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Modulation of Cell Behavior *In Vitro* by the Substratum in Fibroblastic and Leukemic Mouse Cell Lines

(cell membrane/protein polymers/contact inhibition)

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ABSTRACT **Fibroblastic and leukemic mouse cell lines were cultivated on a bovine serum albumin polymer covered with basic or acidic substances. Contact inhibition of fibroblast movement (cell overlapping) and division varied depending on prior treatment of the albumin polymer. The leukemic cells, which normally grow in suspension, attached and spread out on the polymers and could be carried as a monolayer. Preparation of surfaces capable of modulating different parameters of cell behavior *in vitro* is thus possible.**

We have described the use of a polymer of bovine serum albumin as a substratum for the growth of cells *in vitro* (1). This substratum can be easily prepared, and its physicochemical properties can be changed by attachment of various macromolecules to its surface (1). Cells that proliferate on surfaces that vary physicochemically behave differently with respect to contact inhibition of movement (cell overlapping) (2) and of growth (3, 4). A more detailed description of the polymer of bovine serum albumin, and the results obtained with two cell lines (one usually grows as a monolayer and the other grows in suspension) with the polymer as substratum are presented.

MATERIALS AND METHODS

Cell Culture. L (5) and L-1210 (kindly supplied by Dr. I. Gresser, Centre National de la Recherche Scientifique, Villejuif) mouse cell lines were grown in Eagle's minimal essential medium supplemented with 10% calf serum and 50 μg of kanamycin per ml. The L cells were routinely maintained in monolayer on borosilicate glass. The L-1210 leukemic cell line was maintained in suspension in plastic flasks as nonagitated cultures. Before the experiments, the cells were pooled and evenly distributed on the different surfaces. Viable cells were counted by the trypan blue-exclusion method (6) in a hemocytometer after suspension of the cells in phosphate buffered saline (PO_4-saline; pH 7.4) without calcium and magnesium that contained trypsin (0.25 mg/100 ml) and ethylene diaminetetraacetate (EDTA; 0.2 mg/100 ml). Each point on each of the curves represents the mean of duplicate samples. The difference between samples was never more than 5%.

Preparation of the Polymers. To 1 ml of a sterile 30% solution of bovine serum albumin (Poviet Producten Naamloze Vennootschap-Amsterdam), 1.2 ml of a 2.5% aqueous solution of gluteraldehyde was added at 4° with constant stirring. 1 ml of this solution was quickly poured on to the surface of 30-ml plastic Falcon bottles, and the protein was allowed to polymerize for 3 hr at room temperature (24°). Subsequent washings with 3 ml of each of different buffers were as described below.

Measurement of DNA Synthesis. ^{3}H-labeled thymidine ([^{3}H]dT) (15 Ci/mmol) was added to the cultures in a final concentration of 0.5 μCi/ml supplemented with trypsin and EDTA, centrifuged, and washed three times with 10% $HClO_2$ at 4°. The precipitate was dissolved in 1 ml of toluene (Packard Co.) mixed with 14 ml of Liquifluor (Packard Co.), and the radioactivity was measured in a liquid scintillation spectrometer (Packard Co.). Corrections of quenching were made when necessary with an external standard, from a radium source (Packard Co.).

RESULTS

Adaptation of the polymer of bovine serum albumin to cell culture

Washing of the insoluble protein after polymerization is important to obtain good cell growth. Fig. 1 illustrates the growth of L cells on the albumin polymer washed with different buffers. When the albumin polymers were washed with PO_4-saline supplemented with EDTA (pH 7.4), there was little or no cell proliferation; however, when the polymers were washed during the first 24 hr with 0.15 M borate or Tris buffer (pH 8.4) (7), followed by washing with PO_4-saline–EDTA, cells grew at the same rate as on plastic.

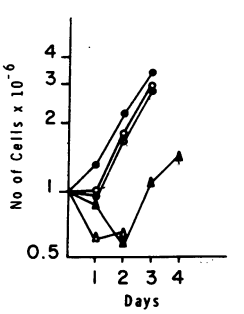

FIG. 1. Proliferation of L cells on plastic (●——●) and on the polymer of bovine serum albumin washed either with PO_4-saline (△——△) or PO_4-saline–EDTA (▲——▲), and with borate or Tris (○——○) buffers plus PO_4-saline–EDTA.

