Proc. Natl. Acad. Sci. USA
Vol. 76, No. 4, pp. 1848–1852, April 1979
Cell Biology

Role of Thy-1 antigen in the *in vitro* differentiation of a rat mammary cell line

(monoclonal antibodies/domes/immunofluorescence)

RENATO DULBECCO*, MAURO BOLOGNA†, AND MICHAEL UNGER

The Salk Institute, San Diego, California 92112

Contributed by Renato Dulbecco, January 15, 1979

ABSTRACT **A line of rat mammary cells, LA7, undergoes differentiation into domes or ridges or projections under partial medium control. Immunofluorescent studies show that at the time of dome formation the LA7 cultures express the Thy-1 antigen at the borders between cells. Exposure of the cultures to certain monoclonal anti-Thy-1.1 sera prevents formation of new domes and causes disappearance of preexisting ones; antisera to whole cells produce no visible effect. Cultures exposed to anti-Thy-1 sera at effective concentrations of antibody against domes show morphological changes and ridge formation. It seems that the specific interaction of these antisera with the Thy-1 antigen redirects the differentiation program of the cells.**

A culture of epithelial cells, rama 25, derived from a dimethylbenzanthracene-induced mammary carcinoma in Sprague–Dawley rats, undergoes differentiation *in vitro*. The differentiated products include fusiform cells, probably related to myoepithelial cells (1). These cells express on their surface Thy-1 antigen (2), which is also present on thymocytes, brain cells, and some fibroblasts (3) and is expressed transiently during muscle differentiation (4). The polygonal rama 25 cells contain this antigen in the cytoplasm, but not at the surface (2).

We have isolated a cell type that is intermediate between the rama 25 cells and their fusiform derivatives, the F-precursor cells (5). A clonal line of these cells (LA7) not only produces fusiform cells at a much higher rate than rama 25 cells, but also undergoes differentiation in various directions under partial control of medium substances, producing projections, ridges, or domes (5). Projections are cell buds protruding into the medium; ridges are thick, often branching, cell strands; domes are blisters enclosing a pocket of liquid between the cell layer and the plastic dish. In cultures of many different epithelial cell types, including rama 25, dome formation can be induced by the substances (such as dimethyl sulfoxide) that induce differentiation of Friend cells (6). The formation of ridges is accompanied by more rapid cell growth than formation of domes.

We report here that the Thy-1 antigen plays an important role in the formation of domes in LA7 cultures: the antigen is present in the cultures when domes are formed, the Thy-1-specific antibodies can prevent dome formation, changing the direction of differentiation.

MATERIALS AND METHODS

The LA7 cells (5) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and 50 ng of both insulin and hydrocortisone per ml. The cells were transferred twice weekly by a 1:4 split. R-29 (rama 29) cells, a fusiform derivative of rama 25 cells, were obtained from Dorothy Bennett.

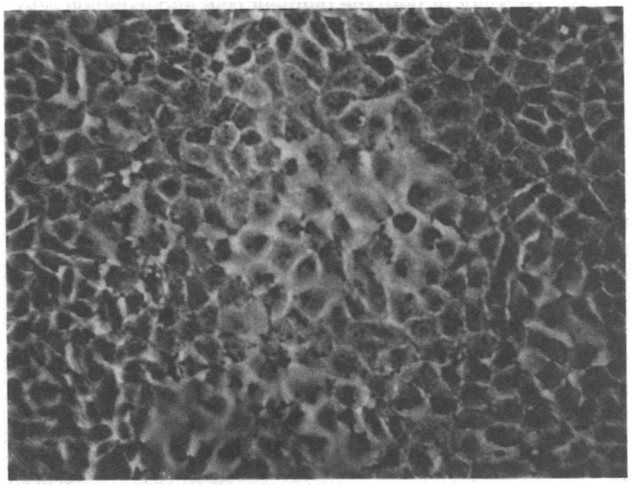

FIG. 1. Phase-contrast photograph of a living 3-day-old LA7 culture. Incipient domes appear paler and well circumscribed; their cells are without droplets and with dark granules. (×110.)

