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Characteristics of Human Adenovirus Type 12 Induced Hamster Tumor Cells in Tissue Culture.* (29307)

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Previous reports(1-4) have described tumor induction in Syrian hamsters with human adenoviruses. In addition, adenovirus type 12 (A-12) has been found to induce tumors in rats(5) and mice(6) but with a lower incidence. In contrast to the findings with other viruses oncogenic for newborn hamsters, *i.e.,* polyoma and Simian virus 40 (SV-40)(7-9), all attempts to isolate the virus from A-12 induced hamster tumors have been negative. The findings that some animals with primary tumors develop neutralizing antibodies(10) and that most animals with primary or transplant tumors develop A-12 type specific complement-fixing antibodies(5) suggest the presence of the virus or viral antigen in some form.

In an effort to detect A-12, a hamster tumor was serially propagated in tissue culture and attempts were made, by a variety of methods, to demonstrate the presence of the virus. Other characteristics studied were the response to superinfection with A-12, morphology and growth characteristics, and transplantability. Detailed karyotype analysis will be presented separately.

Materials and methods. Establishment of tumor cell line. A male hamster which had been inoculated with A-12 within 24 hours after birth developed intraperitoneal tumors. The tumor tissue was removed 42 days after inoculation, minced into small fragments about 0.5-1.0 cm and trypsinized overnight at 4°C with a magnetic stirrer. The suspension was centrifuged at 1200 rpm for 5 minutes, the pellet resuspended in growth medium, the cell suspension inoculated in 60 mm glass petri dishes and incubated at 37°C in a CO_2 incubator. The tumor cell line will subsequently be referred to as hamster tumor, line 1 (H.T.-1).

Growth medium. Eagle's medium (MEM) containing 10% horse serum was used as the initial growth medium and for the first 10 subcultivations; subsequently the serum content was changed to 10% calf serum unless otherwise indicated. All sera were heat inactivated at 56°C for 30 minutes. The medium contained 200 units of penicillin, 200 μg of streptomycin, and 20 units of Mycostatin per ml. All washing was done in phosphate buffered saline (PBS).

Superinfection studies. The MEM plus 10% calf serum was decanted from 2 oz bottles containing approximately 10^6 hamster tumor cells of the 25th passage. A-12 was added, and after incubating for one hour (37°C), the bottles were drained, washed 3 times with 2 ml of PBS, refed with 4 ml of MEM plus 10% horse serum, and incubated at 37°C for the 10 days of the experiment. Cytological observations were made from cover slips. Supernatant fluid and cells were titrated for virus separately, the latter after

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resuspension in 1 ml of medium and freezing and thawing 3 times. Virus assay was performed on HeLa cells fed with MEM plus 10% agamma globulin calf serum.

Newborn hamster cultures. Newborn hamsters were sacrificed; the kidney, skin, and liver were removed and minced into small fragments. These explants were seeded in 2-oz prescription bottles. An embryo-extract-chick plasma clot was used for some explants. After approximately 10 days, monolayers had developed. The strain of virus used and methods for the propagation of HeLa cells and virus titrations were essentially the same as previously described(1,11).

Results. Establishment of tissue culture cell line of adenovirus type 12 induced tumor in vitro. The tumor tissue was prepared as described above. In the fourth day of cultivation small colonies of transparent epithelioid cells were observed, separated by fibroblast-like cells. On the sixth day of culture, the cell sheet was trypsinized. After 5 minutes of trypsinization at 37°C, the cell sheet became detached. After centrifugation, the pellet had a mucoid consistency and resuspension was hard to achieve. From another petri dish, epithelioid cell foci were picked with a capillary pipette and subcultured.

Both the trypsinized and picked cells had a tendency to form islands for the first month in cultivation. Thereafter small characteristic epithelioid cells appeared as a tightly packed monolayer (Fig. 1). Some of the cells showed a transparency which disappeared in the subsequent subcultivations.

Morphological observations of the cells were carried out with cover slips stained with May-Grünwald Giemsa and/or Feulgen. It was observed that on the cover slips small epithelioid cells predominated while on the floor of the petri dish aggregated cell islands were more predominant. As mentioned later, it was found that the serum component of the medium had an influence on the cell morphology.

