CHARACTERISTICS OF AN ESTABLISHED CELL LINE (KU-2) DERIVED FROM HUMAN RENAL CELL CARCINOMA

I. CLONING OF CELLS AND MORPHOLOGICAL STUDY OF CLONES,
II. CELL KINETICS OF KU-2 CELLS,
III. DETECTION OF TYPE C VIRUS IN THE CULTURE

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A KU-2 cell line derived from human renal cell carcinoma was established by an indirect culture system using the nude mouse in November, 1976. These cells have been examined from different points of view including light and electron microscopic observation, and chromosomal analysis. Histopathological characteristics of the KU-2 cell line, even after being transplanted back to nude mouse, remain similar. However, the characterization of this established cell line has not been fully elucidated. In the present experiments, attempts have been made to study the cloning of KU-2 cells and morphological feature of clones, cell growth and kinetics, and detection of the type C virus in the culture. These results suggested that the KU-2 cell line was not homogenous but composed of a heterogenous population of cells based on morphological difference of 6 clones and discrepancy between population doubling time and generation time when calculated from the growth curve and synchronous culture of the KU-2 cells, which may be explained by the cytotoxic effect of excess thymidine. Also the type C virus was negative in the medium.

Key words: Cell line, Human renal cell carcinoma, Cloning, Cell kinetics, Type C virus, Synchronous culture, R-D polymerase activity

INTRODUCTION

A number of cell lines which were derived from human carcinomas are available. Attempts have been made to apply these cell lines in diverse biological inquiries. The difficulty invariably encountered in such an attempt lies in the identification of the cells in culture. Cytomorphological and histochemical studies, and heterotransplantation of cells to verify the reproducibility of tissue architecture may be valid measures for the identification of cell types. However, the population of cells maintained in vitro may have lost some of the characteristics of the original tissue during their growth in an artificial enviroment. Notwithstanding, strenuous endeavors have been devoted to establishing cancer cell lines largely because of the tremendous advantages that could be produced by using tissue culture techniques in the field of oncology.

Previously, we succeeded in establishing the cell line named KU-2 from human renal cell carcinoma which is a very common malignant tumor in urology (1, 2). Much remains to be elucidated before one can fully characterize KU-2 cells. We examined 1) the cloning of the cell and morphology of the clones, 2) cell kinetics of KU-2 cells, and 3) detection of type C

virus in the culture.

Cloning refers to the conduct or procedure of growing a single cell selected from a mixed population of cells that have some characters of interest. A clone is the progeny of the cell which is grown by the process not of sexual reproduction but of vegetative propagation without exchange of genes and which have hence an identical genetic constitution unless they undergo mutation. A clone of cells can be dealt with as a homogeneous population. Cloning of cells, if it becomes a routine procedure, will be of great help in making genetic markers available and can also facilitate characterization of somatic cells.

To understand the dynamic process of cell growth it is essential to make an analysis of cell kinetics to delineate the sequential events that take place within the cell cycle. The manner in which a population of cells grow can be expressed by the logarithmic growth curve. Here, it is understood that the culture is completely random with regard to the cell age in the cell cycle. A nonrandom population of cells can be obtained by the technique of synchronous culture.

The type C virus has been detected in human cancer cell lines. This type of virus which is known as "oncorna" standing for oncogenic RNA, has been shown to cause malignant transformation of host cells. The genome of the virus has been reported to be expressed more readily and with a higher efficiency in transformed cells as the cells are carried in long-term culture as an established cell line.

MATERIALS AND METHODS

1) Cloning of KU-2 cells

A 60 mm plastic Petri dish was inoculated with 20~50 cells disaggregated by treatment with 0.01% EDTA-0.125% trypsin and incubated after addition of 5 ml of culture medium (Eagle's MEM supplemented with 10% fetal calf serum) with or without antibiotics. About 2 weeks later the medium was removed. A clump of colony forming cells was scraped off with a cloning glass rod and transferred to another 35 mm plastic petri dish containing 2 ml medium, which was then incubated. After 2 weeks, the culture was transferred and the same procedure of cloning was repeated at least for 2 months.

