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CHARACTERISTICS IN TISSUE CULTURE OF INFECTIOUS CANINE HEPATITIS VIRUS-INDUCED HAMSTER TUMOR CELLS*

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INTRODUCTION

In a preceding paper, we demonstrated the oncogenicity of the infectious canine hepatitis (ICH) virus in newborn hamsters (KINJO et al., 1968).

Although neither the primary tumors induced in hamsters by the ICH virus or the transplanted tumors contained demonstrable infectious virus, they did, however contain an ICH virus specific tumor antigen. Also the tumor-bearing animals developed complement fixation (CF) antibodies, reactive to both the homologous tumor antigen and the ICH virus tissue culture T antigen (neoantigen).

The data suggested that viral genome which codes for the production of virus specific tumor antigen must be incorporated somehow in the chromosome network of such virus free tumor cells.

Attempts were made by the authors to establish cell lines from the ICH virus-induced hamster tumors and from transplanted tumors. We then attempted to characterize the cells producing the tumor antigen in our in vitro system. This report describes the cultural behavior, oncogenicity and antigen-producing ability of serially subcultured cells derived from the primary ICH virus-induced hamster tumor.

MATERIALS AND METHODS

ICH virus-induced hamster tumor The primary intraperitoneal tumor (designated as HT-7) induced by strain Woc-4 was used. The experimental data of HT-7 were described previously (KINJO et al., 1968).

Cell cultures Primary cultures were prepared from the HT-7 tumor by the following methods. The excised tumor tissue was rinsed initially with Hanks' balanced salt solution and minced with scissors. At least 3 additional rinses were done during the processing. The minced washed tissue fragments were then dispersed at room temperature for 6 hr with a 0.25% trypsin solution. The trypsin-dispersed cells were centrifuged at 1,000 rpm for

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5 min and then resuspended, after the supernate had been discarded, in a sufficient volume of 2 kinds of growth media, to yield a final concentration of about 2×10^6 cells per ml. The prepared cell suspension was then dispensed 10 ml per bottle ($4 \times 10 \times 4$ cm) and 5 ml per small bottle ($3 \times 5 \times 3$ cm). These bottles contained 3 coverslips, and were incubated in a stationary position at 37°C . A cell monolayer was obtained within a week. Serial subcultivations were then made and used in all experiments. The serially cultured cells were referred to as HT-7 cells. A detailed explanation of the subcultivations will be described in the Results.

Media Hanks' balanced salt solution (BSS) containing 0.5% lactalbumin hydrolysate and 0.3% tryptose phosphate broth was used as the basal medium.

A medium supplemented with 20% calf serum was used for the initial growth medium and for the first 9 subcultivations, subsequently the serum content was reduced to 10%. For part of the primary cultures, Medium 199 (Difco), supplemented with 0.3% tryptose phosphate broth and 20% calf serum, was also used.

Medium containing 3~5% calf serum was used as the maintenance medium.

A medium with a reduced calcium chloride content, 0.1 mM, was used for testing calcium sensitivity of the cells.

All of the media contained 200 u/ml of penicillin, 200 $\mu\text{g/ml}$ of streptomycin, and 25 $\mu\text{g/ml}$ of fungizon.

Preparation of tumor antigen from HT-7 cells After a satisfactory heavy growth had been achieved, the HT-7 cells were dispersed with trypsin solution and suspended in a phosphate buffered saline (PBS) to the appropriate concentration, as will be described in the Results. The cell suspension was then sonicated in a Kubota's oscillator (KMS-100) at 10 Kc for 10 min and centrifuged at 9,000 rpm for 30 min. The supernate was used as the tumor antigen.

Other methods used The following methods were applied without description but were reported in the preceding paper (KINJO et al., 1968): methods for preparing the hamster tumor antigen, for testing of tumorous hamster serum, the procedures used for CF test, methods used for virus recovery from tumor cells and other several methods.

RESULTS

1 Characterization of HT-7 cells

The primary cultures prepared from hamster tumor (HT-7) in both kinds of growth media, Hanks' BSS and 199, formed a monolayer with about 7 days incubation (fig. 1). This monolayer consisted of at least 3 morphological distinct cell types, a fibroblastic, an epithelial and a rounded form.

