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Cell Biology

Thy-1: A differentiation marker of potential mammary myoepithelial cells *in vitro*

(immunofluorescence/surface antigen/stem cells)

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ABSTRACT **The rat mammary cell line Rama 25 [Bennett, D. C., Peachey, L. A., Durbin, H. & Rudland, P. S. (1978) *Cell* 15, 283–298] differentiates morphologically *in vitro* from a cuboidal form to a fusiform cell resembling myoepithelial cells. This differentiation occurs in all clonal isolates of the line. By using three different rabbit antisera specific for Thy-1, we have found that antigenic differentiation accompanies morphologic change to the fusiform state. Very few cuboidal cells had Thy-1 detectable on their surfaces in the living state; but after acetone fixation cytoplasmic Thy-1 was detected by immunofluorescence in all cells of the cuboidal type. Thy-1 specificity was established by the fact that immunofluorescence induced by rabbit anti-rat thymocyte serum was abolished by absorption with rat brain but not with erythrocytes, kidney, or liver; immunofluorescence induced by rabbit antiserum to purified Thy-1 glycoproteins from mouse lymphomas was absorbed by Thy-1 positive mouse and rat lymphomas. Surface Thy-1 provides a potentially valuable antigenic marker in the Rama 25 line for studying the differentiation of mammary myoepithelial cells.**

Permanent lines of specialized cell types—e.g., myoblasts (1), neuroblastoma (2), preadipose cells (3), and Friend erythroleukemia cells (4)—have provided useful models for studying factors controlling growth of animal cells and phenomena associated with cell differentiation. A cell line (Rama 25) recently isolated by Bennett *et al.* (5) from a mammary tumor induced in Sprague–Dawley rats by dimethylbenzanthracene may extend the repertoire to mammary cells. This line consists *in vitro* predominantly of cuboidal cells; but despite rigorous cloning it consistently generates numerous progency of fusiform morphology. Hence, fusiform cells appear to derive from the cuboidal cells by a process of variation intrinsic to the nature of these cells. On the basis of morphologic and ultrastructural studies, Bennett *et al.* (5) suggested that the cuboidal cells are mammary stem cells and the fusiform cells are related to myoepithelia (6), which in the developing breast are generated from mammary stem cells. Evolution of fusiform morphology *in vitro* would therefore correspond to a normal differentiation event.

The usefulness of this cell line for studying *in vitro* differentiation of mammary cells will depend on the demonstration of suitable markers for identifying specific cell types. Thy-1 antigen has been reported to be present in cells of the normal murine mammary gland and in mammary tumors (7–9). It is not known, however, which mammary cell types express Thy-1. The possibility that Thy-1 might be a differentiation antigen of certain mammary cell types was suggested by the knowledge that Thy-1 is a differentiation antigen in other cells such as thymocytes, certain peripheral lymphocyte subpopulations, and brains in mice and rats (reviewed in ref. 10). Thy-1 is also expressed transitorily in immature skeletal muscle (11) and has been reported in fibroblasts (12) and skin (13).

With the aim of defining an antigenic marker of mammary differentiation, we chose to determine whether or not Thy-1 was expressed on cuboidal or fusiform sublines of the Rama 25 line derived by Bennett *et al.* (5) and on additional sublines isolated in our own laboratory (unpublished data). We found that Thy-1 provides a serological marker of differentiation in the cuboidal cell line. Indirect immunofluorescence assays revealed that Thy-1 was not detectable on the surfaces of living cells of the presumptive stem line Rama 25 and on those of clonal derivatives of this line, except for infrequent cells of both cuboidal and elongate shapes. In contrast, all of the six fusiform lines studied, which were derived from Rama 25, uniformly expressed abundant Thy-1 on their surfaces.

MATERIALS AND METHODS

Cell Lines. The cuboidal line Rama 25 and its stable fusiform derivatives Rama 4, 29, and 30 were generously provided by D. C. Bennett, Imperial Cancer Research Fund. They were propagated in Dulbecco's modification of Eagle's medium supplemented with 10% calf serum (Pacific Biological, Richmond, CA), insulin (50 ng/ml), and hydrocortisone (50 ng/ml). A cuboidal subclone of Rama 25 (designated LA7-c3) was derived from several successive clonings (unpublished data). BW 5147 [a Thy-1-positive murine lymphoma cell line (14)] and C58[NT]D [a Thy-1 positive rat lymphoma line (11)] were provided by R. Hyman, Salk Institute, and were propagated in culture medium supplemented with 10% horse serum (GIBCO).

