

Effect of vinblastine on distribution of murine leukemia virus-derived membrane-associated antigens

(*gag* and *env* gene-derived membrane antigens/cytoskeleton/cap-like phenomenon/indirect immunofluorescence)

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ABSTRACT The effect of vinblastine on the distribution of murine leukemia virus-derived membrane-associated antigens was examined by using the indirect immunofluorescence of 3.7% formaldehyde-fixed MJD-54 (Moloney murine leukemia virus-infected) cells. On fixed, non-drug-treated cells, p30 antigen was distributed homogeneously and diffusely over the cell membrane. When cells were incubated with 10 μ M vinblastine for 1 hr before fixation, the distribution of p30 antigen was greatly changed, fluorescence now being collected into poles (cap-like formation). In contrast to this distribution pattern for p30 antigen, gp70 antigen was distributed in a micropunctate pattern on the cell surface, with or without vinblastine pretreatment. These observations indicate that the distribution patterns of p30 and gp70 membrane antigens are completely different and that they are differently controlled by cytoplasmic microtubules. In addition, because the p30 membrane antigen visualized in these studies most likely represents viral Pr65^{gag} precursor molecules which are localized directly under and associated with the plasma membrane, these results suggest that, under special conditions of fixation, it is possible to obtain a cap-like phenomenon for cytoplasmic (internal) membrane-oriented proteins.

It now has been generally accepted that the lateral mobility and distribution of externally exposed cell surface proteins are regulated in large part by submembranous cytoskeletal elements (1-6), although there is also some evidence that directed lipid flow in membranes is important as well (7). Such a concept has come mainly from the study of drug effects on the distribution of cell surface proteins. The drugs used include microtubule-depolymerizing agents such as colchicine, nocodazole, and vinblastine (2, 4, 6) and microfilament-depolymerizing agents such as cytochalasin B (4, 8). The distribution of immunoglobulins and concanavalin A receptors on lymphocytes (3, 4, 6) and fibroblasts (1) after treatment with such drugs has been extensively studied by fluorescence microscopy.

In the case of cells chronically infected with murine leukemia virus there are three known classes of cell membrane-associated proteins that are coded for by the viral genome. Two are externally exposed; (i) glycosylated *gag* (group-specific antigen) gene products of M_r 80 \times 10³ and 95 \times 10³ (9-11) which have been described earlier as "Cross cell surface antigens" and shown not to be incorporated into virus particles (12); and (ii) the *env* gene products gp70 and p15E. gp70 exists both at the site of virus buds (13, 14) and over the cell surface (9, 13); p15E is embedded directly in the membrane (15).

In addition, there is an internally exposed cell membrane-associated protein. This is the major *gag* gene product, Pr65^{gag}, which is the precursor to the group-specific viral core proteins (p30, p15, p12, and p10) (10, 16). It has been localized, by elec-

tron microscopic studies, to a position just under the cell membrane (16) and, because it has a hydrophobic NH₂ terminus, p15 (17), it is probably directly associated with the cell membrane. The immunocytochemical distribution pattern of these proteins on the cell membrane and the possible involvement of the cytoskeleton in the regulation of their distribution is not well established.

In this paper we report the distribution pattern of p30 and gp70 antigens on the membranes of murine leukemia virus-infected cells and the effect of vinblastine on their distribution as examined by an improved indirect immunofluorescence technique (18, 19).

MATERIALS AND METHODS

Materials. MJD-54 cells, which are a line of JLSV-9 mouse fibroblasts chronically infected with Moloney murine leukemia virus, were used for all studies. They were originally obtained from K. Manly (Roswell Park Memorial Cancer Institute, Buffalo, NY) and have been maintained in our laboratory in a relatively early (P20-P50) passage over the past 6 years. Vinblastine sulfate (Eli Lilly) was dissolved in sterilized distilled water and diluted with medium to the final concentration used for the experiments. The antisera used were goat anti-Rauscher murine leukemia virus p30 (National Cancer Institute Resources Program, lot 78S-223) goat anti-Rauscher murine leukemia virus gp70 (Resources Program, lot 78S-225) and fluorescein isothiocyanate (FITC)-tagged rabbit IgG against goat IgG (Cappel Laboratories, Cochranville, PA). Both anti-p30 and anti-gp70 antisera were used for immunofluorescence diluted 1:40 in phosphate-buffered saline (P_i/NaCl). FITC-tagged rabbit IgG was used diluted 1:20.