Morphology of HT-1 cells and effect of the serum component of the medium. The HT-1 cells were approximately one-third the size of HeLa cells, and had more (4 to 10) nucleoli. These stained violet-red with May-Grünwald Giemsa.

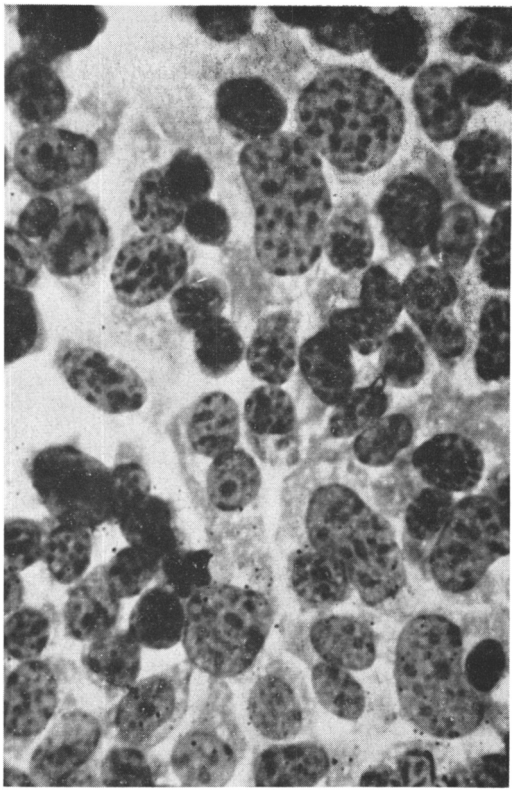

FIG. 1. Photo of cultured HT-1 cells showing small characteristic epithelioid cells in tightly packed monolayer. (×294)

One characteristic of the transparent epithelioid cells that preceded the small epithelioid cells was an intranuclear inclusion-like mass surrounded by a halo. In some of these cells, cytoplasmic inclusion-like masses pushed the nucleus to one side.

The HT-1 cells grew as a monolayer in MEM with 10% horse serum. Upon subsequent subcultivations this monolayer-forming tendency was lost and the cells formed large aggregates containing numerous small epithelioid cells. This phenomenon was noticed around the 10th subcultivation. These cell aggregates floated free in the medium. After centrifugation, trypsinization and cultivation in MEM with 10% calf serum, monolayers were again formed. The cells could be made to grow alternately as monolayers or as balls of aggregated cells detached from the glass, depending on whether calf serum or horse serum, respectively, was employed (Fig. 2).

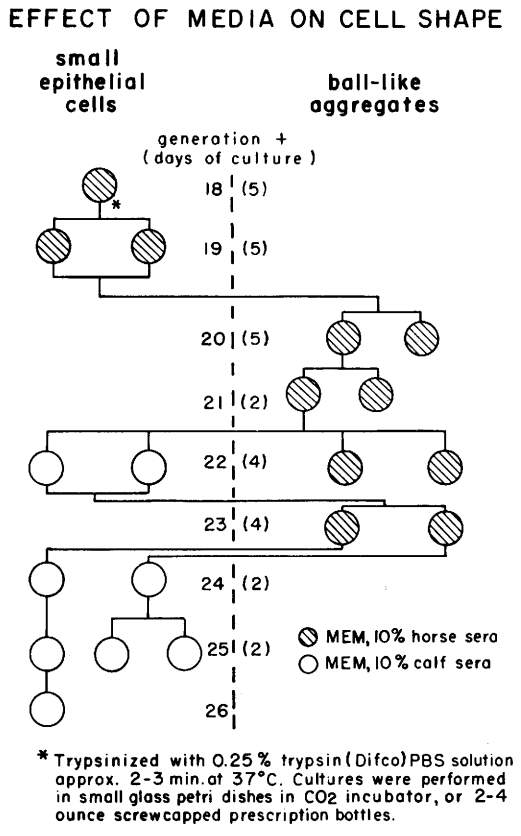

FIG. 2. Reversible conversion of HT-1 cells from monolayer to cell ball form of growth by calf serum and horse serum component of medium respectively.