The cultures were fed twice a week with the same medium. However, clumping of the cell sheets was gradually observed by the 2nd week (fig. 2) and by the 3rd week more than 80% cells were detached from the glass.

At the 3rd week Hanks' growth medium, with only 0.1 mM calcium chloride was substituted for Hanks' or 199 growth medium which contains 1.2 mM or more calcium chloride.

By the 4th to 5th weeks several small cell-colonies were observed, only, in the cultures which had been grown and maintained with the Medium 199, before the 3rd week. These colonies grew rapidly and reached a maximum size, of about 2 mm in diameter, within about 10 days (fig. 3).

Microscopically, the features of these colonies were quite similar to those of transformed colonies of hamster cells induced by oncogenic adenoviruses.

The center of the colony was formed by multiple cell layers consisting of small tightly packed epithelial cells. The margin of the colony consisted of monolayer cells which were considerably pleomorphic, showing polygonal, triangular and fibroblastic appearances (figs. 4 & 5).

These cultures were then dispersed with a trypsin solution and subcultured into new bottles using a low calcium medium supplemented with 20% calf serum as described before. The cell monolayers were easily obtained within 4 days and consisted predominantly of epithelial cells (fig. 6). These cells, after the 3rd passage, regularly formed a complete intact sheet on glass with the use of the low calcium medium.

The growth rate of the HT-7 cells, *in vitro*, has been such as to permit regular weekly subculturing, yielding 3 new bottles from each old one. The doubling time of the HT-7 cells, calculated from an experiment shown in figure 10, was approximately 24 hr.

Cellular metabolism and acid production progressed at a rate requiring fresh medium twice weekly.

No problems of cultivation were encountered to date (6 months). But it was somewhat difficult to prepare a confluent monolayer because the cells lost their contact inhibition properties, and showed a criss-cross cellular pattern and sometimes piled-up in a multilayered colony. The predominant cell population of the HT-7 cells, epithelial cells, was found replaced by fibroblastic cells after 5 passages (figs. 7~9).

2 Effect of calcium chloride on HT-7 cell-growth

As described above, the HT-7 cells were subcultivated and maintained without difficulty by the use of medium with a low calcium chloride content.

To study the effects of calcium chloride on the HT-7 cell-growth, trypsin dispersed cells of the 10th passage level were divided equally into 2 parts. One of which was cultured with Hanks' BSS growth medium at the normal level of calcium chloride (1.2 mM) and another at a low level (0.1 mM).

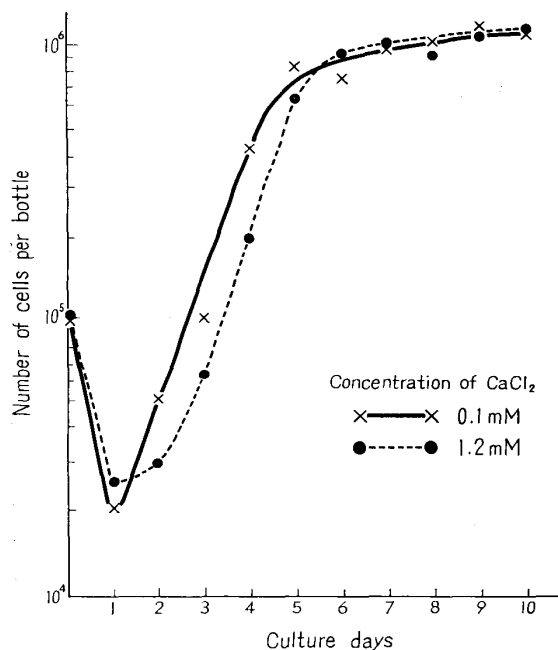
The growth curves of these cells were compared and illustrated in figure 10.

Although the cell growth at the early stages (2~5 days) was better in the cultures with a low calcium chloride content, no difference was observed at the later stages (7~10 days) where maximum cell growth was obtained.

After 10 to 16 passages, the cultures were grown and maintained with both media respectively. No significant difference of growth pattern was observed between the 2 media. Therefore, after the 17th passage level, only the medium with the normal level of calcium chloride (1.2 mM) was used.