For studying dome formation, 2–4 × 10^4 cells in 0.2 ml of medium were seeded in each well of a 96-well microtiter dish in fresh medium or in the center of a 50-mm Nunc plastic dish (drop culture). The following day the medium was removed, replaced with a medium prepared with heat-inactivated serum (30 min at 56°C), and conditioned for 3 days by growing LA7 cultures. The conditioned medium promotes dome formation (unpublished observations). The medium was again replaced with the same conditioned medium (0.1 ml per well or 3 ml per drop culture) before the addition of antibodies. In the drop cultures the cell layer forms a disc about 12 mm in diameter; a continuous layer first forms in the center of the disc. The purpose of drop cultures is to minimize changes of the medium produced by the cells. Premature dome formation was induced by changing the medium to an identical medium containing 250 mM dimethyl sulfoxide.

Antisera. A rabbit antiserum against rat T25 antigen was a gift of Alan Williams (University of Oxford, Oxford, England). It was prepared by immunizing rabbits with purified rat brain T25 (Thy-1 protein) (3). Three monoclonal mouse antisera to the Thy-1.1 antigen, T11A9a, T32B11, and T11D7e, as well as monoclonal mouse antiserum to Thy-1.2 antigen, F7D5, were the gift of P. I. Lake and E. A. Clark (University College, London). T11A9a, T11D7e, and F7D5 are IgM class immunoglobulins; T32B11 is an IgG class. These antisera were supplied as sera of mice with tumors produced by the hybridomas

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* Also Department of Pathology and Medicine, University of California, San Diego, La Jolla, CA 92093.
† On leave of absence from the Department of Pathology, University of L'Aquila Medical School, L'Aquila, Italy.