Abbreviation: PO_4-saline, phosphate buffered saline.

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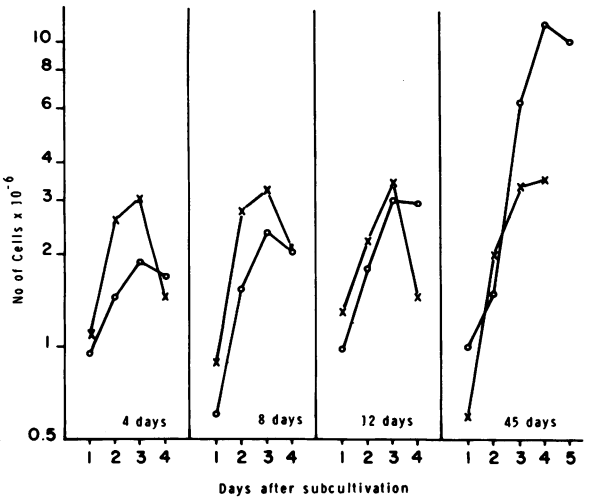

FIG. 2. Proliferation of L cells on plastic (×——×) and on albumin polymer (O——O) left 4, 8, 12, and 45 days in PO_4-saline–EDTA after a 24-hr wash with borate buffer.

Polymers washed with PO_4-saline without EDTA did not support cell growth.

The time of addition of the buffer after polymerization had been completed was also important. Proliferation was fastest during the first 24 hr of culture when the buffer was added between 3 and 3.5 hr after polymerization.

The proliferation of L cells also depended on the length of time the polymers were left in PO_4-saline–EDTA. The albumin polymer was washed during the first 24 hr with borate buffer, then left for various periods in PO_4-saline–EDTA (Fig. 2). Maximum cell concentration per flask increased with time when the polymer was left in PO_4-saline–EDTA. After 45 days in PO_4-saline–EDTA, the cell concentration per flask increased almost 4-fold, compared to the cultures maintained on a plastic surface.

We can summarize the basic procedure adopted for treatment of polymer of bovine serum albumin as follows: 3–3.5 hr after polymerization, borate buffer is added and left for 24 hr. The borate buffer is then replaced with PO_4-saline–EDTA, which is changed every third day. The polymerized surface is ready for use after being covered with PO_4-saline–EDTA for 12 days. Before seeding the cells, the surface is washed twice with complete medium (see *Methods*). To attach a substance to this surface the same procedure is followed with the additional steps described below.

Modulation of growth by the substrarum

Substratum-Dependent Cells (the L Cell Line). The L cell line that grows on the albumin polymer tends to form thick clusters of round cells (Fig. 3 *left*). This is in contrast with the well-known behavior of this type of cell in monolayer on Pyrex or on plastic (Fig. 3 *middle*). Since the membranes of most cell lines are mainly negatively charged, we tried to cover the polymer with a positively charged substance to see whether cells would then form a monolayer. This was done as follows: after polymerization, the bottles coated with bovine serum albumin were left in borate buffer for 24 hr; then, borate buffer containing 1 mg of poly(lysine) (molecular weight 130,000)/ml was added and was replaced 24 hr later with PO_4-saline–EDTA. The saline–EDTA was renewed each fourth day for 12 days. The polymer was washed twice with complete medium (see *Methods*), then 2×10^4 L cells per flask were seeded. L cells adhered well to the polymer treated with poly(lysine), and when confluent, formed a fibroblastic monolayer with a criss-cross pattern (Fig. 3 *right*). This finding led us to treat the albumin polymer with different substances, charged positively or negatively; the same pro-