The data presented here and the preceding item suggested that calcium sensitivity of the HT-7 cells was limited to only the primary or early passage levels of HT-7 cell cultures.

FIGURE 10 *Effect of calcium chloride on the growth of the HT-7 cells at the 10th passage level*



Hanks' medium with 0.1 mM and 1.2 mM calcium chloride, with 0.5% lactalbumin hydrolysate and 10% calf serum was used.

3 Attempts to recover ICH virus from HT-7 cells

The presence of ICH virus in the cultures HT-7 cells was tested by the inoculation of dog kidney cells (DKC) and Vero cells, which were susceptible to ICH virus infection, with the supernatant fluid, cell culture extracts and viable cells.

All cultures were examined, for at least 10 days, for the appearance of ICH virus CPE. All negative cultures then were frozen and thawed 2 to 3 times, and supernates were inoculated onto fresh DKC.

No virus was isolated by these methods from the HT-7 cells of several passage levels.

4 Presence of tumor antigen in HT-7 cells

Cells at the various passage levels were dispersed by a 0.25% trypsin solution and then centrifuged. The sedimented cells were then resuspended in PBS to make cell concentrations, as shown in table 1, ranging from 3.5×10^5 ~ 6.8×10^6 cells per ml.

After these cell-suspensions were sonicated and centrifuged as described in Materials and methods, the supernates were tested for the presence of ICH virus tumor antigen by the CF test using sera from hamsters carrying transplanted tumors. The sera used were diluted so as to contain 4 units of CF antibody titer.

The results obtained were summarized in table 1. The CF titer of tumor antigen was usually low except the case of the pooled sample of passages Nos. 4~6 whose titer was 1 : 16.

TABLE 1 *Antigenicity of the HT-7 cells at different passage levels as determined by the CF test against the serum from the tumor-bearing hamster*

NO. OF PASSAGE IN VITRO	CULTURED DAY AFTER PASSAGE	NO. OF CELLS PER MILLILITER *1	CF ANTIGEN TITER *2
1	48	2.0×10^6	4
2	8 } *3	1.3×10^6	4
3	5 }		
4	7	1.6×10^6	1
5	8	1.2×10^6	2
6	6	3.5×10^5	1
4	17 } *3	2.8×10^6	16
5	12 }		
6	13 }		
7	5	5.0×10^5	1
9	9	6.8×10^5	4
12	4	7.5×10^5	4
15	7	2.5×10^6	8
21	7	6.8×10^6	8

*1 These cell suspensions were sonicated and centrifuged, and the supernates were used as CF antigens.

*2 Reciprocals of the highest antigen dilutions showing positive CF reaction against 4 units of tumor antibody

*3 Cell materials were pooled.

It seemed that there was no close correlation between the cell concentration and the CF antigen titer under the test conditions. For example, 1.6×10^6 cells of the 4th passage showed an antigen titer of 1 : 1, while 6.8×10^5 cells of the 9th passage, less than half number of cells of the former, showed a 4 times higher antigen titer, 1 : 4. However, it could be said that the tumor antigen reactive, with ICH virus specific sera from tumor-bearing hamsters, was always present in the HT-7 cells regardless of the number of passages.

Another attempt was made to demonstrate the presence of a tumor antigen in the HT-7 cells by the fluorescent antibody (FA) test. Cells from the 2nd, 3rd and 9th passages were grown on coverslips, fixed in acetone, and then exposed first to serum from a transplanted tumor-bearing hamster and then to a fluorescence-conjugated rabbit anti-hamster globulin serum.

Intense fluorescent staining was found in nearly all cells of the 3 passage levels tested. It was concentrated in the out-line of the nuclear membrane. In a few small rounded type cells nuclear fluorescence was also observed (fig. 11).

Both the CF and FA tests indicated the persistence of a viral specific tumor antigen, through at least 9 passages, in vitro.

