (Fig. 3) present a striking contrast in morphology: the resolution attainable is evident, and individual RLVs in the process of budding, or adhering to the cell surface after envelopment, can be identified by their coating of 10-nm knobs. These knobs appear to be randomly organized on the virus surface, but this could be due in part to their known fragility (36), which might result in the loss of some knobs from an originally more regular array. In thin sections of V_9-RLV cells, periodic densities probably representing individual knobs are often evident on the outer surfaces of the envelopes of budding and mature viruses (Fig. 3), although these knobs are more readily apparent in negatively-stained and freeze-dried preparations of purified virus (Fig. 4). Ferritin-antibody labeling of V_9-RLV cells, using an anti-gp70 serum and followed by freeze-drying, appears to completely cover the virus buds, obscuring knob detail (A. Demsey, unpublished data).

Knoblike structures are also evident randomly dispersed over the surfaces of V_9-RLV cells (Fig. 3). Similar observations were made

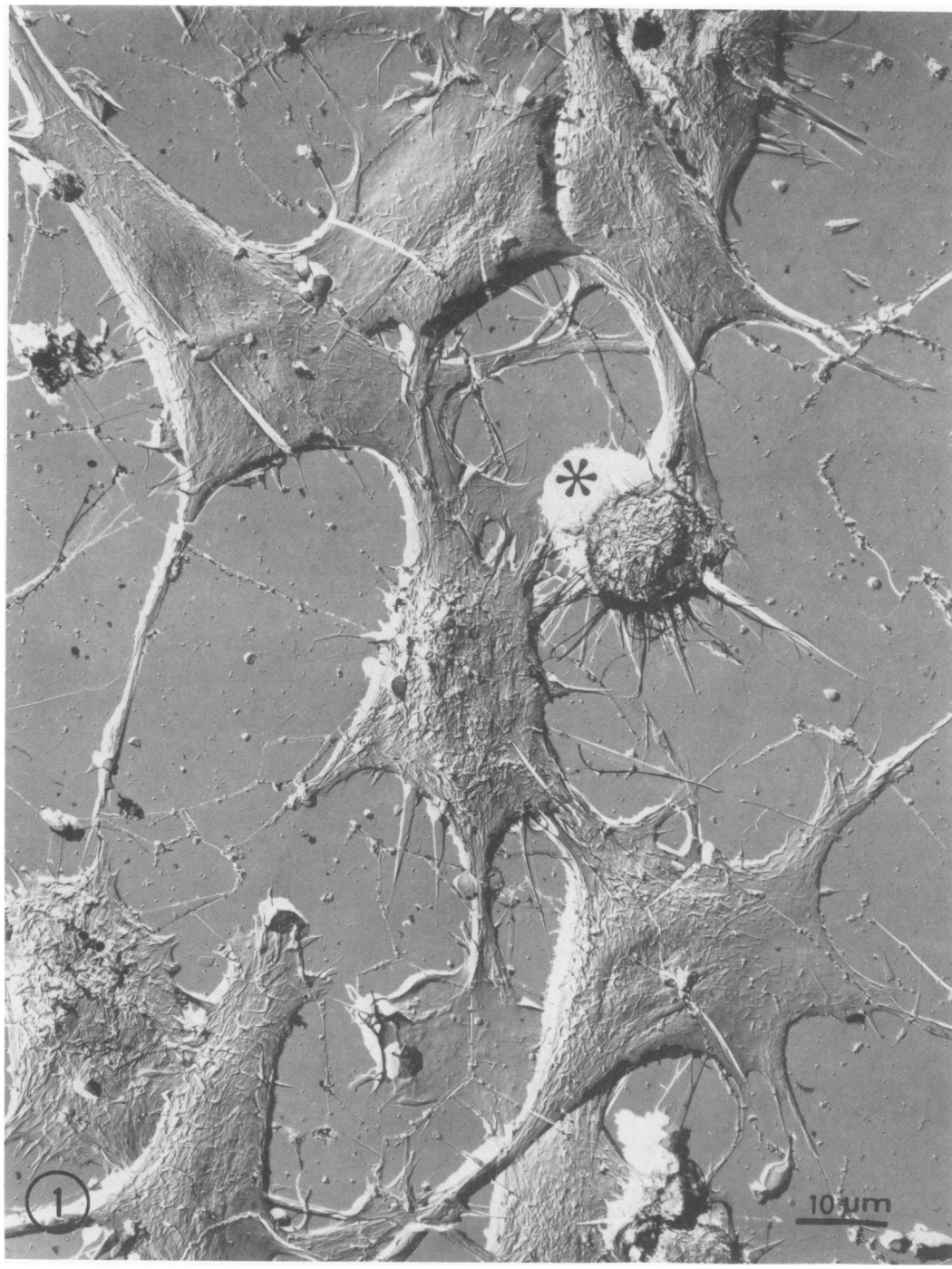

FIG. 1. *Survey electron micrograph of a platinum-carbon replica of freeze-dried intact V_9 cell monolayer. The asterisk marks a cell undergoing mitosis. ×1,300.*