Cell Culture. MJD-54 cells were seeded on sterile 18 \times 18 mm glass coverslips and grown in Dulbecco's modified Eagle's minimal essential medium (DME medium; GIBCO) supplemented with 10% fetal calf serum (Flow Laboratories, McLean, VA) in a 5% CO₂/95% air incubator for 1-2 days. After this period, the medium was replaced with DME medium containing 0.3% fetal calf serum and the cells were maintained in this for 1 day. This step of maintaining cells in low-serum medium was found to be important for the immunofluorescence technique because such cells spread well onto the substratum and have a flattened cytoplasm that makes observation of the fluorescence very clear compared to cells maintained in high-serum medium (18, 19).

Indirect Immunofluorescence. Cells were preincubated with or without 10 μ M vinblastine for 1 hr at 37°C in DME medium plus 0.3% fetal calf serum. After incubation, cells were

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Abbreviations: FITC, fluorescein isothiocyanate; P_i/NaCl, phosphate-buffered saline; DME medium, Dulbecco's modified Eagle's minimal essential medium.

rinsed once in DME medium and fixed with freshly made 3.7% formaldehyde (Fisher certified reagent, Fairlawn, NJ) in $P_i/NaCl$ for 20 min at room temperature. We chose this concentration because the cells did not show strong p30 fluorescence when they were fixed with lesser concentrations such as 1% (data not shown). The fixed cells were then rinsed well with $P_i/NaCl$ to wash away the formaldehyde, and the first antiserum (anti-p30 or anti-gp70) was added. The incubation of cells with antiserum was for 40 min at 37°C in 5% $CO_2/95\%$ air in a humidified incubator. Next, the cells were rinsed in $P_i/NaCl$ for 15 min at room temperature with continuous agitation to remove unbound antiserum. Incubation of cells with the second antiserum (FITC-tagged rabbit IgG) and the following rinse of cells in $P_i/NaCl$ were performed in the same way. During all of the procedures the cells were processed without air drying. Finally, coverslips were mounted on glass slides with Aquamount (Lerner Laboratories, Stamford, CT) and viewed with a Leitz fluorescence microscope. Photographs were taken at the same exposure on Kodak Tri-X film; prints were also made at the same exposure. In those experiments in which acetone was used, cells prefixed in 3.7% formaldehyde were passed through acetone/ H_2O , 1:1, for 2 min, acetone for 5 min, and acetone/ H_2O , 1:1, for 2 min at $-20^\circ C$ serially (18). Indirect immunofluorescence was then detected as described above.

Electron Microscopic Study. Cells were grown and maintained as described above in T-75 flasks (Costar, Cambridge, MA). After treatment (or not) with 10 μM vinblastine for 1 hr, fixation with formaldehyde, and incubation with anti-p30 antiserum as for immunofluorescence studies, the cells were scraped free with a rubber policeman and pelleted by centrifugation. Cell pellets were further fixed with 2% glutaraldehyde/0.1 M sodium cacodylate, pH 7.4. Postfixation with OsO_4 , dehydration, embedding, sectioning, and staining with lead citrate were done as described (20).