TABLE 2 *Oncogenicity of the HT-7 cells at different passage levels in hamsters*

EXP. NO.	NO. OF PASSAGE	CULTURED DAY AFTER PASSAGE	NO. OF CELL INOC. / VOLUME (ml)	ROUTE OF INOC.*1	AGE OF HAMSTER INOC. days	NO. WITH TUMOR / NO. INOC.	DAY OF TUMOR APPEARANCE *2
29	1	7	$3.0 \times 10^3/0.1$	IP	2	7/7	23 (3), 28 (2), 41 (2)
30	"	"	"	"	5	3/3	23 (2), 41 (3), 44 (1)
"	"	"	"	SC	5	3/3	
35	"	41	"	"	14	6/6	20 (4), 36 (2)
37	4	6	$1.6 \times 10^5/0.1$	"	34	2/2	13 (2)
40	9	7	$2.0 \times 10^4/0.2$	"	16	3/3	16 (3)
"	"	"	$2.0 \times 10^3/0.2$	"	"	3/3	22 (1), 26 (2)
"	"	"	$2.0 \times 10^2/0.2$	"	"	3/3	35 (2), 40 (1)
42	12	4	$7.5 \times 10^4/0.3$	"	20	3/3	20 (3)
48	15	7	$2.5 \times 10^3/0.2$	"	24	3/3	13 (2), 17 (1)

*1 IP: Intraperitoneally SC: Subcutaneously

*2 23 (3) means 3 hamsters developed tumors at day 23.

5 Oncogenicity of HT-7 cells

To confirm the continued oncogenic potency of the HT-7 cells maintained in vitro, hamsters were injected subcutaneously or intraperitoneally with trypsinized cells from several passage levels.

The results obtained were shown in table 2. All hamsters receiving the cell injection developed tumors at the site of inoculation (fig. 12).

Experiment 40, in the table, was done to determine the minimum cell numbers needed to produce tumors in suckling hamsters of the same litter. However, the highest dilution of cells used (2×10^2 cells/0.2 ml) also produced tumors though the latent periods were prolonged.

The experimental data suggested that the HT-7 cells persistently retained their oncogenic properties after many generations in vitro.

Five tumor-bearing hamsters were selected from the above experimental animals and tested for tumor antigen and antibody development. All tumors from these animals contained tumor antigens detectable by the CF test. Also, the sera from these animals had a CF antibody reacting with the hamster tumor antigen (tab. 3).

TABLE 3 *Level of complement-fixing tumor antibody in sera of hamsters carrying the HT-7 cell-induced tumors and of tumor antigen in the tumors themselves*

HAMSTER NO.	NO. OF PASSAGE USED	NO. OF CELL INOC.	ROUTE OF INOC.	DAY OBSERVED	TUMOR WEIGHT	TITER OF CF	
						Antibody	Antigen
HT-29-1	1	3.0×10^3	IP	61	70 ^g	64	16
HT-35-1	"	"	SC	56	33	128	128
" -2	"	"	"	"	7	16	32
" -3	"	"	"	"	2	16	32
HT-40-1	9	2.0×10^4	"	32	16	32	32

CF antigen or antibody titers were expressed as the reciprocals of the highest antigen or antibody dilutions showing positive reactions against 4 units of tumor antibody or antigen.

In addition, a serum sample from one of the hamsters was selected and tested for its specificity to the ICH virus by the box-CF test using ICH virus tissue culture T antigen (neoantigen). The serum reacted similarly with both the tumor and ICH virus tissue culture T antigens (tab. 4). The results showed that the antibody reactive with the tumor antigen might be ICH virus specific.

Histopathological findings of the HT-7 cell-induced tumor (Exp. 40) showed to be similar to those induced by ICH virus or transplantation of the HT-7 tumor (KINJO et al., 1968).

The primary tumor was shown to be mostly a peritheliomatous growth and rarely a fibrosarcomatous growth, but the HT-7 cell-induced tumor was dominated by a fibrosarcoma structure. The tumor cells were basically spindle-shaped.