on replicas of the surfaces of suspension-cultured STU-Eveline cells (25, 27). This appearance is consistent with recent indications that gp70 and other MuLV-specific antigens are located not only on virus buds, but also on the generalized cell surface (11, 14, 15, 27, 37). A number of particles on the cell surface may also correspond to normal cell surface constituents,

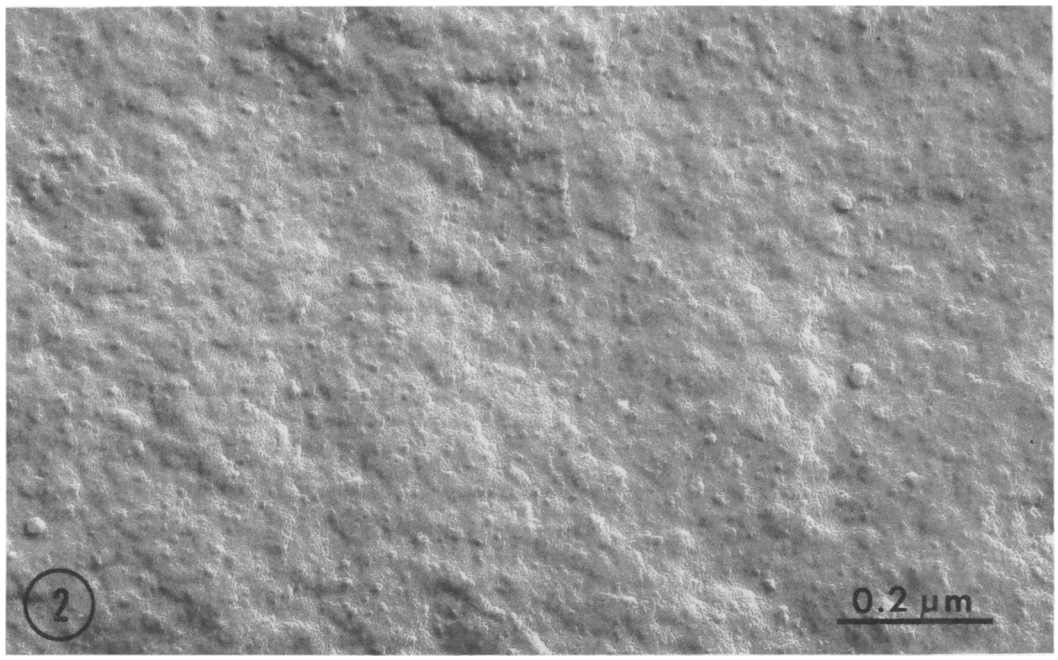

FIG. 2. *High magnification of a replica of a freeze-dried V_9 cell surface. Some particulation is evident.* ×*96,000.*

since uninfected V_9 cells display some surface particulation. The amount of cell surface particulation specifically associated with viral infection can, to some extent, be determined by comparing particle number and size distribution per unit area of V_9 and V_9-RLV cell surfaces (Fig. 5). Such analysis indicates that the V_9 surface possesses approximately 45% as many particles as the V_9-RLV cell surface and less than 40% as many particles in the range of 10 nm, the size of the MuLV knobs. Furthermore, knob-sized particles on the V_9-RLV surface appear much more prominent than other surface particles, particularly when viewed in stereo pairs.

