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LOCALIZATION OF T25 GLYCOPROTEIN IN WILD-TYPE AND THY 1⁻ MUTANT CELLS BY IMMUNOFLUORESCENCE AND IMMUNOELECTRON MICROSCOPY*

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The steps in the biosynthesis of cell membrane glycoproteins and their transport to the cell surface are not well understood. We have been studying a series of mutant cell lines which have blocks in their cell surface expression of T25, a membrane glycoprotein bearing the Thy 1 alloantigen (1, 2). Biochemical characterization of the nature of the blocks should provide information about specific steps in cell membrane glycoprotein biosynthesis.

Five classes of Thy 1⁻ mutants have been identified by complementation analysis (2, 3). In somatic cell hybridization studies, mutants of each class complement mutants of all other classes but do not complement other mutants of the same class. The genetic studies are consistent with the hypothesis that one mutant class (class D) defines the structural gene coding for the Thy 1 alloantigenic determinant. The four remaining classes (A, B, C, and E) define genes other than that coding for the Thy 1 determinant but whose action is necessary for expression of Thy 1 on the cell surface.

The Thy 1 alloantigenic determinant is borne on a glycoprotein of approximate mol wt 25–30,000 (4) which, in the mouse, has been termed T25 (5, 6). Mutants of classes A, B, C, and E synthesize T25 molecules which are structurally different from the wild-type glycoprotein and which are not expressed on the cell surface, as determined by both biochemical and serological criteria (2, 7).[1] Pulse-chase experiments showed that the mutant molecules are degraded faster than the wild-type molecules;[1] however, the increased rate of degradation of the mutant molecules does not seem sufficient to account for the 500–1,000-fold deficit of T25 on the cell surface.

The existence of separate mutant classes, each of which synthesizes a mutant T25 molecule which does not reach the cell surface, raises questions about the

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[1] I. Trowbridge, R. Hyman, and C. Mazauskas. *Cell.* In press.

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localization of T25 in mutant vs. wild-type cells and among the different mutant classes themselves. The T25 glycoprotein has been isolated from cultured mouse lymphoma cells and has been used to prepare a rabbit antiserum which specifically reacts with T25. This antiserum has allowed us to use immunohistochemical methods to detect T25 in wild-type and mutant cells. In the present study we have used immunofluorescence and immunoelectron microscopy to verify the absence of T25 from the cell membrane of mutant cells and, in preliminary experiments, to demonstrate directly the presence of T25 molecules within mutant cells.

Materials and Methods

Cell Lines. The wild-type AKR/J lymphoma line BW5147 (Thy 1^+) and the class A mutant BW5147 (Thy 1^-a) have been described previously (3, 7). (This mutant cell line was referred to as BW5147 (Thy 1^-) in previous publications). The cells were grown in suspension culture in Dulbecco's modified Eagle's medium with 10% horse serum (8).

Rabbit Anti-T25. Rabbit antiserum against T25 glycoprotein was prepared by repeated subcutaneous injections of the purified glycoprotein in complete Freund's adjuvant. A detailed account of the purification of the antigen and the properties of the antiserum will be given elsewhere. Briefly, T25 glycoprotein was purified from BW5147 (Thy 1^+) cells by solubilization of a crude membrane pellet with sodium deoxycholate, fractionation on a pea lectin-Sepharose column followed by gel filtration (9). The antiserum against T25 glycoprotein was cytotoxic for Thy 1^+ lymphomas but not Thy 1^- mutant cells. One major radioactive species with a 25,000 mol wt and corresponding to T25, was specifically precipitated by the antiserum from BW5147 (Thy 1^+) cells labeled either by the lactoperoxidase surface-labeling technique or metabolically labeled with [^{3}H]mannose or [^{3}H]leucine.

To demonstrate that intracellular staining was specific for T25, the rabbit anti-T25 antiserum was absorbed with intact BW5147 (Thy 1^+) cells to remove the anti-T25 antibodies, the remaining serum being used as a control. A pellet of 2×10^8 viable cells was incubated with 0.25 ml of a 1:10 dilution of the antiserum in phosphate-buffered saline for 30 min at room temperature. The cells were then centrifuged into a pellet and the supernate removed. This process was repeated three times.