RESULTS AND DISCUSSION

A primary goal of this study was to localize by immunofluorescence the p30 antigen on the cell membrane. In order to do this we found that first we had to fix the MJD-54 cells with 3.7% formaldehyde and then stain with anti-p30 antiserum. In this case, a homogeneous or diffuse pattern of fluorescence could be observed (Fig. 1A); when viable cells or cells fixed in 1% formaldehyde were used, only weak p30 fluorescence was observed (data not shown). We contend that the homogeneous fluorescence pattern shown in Fig. 1A represents p30 antigen associated with the cell membrane because, when complete permeabilization of cells is achieved with acetone after formaldehyde fixation, the p30 fluorescence pattern is different (Fig. 1B)—i.e., a granular fluorescence is noted over the cytoplasm with a concentration of p30 antigen in the perinuclear region. This perinuclear pattern appears similar morphologically to the cytochemical staining of the Golgi region seen in cultured mouse 3T3-L1 cells (21).

When the MJD-54 cells had been preincubated with 10 μM vinblastine for 1 hr before fixation and then processed for indirect immunofluorescence with anti-p30 serum, the distribution of fluorescence (Fig. 1C) was drastically changed from the case of nontreated cells (Fig. 1A). Fluorescence now was collected into a limited region or pole of the cell, and other regions no longer displayed fluorescence staining. This collection of fluorescence into a limited region appears similar morphologically to the capping phenomenon described for lymphocytes and other cell systems (2–5, 7, 8), although the mechanism of cap-like formation of p30 antigen may be different, as discussed below. The location of such putative p30 antigen caps on the