TABLE 4 *Box complement fixation tests of serum from hamsters carrying the HT-7 cell-induced tumor versus the corresponding hamster tumor antigen and ICH virus tissue culture T antigen (neoantigen)*

ANTIGEN	ANTIGEN DILUTION	HT-35-1 HAMSTER SERUM DILUTION					
		4	8	16	32	64	AC * ²
HT-35-1 hamster tumor antigen	4	4* ¹	4	4	4	4	0
	8	4	4	4	4	4	0
	16	4	4	4	4	4	0
	32	4	4	4	3	1	0
	64	4	4	1	±	0	0
Tissue culture T antigen * ³	4	4	4	4	4	±	0
	8	4	4	4	2	0	0
	16	4	4	3	±	0	0
	32	3	3	2	0	0	0
	SC * ²	2	1	0	0	0	0

*¹ Degree of fixation read on a scale ranging from 4 (no hemolysis) to 0 (complete hemolysis).

*² AC: Antigen control SC: Serum control

*³ Methods of preparation were previously described (KINJO et al., 1968).

DISCUSSION

This paper reported successful, *in vitro*, subcultivation of ICH virus-induced hamster tumor and described their characteristics during serial cultivations.

The cultivation of tumor cells derived from type 12 adenovirus-induced hamster tumors has already been described (STROHL et al., 1963; KITAMURA et al., 1964; FREEMAN et al., 1965; ROUSE et al., 1966; KATZ & SMITH, 1967).

As was mentioned by ROUSE et al. (1966), all these cells have certain properties in common: (1) they induce progressively growing tumors following transplantation to hamsters; (2) they induce the formation of antibodies which react in CF tests with extracts of adenovirus-induced tumors or crude adenovirus antigens, and in fluorescent antibody (FA) tests with the tumor cells; (3) they contain the corresponding antigen(s) demonstrable by CF and FA tests; (4) they are devoid of demonstrable infectious type 12 virus, as are the hamster tumors themselves.

In the present experiments, we also confirmed the above properties for the HT-7 cells.

During our experiments for the establishment of a cell line from ICH virus-

induced tumor, we observed that the primary cells grew as confluent monolayers within a week. But by the 2nd and 3rd weeks clumping of the cell sheet and detachment of the cells from the glass occurred.

A similar phenomenon in cell lines derived from the type 12 adenovirus-induced hamster tumors has been reported by FREEMAN et al. (1965), and by KATZ & SMITH (1967). They showed that the clumping was caused by the calcium concentrations used in the tissue culture media, and it could be reversed by using a low calcium medium (0.1 mM).

In vitro transformation by oncogenic adenoviruses a similar phenomenon has also been reported (FREEMAN et al., 1965; FREEMAN et al., 1966; KUSANO & YAMANE, 1967).

In general, it has been proposed that, in vitro, adenovirus-induced tumor cells are calcium sensitive.

Referring to the above findings, we tried to use the low calcium medium after the 3rd week, when about 80% of the cells have been detached from the glass. One to 2 weeks later, we found several small cell-colonies on the glass. These colonies developed only in the cultures which had been grown and maintained with Medium 199, before the use of the low calcium medium. The explanation of why such cell growth could not occur in Hanks' BSS is obscure. These cell-colonies grew rapidly, and in vitro looked like transformed colonies. The cells in colony were predominantly small polygonal cells and they lacked the property of contact inhibition and thus formed multiple cell layers. These cells had oncogenic potency toward hamsters and were easily subcultured.

Thereafter, serial passages were made using the low calcium medium without difficulty. At the 10th to 16th passage levels, calcium sensitivity of the cells was examined. The results found showed that the cells can grow in medium with the usual calcium content. After the 17th passage, only medium with the usual level of calcium chloride (1.2 mM) was used. Thus, we successfully maintained the tumor cells in vitro for more than 20 serial passages.

These cells retained their oncogenic potency up to at least the 15th passage which was the latest passage level tested. A relatively small number of these cells, such as 200 cells, was enough to produce tumors in 100% of the hamsters inoculated.

Furthermore, almost all the cultured cells contained a tumor antigen detectable by the fluorescent antibody test. From both findings, it is likely that the majority, if not all, of the HT-7 cells carry the genetic determinants for tumor production in hamsters and synthesis of tumor antigen. These genetic determinants are constantly passed onto their daughter cells.