Antisera. (*i*) Rabbit anti-rat thymocyte serum (ATS) with specificity for the Thy-1 glycoprotein (T25) was prepared, absorbed with rat tissues, and characterized serologically as described (11); in the present experiments it was used at a dilution of 1:50. (*ii*) Fluoresceinated immunoglobulin fraction of goat anti-rabbit IgG (Cappel Laboratories, Downington, PA, No. 8578) was used at a dilution of 1:100 in an indirect immunofluorescence assay. (*iii*) A rabbit antiserum to T25 glycoprotein (purified from BW 5147) was generously provided by I. S. Trowbridge, Salk Institute, and used at a dilution of 1:20 (15). (*iv*) Another rabbit antiserum to T25 glycoprotein (purified from rat brain) was generously provided by A. F. Williams, Medical Research Council, Oxford, and was used at a dilution of 1:20 (16).

Immunofluorescence. Cells were grown on Propper glass coverslips (size RD, thickness 1) in Falcon tissue culture dishes in 10 ml of medium or directly on 90-mm Nunc or Falcon tissue culture dishes. For assaying directly in culture dishes, the medium was removed and selected areas were surrounded by a rubber O-ring coated with silicon grease. Antisera were applied

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Abbreviation: ATS, rabbit anti-rat thymocyte serum.

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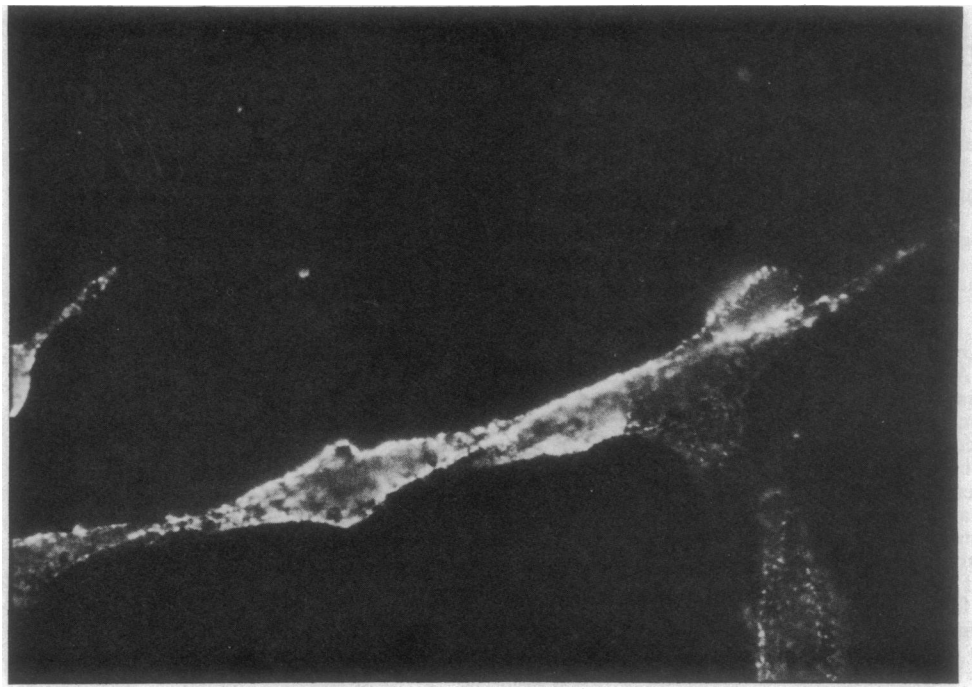

FIG. 1. A strand of elongated myoepithelial-like cell of the Rama 29 line. Thy-1 was revealed on the surface of the living cell by sequential exposure to ATS and fluoresceinated goat IgG anti-rabbit IgG (2) at room temperature in the presence of 10 mM sodium azide. (×350.)

to cells in the enclosed ring. At the end of the procedure the O-ring was removed and a coverslip was applied.