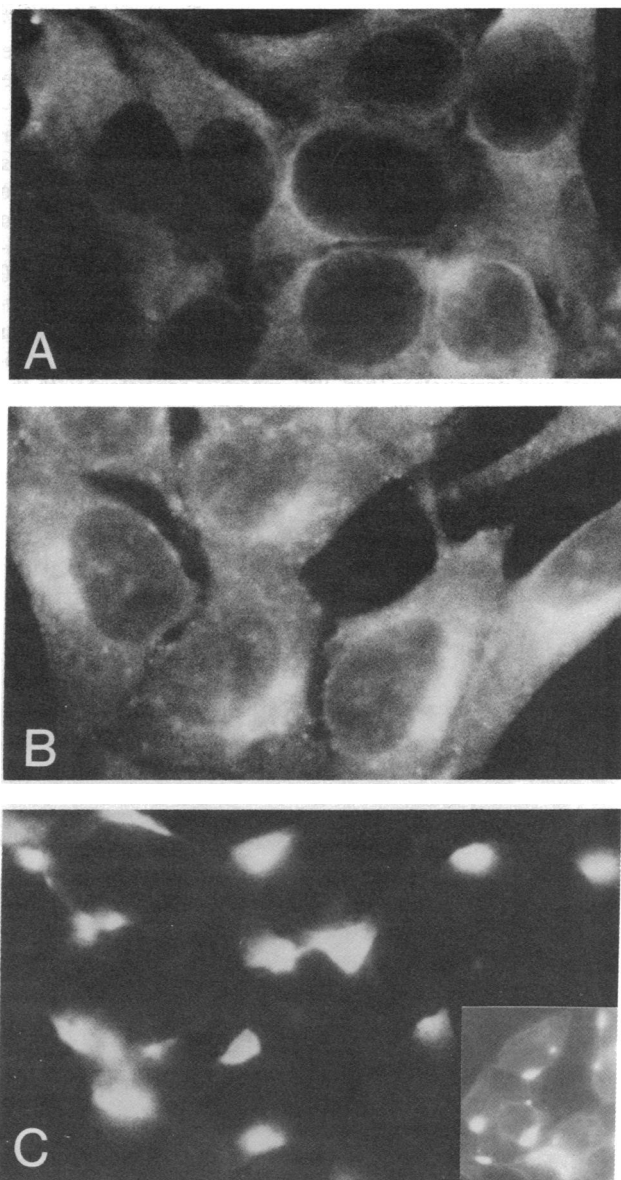


FIG. 1. (A) Non-drug-treated MJD-54 cells were fixed with 3.7% formaldehyde and stained with anti-p30 antiserum by the indirect immunofluorescence method. (B) Non-drug-treated cells were fixed with 3.7% formaldehyde, subjected to acetone treatment, and then stained with anti-p30 antiserum. (C) Vinblastine-treated cells were fixed with 3.7% formaldehyde and stained with anti-p30 antiserum. (Inset) Lightly exposed photograph to portray the cell contour. As controls, we used nonimmune goat serum and anti-p30 antiserum preabsorbed with disrupted Rauscher leukemia virions at 1:40 dilution under the conditions of A, B, and C and found no fluorescence. We also found no fluorescence when anti-p30 antiserum was used against third-passage uninfected BALB/c mouse embryo fibroblasts (kindly provided by R. Shames). (A, B, C, $\times 1300$; Inset, $\times 400$).

cell membrane varied from one cell to another and the number of cap-like structures per cell varied as well. Some cells formed one cap and others had two or three caps. This cap-like formation of p30 antigen was observed in about 80% of vinblastine-treated cells ($\pm 10\%$). Furthermore, cap-like formation was also observed after treatment with 10 μM colchicine or nocodazole (data not shown). Thus, it appears that the distribution of p30 antigen on the cell membrane might be considered to be regulated in part by cytoplasmic microtubules.

To determine whether this cap-like formation observed with p30 antiserum was a general property of MuLV cell membrane

antigens, the distribution of gp70 antigen was examined by the same indirect immunofluorescence technique. On the surface of non-drug-treated cells fixed with 3.7% formaldehyde, the distribution pattern of gp70 fluorescence (Fig. 2A) was characteristic and completely different from the one observed for p30 fluorescence—i.e., the gp70 antigen appeared as numerous micropunctate dots over the entire surface. This micropunctate fluorescence might correlate to the microaggregate distribution of gp70 previously observed by immunoferritin electron microscopy (14). The same clear images of the distribution of gp70 also were seen with cells fixed in 1% formaldehyde. Furthermore, when the cells were made permeable with acetone after fixation, the gp70 fluorescence appeared to be mostly cytoplasmic and perinuclear (Fig. 2B), which is consistent with the results obtained for p30 antigen (Fig. 1B).