Attempts to isolate infectious ICH virus, in DKC, from these cultured cells

yielded negative results. In regard to this point, we are planning to use the technique of cell fusion or heterokaryon formation as described previously (KINJO et al., 1968). The many intensive attempts to isolated infectious virus from established cell lines derived from adenovirus induced hamster tumors have always proved negative (STROHL et al., 1963; FREEMAN et al., 1965; ROUSE et al., 1966; KATZ & SMITH, 1967). KATZ & SMITH (1967) also failed to demonstrate the viral DNA in tumor cells. These results suggest that virus replication is not necessary for tumor induction by these cells in hamsters.

FUJINAGA & GREEN (1966) have demonstrated a specific viral messenger RNA in the polyribosomes of the adenovirus tumor and transformed cells. Their results indicated the presence of functional DNA of viral origin within the tumor cells.

An interesting future study problem is the relationship between the presence of viral genome or a fraction of it in cultured tumor cells and oncogenicity of these cells.

On the other hand, the sera from hamsters carrying tumors, which were induced by inoculation of virus-free cultured tumor cells, reacted not only with homologous tumor antigen but with ICH virus tissue culture T antigen (neo-antigen) in the CF test. The latter suggests that the serum antibody of a tumor-bearing hamster is ICH virus specific.

Histopathological findings from the HT-7 cell-induced hamster tumors were shown to be similar to those induced by the ICH virus (KINJO et al., 1968), but a fibrosarcoma structure, was dominant and the tumor cells were basically spindle-shaped.

Considering that the predominant cell type of the HT-7 cells, epithelial, have been gradually replaced by a fibroblastic form, the above histological changes give us an interesting future problem to analyze the histogenesis of the ICH virus-induced tumor.

In the present experiments we confirmed that the HT-7 cells derived from ICH virus-induced hamster tumor possess the same properties as the human adenovirus type 12-induced hamster tumor cells.

KATZ & SMITH (1967) used 8 types of adenoviruses to inoculate adenovirus type 12-induced tumor cells and then they classified these viruses into 4 groups by their patterns of cellular response. They found that this classification was closely correlated with the oncogenicity.

A study of the susceptibility as well as the resistance of the HT-7 cells to superinfection with ICH virus and human adenoviruses may be an effective approach to analyze the relationship between the oncogenicity of the ICH virus and human adenoviruses.

SUMMARY

Characteristics of ICH virus-induced hamster tumor cells serially propagated, in vitro, (referred HT-7 cells) were described.

The predominant cell seen in the population of the HT-7 cells at the early passage levels, was the epithelial. However, these cells were replaced, following several passages, by fibroblastic cells. They propagated well in vitro and showed a lack of contact inhibition.

Subcultivation of the HT-7 cells was accomplished by growing the cells in the medium with a low calcium content, 0.1 mM. All attempts to isolate infectious virus from the HT-7 cells by using supernatant fluids, lysed cells, and viable cells to inoculate dog kidney cells and Vero cells were negative. The HT-7 cells continuously possessed a virus-specific tumor antigen and their oncogenic potency toward hamsters.

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EXPLANATION OF PLATES

PLATE I

- Fig. 1 Primary culture prepared from ICH virus-induced hamster tumor (HT-7 cells)
Seven days after cultivation with medium 199
Giemsa staining $\times 150$
- Fig. 2 Same as figure 1
Three weeks after cultivation
Clumps and ball-like aggregates of the cells were noticed.
Giemsa staining $\times 150$
- Fig. 3 Primary culture of the HT-7 cells
After the 4th week of cultivation a low calcium (0.1 mM) medium was substituted for medium 199. Several small cell-colonies were clearly observed on the glass surface of the culture bottles.
Unstained preparation $\times 1$
- Fig. 4 A quarter of a cell colony of the fig. 3
The center of the colony (left lower part of the figure) consisted of small tightly packed cells forming multiple cell layers. Photograph was taken 45 days after cultivation.
Unstained preparation $\times 75$
- Fig. 5 One of cell colonies of the figure 3
A multilayer of cells in the center of the colony and a monolayer of cells in the margin were clearly observed. The cells were pleomorphism, ranging from a polygonal to a fibroblastic morphology.
Giemsa staining $\times 150$