Replicas of MuMT-73 cells (Fig. 6) similarly reveal numerous MuMTV buds on the cell surface. These viruses are characterized by the presence of spikes that are smaller than MuLV knobs, are more regularly arranged, and are more readily visualized in thin sections. The generalized cell surface is also covered with particles similar in appearance to virus spikes, although their small size sometimes renders them difficult to visualize.

Knobs, spikes, and similar particles on the cell surface apparently do not represent external manifestations of intramembranous particles demonstrable by freeze-fracturing (20, 34), since intramembranous particles are lacking within the envelopes of budding MuMTV (30) and MuLV (Fig. 7).

DISCUSSION

Although the general cell morphology revealed by other replica techniques, such as critical-point drying and air drying, has been adequate for many studies (2, 7, 10, 19, 31), these techniques do not appear to afford the resolution obtainable with the method of freeze-drying that we have outlined. Freeze-drying has been used previously to examine the surfaces of cells (8, 9), but structural detail did not appear to be preserved as well as in our preparations, perhaps because of our more precise control of sublimation temperatures or because of the relatively short periods of fixation used by other investigators. We have found either a standard glutaraldehyde prefixation-OsO_4 postfixation or fixation with OsO_4 alone to be satisfactory.

Recent reports (23, 29) have suggested that MuMTV may possess some sort of coat exterior to the viral spikes. However, we have failed to substantiate this in our system, and the appearance of spikes as well as knobs on virus surfaces after freeze-drying is consistent with indications of the presence of these structures on purified virus by negative staining (18, 23).

