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Induction of sarcomas in athymic mice

(human xenografts/murine leukemia virus/transformation/ATP phosphohydrolase)

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ABSTRACT **During the course of serial passage of 50 human xenografts in the athymic mouse over a period of 5 years we have observed two cases of induction of sarcomas in the murine stromal tissue associated with the human xenografts. Both times the growth of the murine sarcomas overtook that of the human xenograft. This change was monitored by analysis of the lactate dehydrogenase isozyme profile and histology of each passage of the human xenografts in the athymic mice. The two murine sarcomas were subsequently established in tissue culture. The sarcoma cell lines were found to be malignant by morphological and growth characteristics and were tumorigenic. They contained large amounts of murine leukemia virus when assayed for reverse transcriptase activity by infection of mouse SC-1 cells and BALB/c and NIH Swiss fibroblasts with filtered supernates, and some type C virus particles were observed by electron microscopy in tumor tissues. However, we were unable to demonstrate the presence of murine sarcoma virus by *in vitro* transformation of fibroblasts or sarcoma formation *in vivo* with cell free filtrates. Preliminary biochemical data indicate that the sarcomas are extremely high in plasma membrane ATP phosphohydrolase.**

We found previously that induction of murine leukemia virus (MuLV) was associated with transplantation and passage of a human xenograft, an oat cell carcinoma, in athymic mice (1). We have also shown that chronic antigenic stimulation of the athymic mice by pinworm infection or xenografts of several types of human tumors led to an increase in levels of the MuLV envelope protein gp70 in the serum, followed in many cases by the induction of lymphoma (2). We now describe a third phenomenon: induction of murine sarcomas in the stromal tissue associated with human xenografts. Out of 50 human tumors maintained in the athymic mice, there were two such occurrences: one during passage of a human adenocarcinoma (T362) and the other during passage of a human hepatoma (Li-7).

MATERIALS AND METHODS

Mice. BALB/c athymic mice (*nu/nu*) were bred under pathogen-free conditions in the Athymic Mouse Facility at University of California, San Diego.

Human Tumor Xenografts and Transplantation. Human adenocarcinoma (T362) was established in athymic mice in our colony. Human hepatoma (Li-7) was obtained as a xenograft in athymic mice in its 50th passage from the National Cancer Center Research Institute of Japan. The pathology and transplantation of Li-7 were reported by Shimosato *et al.* (3). Tumor transplantation was carried out as described (4).

Histology. Tissues for light microscopy stained by hemotoxylin/eosin were prepared as described (5).

Lactate Dehydrogenase Assay. Frozen tumor tissues were extracted by 50 mM sodium phosphate, pH 7.5. Lactate dehydrogenase activity was determined spectrophotometrically in an aliquot of the tumor extract (6). A suitable aliquot of the extract was subjected to gel electrophoresis (Corning Medical, Palo Alto, CA) in 50 mM sodium barbital containing 0.035% EDTA, pH 8.6. The profiles of isozyme distribution were determined by using a Corning fluorometer/densitometer.

Tissue Culture. Primary cultures of tumors were initiated by mincing tumors and incubating in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum at 37°C in an atmosphere of 95% air and 5% CO_2. Medium also contained penicillin, ampicillin, and streptomycin.

Electron Microscopy. Tissues for electron microscopy were prepared according to standard procedure (1).

Reverse Transcriptase Assay. The RNA-dependent DNA polymerase release assay was performed as described by Welsh *et al.* (7).

Transformation Assay. Focus assays as described by Aaronson *et al.* (8) were used to determine the presence of murine sarcoma virus (MuSV), using monolayers of BALB/c 3T3 and embryonic fibroblasts.

Plasma Membrane Preparation. Plasma membranes from tumors were prepared as described by Knowles *et al.* (9).

ATP Phosphohydrolase Assays. ATP phosphohydrolase activities were determined at 37°C in a reaction mixture containing 30 mM histidine/Tris at pH 7.4, 5 mM $MgCl_2$, 5 mM Tris ATP, and 30–100 μg of membrane protein (10). Inorganic phosphate released was determined colorimetrically.