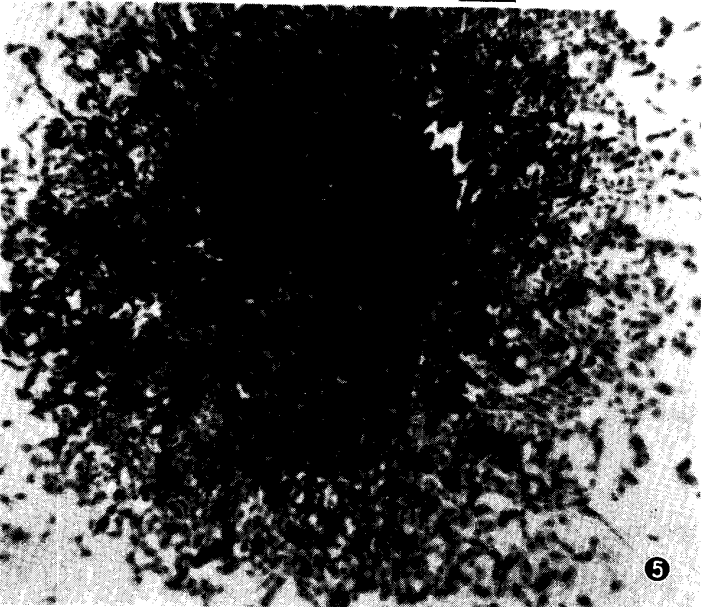
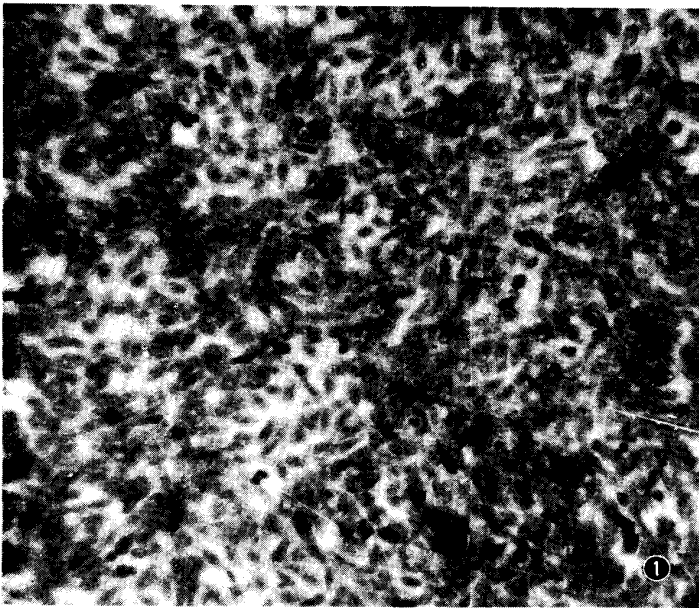


PLATE II

- Fig. 6 Secondary cultures of the HT-7 cells prepared from cell-colonies shown in figure 3
Cell monolayers predominantly consisted of epithelial cells. Four days after subcultivation
Giemsa staining $\times 150$
- Fig. 7 HT-7 cells of the 4th passage
Four days after cultivation
The arrangement of the cells was somewhat irregular and a criss-cross pattern of cells was observed.
Giemsa staining $\times 150$
- Fig. 8 HT-7 cells of the 6th passage
Four days after cultivation
The criss-crossing of the cells was clear and partial piling-up of the cells could be observed.
Giemsa staining $\times 150$
- Fig. 9 Another preparation of the HT-7 cells at the 6th passage level
The fibroblastic cell morphology was predominant in this figure. Criss-crossing and piling-up of the cells were also observed.
Giemsa staining $\times 150$
- Fig. 11 Fluorescent antibody-stained preparation of the HT-7 cells at the 9th passage level
Fluorescent staining is intense at the margins of nuclei. In a few small rounded type cells, nuclear fluorescence was also observed.
 $\times 600$
- Fig. 12 Hamster (HT-29-1) with tumor induced by intraperitoneal inoculation of the primary HT-7 cells
Photograph was taken 60 days after inoculation.