Both cell balls and cell monolayers gave rise to progressively growing tumors when transplanted to weanling hamsters. Karyotypic analysis showed polyploidy with a tendency for deletion of acrocentric chromosomes (12).

Transplantability. Progressively growing tumors developed following inoculation of weanling hamsters with at least 1×10^6 cells subcutaneously, 1×10^5 cells in the cheek pouch, and in newborns following inoculation of 1×10^4 cells subcutaneously.

A-12 virus isolation attempts from tumor tissue culture cells. As shown in Table I, several methods were used in an effort to demonstrate virus:

(a) Subculture of supernatant fluids: Tissue culture media from various passage levels were collected, frozen, and subsequently assayed for virus by the direct inoculation of 0.3 or 0.1 ml per tube of HeLa cells ($4\text{-}5 \times 10^4$ HeLa cells plus 1.0 ml MEM with 10% agamma calf serum per tube) and observed until uninoculated controls degenerated, *circa* 2 weeks. Media changes were made with MEM plus 5% agamma calf serum.

(b) Subculture of lysed cells: At several different passage levels, $1\text{-}5 \times 10^6$ cells were suspended in 1.0 ml of media, disrupted by 3 cycles of freezing and thawing, and inoculated into HeLa tubes as described under (a).

(c) Electron microscopy: Tissue culture cells in their 32nd passage were examined for adenovirus particles by electron microscopy.

(d) Mixed culture of tumor and HeLa cells: Trypsinized tumor cells and HeLa cells were mixed and subcultured together. The two types of cells were in direct contact and readily distinguishable as illustrated in Fig. 3. The mixed cultures were subcultured for 16 passages in which the proportion of each cell type remained approximately the same. The cultures were observed for CPE and super-

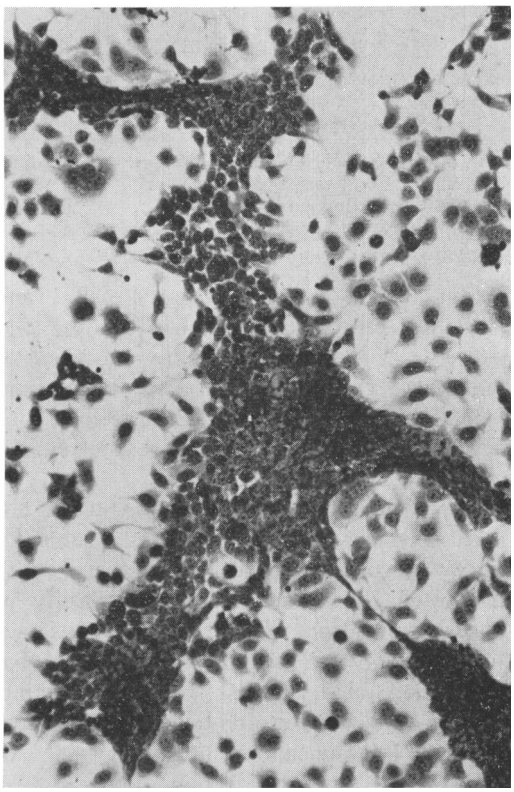

FIG. 3. HT-1 (dense areas) and HeLa cells (sparse areas) growing in 9th passage of mixed culture. (×123)

TABLE I. Direct and Indirect Methods Employed in Attempts to Detect A-12 in HT-1 Cell Cultures.

Method	A-12 TC generation	Days in continuous culture	Source	Test days	Final indicator cell	Result‡
Direct	1	0	fluid	14	HeLa†	—
	2	18	fluid	1, 2, 5	HeLa†	—
	3	17	fluid	2	HeLa†	—
	21	141	cell	1, 2	HeLa*	—
			fluid	1, 2	HeLa*	—
	29	138	cell	7	HeLa*	—
			fluid	5	HeLa*	—
	30	142	cell	2	HeLa*	—
			fluid	2	HeLa*	—
	31	142	cell	2	HeLa*	—
			fluid	1, 2	HeLa*	—
	33	141	cell	2	HeLa*	—
			fluid	2	HeLa*	—
	36	150	cell	5	Electron microscopy	—
				Mixed with		
Mixed culture	1	76	cell	HeLa (2, 5, 7, 9) monolayer	HeLa*	—
	13	74	cell	HeLa (2, 4, 8, 12) suspension	HeLa*	—
	16	105	cell	HeLa (3, 10, 30, 32)* "	HeLa*	—
	17	109	cell	HeLa (3, 10, 30, 32)* "	HeLa*	—
	23	108	cell	HeLa (5,8,10,12,17, 21,27,28,30)* "	HeLa*	—
	22	98	cell	Primary NBH skin, kidney (18)* monolayer	HeLa*	—
	32	138	cell	Human fetal cell (6, 11)* monolayer	HeLa*	—
	23	108	cell	HeLa (45)* suspension	Electron microscopy	—