II) Cell kinetics of KU-2 cells

　a) Growth curve

Two 35 mm plastic petri dishes were inoculated with approximately 5×10^4 cells per dish and incubated with 2 ml of culture medium added. Cell counts were taken daily from the next day until day 11 by means of a Coulter counter. The cell number, which was the mean for duplicate dishes, was plotted on a semilogarithmic graph, from which the population doubling time was then calculated.

　b) Cell cycle analysis

For analyzing the cell cycle of the cells an attempt was made to synchronize their growth in the S phase by treating them twice with excess thymidine. Approximately 3×10^4 cells were plated in a Petri dish with a 15 mm coverslip, which was then incubated with 1 ml of culture medium added. Three days later when the cells were in the logarithmic growth phase the medium was removed and replaced with 1 ml of medium containing 2.5 mM thymidine. After 25 hr of incubation in the presence of thymidine, the cells were washed 3 times and incubated for another 10 hr. Then with the medium renewed the cells were incubated for either 14 or 24 hr and washed 3 times. After renewal of the medium, the cells were sampled at intervals of 2 hr. Cells sampled were pulse-labeled with ^{3}H-thymidine (^{3}H-T) and the amount of the label incorporated was measured, from which the duration of the DNA synthesis phase (S) was estimated. The mitotic phase (M) was estimated by direct observation of mitosis. Briefly, for pulse-labeling with ^{3}M-thymidine, the culture medium was removed from the dish and replaced with 0.5 ml of a ^{3}H-thymidine solution (1 μCi/ml) prewarmed to 37°C. After incubation at 37°C for 20 min, the coverslip was taken out, fixed in a 1 : 3 mixture of acetic acid and methanol, washed 5 times with 0.85% saline and 2 times with methanol, allowed to dry and subjected to radioactivity counting in a liquid scintillation counter. For

the observation of mitosis, the coverslip was removed from the dish, fixed in methanol, washed in water and stained with Giemsa stain. The mitotic index was calculated from the total cell count and the number of cells in the M phase.

III) Detection of type C virus in the culture by R-D polymerase activity assay

a) Preparation of specimen

A 100 mm plastic petri dish was inoculated with KU-2 cells and incubated with 10 ml of culture medium added. Three days later, the medium was discarded and replaced with 8 ml of fresh medium. After 24 hr of incubation, the medium was used as the specimen for the assay. The specimen was centrifuged at 2,000 rpm for 10 min. The supernatant was fractionated by centrifugation in a gradient of 20% sucrose at 24,000 rpm for 2 hr at 4°C. The sediment was suspended in 100 μl of NET buffer (0.1 M NaCl, 0.001 M EDTA, 0.01 M Tris-HCL, pH 7.6). A suspension of viruses produced by AC cells - a cell line derived from rat glioma which is known to produce the C type virus - provided by Takeda Chemical Industry's LTD, served as the control.

b) Assay for R-D polymerase activity

A modification of the original method of Aaronson (3) using an exogenous template was employed for the assay. The reaction mixture was composed of 5 mM dithiothreitol, 40 mM Tris-HCL (pH 7.6), 60 mM KCL, 5 mM magnesium acetate, 1 mM manganese acetate, 0.1% Triton x-100, 0.4/ ml polyadenylate oligodeoxythymidylate,0.1 mM ³H-thymidine 5'-triphosphate, (³H-TTP), and 0.1 mg/ml bovine serum albumin. To the mixture was added 10~30 1 of specimen as described above to make a total volume of 0.1 ml. The mixture was incubated at 37°C for 60 min, cooled on ice and after the addition of 10% trichloroacetic acid (TCA) containing 10 mM sodium pyrophosphate and 10 mM monobasic sodium phosphate to stop the reaction, it was cooled again for 20 min. The sediment thus formed was collected on glassfiber paper (Whatman, GF/C), washed 5 times with 5 ml of cold 10% TCA and allowed to dry before being subjected to radioactivity counting in a liquid scintillation counter.

RESULTS

1) Morphology of clones

Six pure cell lines were obtained from KU-2 hybrid cells after 2 months of cloning clones. No. 1~3 were treated with antibiotics and No. 4~6 were not treated. Fig. 1 shows some morphological features of these clones as seen under the phase contrast microscope. The clones are of epithelial origin with granules in the cytoplasm and with a high N/C ratio. When in sparse suspension the clones were morphologically similar to each other. In confluent monolayers they were somewhat distinguishable from other clones. There were round cells intermingled with spindle-shaped cells in clones No. 1 and 2, predominance of spindle-shaped cells in clone No. 3 and of a comma-like or triangular cells in clones No. 4 and 5, and/or more homogeneous round cells in clone No. 6.

2) Growth rate and cell cycle

Fig. 2 shows the growth curve of KU-2 cells. The saturation density and the population doubling time when calculated from the growth curve, was 2.9×10^5 cells/ cm^2 and approximately 24~26 hr, respectively.