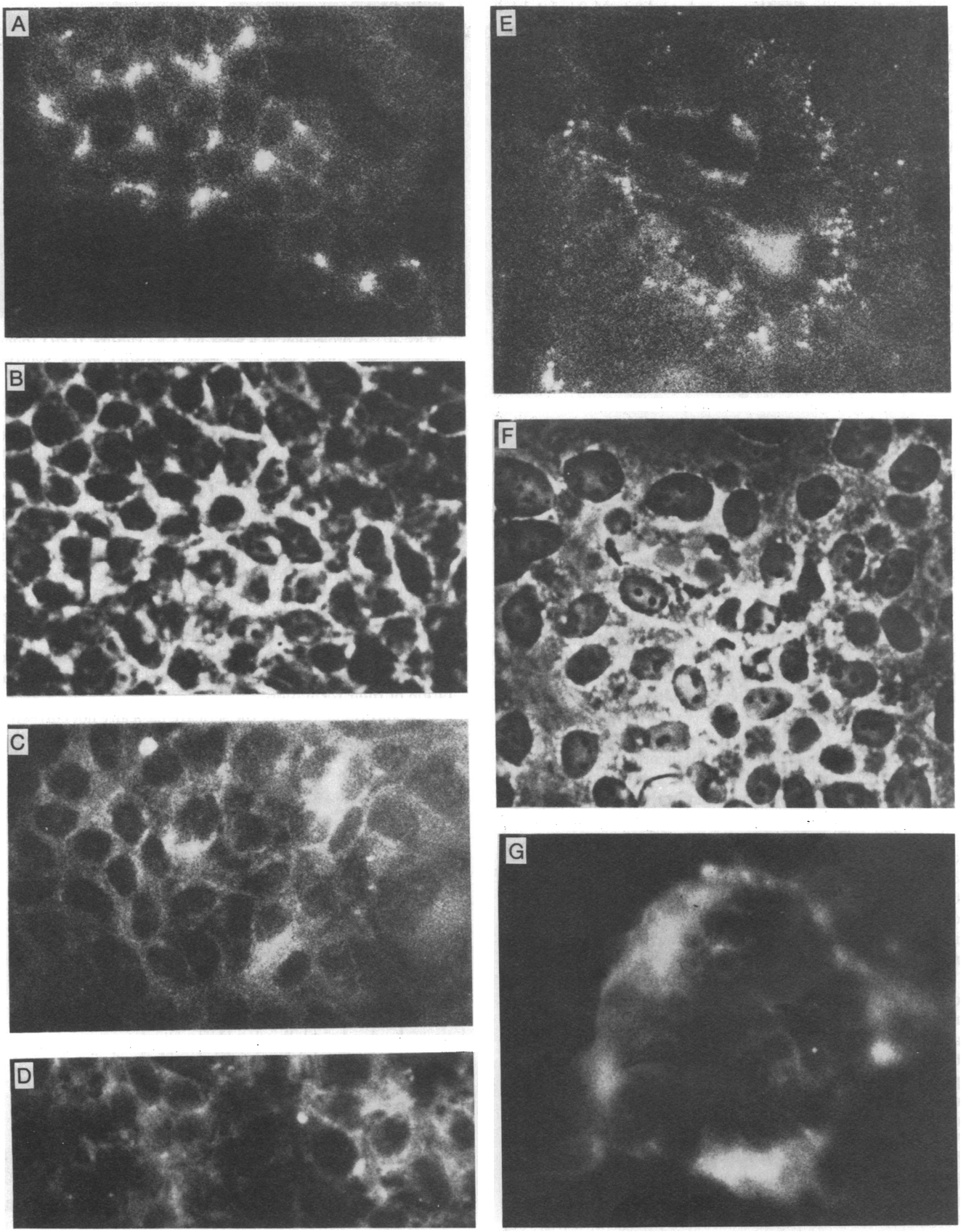

FIG. 2. Immunofluorescent staining of Thy-1 antigen in 3-day-old LA7 cultures. (*A* and *E*) Immunofluorescence in incipient domes, live cultures; (*B* and *F*) corresponding phase-contrast photographs; (*C*) immunofluorescence, acetone fixation, area outside domes. (*D* and *G*) Methanol-fixed: (*D*) area outside domes; (*G*) dome. (*A*, *C*, and *E*) Anti-T-25 serum; (*D* and *G*) monoclonal anti-Thy-1.1 serum T11A9. (× 564.)

releasing the antibodies; they were kept diluted 1:2 in fetal calf serum at 4°C. Antiserum to R-29 cells was obtained by immunizing BALB/c mice with living cells (2×10^7 cells per mouse intraperitoneally every 2 weeks for 10 weeks).

Immunofluorescence was carried out by the indirect method as described (5). For methanol fixation, the cultures were washed three times with ice-cold phosphate-buffered saline and then exposed to 100% methanol overnight at 4°C. Immunofluorescence end points were determined as the highest serum dilutions giving clearly recognizable labeling with live cells. Cytotoxic tests with C58[NT]D cells (4) used 10^5 cells in suspension in 0.15 ml of Hepes-buffered medium with 5% fetal calf serum and with rabbit complement, 1 hr incubation at 37°C, and termination by immersion in an ice bath. Dead cells were identified by trypan blue uptake. Cytotoxic tests with R-29 or LA7 cells were carried out with $2–4 \times 10^4$ cells attached to the plastic in wells of microtiter trays, in 0.2 ml of medium with 10% calf serum and rabbit complement for at least 4 hr at 37°C. Surviving live cells were recognized after digluteraldehyde fixation and Giemsa staining.

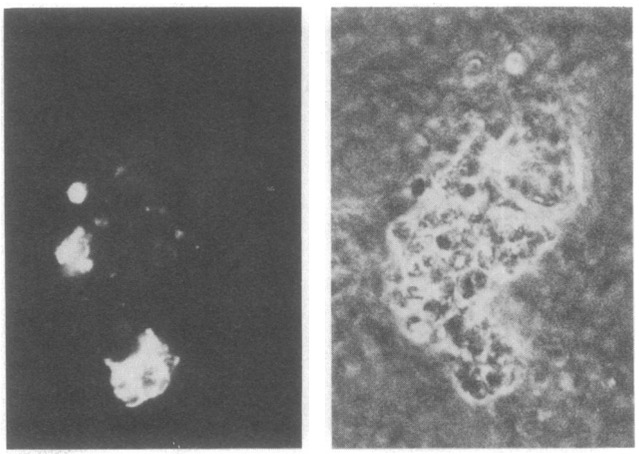

FIG. 3. A projection in a week-old LA7 culture. (*Left*) Immunofluorescence with anti-T-25 serum; (*Right*) phase contrast. (×204.)