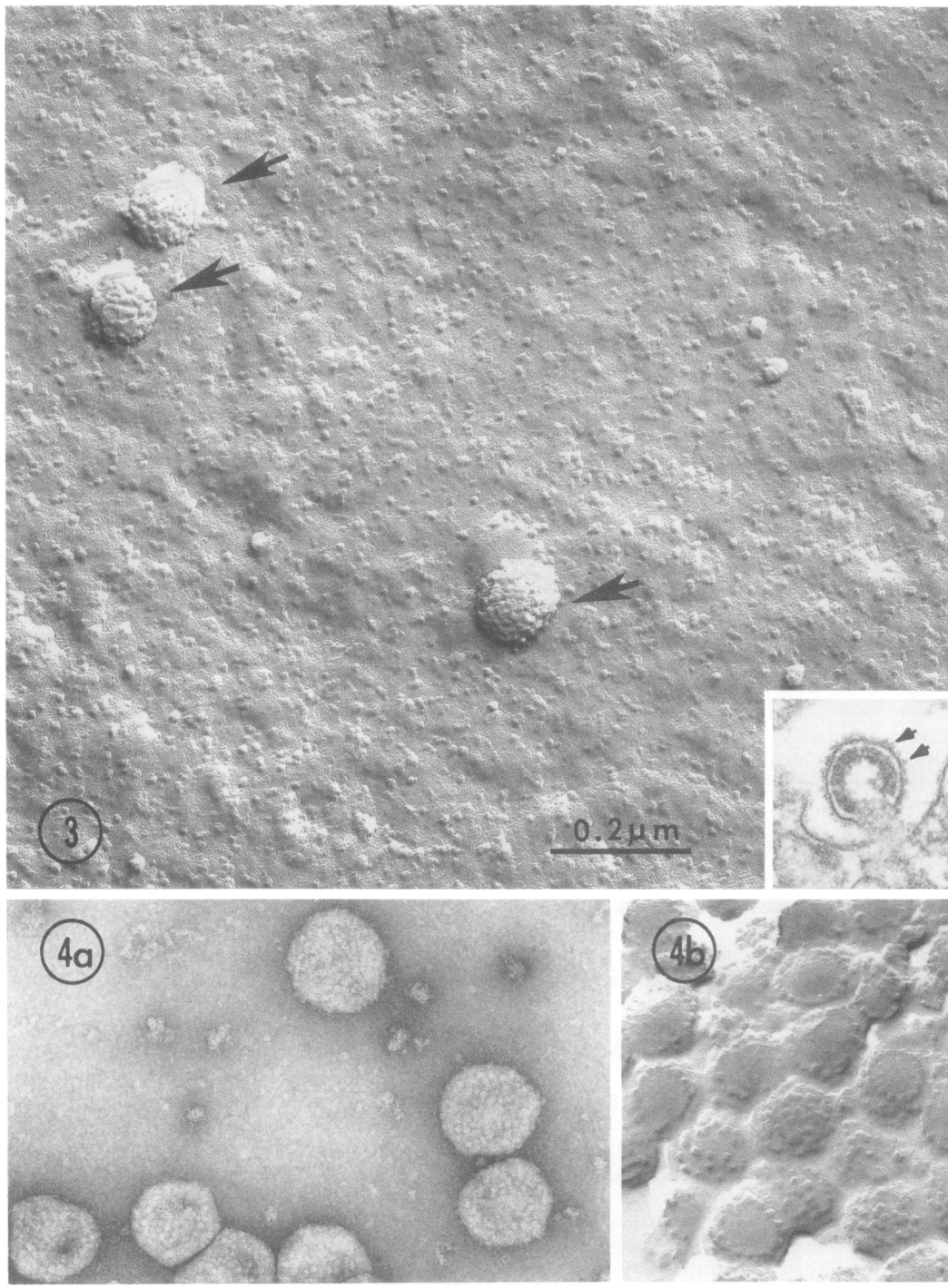

FIG. 3. *High magnification of the surface of a freeze-dried V_s-RLV cell. Budding MuLV (arrows) are covered with knobs, and the rest of the cell surface is covered with similar particles. ×96,000. (Inset) Thin section of a virus particle budding from the surface of a V_s-RLV cell. Densities probably representing knobs are evident (arrows). ×96,000.*

FIG. 4. *(a) Purified RLV negatively stained. ×96,000. (b) Freeze-dried preparation of purified RLV with knobs evident. ×96,000.*

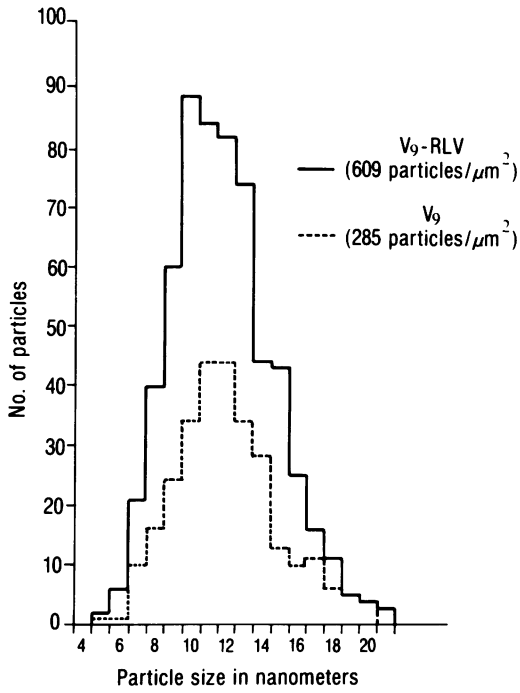

FIG. 5. *Size distribution of particles discernible on the surfaces of V_9 and V_9-RLV cells after freeze-drying. Knobs on viruses were not included. Particles are more than twice as numerous on V_9-RLV cells, particularly in the 10-nm size range.*

Although the possibility must be considered that the appearance of the cell surface, after freeze-drying, may represent preparative artifact, the freeze-fracturing studies noted, as well as indications from a variety of other experimental studies including freeze-drying of erythrocyte ghost membranes, artificial membrane liposomes, and unfixed cells, suggest that freeze-drying affords an accurate representation of cell surface organization (Demsey et al., manuscript in preparation).