Synchronous culture of KU-2 cells yielded 7~8 hr length of S phase and 1~ 2 hr of M phase (see Fig. 3). The G_2 phase was estimated to be 2 hr from Fig. 3. The G_1 phase was difficult to estimate because of an inadequate degree of synchrony attained, but theorectically appeared to be

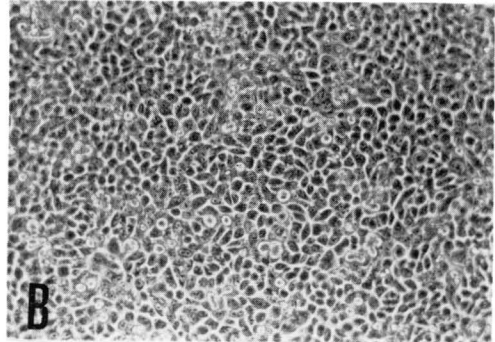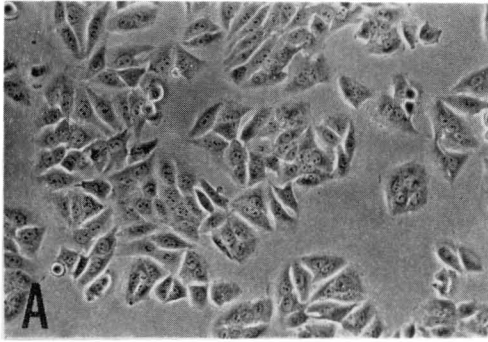Table 1. R-D polymerase activity in culture media of AC and KU-2 cells

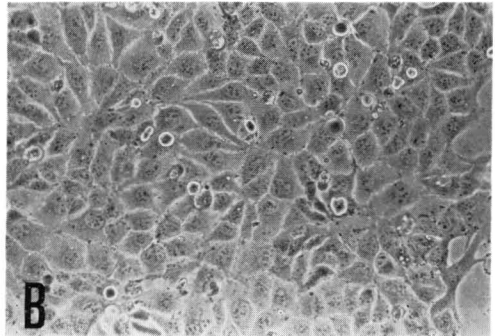| cell lines	vol of suspension (1)	³H-TTP incorporation (p moles)	
		Ext 1	Ext 2
AC[1]	10	598	241
KU-2-p93[2]	10	1	N. D.[3]
KU-2-p104[2]	30	N. D.	1

1) type C virus producing cell line from rat glioma
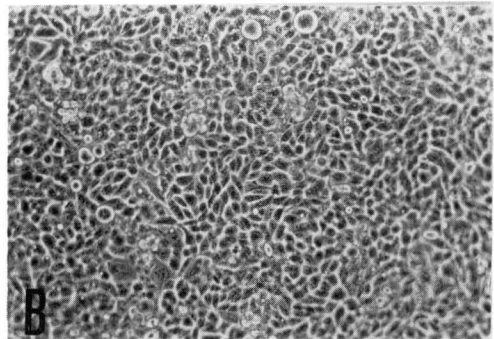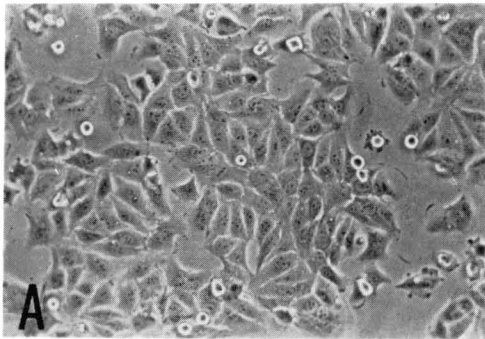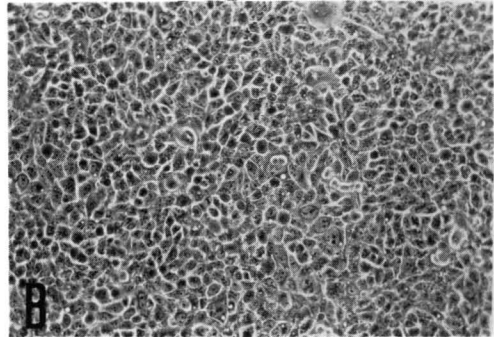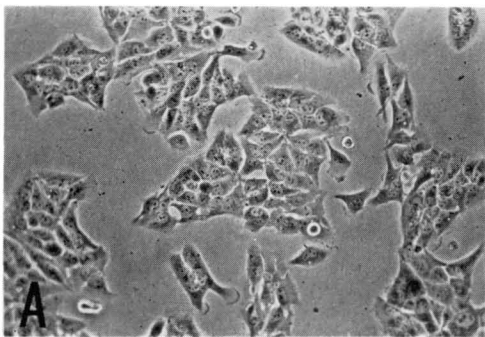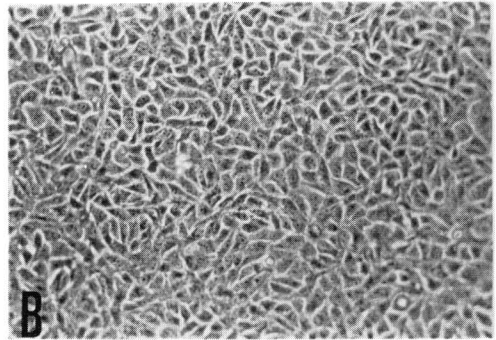2) different passage number
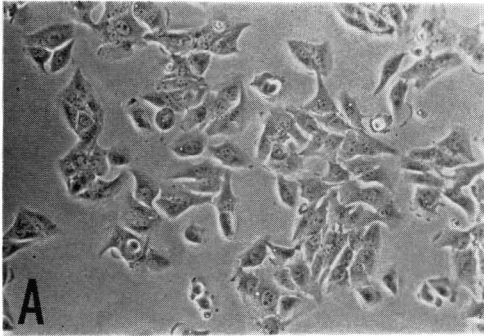3) not done