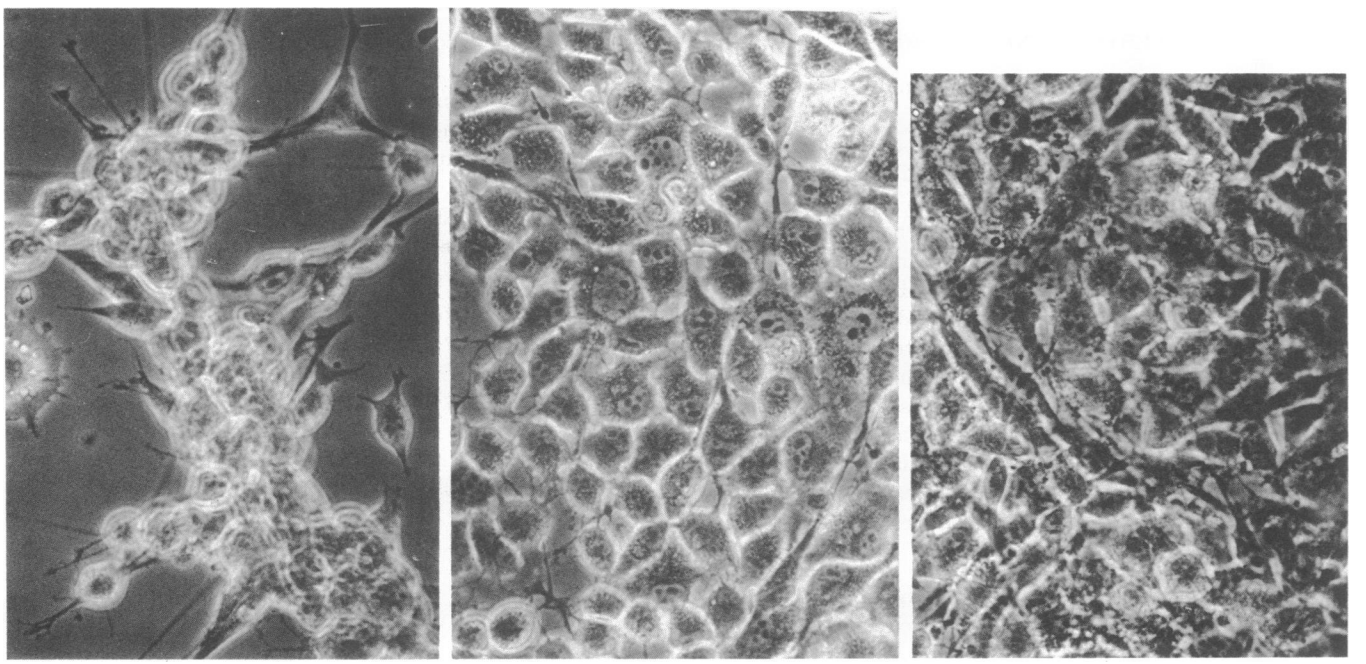

FIG. 3. (*Left*) Morphology of L cells on albumin polymer when the growth curve reached a plateau; (*middle*) morphology of L cells on plastic when the growth curve reached a plateau; (*right*) morphology of L cells on albumin polymer treated with poly(lysine) when the growth curve reached a plateau. All figures ×254.

cedure described for poly(lysine) was used. Table 1 summarizes the results with respect to formation of aggregates or monolayers at the time the growth curves reached a plateau. It shows that monolayers, like those illustrated in Fig. 3 *right*, were only obtained with positively charged substances and that treatment with negatively charged substances resulted in piling up of cells as in Fig. 3 *left*. Treatment of the surface of the albumin polymer with basic amino acids instead of basic poly(amino acids) resulted in the formation of aggregates.

Changing the concentration of poly(lysine) in the borate buffer used to treat the albumin polymer showed (Fig. 4) that cells reached a higher concentration with 1 mg/ml than with 0.1 mg or 0.01 mg/ml. Poly(lysine) and poly(ornithine) of different molecular weights were also tested. Fig. 5 shows that proliferation of cells was better on the polymer than on plastic and the differences in molecular weight did not play an important role. Slightly better results were obtained with poly(ornithine) (molecular weight 120,000) than with poly(lysine) (molecular weight 139,000). Crude histones were also used at different concentrations with the same procedure described for poly(lysine); the best growth was obtained with 0.1 mg/ml in borate buffer. Fig. 6 illustrates the growth of L cells on albumin polymer treated with 0.1 mg/ml of crude, arginine-rich and lysine-rich histones. It was found that cells do not grow on polymer treated with lysine-rich histone.