RESULTS

Dome Formation by LA7 Cells. Cultures of LA7 cells begin to form domes soon after they become confluent. At this stage the cells are rather flat and expanded, have beaded edges, and stain weakly with Giemsa. The incipient domes consist of small patches of cells with more expanded and thin cytoplasm (in light microscopy); on these cells and between them, irregular small granules or ridges stand out dark in phase-contrast microscopy (Fig. 1). Interspersed among these cells are occasional cytoplasmic debris or nucleus-less sheets. About 24 hr after the appearance of incipient domes, the cultures contain many elevated domes with the classical blister morphology, and in the subsequent 24 hr the cultures become almost completely covered by them. By time-lapse photography, elevated domes can be seen to form at sites formerly occupied by incipient domes. Elevated domes comprise between 10 and 100 cells; as previously shown (5), these domes are frequently connected to solid strands of cells that protrude into the medium (projections). Time-lapse photography shows that in the first few days elevated domes pulsate every few hours, as previously shown for other cultures (7), reforming at the same location. Later, some domes become permanent. After several additional days they become infiltrated with fusiform cells that either preexisted in the culture or were formed from projections. Occasionally domes of this kind become detached as small balloons floating in the medium. Similar findings were reported for a mouse mammary cell line (8).

Presence of Thy-1 Antigen on Surface of LA7 Cells. Living or fixed cultures containing domes were examined by immunofluorescence. In all cases the antigen could be recognized, although in small quantities (Fig. 2). In living cultures the antigen was at the borders between cells in domes (mostly incipient); the brightest spots were where the borders of several cells merged (Fig. 2 *A* and *E*). Some nucleus-less cytoplasmic sheets, usually in incipient domes, displayed the most stain (Fig. 2*E*). Very bright fluorescence was present at the tips of projections (Fig. 3). In cultures fixed with acetone or methanol (which affords the best preservation) (Fig. 2 *C*, *D*, and *G*), fluorescence was observable in the cell cytoplasm and at the intercellular borders; it was usually somewhat more intense in domes, but was not confined to them.

Effect of Thy-1 Antisera on Dome Formation and Persistence. The effects of antisera added just before domes began to form spontaneously are shown in Fig. 4 and Table 1. Two Thy-1.1 monoclonal sera (T119a and T32B11) prevented dome formation at high dilutions. Various kinds of control sera (i.e., normal mouse serum, fetal calf serum, and a monoclonal antiserum to Thy-1.2, which is absent in rats) resulted in no visible effect. Several other monoclonal Thy-1.1 antisera were tested; some had weaker effects on domes, and T11D7e had none. In different assays, the anti-dome titer of a given antiserum showed some differences (up to 5-fold), probably depending on the state of the culture or the number of cells. There was no cell killing either within or outside domes. The two monoclonal anti-Thy-1.1 sera prevented induction of premature formation of domes by dimethyl sulfoxide and caused the disappearance of existing domes (about 50% reduction in 2 hr). The titer for disappearance of domes, however, was 33–50% that for inhibition of their formation.

The anti-dome titers of the sera are basically not correlated with their cytotoxic titers towards Thy-1-positive cells (such as the C58[NT]D rat lymphoma or the R-29 cells) (Table 1). It is