Clone No. 1

Clone No. 2

Clone No. 3

Clone No. 4

Clone No. 5

Clone No. 6
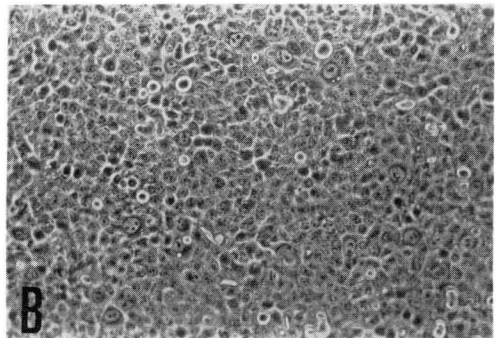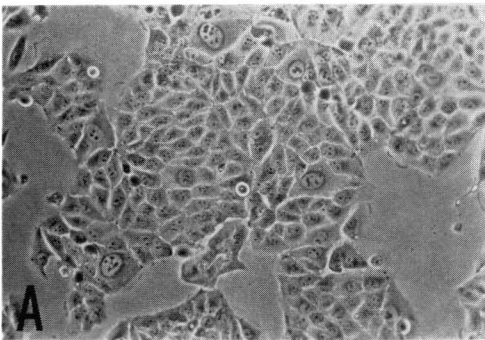

Fig. 1.   Serial phase contrast microscopic view of each clone in sparse suspension
or in confluent monolayers (a,b). Reduced from ×100
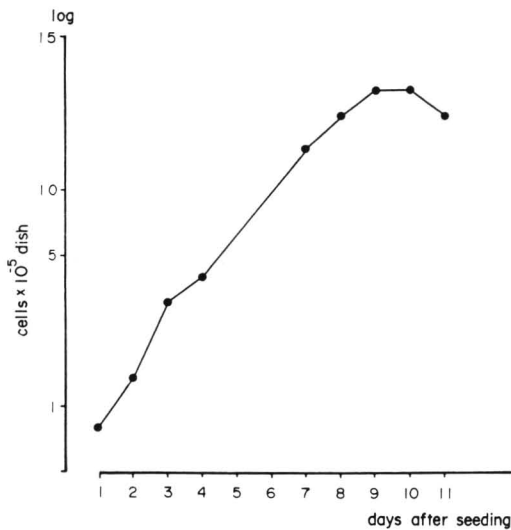
Fig. 2.   Growth curve of KU-2 cells

Fig. 3.   Synchronization of KU-2 cells
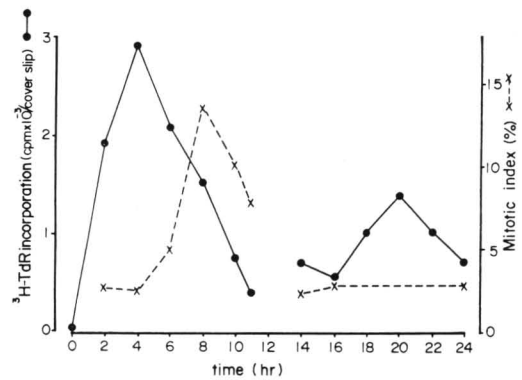following thymidine blockade

in the range of 6∼7 hr. The generation
time was thus 16∼17 hr.
3) R-D polymerase activity in culture
media
No R-D polymerase activity was detected

in KU-2 cells as shown in Table 1. There
was no incorporation of ^{3}H-thymidine in
the medium after different passages, where-
as the radiolabel was incorporated at a
high concentration in AC cells.