Growth of cell monolayers on positively and negatively charged substances was compared in the following way: after polymerization, the bovine serum albumin was left for 24 hr in borate buffer; for the next 24 hr, one group was left in borate buffer with 1 mg of crude histones per ml, another group was left in borate buffer with 0.1 mg of poly(lysine) per ml, and a third group was left in borate buffer with 1 mg of DEAE-dextran per ml. The supernatants were then removed, and the polymers were left in PO_4-saline–EDTA for 12 days. A fourth and a fifth group were left in borate buffer during the first day after polymerization, in borate buffer supplemented with 1 mg of poly(glutamic acid) (molecular weight 75,000)/ml or 1 mg of heparin per ml, respectively, during the second day, and in borate buffer supplemented with 1 mg of DEAE-dextran per ml during the third day. Then they were washed with PO_4-saline–EDTA for 12 days. With this treatment, one can obtain a surface covered with poly(glutamic acid) on which cells grow in a monolayer. L cells were then seeded on the different polymers after washing with Eagle's minimal essential medium. Cell counts and measurement of DNA synthesis were performed daily in the cultures proliferating both on plastic and on albumin polymers, treated as described. Fig. 7 shows that higher cell densities were obtained on albumin polymers treated with basic substances than on the controls (plastic). However, on albumin polymers treated with negatively charged substances, cell proliferation and DNA synthesis were less pronounced.

TABLE 1. *Formation of aggregates (A) or monolayers (M) by L cells growing on a polymer of bovine serum albumin treated with different substances*

Polymers of bovine serum albumin treated with:	
—	A
Dextran sulfate	A
Poly(cysteine)	A
Poly(glutamic acid)	A
Poly(tryptophan)	A
Poly(phenylalanine)	A
Poly(tyrosine)	A
Poly(leucine	A
Poly(histidine)	M
Poly(arginine)	M
Poly(lysine)	M
Poly(ornithine)	M
Crude histones	M
DEAE-dextran	M

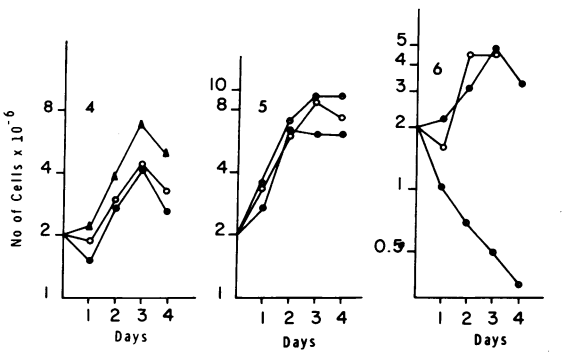

FIG. 4. Growth of L cells on albumin polymer treated with different concentrations of poly(lysine) (1000 ▲——▲, 100 ○——○, and 10 ●——● μg/ml).

FIG. 5. Growth of L cells on plastic and on albumin polymer treated with poly(ornithine) of different molecular weights (120,000, ◓——◓; 4000, ○——○; plastic, ●——●).

FIG. 6. Growth of L cells on albumin polymer treated with crude (●——●), arginine-rich (○——○), and lysine-rich (◐——◐) histones.

Cells Growing in Suspension (L-1210 Leukemia). L-1210 leukemia cells were seeded on a polymer of bovine serum albumin treated with histones in the same manner as described above. A week later, the cells progressively lost their rounded shape (Fig. 8 *middle*) and spread out on the polymer, assuming a fibroblastic form. The medium of identical cultures maintained both in bottles coated with bovine serum albumin and in uncoated plastic bottles, was aspirated and the cells were counted. Medium was then blown strongly with a pipette against different surfaces and the cells collected were counted. There were no cell aggregates. No cells were recovered from the plastic surfaces. However, after washing the surfaces with bovine serum albumin with medium, the number of cells represented 60–80% of the total count (supernatants plus attached cells). The fibroblastic cells progressively invaded the whole surface of the flask (Fig. 8 *right*) while the round cells disappeared. The new cell type was transferred by trypsinization, and after 5 or 6 subcultivations no more round cells could be detected in suspension. The fibroblastic cell type isolated on the polymer is now in its 19th subcultivation after 4 months in culture. At different times, the fibroblastic cell type was subcultivated on plastic surfaces but ceased dividing after five or six transfers. Repeated attempts to adapt the cells again to growth in suspension were unsuccessful.