RESULTS

Induction of Mouse Sarcomas as Determined by Histologic Observation and Change in Lactate Dehydrogenase Isozyme Pattern. Fig. 1 shows an increase in the murine stromal tissue associated with the human adenocarcinoma xenograft T362 from the 5th passage to the 12th passage. Although the stroma appeared normal at the 5th passage, it had completely replaced the adenocarcinoma by the 12th passage. At passage 5 (Fig. 1*A*) the stroma was composed of spindle cells with elongated nuclei and there were no more than two or three stromal nuclei abreast at any point. By passage 10 (Fig. 1*B*) the stroma had obviously begun to proliferate. The stromal cells were still spindle cells, but the nuclei showed pleomorphism, and had acquired nucleoli. Some nuclei were also hyperchromatic. In passage 11 (Fig. 1*C*) there was even more proliferation of the stroma and a suggestion of a storiform pattern of the fibroblast bundles was present. Most of the stromal nuclei were larger than those in passage 5 and they were often oval. By passage 12 (Fig. 1*D*) the

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Abbreviations: MuLV, murine leukemia virus; MuSV, murine sarcoma virus.

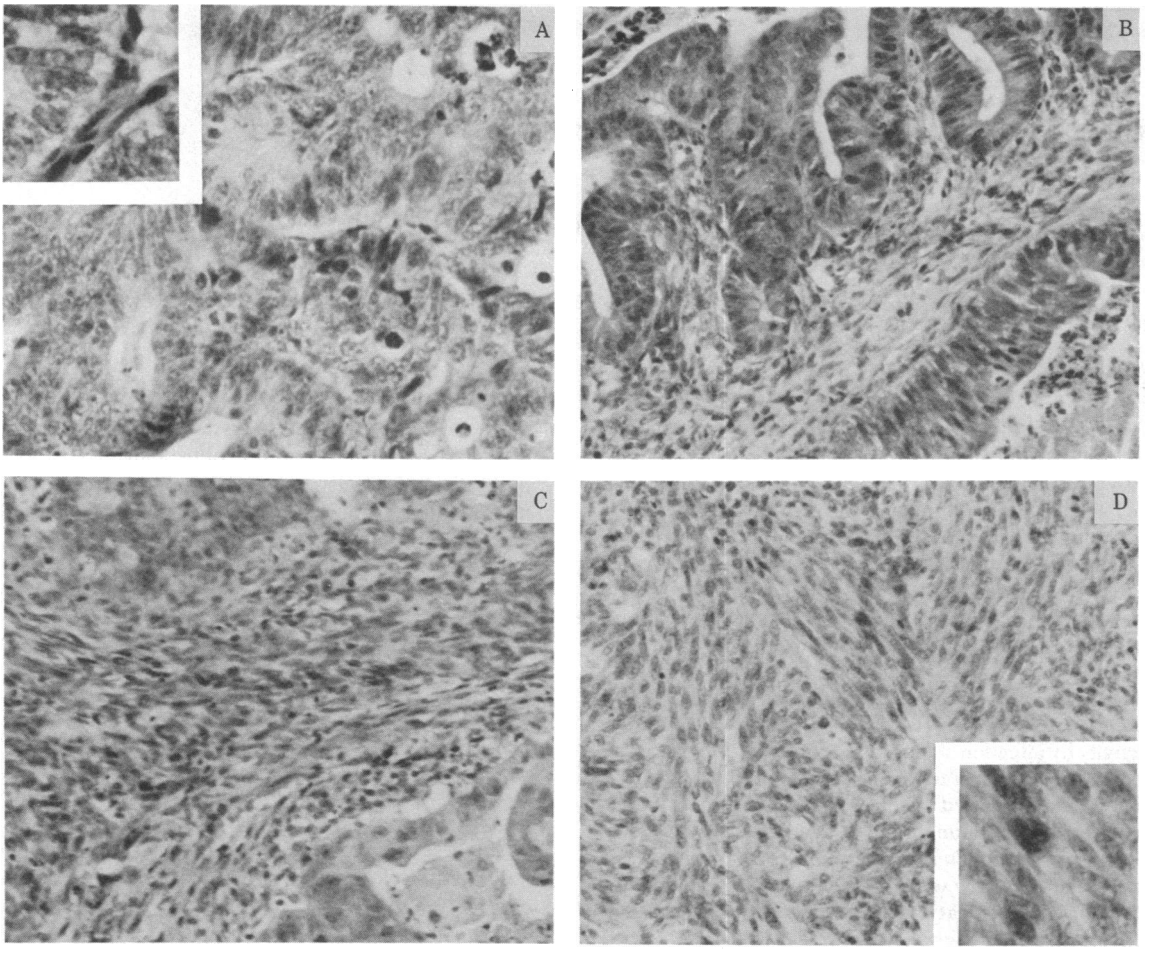

FIG. 1. Histology of cells during sarcoma development. (*A*) Adenocarcinoma (T362) with normal mouse stroma from passage 5. *Inset* shows fusiform fibroblasts between the adenocarcinoma cells. (*B*) Passage 10. The stroma has proliferated. Several cell layers lie between the carcinoma cells. (*C*) Passage 11. Further stromal proliferation has taken place. (*D*) Passage 12. The stromal proliferation has completely replaced the adenocarcinoma. *Inset* shows the stromal cells. They are large and plump and have hyperchromatic nuclei with nucleoli. (×25; *Insets* ×250.)