* 10% α-gamma-globulin calf sera was used for media component.
† Three blind passages.
‡ Final reading on 10th day.

Note: Cells in direct method and from the mixed culture were frozen and thawed 3 times, centrifuged at 1000 rpm for 5 minutes and supernate 0.3 and 0.1 ml was inoculated into HeLa tube.

natant media and/or disrupted cells were tested for the presence of virus as described under (a). Electron microscopy was done of the mixed cultures at the 14th generation. Similarly, mixed cultures were also done with newborn hamster kidney and human fetal cells.

By all methods described, no virus has been detected in the tumor cells (Table I).

(e) Inoculation of newborn hamsters with irradiated cells: To test the possibility of host modification of virus(13) by passage in hamsters and also the possibility that irradiation might unmask a latent infection, tumor cells were irradiated (2000 r) and 1×10^6 cells inoculated into newborn female hamsters. No tumors were induced.

Growth rate. Tumor cells of the 23rd passage were trypsinized and 2×10^3 and 4×10^4 cells were dispensed into petri dishes; HeLa cells were similarly treated for controls. Cells were incubated at 37°C under CO_2 in MEM + 10% calf serum and periodic cell counts were made. Difficulty was encountered in counting the tumor cells since they tended to form cell aggregates instead of single cell suspensions after trypsinization. The results (Fig. 4) show a rapid growth rate and approximately a 40-fold increase from the beginning of the culture, regardless of initial number of cells.

Response to (super) infection. Approximately 10^6 HT-1 cells were inoculated with A-12 and studied for cytological changes, intracellular and extracellular virus. HeLa cells and monolayers of primary newborn hamster kidney, skin and liver were similarly inoculated and studied as controls.

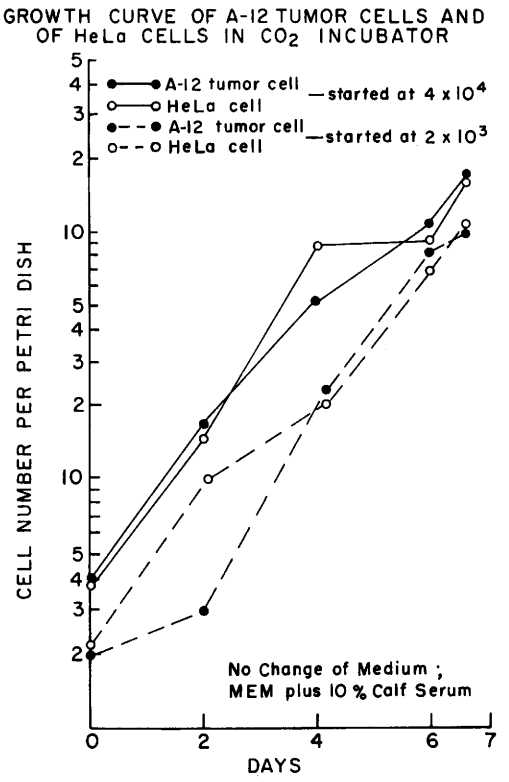

FIG. 4. Growth rate curves of 23rd passage HT-1 cells in tissue culture.

Characteristic adenovirus cytopathic effects and inclusion bodies were observed in HeLa cells after 51 hours while the tumor cells and the 3 normal hamster tissue cultures showed no typical adenovirus CPE during the observation period. No viral growth was demonstrable in either cells or supernatant fluid of cultures of hamster tumor or newborn hamster kidney, skin or liver. In HeLa cells intracellular virus growth was found after 28 hours and extracellular virus growth after 76 hours (Fig. 5).