Immunolabeling Reagents. For immunofluorescence staining, an indirect procedure was used, employing affinity purified goat antibodies to rabbit IgG. The goat antibodies were conjugated with fluorescein isothiocyanate or with lissamine rhodamine B sulfonyl chloride (10). For indirect immunoferritin staining, the goat antibodies were conjugated with six times recrystallized ferritin by the procedure of Kishida et al. (11).

Surface Immunofluorescent Labeling of T25 Protein. Both the wild-type and mutant cells were first treated in suspension with rabbit antisera to T25 protein in phosphate-buffered saline containing 0.2% bovine serum albumin, pH 7.4 for 30 min at 0°C. After washing, the cells were then reacted with fluorescein-conjugated goat antibodies to rabbit IgG for 30 min at 0°C. After washing the cells free of unbound protein, they were then fixed with 2% formaldehyde in 0.1 M phosphate buffer, pH 7.4, for 30 min at 0°C, and examined by fluorescent and Nomarski optics.

Intracellular Immunofluorescent Labeling of T25 Protein. Wild-type and mutant cells were fixed with 0.2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 for 45 min at 0°C. Fixed cells were then kept overnight in 0.1 M phosphate buffer at 4°C, after which they were infused in 1.2 M sucrose, frozen, and sectioned in the frozen state. For immunofluorescent staining, such sectioning was carried out at about −30°C to section thicknesses of about 0.2 μm, whereas for immunoferritin staining, sections were cut at −80°C to thicknesses of approximately 700 Å (12–14). Both types of sections, after thawing, were then stained with rabbit antisera to T25. As controls, some sections were treated with normal rabbit IgG. The thicker sections were then treated with rhodamine-conjugated goat antibodies to rabbit IgG and processed for fluorescence observations. (Rhodamine conjugates were used here rather than fluorescein conjugates because of the autofluorescene of the glutaraldehyde-treated cells.) The thinner sections, on electron microscope grids, were labeled with ferritin-conjugated goat antibodies to rabbit IgG (13), and observed by electron microscopy.

Microscopy. Fluorescence observations were made with Zeiss Photoscope III by using a ×63 oil immersion lens and an epi-illuminator. Fluorescein and rhodamine fluorescences were excited with an Osram HBO 50 W bulb, and the filter combinations CZ 487710 and CZ 487714, respectively, were used for observations. Photography was performed with Kodak Plus X film.

Electron microscopic observations were made with a Phillips Model 300 electron microscope at 60 kV.

Results and Discussion

In this study we have used immunofluorescent and immunoelectron microscopic methods to directly visualize T25 in wild-type and Thy 1⁻ mutant cells. The results confirm the interpretation based on serological and biochemical methods. The wild-type cell line BW5147 (Thy 1⁺) when stained in the living state by indirect immunofluorescence, by using rabbit anti-T25 as the primary reagent, shows T25 clustered over the cell surface (Figs. 1a and b). No surface fluorescence is seen when the class A mutant cell line BW5147 (Thy 1⁻a) is stained in a similar manner (Figs. 2a and b). In frozen sections examined by immunofluorescence, the wild-type cell line shows staining predominantly at the cell membrane with areas of weak diffuse cytoplasmic fluorescence (Fig. 3). In contrast, the mutant cell line shows only diffuse patchy fluorescence throughout the cytoplasm (Fig. 4). This cytoplasmic staining was absent when the anti-T25 antiserum was first absorbed with intact Thy 1⁺ cells (data not shown). Therefore there was no evidence that the anti-T25 serum contained antibodies which recognized contaminating intracellular specificities. Controls using normal rabbit serum in place of the antibodies to T25 were always negative.

A similar distribution of T25 was seen by immunofluorescence in mutants of classes C and E and their corresponding wild-type lines (data not shown).