Indirect immunofluorescence was performed as described (11). Briefly, the living cells were washed twice with culture medium containing 25 mM Hepes and 5% fetal calf serum (H medium). Antibody was diluted in H medium and, after 30 min at 23°C, the cells were washed three times with H medium. Fluoresceinated anti-immunoglobulin was added in H medium and, after 30 min at 23°C in the dark, the cells were washed three times with H medium. The coverslips were inverted over a drop of 90% glycerol/10% phosphate-buffered saline and examined at 440 nm by a Zeiss fluorescent microscope with epi-illumination. Acetone fixation of cells attached to coverslips was carried out by brief immersion (5 sec) in ice-cold acetone. All other steps were the same as for living cells. In all procedures with living cells, 10 mM sodium azide was present to prevent potential redistribution of membrane antigen by bivalent antibodies.

Table 1. Indirect immunofluorescence on living mammary cell lines with rabbit anti-Thy-1 sera

Anti-serum	Cell line: Rama 25	Rama 4	Rama 29	Rama 30	BW 5147
ATS*	−	+	+	+	+
αT25†	−	+	+	+	+
αT25‡	−	+	+	+	+
αT25§	−	−	−	−	−

+ Indicates unequivocally positive fluorescence; − indicates no fluorescence.

* Rabbit antiserum to rat thymocytes. Prior absorption with rat brain tissue abolished positive reactions.

† Rabbit antiserum to Thy-1 glycoprotein purified from mouse lymphoma BW 5147 cells (15).

‡ Rabbit antiserum to Thy-1 glycoprotein purified from rat brain (16).

§ Rabbit antiserum to Thy-1 glycoprotein purified from mouse lymphoma BW 5147 cells that had been absorbed with C58[NT]D cells to remove anti-Thy-1 activity.

In order to establish the Thy-1 specificity of intracellular staining, aliquots of rabbit antisera to T25 glycoprotein were absorbed with intact BW 5147 cells or C58[NT]D cells. Approximately 2×10^8 cells were suspended for 30 min at 23°C in 1 ml of antiserum (diluted 1:20 in H-medium). The cells were removed by centrifugation and fresh cells were added to the supernate. This process was repeated five times.

RESULTS

Because the evolution of fusiform cell types is crucial to the present work, we first tested whether or not this evolution could be reproduced in our laboratory. Ten clonal sublines from the Rama 25 line were isolated and recloned two to four times. In agreement with the results reported by Bennett *et al.* (5) all the clones generated fusiform cells, although at very different rates (unpublished data). Because LA7-c3, a cuboidal subline isolated by four serial clonings, rarely produces fusiform cells, it was selected for immunofluorescence studies.

Immunofluorescence of Living Cells. Table 1 summarizes the results of immunofluorescence assays for Thy-1 on the parental stem cell line, Rama 25, three of its stable fusiform derivatives, Rama 4, 29, and 30, and the mouse lymphoma BW 5147. Each of the fusiform lines was morphologically homogeneous; all resembled myoblasts and tended to form chains. Indirect immunofluorescence examination in the living state

Table 2. Indirect immunofluorescence on acetone-fixed mammary cell lines with rabbit anti-Thy-1 sera

Anti-serum	Cell line: Rama 25	LA7-c3	Rama 4	Rama 29	Rama 30	BW 5147
ATS	+	+	+	+	+	+
αT25	+	+	+	+	+	+
αT25	+	+	+	+	+	+
αT25	−	−	−	−	−	−

The same antisera and scoring as that detailed in Table 1 was used.