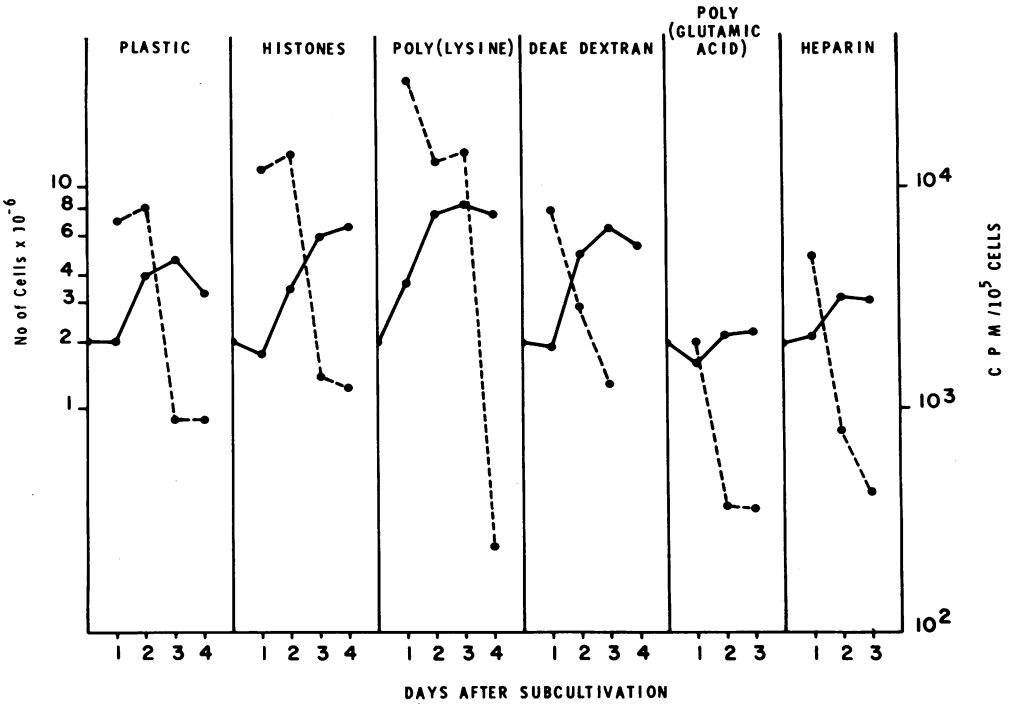

FIG. 7. Cell proliferation (●——●) and DNA synthesis (●- - -●) of L-cell cultures growing on plastic and on albumin polymer treated as indicated.

DISCUSSION

Carter (8) showed that cells in culture tend to migrate to the substratum with which they have a better affinity. This property, called haptotaxis, can explain the aggregation or formation of monolayers of L cells on different surfaces. Cells on the polymer of bovine serum albumin either untreated or treated with negatively charged substances have a greater affinity towards each other than towards the solid substratum, and thus will overlap and grow on top of each other. However, when cells are seeded on plastic or on the polymer treated with positively charged substances, they have a higher affinity towards the solid substratum than towards each other, and form a monolayer. Therefore, overlapping of cells can be modulated by the surface on which cells grow, and these results agree with Carter's experiments (8). It is interesting, however, that cells growing in clusters could grow to much higher densities than cells in monolayer. Results indicate that cultures on the albumin polymer left for 45 days in PO_4-saline–EDTA were able to reach a cell density per dish four times higher than the one obtained on plastic, without renewel of medium (9–11) or adjustments of pH (12, 13); this shows that the inhibition of cell division was not dependent on cell density. Since EDTA is necessary, certain growth-limiting ions may be present in the polymer. When growing