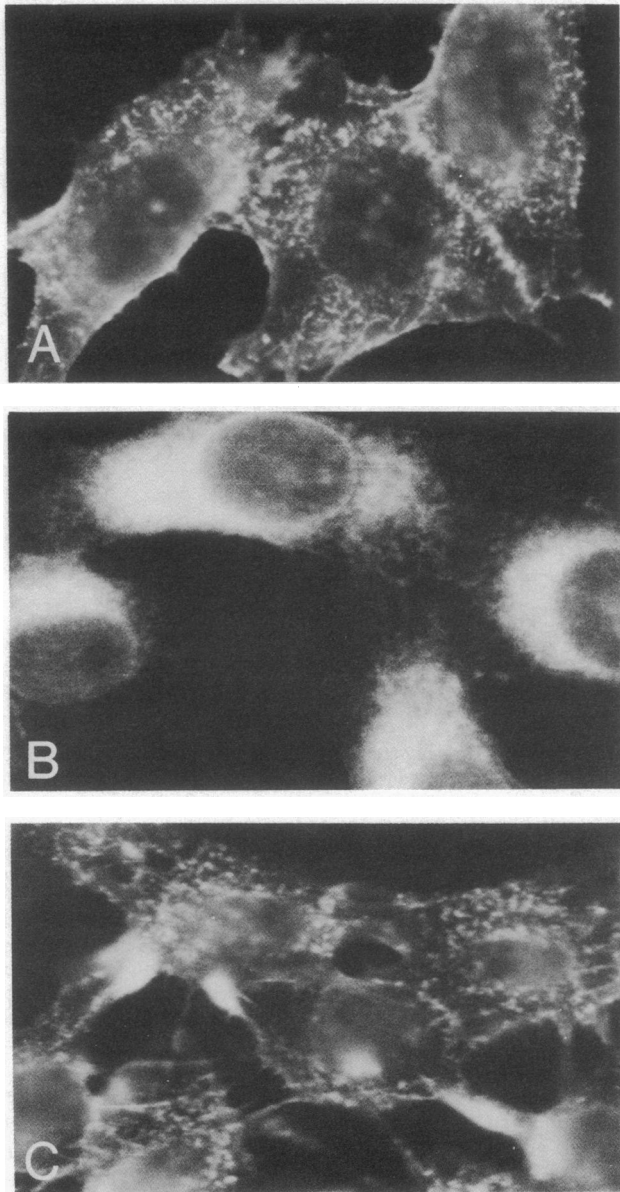


FIG. 2. (A) Non-drug-treated MJD-54 cells were fixed with 3.7% formaldehyde and stained with anti-gp70 antiserum by the indirect immunofluorescence method. (B) Non-drug-treated cells were fixed with 3.7% formaldehyde, subjected to acetone treatment, and then stained with anti-gp70 antiserum. (C) Vinblastine-treated cells were fixed with 3.7% formaldehyde and stained with anti-gp70 antiserum. ($\times 1300$.)

The effect of vinblastine on the distribution of gp70 antigen was also examined. Cells were incubated with 10 μ M vinblastine, fixed with 3.7% formaldehyde, and processed for indirect immunofluorescence. In this case, however, the treatment of cells with vinblastine did not affect the distribution pattern of antigen (Fig. 2C); only the micropunctate gp70 fluorescence was seen on >90% of the surface of drug-treated cells. In the other cells (<10%), larger clusters of fluorescence were observed in addition to the micropunctate fluorescence. Identical results were also found for cells treated with 10 μ M colchicine or nocodazole (data not shown). These results suggest that cytoplasmic microtubules have virtually no regulatory role on the distribution pattern of gp70 surface antigen under the conditions used in these experiments.

We next wanted to know whether the p30 fluorescence detected on cells fixed in 3.7% formaldehyde was due to glycosylated gag products or to Pr65^{gag} moieties. It is well established, both by surface iodination (9) and by the immunofluorescence of viable cells (22), that glycosylated products of gag are exposed at the surface of murine leukemia virus-infected mouse fibroblasts. However, because only a weak p30 fluorescence was detected on viable MJD-54 cells or cells fixed in 1% formaldehyde, we doubted that the p30 fluorescence detected on the fixed cells came mainly from glycosylated gag products. To confirm this point, we performed two experiments based on the results of Schultz *et al.* (23). First we treated MJD-54 cells with tunicamycin at 10 μ g/ml, which has been reported to inhibit the appearance of glycosylated gag products at the cell surface after a 1-hr treatment (23). Then the cells were fixed only in 3.7% formaldehyde and examined for p30 fluorescence. We found that the p30 staining persisted even after a 16-hr exposure to tunicamycin, which argues either that glycosylated gag products still remain on the surface of the cells even after the tunicamycin treatment or that the p30 staining is something other than a glycosylated gag product. In a second experiment, we exposed cells to 0.05% (or 0.25%) trypsin before fixation with 3.7% formaldehyde to remove glycosylated gag products from the surface of infected mouse fibroblasts (ref. 23; A. M. Schultz, personal communication) and found that the p30 fluorescence persisted. Thus, again, either the conditions of trypsin treatment were insufficient to remove glycosylated gag product from the cell surface or the p30 fluorescence that we observed is some molecule other than a glycosylated gag product.