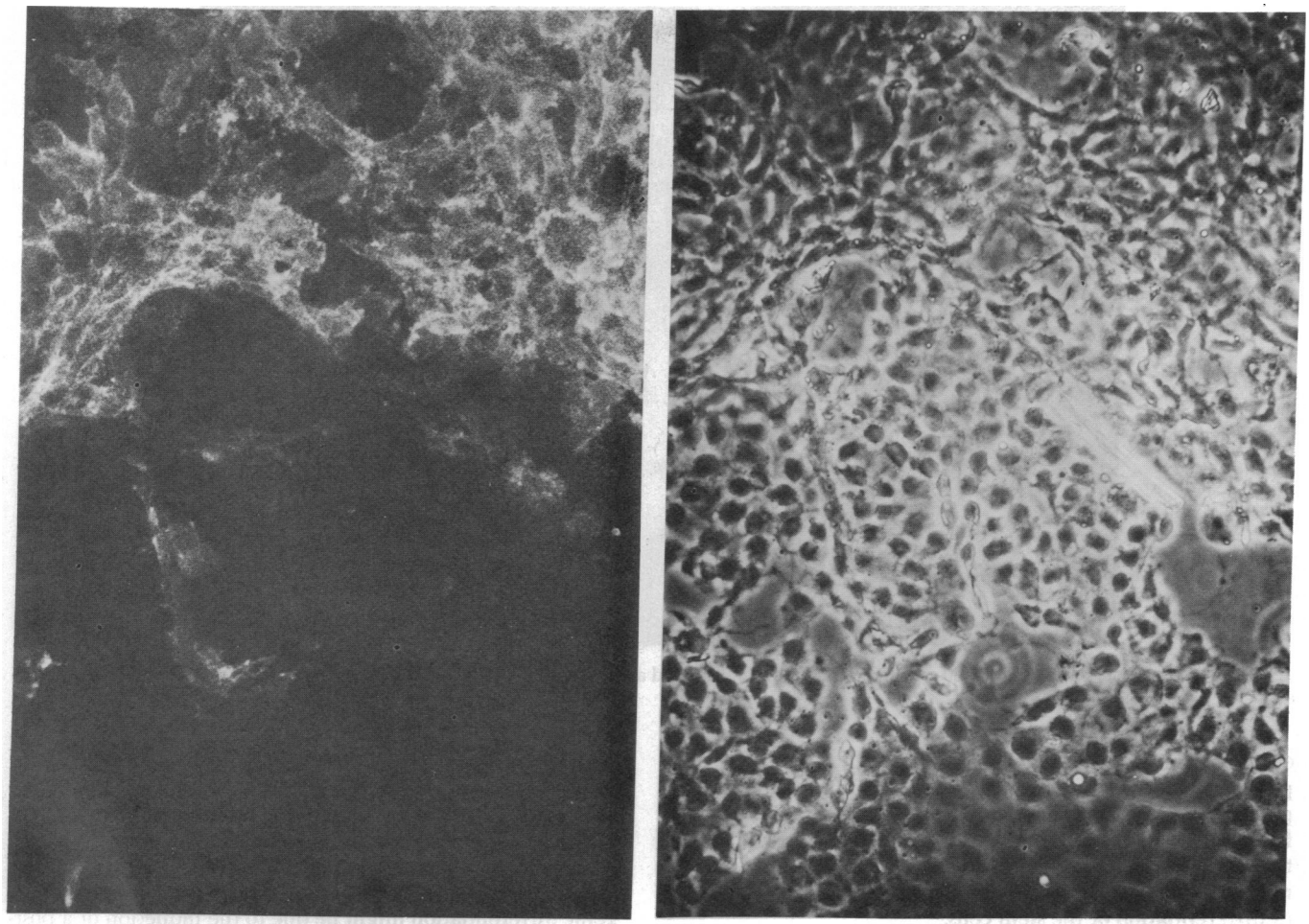

FIG. 2. Mixed culture of myoepithelial-like cells (Rama 29) and cuboidal cells (LA7-c3) examined in the living state as in Fig. 1. The two cell types are recognized in the phase contrast photograph (*Right*) of the same field. The fusiform (myoepithelial-like) cells form a colony in the upper part of the figure and infiltrate the colony of cuboidal cells below. Only the myoepithelial-like cells exhibit fluorescence (*Left*). (×230.)

with ATS revealed very bright patches of fluorescence distributed over the surface of all cell bodies and their fine processes (Fig. 1). Bright fluorescence was seen with ATS that had been absorbed with rat liver, kidney, and erythrocytes; but no fluorescence was seen with ATS that had been absorbed with rat brain tissue. The pattern of fluorescence induced on the fusiform cells with anti-T25 antiserum was similar to that with ATS, but no fluorescence was induced with similar dilutions of normal rabbit serum. Sparse and confluent cultures displayed equivalently bright fluorescence with ATS, without any apparent difference in the surface distribution of the antigen.

The predominant cellular morphology in the parental stem cell line Rama 25 was cuboidal, but occasionally elongate forms were seen singly and in small groups (see Fig. 2). The majority of Rama 25 cells were nonreactive when tested in the living state by indirect immunofluorescence with either ATS or anti-T25 antiserum. However, occasional positive cells, of both cuboidal and elongate morphologies, were identified singly and in groups. Absorption of ATS with rat brain removed all visible reactivity.