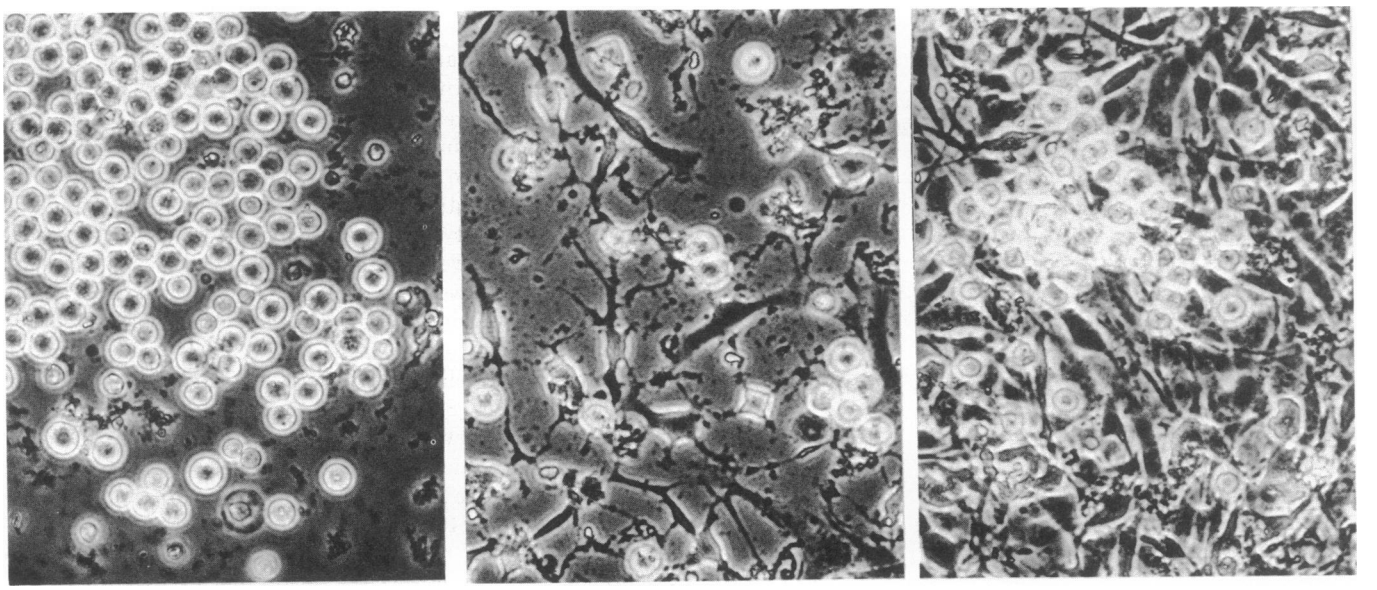

FIG. 8. L-1210 cells cultivated on albumin polymer treated with histones: (*left*) 24 hr after subcultivation, (*middle*)) 8 days after subcultivation, (*right*) 15 days after subcultivation. All figures ×250.

in a monolayer, cells can also proliferate to different densities when the substratum is changed. This is evident from the growth curves of cultures growing on the polymers of bovine serum albumin treated with different concentrations of poly-(lysine) and from the comparison between growth on the polymers and on plastic. We previously suggested that the loss of inhibition of the cell cycle during transformation can be expressed in different ways depending on the affinity of the cells with the substratum (14, 15). The present work supports this finding and indicates that inhibition of the cell cycle is also dependent on the substratum, in addition to the other parameters described (9–13, 16, 17). Dependence on the substratum could explain the discrepancy reported between behavior *in vitro* and *in vivo* (18) and shows that during "transformation" experiments, the cell environment must be considered.

It is interesting that the substances that give the best cell growth [crude and arginine-rich histones, as opposed to lysine-rich histones; and poly(ornithine), poly(lysine), or DEAE–dextran] are the same substances that stimulate cell permeability (19). On the contrary, poly(glutamic acid), which decreases the stimulating effect of the basic poly(amino acids) on cell permeability (19), also neutralizes the effect of DEAE–dextran in our cultures. These substances could possibly have a target at the cell membrane that is responsible for both cell permeability and the capacity to reach high cell densities (20).

Although results were the same with substances of two different molecular weights, the molecular weight must be important to obtain the effect described above, since the aminoacid monomers did not have the same effect even when used at 10 times the concentration of the respective polymers.

The substratum described above is not only useful for study of the modulation of growth of cells that need a surface for attachment, but also for modulation of the behavior of cells that normally grow in suspension. Results obtained with L-1210 cultures could be due to a selection of cells capable of adhering to a solid surface or to an adaptation of all cells in the culture to the new environment. The results we obtained with L-1210 cultures *in vitro* indicate a similarity to the growth of these cells *in vivo*, depending on the site of inoculation. When L-1210 cells are injected intraperitoneally, they grow as ascitic tumors; when injected subcutaneously, they grow as solid tumors.

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