stromal proliferation had completely overtaken the adenocarcinoma. Bundles of fibroblasts interwove in an obvious storiform pattern and all the individual nuclei were plump and almost all were oval. Nucleoli were easy to find and mitotic figures were occasionally present. Taken together, these features all show a continuous progression of stromal proliferation and change to pleomorphism and atypicality of the individual cells.

Fig. 2 shows the lactate dehydrogenase isozyme profiles that correspond to the histology in Fig. 1 *B–D*. In passage 5 (not shown) and 10 (Fig. 2*A*) mouse lactate dehydrogenase was virtually absent. The isozyme profiles for these two passages were very similar, in contrast to their histologies (Fig. 1 *A* and *B*), in which differences in the stroma could be distinguished. This observation suggests that the lactate dehydrogenase isozyme profile is not as sensitive a method for detecting small changes in the tumor as is the histological diagnosis. However, by passage 11 (Fig. 2*B*) the mouse lactate dehydrogenase made up 50% of the total activity, which correlated well with the histological findings (Fig. 1*C*). By passage 12 the tumor lactate dehydrogenase was 100% mouse enzyme (Fig. 2*C*), again correlating with the histological data (Fig. 1*D*).

Similar data for both the histology and lactate dehydrogenase isozyme profile were obtained when the properties of the human hepatoma Li-7 changed between the 59th and the 63rd passage.

Cell Culture Studies. Primary cultures were established from sarcoma cells from the 12th passage of T362 and the 63rd passage of Li-7. A fibroblast line grew out in each case, respectively designated T362 (m) and Li-7 (m). They were shown to be murine cells by their lactate dehydrogenase profiles, which were similar to the profile in Fig. 2*C*. Their morphology and growth behavior, though different from each other, were characteristic of malignant cells as shown in the photomicrograph of Li-7 (m) (Fig. 3). Both lines exhibited tumorigenicity by their ability to grow in a new host.

Presence of Virus in the Sarcomas. Some type C virus particles were observed when T362 (m) and Li-7 (m) tumor tissues were examined by electron microscopy (Fig. 4). The supernatant fluids of cultured cells T362 (m) and Li-7 (m) contained high levels of RNA-dependent DNA polymerase as shown in Table 1. Xenotropic and ecotropic MuLV were detected in both tumors (data not shown). However, we were unable to detect MuSV with either the *in vivo* or the *in vitro* assay.

ATP Phosphohydrolase Activity of the Plasma Membranes of the Sarcomas. We have been studying the various membrane-bound ATPases of several human xenografts (10, 11), including hepatoma Li-7. Data in Table 2 illustrate the dramatic increase of the ATP phosphohydrolase activity of the plasma membranes during the period when the human hepatoma was overtaken by the mouse sarcoma. The activity then decreased to a steady level of 2 μmol of P_i/min per mg of protein. Similarly, we also observed very high ATP phosphohydrolase activity in the plasma membranes of T362 (m) mouse sarcoma.