Immunofluorescence of Fixed Cells. Table 2 summarizes the results of immunofluorescence assays for Thy-1 on the parental stem line, Rama 25, a cuboidal subclone, LA7-c3, and three fusiform derivatives of the parental line. A mouse lymphoma, BW 5147, was used as a Thy-1 positive control. After acetone fixation the fluorescence of fusiform cells exposed to ATS and anti-T25 remained bright but the pattern was less sharply defined (Fig. 3). In the cuboidal stem line cultures as well as in the LA7-c3 subline, a distinctive fluorescence, faint over the cytoplasm and prominent over the nucleus (Fig. 4), was induced on all cells of cuboidal morphology by all antisera with Thy-1 specificity. The frequency of intensely bright cells (both cuboidal and elongate) in acetone-fixed preparations was about the same as observed in the living state. Brightly fluorescent cells of a stellate morphology, intermediate between cuboidal and elongate, were seen for the first time after acetone fixation. Fluorescence induced on acetone-fixed cells by ATS was abolished by prior absorption with rat brain, and that induced by rabbit antiserum to mouse lymphoma T25 glycoprotein was abolished by absorption with Thy-1 positive lymphoma lines (BW 5147 and C58[NT]D). No similar patterns of fluorescence were observed when acetone-fixed Rama 25 cells were exposed to normal rabbit serum.

DISCUSSION

We have confirmed the results of Bennett *et al.* (5) that a mammary cell line (Rama 25) predominantly cuboidal in morphology generates cells of fusiform morphology. Given the regularity of the phenomenon in a considerable number of independent experiments, it seems likely that this is a differentiation event, possibly related to that occurring *in vivo*.

Expression of Thy-1 antigen on the cell surface is a faithful indicator of this differentiation, being present not only in four lines of fusiform cells isolated by Bennett *et al.* (5), but also in all clones of similar morphology independently generated in

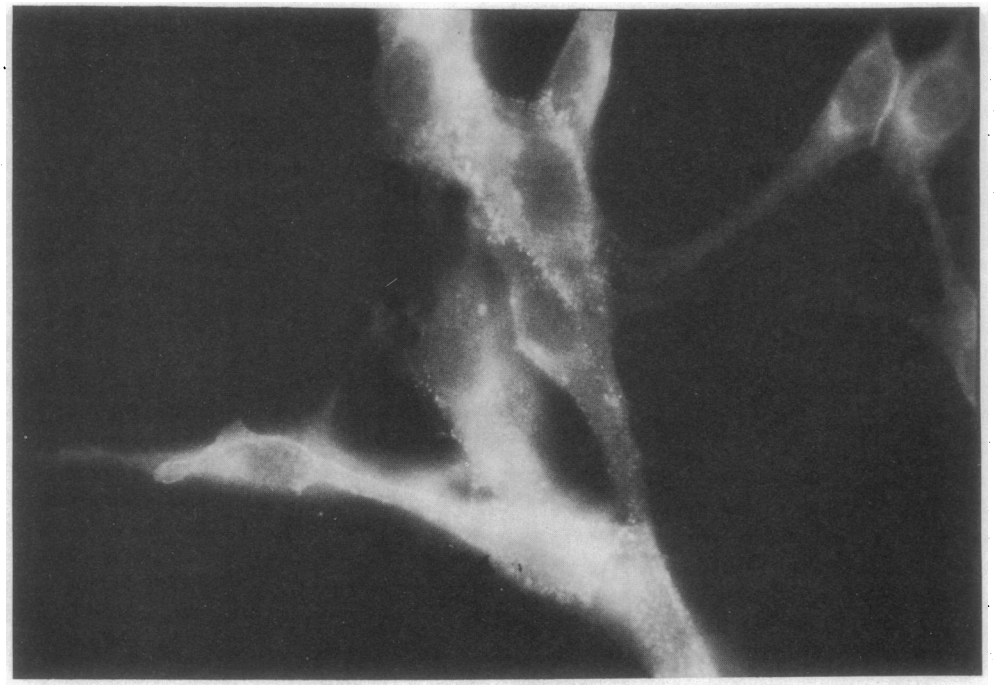

FIG. 3. Myoepithelial-like Rama 30 cells fixed in acetone and stained as described in Fig. 1. Fluorescence is seen both at the plasma membrane and in the cytoplasm. (×630.)

our laboratory. In cultures of the stem cell line, Thy-1 was expressed on the surface of occasional cells of cuboidal morphology suggesting that these cells are direct precursors of fusiform cells; however, additional work is required to establish this connection firmly. If our provisional interpretation is correct, Thy-1 will be a convenient marker for studying one line of differentiation of the stem cells.