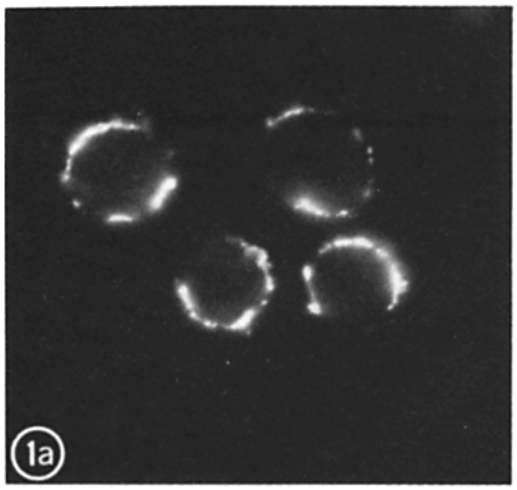
The development of ultrathin frozen sectioning methods of specimen preparation for immunoferritin electron microscopy (12, 13) has allowed us to carry out preliminary experiments at the ultrastructural level to study the distribution of T25 within the cytoplasm as well as on the surfaces of these cells. With the wild-type cells, the immunoferritin staining for T25 is mostly on the surface, with only light staining in the cytoplasm (Fig. 5). In the mutant cell line, little T25 is seen near the cell surface, but substantial amounts are found throughout the cytoplasm (Fig. 6). These results are therefore entirely consistent with the immunofluorescent results described. Beyond that, however, it is of interest that the immunoferritin staining is often observed in clusters (Fig. 6, arrows) extending up to the cytoplasmic face of the surface membrane. This suggests a partially vesicular distribution of T25 protein inside the mutant cells, possibly extending beyond the endoplasmic reticulum up to the plasma membrane.

FIG. 1. a) Indirect fluorescein immunofluorescence staining of the T25 glycoprotein on the surfaces of intact BW5147 (Thy 1⁺) cells, and b) the same field viewed with Nomarski optics.

FIG. 2. a) The absence of surface staining for the T25 glycoprotein on intact BW5147 (Thy 1⁻a) cells, and b) the same field viewed with Nomarski optics.

FIG. 3. Fixed frozen sections of BW5147 (Thy 1⁺) cells stained by indirect rhodamine immunofluorescence for the T25 glycoprotein. Note the largely surface staining of the cells.