It is probable that virus knobs and spikes represent molecular oligomers, as may be the case for intramembranous particles (21), which are similar in size. Knobs in particular are considerably larger than would be expected for individual protein molecules, and there are suggestions that the individual knobs may in fact consist of multiple gp70 molecules (5). MuMTV spikes may also consist of several molecules (23). Our results indicate that MuLV knobs and MuMTV spikes are formed on the cell surface prior to their aggregation into budding virus and are randomly dispersed.

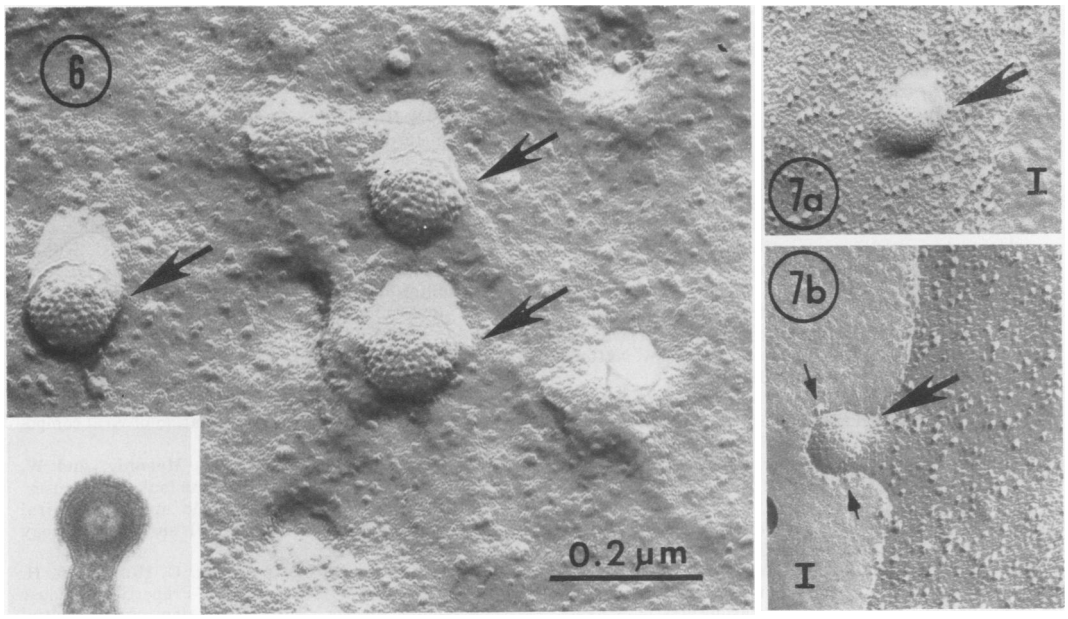

FIG. 6. *Replica of a freeze-dried MuMT-73 cell surface. Regularly arranged spikes are evident on the MuMTV surfaces (arrows). ×96,000. (Inset) Thin section of a virus particle budding from a MuMT-73 cell and displaying prominent spikes. ×96,000.*

FIG. 7. *Replicas of freeze-fractured STU-Eveline cells revealing the cytoplasmic or protoplasmic fracture face (PF) of the surface membrane. "I" indicates ice face. Intramembranous particles are excluded from budding MuLV (large arrows). Structures surrounding the virus bud in (b) (small arrows) are suggestive of knobs. ×96,000.*

We have recently reported an application of this technique to an L1210(V) gln$^-$ cell line that is coinfected with MuLV and MuMTV; the surface projections on individual budding MuLV and MuMTV are homogeneous, suggesting a high degree of morphogenetic specificity for the envelopes of both viruses (A. Demsey, T. A. Calvelli, D. Kawka, C. W. Stackpole, and N. H. Sarkar, Virology, in press). Similarly, this freeze-drying technique should permit us to visualize many other aspects of the dynamic morphogenesis of murine oncornavirus envelopes without the need for using perturbing externally applied labels to identify membrane constituents.

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