Acetone fixation of cells that were negative for surface Thy-1 revealed faint but unequivocal cytoplasmic reactivity with anti-Thy-1 antisera, which was totally absorbed by appropriate Thy-1 bearing cells and tissue. It seems therefore that in the cuboidal stem cells Thy-1 antigen is synthesized but remains in the cytoplasm without becoming exposed on the surface of the plasma membrane. A similar situation occurs in mutant lines of mouse T-lymphocytes that contain defects in glycosylation; these mutants synthesize Thy-1 but fail to insert the molecule into the cell membrane (14, 16). These data suggest that antigenic differentiation might be due to the induction of a post-translational event allowing protein synthesized in both stem cells and fusiform derivatives to reach the surface in the differentiated cells. Alternatively a new protein could be synthesized in the differentiated cells, which is antigenically related

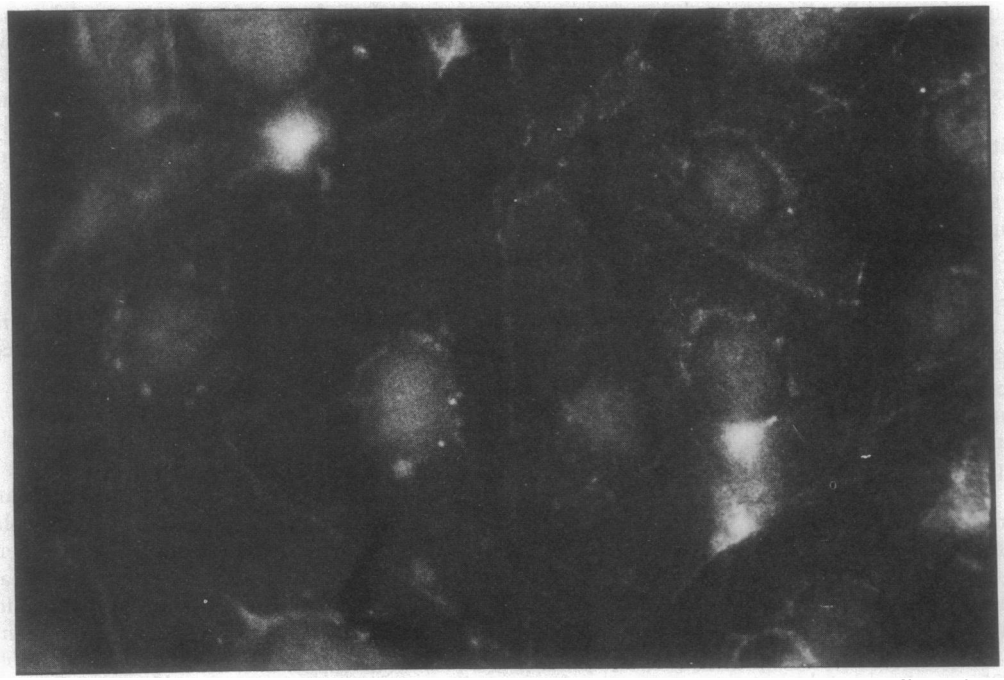

FIG. 4. Cuboidal Rama 25 cells fixed in acetone and stained as in Fig. 1, showing the cytoplasmic, especially perinuclear, fluorescence. (×630.)

to that present in the stem cells but different in its ability to reach the cell surface. Some of these questions may be answered by characterizing the molecular nature of the antigens present in the two cell types.

The regular presence of Thy-1 antigen on the surface of the fusiform Rama cells supports the hypothesis that they are myoepithelial cells, because the antigen exists, at least during some stages of development, in skeletal muscle (11) and in murine smooth muscle cell lines (J. Lesley, personal communication). However it remains to be established whether or not myoepithelial cells express Thy-1 *in vivo*.

Our findings raise questions in relation to the differentiation of the Rama 25 stem cells. For examples: How many steps are there in the differentiation of cuboidal stem cells to the fusiform, presumptively myoepithelial, derivative? What is the nature of the association of the antigenic to the morphological differentiation? Is there a causal relationship between the two phenomena and in which direction? Or are they merely independent expressions of a common cellular change?

The sharing of a surface antigen (Thy-1) by breast and certain lymphoid cell subpopulations is intriguing. Expression of Thy-1 antigen on the surface of cultured prothymocytes is rapidly induced by subnanogram quantities of thymopoietin, a thymus-derived polypeptide (17). It remains to be ascertained what controls the appearance of Thy-1 antigen in the mammary cell system and what is its function.

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