Because it appears unlikely, based on the above experiments, that the p30 membrane-associated fluorescence comes from glycosylated gag moieties, by elimination it is likely that they represent Pr65^{gag} molecules which are located just under the cell membrane (16). If this is the case, then it means that the fixation with 3.7% formaldehyde that we used slightly altered the surface membrane so that the underlying cytoplasmic face of the membrane was made permeable to p30 antibody molecules. This is certainly possible because it recently has been shown (24) that the source of the formaldehyde (Serva, Heidelberg, Federal Republic of Germany) and the temperature (4°C) of formaldehyde fixation are critical in determining the degree of impermeability to antibody. We have found that, in our system with MJD-54 cells fixed only in 3.7% formaldehyde, a microtubular network (in the case of non-drug-treated cells) or paracrystals (in the case of vinblastine-treated cells) can be seen with antitubulin antiserum in about half of the cells (data not shown). On the other hand, fluorescence was not detected with anti-tubulin antiserum on cells fixed with 1% formaldehyde. Although the microtubule and paracrystal staining was significantly lower in intensity than when the cells were completely permeabilized with acetone, the above results do indicate that some degree of permeabilization has occurred in

cells fixed with 3.7% formaldehyde. Both the diffuse pattern (Fig. 1A) and the cap-like pattern (Fig. 1C) seen with p30 antiserum also were observed with p15, p12, and p10 antisera (data not shown). These observations strongly suggest that the p30 fluorescence we are following on 3.7% formaldehyde-fixed cells comes mainly from molecules with all the determinants of Pr65^{gag}, located just beneath the cell membrane.

The next question posed by the cap-like formation of p30 antigen seen in Fig. 1C is whether cytoplasmic microtubules regulate the distribution of membrane-associated p30 antigen directly or indirectly. In this regard, we found that the cap-like fluorescence of p30 antigen was completely inhibited when cells were preincubated with both vinblastine (10 μ M) and cytochalasin B (10 μ g/ml) (data not shown). Furthermore, electron microscopic observation of cap-like cells that had been treated with vinblastine and processed in the same way as for immunofluorescence showed a submembranous dense region limited to some portion of the cell membrane (Fig. 3A). These submembranous densities were not observed in non-drug-treated cells and appeared to be composed of a filamentous meshwork (the width of filaments was \approx 6 nm) (Fig. 3B). We also examined actin distribution, on cells fixed only with 3.7% formaldehyde, by the same indirect immunofluorescence method (the anti-actin antiserum was a generous gift from K. Burridge, Cold Spring Harbor Laboratory). On non-drug-treated cells, actin fluorescence appeared as diffuse staining, and tiny bleb-like fluorescence was observed as well. In the case of cells pretreated with vinblastine, actin staining was not homogeneous but deviated to some region of the cell membrane or cytoplasm. Also, in 20% of drug-treated cells, a typical cap-like pattern of actin fluorescence was observed which was similar to the cap-like fluorescence of p30 antigen (Fig. 1C). Thus, from these

observations microfilaments also seem to be involved in the regulation of the distribution pattern of p30 membrane-associated antigen.

So far, we have shown that, under special conditions of fixation, it is possible to obtain a cap-like phenomenon for cytoplasmic proteins (probably membrane-associated Pr65^{gag}). It is not yet clear, however, how such cap-like structures are induced in vinblastine-treated cells. There are at least two possibilities.

(i) p30 antigens are spontaneously accumulated in such cap-like areas by the drug treatment itself. In this case, this means that the cap-like structures are already formed prior to fixation. (ii) p30 antigens are distributed diffusely on vinblastine-treated cells before fixation; however, they are aggregated into cap-like structures by formaldehyde fixation.

Because we cannot yet distinguish between these two situations, we have proposed calling the phenomenon shown in Fig. 1C "cap-like" instead of "capping." Capping is already defined as the redistribution of membrane antigens caused by cross-linking with multivalent ligands.