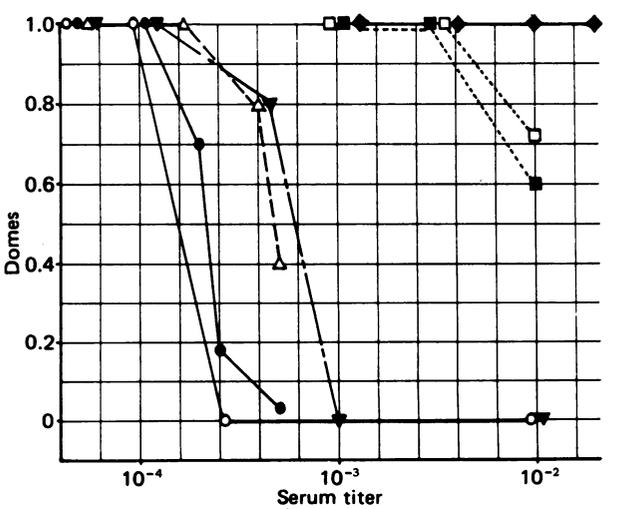

FIG. 4. Effect of antiserum on dome formation in microtiter wells. Abscissa, final concentrations (titers) of the sera; ordinate, proportion of domes present 24 hr after domes had begun to appear. ● and ○, Two experiments with monoclonal anti-Thy-1.1 T11A9a (IgM); △ and ▼, two experiments with monoclonal anti-Thy-1.1 T32B11 (IgG); ■, mouse anti-R-29 cell serum; □, mouse anti-rama 25 cell serum; ◆, several sera: monoclonal anti-Thy-1.2 F7D5 and normal mouse, calf, and rabbit sera.

Table 1. Titers of antisera

Cells	Antiserum* T11A9a	T32B11	T11D7e	Anti-R-29
LA7				
Anti-dome	2×10^{-4}	7×10^{-4}	$>10^{-2}$	$>10^{-2}$
Cytotoxic	$>10^{-2}$	$>10^{-2}$		7×10^{-5}
R-29				
Cytotoxic	8×10^{-5}	4×10^{-4}	10^{-4}	10^{-4}
Immunofluorescent	3×10^{-5}		10^{-4}	10^{-4}
C58[NT]D				
Cytotoxic	5×10^{-6}	2×10^{-5}	7×10^{-6}	

Anti-dome titer = $(2 \times 10^{-4})/(>10^{-2}) = <2 \times 10^{-2}$ for T11A9a, $(>10^{-2})/(10^{-4}) = >10^{2}$ for anti-R-29 LA7 cytotoxic titer. T11A9a/anti-R-29 = $(<2 \times 10^{-2})/(>10^{-2}) = <2 \times 10^{-4}$.

* Concentrations at which the 50% survival was obtained, except for immunofluorescence.

striking that the monoclonal sera have very low cytotoxicity titers towards LA7 cells. This implies that Thy-1 molecules are poorly expressed on the surface of these cells, as also shown by the weak immunofluorescence. Also striking is that two R-29 antisera, although highly cytotoxic for LA7 cells, had little effect on domes. Hence the interaction of antibody molecules with many surface antigens is irrelevant in dome formation; apparently the Thy-1 antigen has a special function in this differentiation.

Morphological Culture Changes Induced by Antisera. The confluent cell layer formed after addition of antiserum at concentrations capable of inhibiting dome formation to sparse cultures of LA7 cells was morphologically different from that of a doming culture: the cells were less spread out, formed wavy lines of reciprocal contact, and did not have beaded edges. These cultures had pronounced ridges. Antiserum addition to drop cultures with domes in the central part caused the morphological changes only in an outer ring that became confluent after the antiserum was added; the two parts were sharply delineated (Fig. 5). Cell counts showed that cell multiplication was not inhibited by the antiserum. Sera without anti-dome effect did not affect morphology.

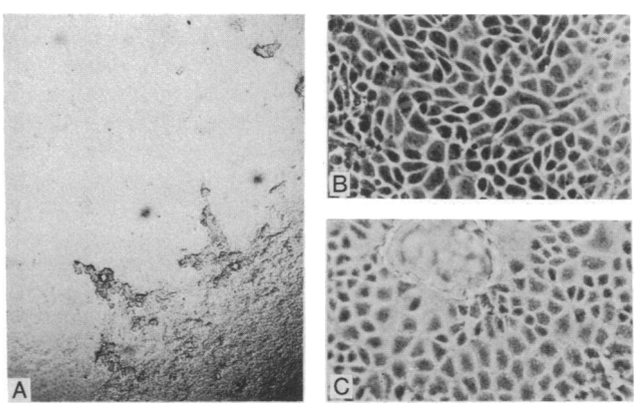

FIG. 5. (*A* and *B*) Effect of T11A9a monoclonal anti-Thy-1.1 serum on a LA7 cell drop culture. Serum was added when the center part of the cell layer was confluent and domes started appearing. (*A*) Low power, not phase photograph of the living culture with the altered halo lower right, surrounding a relatively unaltered center without domes. Prominent ridges in halo and at the transition (×41.) (*B*) Phase contrast photograph of the living altered cell layer, outside ridges. (×102.) (*C*) Phase contrast photograph of the living cell layer in a control culture without antiserum: elevated dome upper left. (×102.)