FIG. 4. Fixed frozen sections of BW5147 (Thy 1⁻a) cells stained by indirect rhodamine immunofluorescence for the T25 glycoprotein. Note that most of the staining is cytoplasmic.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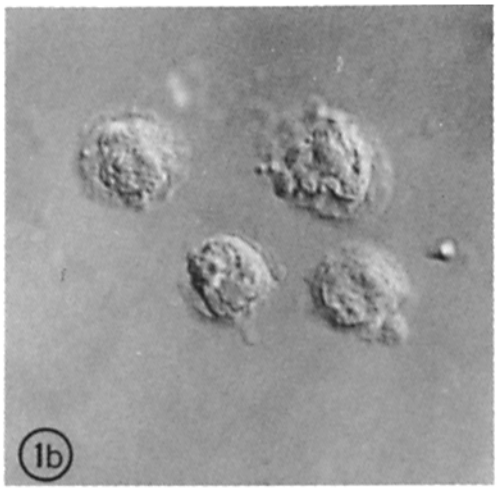
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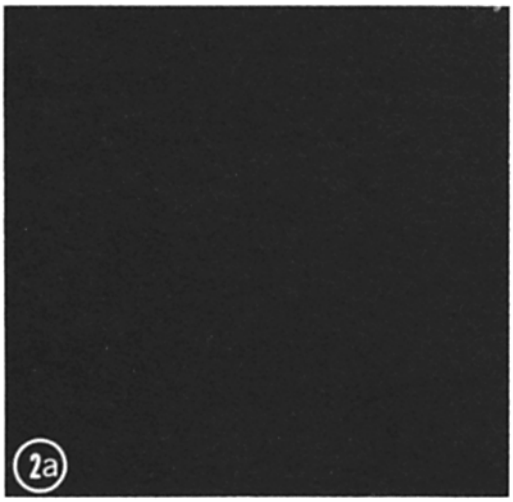
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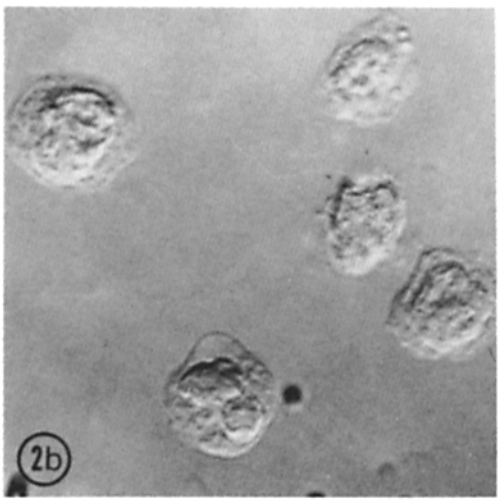
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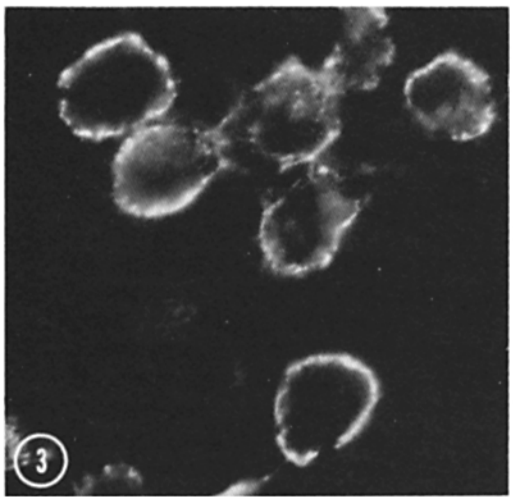
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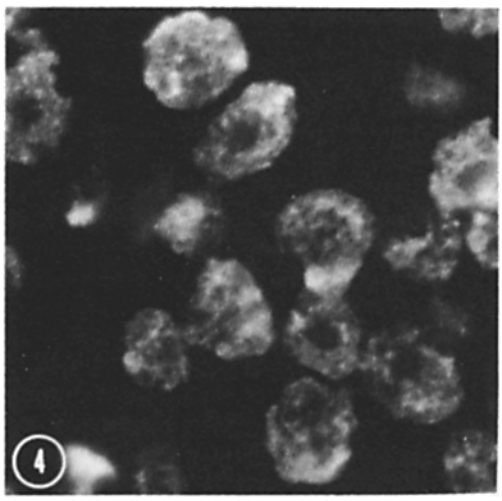
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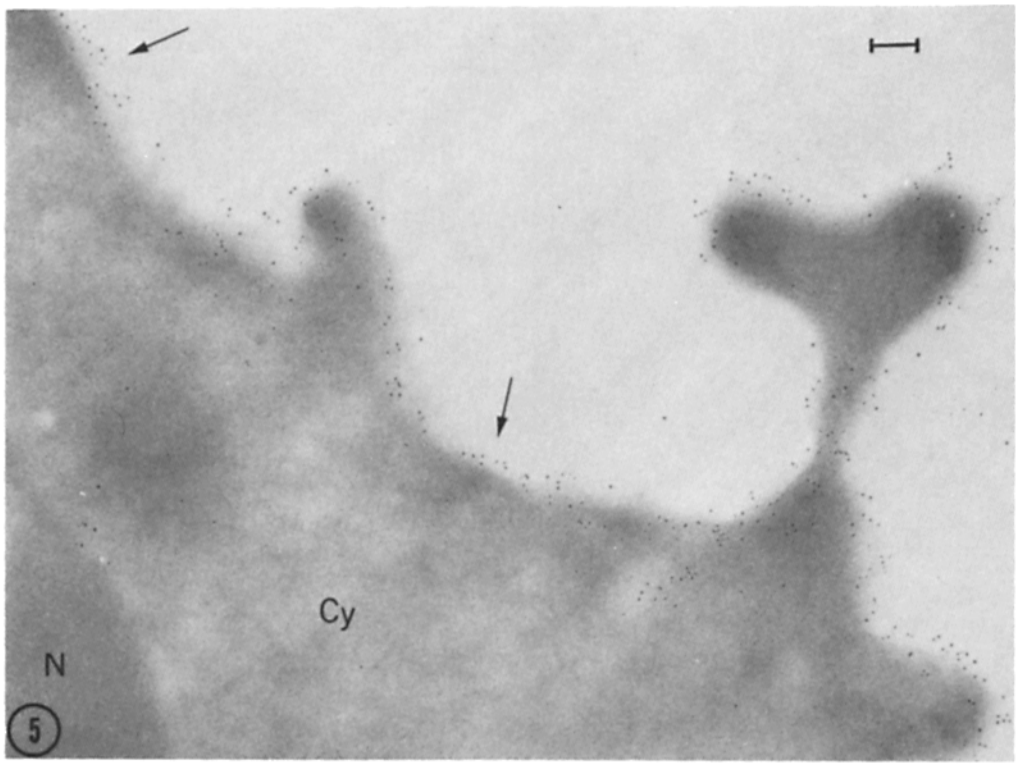
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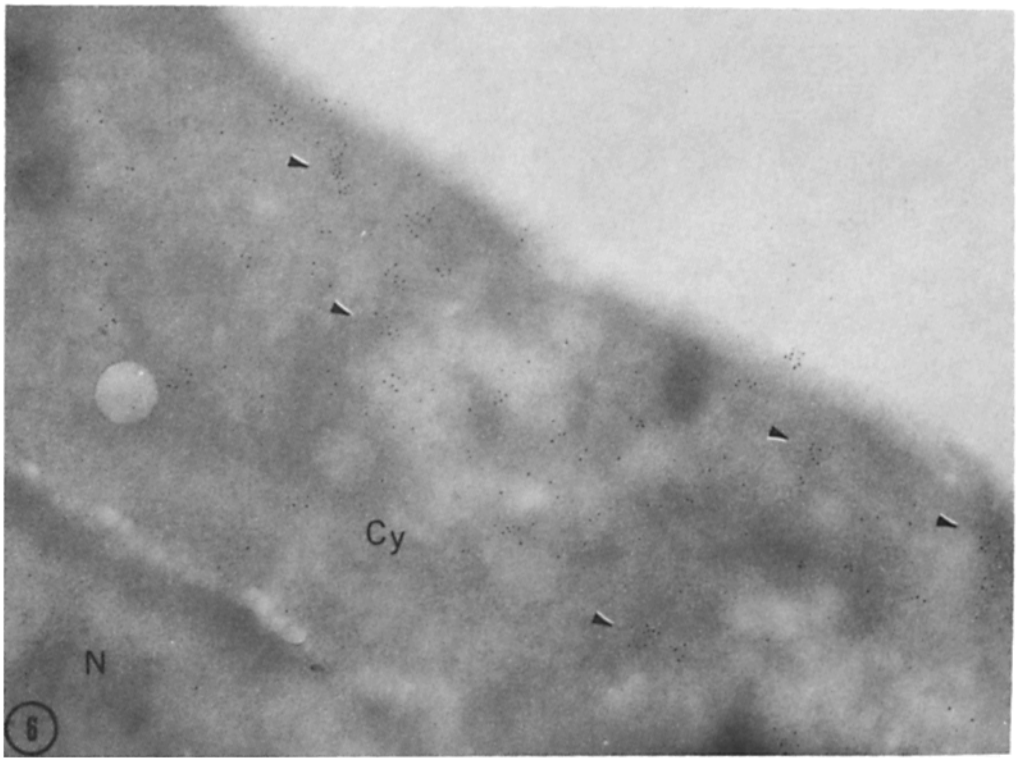
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The genetic behavior of the class A, B, C, and E mutants and the fact that each mutant of these classes synthesizes an incomplete T25 molecule suggested the hypothesis that the mutants had defects in posttranslational steps of biosynthesis. Since T25 is a glycoprotein, the most likely block seemed to be a defect in carbohydrate biosynthesis (2, 7). Evidence supporting this idea comes from two experimental approaches. First, biochemical studies on the radiolabeled glycopeptides of mutant and wild-type T25 glycoproteins show that the oligosaccharides of the mutant molecules differ from those of the wild-type molecules.[1] Second, mutants with a Thy 1^- phenotype and which complement with class E mutants have been selected by prolonged exposure of mutagenized wild-type cells to cytotoxic concentrations of concanavalin A (I. Trowbridge, and R. Hyman, unpublished results). The class E mutants show a pleiotropic defect which affects the glycosylation of many glycoproteins, although only T25 is absent from the cell surface.[1]