Here it must be reemphasized that, in contrast to the p30 distribution pattern shown above, we found that the distribution pattern of gp70 fluorescence was micropunctate in appearance for the cells fixed in 3.7% formaldehyde. Furthermore, the pretreatment of cells with vinblastine did not cause a change in that distribution pattern. Also, when we examined gp70 fluorescence with viable cells with or without vinblastine pretreatment, we again observed a micropunctate appearance in >90% of both nontreated and drug-treated cells. Thus, we believe that the gp70 molecules represent a class of murine leukemia virus membrane proteins, the distribution of which is not regulated by microtubules. This differential behavior of *gag* and

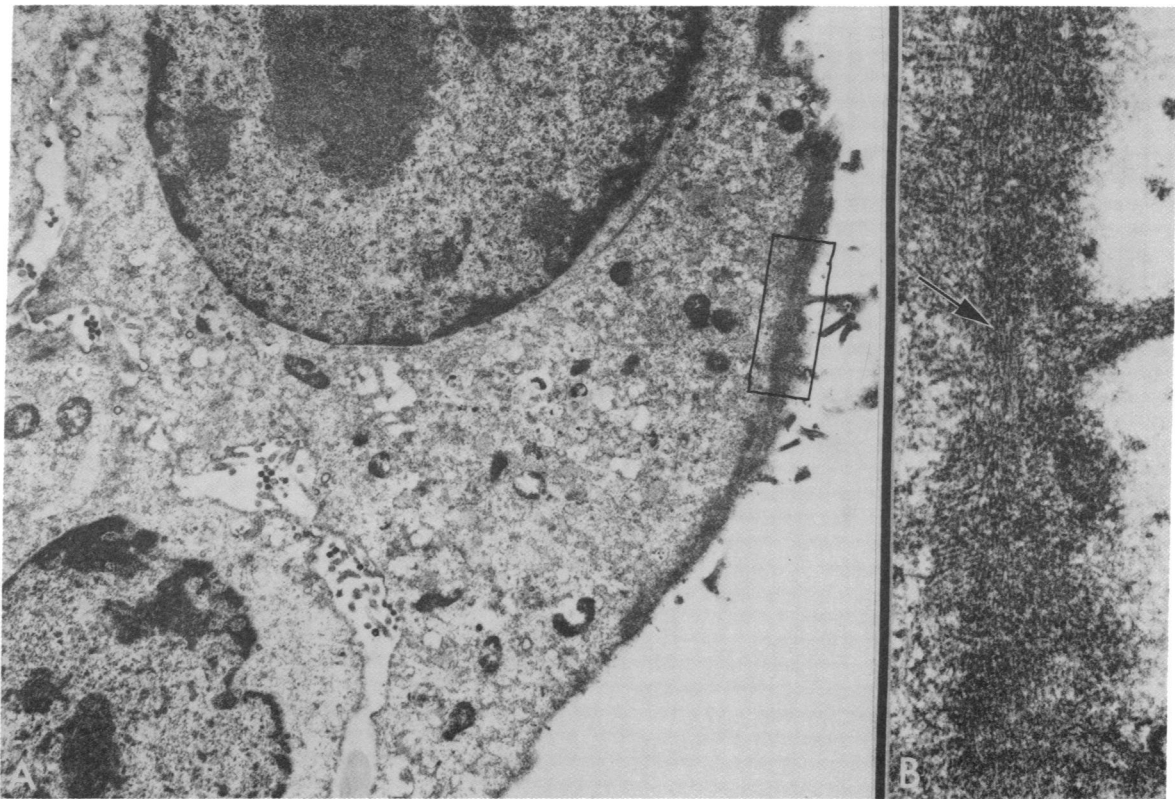


FIG. 3. (A) Cap-like cells. After MJD-54 cells were treated with vinblastine, fixed with 3.7% formaldehyde, and incubated with anti-p30 antiserum as for the immunofluorescence study, they were processed for electron microscopy. (\times 8800.) (B) Higher magnification of submembranous dense region shown in A. (\times 48,750.)

env gene-derived membrane-associated antigens probably has important implications for the mechanism of murine leukemia virus morphogenesis.