The effects of the antisera were reversed by washing the cultures and replacing the medium with regular conditioned medium: the usual cell morphology was restored, domes reappeared, and ridges became less prominent.

DISCUSSION

The ability of certain monoclonal antisera to Thy-1.1 antigen to prevent, in a reversible way, the formation or persistence of domes in LA7 cell cultures shows that the Thy-1 antigen plays an important role in this type of differentiation. It is not known why all Thy-1.1 antisera do not produce this effect. The different effects may be used as a tool for probing the role of the Thy-1 antigen in this differentiation.

It is striking that antisera against R-29 cells are at least 1/5000th as effective in inhibiting dome formation, on the basis of cytotoxic titers for LA7 cells, than the two anti-Thy-1 sera. The difference may be attributed to either the specificity of the antibodies or to the fact that monoclonal sera have an extraordinarily high titer of a single specificity, the specificity itself being unimportant. The identification of the antigen by immunofluorescence at the cell borders suggests that it determines special features of cell contacts within and outside domes.

It is intriguing that the Thy-1 antigen is most evident on the surface of the cells of incipient domes. These cells show prominent surface granules, and some appear to be generating cell debris even if not exposed to antiserum. Perhaps the initial process of dome formation involves a marked rearrangement of the cell surface, occasionally causing the death of the cells.

An important aspect of the action of the antisera is to alter, in part reversibly, the morphology of the developing cell layer with formation of ridges, which are an alternative state of differentiation of LA7 cells (5). Hence the antisera seem to alter the developmental program of the cells. Where this program has already been actuated (as in the center of drop cultures) ridges are not formed (although domes disappear), showing that certain aspects of differentiation, once actuated, are rather stable. The higher antiserum concentration required for abolishing domes than for preventing their formation may be due to the same effect. Because Thy-1 antisera alter the cell layer outside the domes, formation of domes must have additional requirements in addition to the presence of the antigen at the cell surface. Domes are probably brought about by a dual mechanism: a pumping action transporting water and ions to the area underneath the cells (9, 10) and some special interaction between cells (or between cells and plastic) that allows the cell layer to be lifted by the hydrostatic pressure. The Thy-1 antigen may contribute to the latter mechanism. The antisera may act by altering the interactions between cells, in a similar way to an antiserum against a surface protein of chicken brain cells, which prevents the adhesion of the cells *in vitro* (11). A role of Thy-1 in cell interactions leading to differentiation within the thymus has been suggested (12). The antigen may perhaps play a similar role in myoblasts, in which it is present during fusion but disappears when fusion is completed (4).

The presence of the Thy-1 antigen on the surface of LA7 cells is probably related to their role as precursors of fusiform cells (5), which are strongly Thy-1 positive (2). In fact the projections, which contain the direct precursors of the fusiform cells (5), contain strongly Thy-1-positive cells at their tips; moreover, projections are formed from domes. However, antibodies to Thy-1 do not inhibit the formation of projections.

We thank Dr. P. I. Lake and Dr. E. A. Clark for the generous gift of monoclonal sera used in this work and Dr. A. Williams for the anti-T25 serum. We acknowledge the competent technical help of S. Okada.

This investigation was supported by Grant 1-R01 CA21993-01, awarded by the National Cancer Institute, and by grants from the Hammer Foundation, the Educational Foundation of America, and the Pardee Foundation. M.B. is a recipient of an educational assistance fellowship from Merck, Sharp & Dohme International. M.U. is an American Cancer Society Postdoctoral Fellow, Grant PF 1212.

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