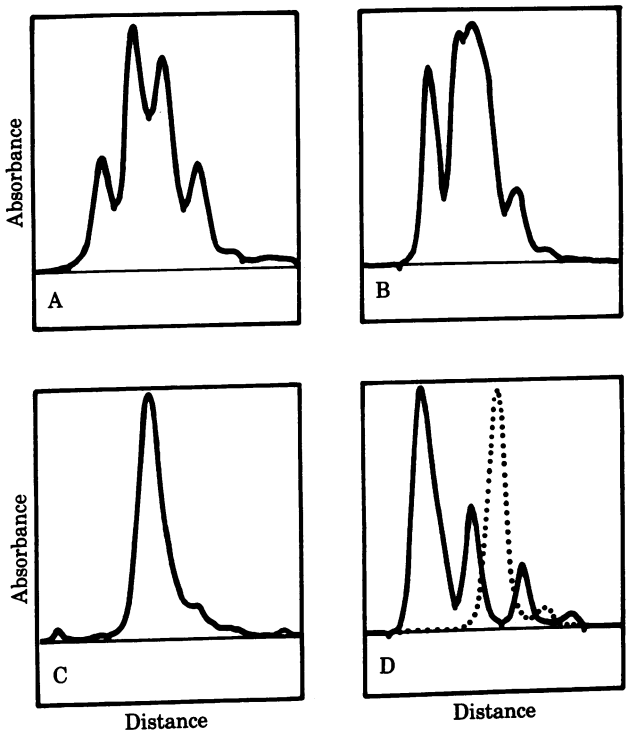

FIG. 2. Lactate dehydrogenase isozyme profiles. (*A*) Isozyme profile of T362 homogenate from passage 10; 100% human by analysis. (*B*) Isozyme profile of T362 homogenate from passage 11; 50% mouse isozyme by analysis. (*C*) Isozyme profile of T362 homogenate from passage 12; 100% mouse isozyme by analysis. (*D*) Isozyme profile of human liver (——) and mouse connective tissue (·····) for comparison.

DISCUSSION

The ability of athymic mice to tolerate human tumor xenografts has been exploited by many laboratories for cancer research. During transplantation of human tumor xenografts, there is usually a small amount of murine stromal tissue associated with the human tumor. In general, the mouse lactate dehydrogenase contributes less than 10% to the total enzyme activity. Recently we observed that the percentage of mouse lactate dehydrogenase in the tumor extract began to increase in 2 out of 50 human tumors being maintained in our athymic mice colony. An increase in the murine stromal cells was also observed histologically. This phenomenon has been reported by Houghton and Taylor (12), who observed induction of a murine tumor in one out of six adenocarcinoma xenografts passaged in athymic mice. Goldenberg and Pavia (13) have shown that the mouse stromal cells from some human xenografts when grown *in vitro* were malignant, although malignant murine sarcoma was never observed by them *in vivo*. They also did not detect type C virus particles in these cells. In a different system, Huebner *et al.* (14) have shown that cultured human cancer cells when transplanted into anti-thymocyte-treated newborn rats caused induction of rat sarcomas. Oncornavirus activity was not observed in this system. In our system, murine virus was found as evidenced by the presence of high reverse transcriptase activity and intercellular type C virus particles, but we have as yet no evidence of MuSV activity.

If MuSV was not responsible for the induction of the sarcomas, how did these malignancies arise from contact with the human xenografts? One possibility is that transfection (15) occurred *in vivo*. The second possibility is that these two human tumors were producing large amounts of transformation growth factors. Todaro *et al.* (16) have shown that transformation growth factors when continuously present in the growth media of target normal cells caused morphological changes of normal cells to a malignant phenotype.

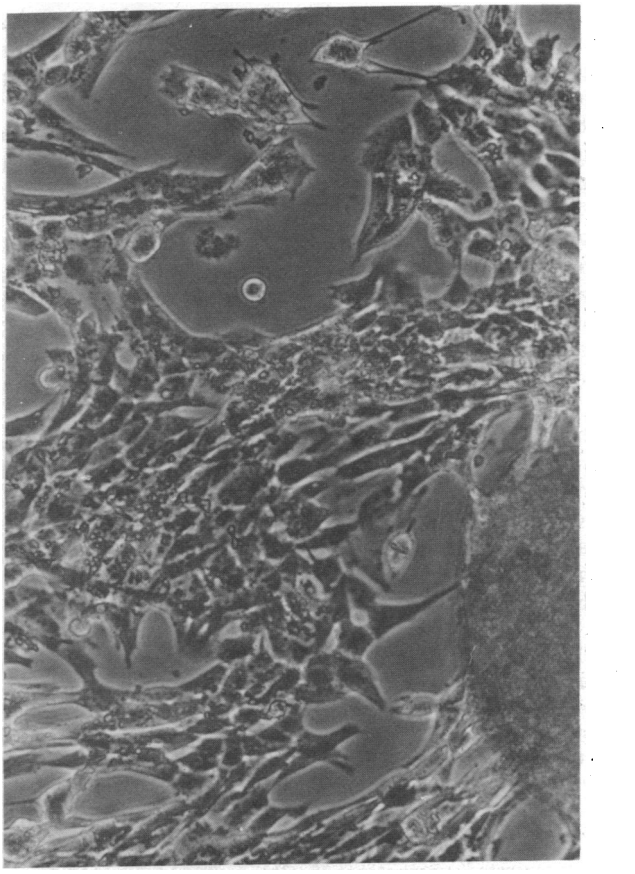

FIG. 3. Photomicrograph of Li-7 (m). Multilayered growth of Li-7 cells in tissue culture with nonaligned random orientation typical of transformed cells. (×375.)