Finally, we wish to point out the need, in immunofluorescence, to examine carefully the technical problem of formaldehyde fixation in regard to membrane permeability or impermeability to antibody molecules (IgG). On 1% formaldehyde-fixed cells, only micropunctate fluorescence for gp70 was visualized; cytoplasmic microtubules were not stained to any degree. On the other hand, for 3.7% formaldehyde-fixed cells, both micropunctate fluorescence of gp70 and, to some degree, a cytoplasmic microtubule network were observed. Because gp70 is an externally localized protein and microtubules represent internal proteins, these results indicate that we can control membrane permeability to antibody molecules by changing the concentration of the formaldehyde used for fixation. Complete permeabilization is achieved if acetone treatment is used after formaldehyde fixation. Furthermore, we have confirmed that the nonpermeabilizing or permeabilizing effect of formaldehyde fixation described above is not due to the source of the formaldehyde because the same results were obtained with 1% or 4% paraformaldehyde fixation, respectively (data not shown).

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1. Ash, J. F., Louvard, D. & Singer, S. J. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5584-5588.
2. Berlin, R. D. (1975) *Ann. N.Y. Acad. Sci.* **253**, 445-454.
3. Bourguignon, L. Y. W., Tokayasu, K. T. & Singer, S. J. (1978) *J. Cell. Physiol.* **95**, 239-258.
4. Edelman, G. M., Yahara, I. & Wang, J. L. (1973) *Proc. Natl. Acad. Sci. USA* **70**, 1442-1446.
5. Oliver, J. M., Ukena, T. E. & Berlin, R. D. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 394-398.
6. Oliver, J. M., Gelfand, E. W., Pearson, C. B., Pfeiffer, J. R. & Dosch, H. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 3499-3503.
7. Stern, P. L. & Bretscher, M. S. (1979) *J. Cell Biol.* **82**, 829-833.
8. Yahara, I. & Kakimoto-Sameshima, F. (1979) *Exp. Cell Res.* **119**, 237-252.
9. Buetti, E. & Diggelmann, H. (1980) *J. Virol.* **33**, 936-944.
10. Edwards, S. A. & Fan, H. (1979) *J. Virol.* **30**, 551-563.
11. Tung, J. S., Yoshiki, T. & Fleissner, E. (1976) *Cell* **9**, 573-578.
12. Ledbetter, J. A., Nowinski, R. C. & Eisenman, R. N. (1978) *Virology* **91**, 116-129.
13. Schwarz, H., Hunsmann, G., Moennig, V. & Schafer, W. (1976) *Virology* **69**, 169-178.
14. Yeger, H. & Kalnins, V. I. (1978) *Virology* **91**, 489-492.
15. Oroszlan, S. & Nowinski, R. C. (1980) *Virology* **101**, 296-299.
16. Yeger, H., Kalnins, V. I. & Stephenson, J. R. (1978) *Virology* **89**, 34-44.
17. Barbacid, M. & Aaronson, S. A. (1978) *J. Biol. Chem.* **253**, 1408-1414.
18. Solomon, F., Salzman, A. & Magendantz, M. (1979) *Cell* **18**, 431-438.
19. Tucker, R. W., Sanford, K. K. & Frankel, F. R. (1978) *Cell* **13**, 629-642.
20. Luftig, R. B., McMillan, P. N. & Bolognesi, D. P. (1974) *Cancer Res.* **34**, 3303-3310.
21. Novikoff, A. B., Novikoff, P. M., Rosen, O. M. & Rubin, C. S. (1980) *J. Cell Biol.* **87**, 180-196.
22. Yoshiki, T., Mellors, R. C., Hardy, W. D., Jr. & Fleissner, E. (1974) *J. Exp. Med.* **139**, 925-942.
23. Schultz, A. M., Rabin, E. H. & Oroszlan, S. (1979) *J. Virol.* **30**, 255-266.
24. Deppert, W., Hanke, K. & Henning, R. (1980) *J. Virol.* **35**, 505-518.