Hickman et al. (15) have shown that IgA and IgE-secreting murine myeloma cells treated with tunicamycin, an inhibitor of glycosylation, show greatly reduced secretion of myeloma protein. By immunofluorescence unglycosylated protein is found inside the cells where it is present in a punctate distribution. Vesicles formed by the endoplasmic reticulum are seen in the tunicamycin-treated cells, and it was suggested that the unglycosylated myeloma protein is present mainly in these vesicles, but no immunoelectron microscopic observations were made.

To gain further information from immunoelectron microscopy on the specific nature of the blocks in different classes of Thy 1^- mutant it will be necessary to use newly developed methods for positive staining of frozen sections[2] to delineate better the internal membranes and ultrastructure of the cell. Furthermore, the use of synchronized cell cultures may permit the intracellular pathway of T25 biosynthesis to be elucidated.

Summary

The wild-type BW5147 (Thy 1^+) cell line and its Thy 1^- mutant derivative BW5147 (Thy 1^-a) were examined by immunofluorescence and immunoelectron microscopy for the presence of T25, the glycoprotein which bears the Thy 1 alloantigen. The wild-type cell had T25 predominantly localized on the cell surface. In the mutant cell line, T25 accumulated intracellularly and was present in a clustered distribution throughout the cytoplasm. T25 was not present on the surface of the mutant cell line in significant amount.

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FIG. 5. Immunoferritin staining for the T25 glycoprotein on fixed, ultrathin frozen sections of BW5147 (Thy 1^+) cells. Note the largely surface localization of the ferritin stain.

FIG. 6. Immunoferritin staining for the T25 glycoprotein on fixed, ultrathin frozen sections of BW5147 (Thy 1^-a) cells. The ferritin stain is largely cytoplasmic and often seen in small clusters, up to the plasma membrane.

[2] K. Tokuyasu. Manuscript submitted for publication.

References

1. Hyman, R., and I. Trowbridge. 1977. Analysis of lymphocyte surface antigen expression by the use of variant cell lines. *Cold Spring Harbor Symp. Quant. Biol.* 41:407.
2. Hyman, R., and I. Trowbridge. 1978. Analysis of the biosynthesis of T25 (Thy-1) in mutant lymphoma cells: a model for plasma membrane glycoprotein biosynthesis. *Cold Spring Harbor Conference on Differentiation of Normal and Neoplastic Hematopoietic Cells*. In press.
3. Hyman, R., and V. Stallings. 1974. Complementation patterns of Thy-1 variants and evidence that antigen-loss variants "pre-exist" in the parental population. *J. Natl. Cancer Inst.* 52:429.
4. Barclay, A., M. Letarte-Muirhead, A. Williams, and R. Faulkes. 1976. Chemical characterization of the Thy-1 glycoproteins from the membranes of rat thymocytes and brain. *Nature (Lond.)*. 262:563.
5. Trowbridge, I., I. Weissman, and M. Bevan. 1975. Mouse T-cell surface glycoprotein recognized by heterologous anti-thymocyte sera and its relationship to Thy-1 antigen. *Nature (Lond)*. 256:652.
6. Trowbridge, I., and C. Mazauskas. 1976. Immunological properties of murine thymus-dependent lymphocyte surface glycoproteins. *Eur. J. Immunol.* 6:557.
7. Trowbridge, I., and R. Hyman. 1975. Thy-1 variants of mouse lymphomas. Biochemical characterization of the genetic defect. *Cell*. 6:279.
8. Horibata, K., and A. Harris. 1970. Mouse myelomas and lymphomas in culture. *Exp. Cell Res.* 60:61.
9. Trowbridge, I., M. Nilsen-Hamilton, R. Hamilton, and M. Bevan. 1977. Preliminary characterization of two thymus-dependent xenoantigens from mouse lymphocytes. *Biochem. J.* 163:211.
10. Brandtzaeg, P. 1973. Conjugates of immunoglobulin G with different fluorochromes. I. Characterization by anionic-exchange chromatography. *Scand. J. Immunol.* 2:273.
11. Kishida, Y., B. Olsen, R. Berg, and D. Prockop. 1975. Two improved methods for preparing ferritin-protein conjugates for electron microscopy. *J. Cell Biol.* 64:331.
12. Tokuyasu, K. 1973. A technique for ultracryotomy of cell suspensions and tissues. *J. Cell Biol.* 57:551.
13. Tokuyasu, K., and S. J. Singer. 1976. Improved procedures for immunoferritin labelling of ultrathin frozen sections. *J. Cell Biol.* 71:894.
14. Bourguignon, L., K. Tokuyasu, and S. J. Singer. 1978. The capping of lymphocytes and other cells, studied by an improved method for immunofluorescent staining of frozen sections. *J. Cell Physiol.* In press.
15. Hickman, S., A. Kulczycki, R. Lynch, and S. Kornfeld. 1977. Studies of the mechanism of tunicamycin inhibition of IgA and IgE secretion by plasma cells. *J. Biol. Chem.* 252:4402.