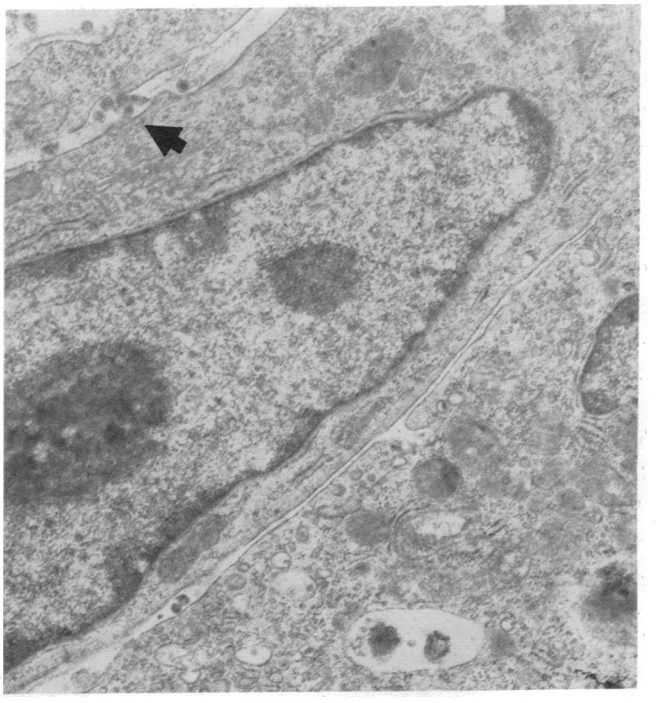

FIG. 4. Electron micrograph of Li-7 (m). Arrow denotes type C virus particles. (×4360.)

Preliminary biochemical studies showed that the plasma membranes of the sarcomas possessed a very high ATP phosphohydrolase activity, a property common to many other tumor plasma membranes we have investigated (9, 10). However, the activities in the plasma membranes of the sarcomas were much higher than found for the other tumors and were many times higher than the plasma membrane ATP phosphohydrolase activity of a normal tissue, such as liver (0.3 μmol of P_i/min per mg of protein). We have also found (data not shown) that the majority of the ATP phosphohydrolase activity of the sarcoma plasma membranes could be attributed to an ADP diphosphohydrolase (an enzyme that hydrolyzes both ATP and ADP). The significance of such a high level of this enzyme activity in the plasma membranes of the sarcoma is yet to be determined.

The generation of the mouse sarcoma from fibroblasts adhering to the human tumors promises to be a useful system in an understanding of the transformation process. It should be emphasized that transformation of mouse fibroblasts did not occur in the athymic mouse except in the proximity of the human tumor. We also stress that care and caution must be utilized when evaluating results with human tumors growing in the athymic mouse.

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Table 1. Release of reverse transcriptase activity from filtered concentrated supernatants of cell lines T362 (m) and Li-7 (m)

Addition	Activity, cpm: With poly(rA)·oligo(dT)	Activity, cpm: Without poly(rA)·oligo(dT)
Supernatant from T362 (m) cells	10,869	130
Supernatant from Li-7 (m) cells	59,705	227
Purified 4070A virus	76,805	346

Media from cell culture of T362 (m) and Li-7 (m) were concentrated as described (1) and assayed for reverse transcriptase activity. The assay mixture contained 1.5 mM $MgCl_2$, 50 mM Tris·HCl at pH 8.1, 0.03–0.12% Nonidet P-40, 20 mM dithiothreitol, 100 μM dATP, dCTP, and dGTP, 2.5 mM [*methyl*-^{3}H]dTTP, and $(rA)_n \cdot (dT)_{12-18}$ at 10 μg/ml when added. After incubation at 37°C for 30 min, the reaction mixture was filtered through DEAE-cellulose filters and polymerization was quantitated by measuring the radioactivities on the filters in Aquasol in a liquid scintillation counter.

Table 2. Plasma membrane ATP phosphohydrolase activity of Li-7 and T362 from different passages

Passage of Li-7	Mouse lactate dehydrogenase, %	ATP phosphohydrolase, μmol P_i/min per mg protein	Passage of T362	Mouse lactate dehydrogenase, %	ATP phosphohydrolase, μmol P_i/min per mg protein
53rd	0	0.77	18th	100	2.79
61st	41	4.72	21st	100	2.68
62nd	80	3.34			
66th	100	1.72			
67th	100	1.31			
68th	100	2.02			

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