Discussion. Extensive efforts by many laboratories to isolate viruses from human tumors have resulted in questionable or negative results. The results of specific attempts to isolate adenoviruses from human tumors might help to clarify the role of adenoviruses in naturally occurring tumors of humans. For these reasons, extensive efforts have been made to demonstrate the presence of the virus in hamster tumors specifically induced by A-12, in the hope that these methods, if successful, would be applicable to human tumors. The virus isolations by Gerber(14) and Sabin and Koch(15) from SV-40 induced tumors by either mixed culture with indicator cells, or by prolonged cultivation *in vitro,* respectively, suggested the use of these procedures for detection of virus in A-12 induced tumors. Our negative results suggest the absence of the virus or alternatively, a low order of sensitivity of the indicator cells employed, *i.e.,* HeLa. The latter alternative is under investigation since human fetal kidney cells appear to be more sensitive to A-12 than do HeLa cells(6).

It must be emphasized that these negative attempts at virus isolation cannot be regarded as conclusive. The appearance of A-12 specific complement fixing antibodies in hamsters bearing transplants of A-12 induced tumor, originally reported by Huebner *et al*(4,5), and confirmed in this laboratory(16) is strongly indicative that virus, or at least one viral component, is replicating in the tumor cells. This offers strong inducement for fur-

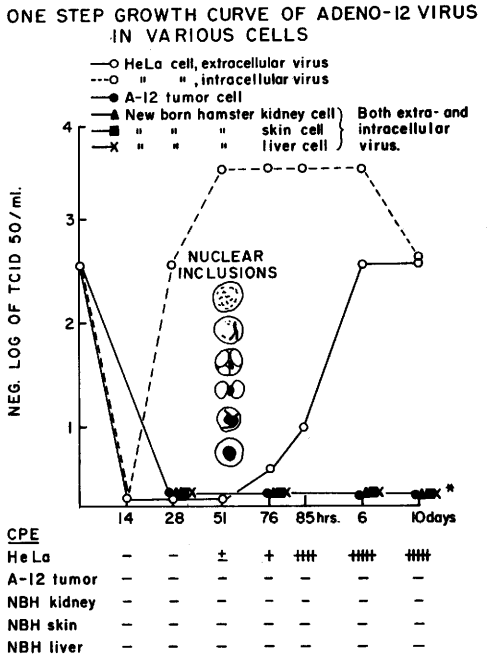

FIG. 5. Response of HT-1, HeLa, newborn hamster kidney, skin and liver cultures to (super) infection with A-12.

ther attempts at isolation of virus or viral DNA from A-12 induced tumors.

The morphology, rapid growth rate and transplantability of HT-1 are consistent with findings by others with tumor cells grown *in vitro*. The apparent resistance of HT-1 cells to overt infection with A-12 appears similar to the response of the normal cells of the Syrian hamster in culture.

The essential findings contained herein were presented at the 14th Annual Meeting of the Tissue Culture Association(17). Since the preparation of this manuscript, a report has appeared by Strohl *et al*(17) indicating results comparable to some of those described herein.

Summary. Characteristics of a human adenovirus type 12 induced hamster tumor serially propagated *in vitro* are described. These include small cell size, epithelioid appearance, rapid growth rate, resistance to superinfection with A-12, and transplantability to weanling hamsters. These cells grew either as monolayers or as balls of aggregated cells detached from the glass, depending on whether calf serum or horse serum was added to the Eagle's medium. Attempts to demonstrate virus activity by subculture of supernatant fluids and lysed cells into HeLa cells, mixed culture with human and hamster cells, electron microscopy, and inoculation of newborn hamsters with irradiated tumor cells were negative.

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Interchangeability of Adrenocortical Hormones in Initiating Mammary Secretion *in vitro*.* (29308)

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Adrenocortical hormones have been studied in a variety of experiments for their influence on mammary gland growth and function. Studies in mice, particularly with regard to induction of milk secretion, have involved the use of the glucocorticoid, cortisol(1,2). Cortisol has also been the corticoid employed for various mouse mammary gland studies *in*

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