DISCUSSION

The history of cloning of cell lines can be traced back to the capillary cloning technique reported by Sanford (4) in 1948, who succeeded in isolating single cells by sucking them into a capillary pipette and growing then in culture. This marked a major breakthrough in the development of single cell cloning.

Puck (5), on the other hand, devised a method for plating single cells to enable colonies to be obtained. The method consists of inoculating a vessel with a very dilute suspension of cells and leaving it undisturbed. The clones thus gained are designated clonial clones to distinguish them from single cell clones. The recent advance in tissue culture techniques has facilitated cloning of cells, making it essential for the genetic, biochemical, pathologic and immunological studies of cells.

Our interest in this study was limited to the cloning of KU-2 cells and the morphology of clones. Most recently, Nagakura and colleagues (6~8) have reported the heterogenicity in tumorigeneity and in susceptibility to NK cell-mediated cytotoxicity and/or bioeffects by $1 \alpha, 25(OH)_2D_3$ and in amount of $1 \alpha, 25(OH)_2D_3$ receptors using ten clone lines of KU-2 cells.

The synchronous culture technique as a means of analyzing the cell cycle consists of synchronizing the growth of a population of cells in culture to place them in given phase of cycle. This method enables the replicative and metabolic processes within single cells to be extended over the population as a whole. When a population of cells in logarithmic growth phase are treated with a specific inhibitor of DNA synthesis, then cessation of DNA synthesis results in cells which are in the S phase, while the other cells are allowed to pass through the cell cycle until eventually they coincide at the starting point of the S phase. Cells must usually be manipulated twice to achieve synchrony of their growth at the begining of S phase (9). After the first attempt at synchronization, the cells are incubated in fresh inhibitor-free medium for a somewhat longer span of time than the duration of the S phase. This allows the majority of cells to go past S into other phases. Subsequent incubation of the cells in the presence of an inhibitor of DNA synthesis results in most of the cells coinciding at the beginning of the S phase.

There are some problems with the attempt at synchronizing cultures in the S phase, according to Yamada (10). First, the timing of treatment with an inhibitor of DNA synthesis to achieve satisfactory synchrony of growth will vary with the type of cell line used. Secondly, the cytotoxic effect of such inhibitor agents interferes with the process of synchronization. Thirdly, the use of excess thymidine may results in an exceedingly large thymidylic acid pool, so large as to make the specific radioactivity of ^3H-thymidine incorporated as the measure of the rate of DNA synthesis too low for the quantitation of the latter parameter. In fact, in our experiment some dividing cells emerged as they were synchronized into the S phase by treatment with excess thymidine. This indicates the possibility of some cells so synchronized entering M earlier than the S phase. The fact that the generation time of the clones was shorter than the population doubling time may be explained by the cytotoxic effect of excess thymidine. The exceedingly large thymidylic acid pool made available during the first attempt at synchronization appears to be accountable for the unsatisfactorily low degree of synchrony achieved.

The availability of molecular biological techniques has led to great achievements in the viral oncological study of human cancers. Viruses which have been implicated in human carcinogenesis include DNA viruses such as EB and type II hepes viruses and RNA viruses such as tupe C and B viruses. Of these, the type C virus (oncorna), which in 1911 was shown by Rous (11) to cause chicken sarcomas, was subsequently detected in a number of other animal species.

The discovery of the new enzyme, RNA-dependent DNA polymerase or reverse

transcriptase described by Temin & Mizutani (12) in 1970, led to recognize the possibility that the genome of the RNA viruses is transcribed into DNA and becomes incorporated as such into the genome of host cells. Consequently, the detection of human cancer by using this enzyme includes.: 1) the direct detection of the enzyme activity in human cancer cells or tissues, 2) detection of specific DNA which is likely to be synthesized by the virus, and 3) in vitro synthesis of virus-specific DNA with viral RNA as the template and detection of virus-specific RNA in human cancer cells, on the assumption that a virus known to be oncogenic in animals can also cause cancer in man (13〜15).

Our assay for R-D polymerase activity by a modification of Aaronson's method proved negative, excluding the possibility of type C virus or its genome being present in KU-2 cells.

The detection of type C virus may help in identifying the mechanism of viral oncogenesis or transformation of cells.

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和文抄録

ヒト腎細胞癌由来の樹立細胞株 **KU-2** の性状について
（1）クローン分離とクローン株の形態学的観察
（2）生長度測定と細胞周期
（3）C型ウイルスの検出

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KU-2 細胞株はヒト腎細胞癌のヌードマウス移植を経由した間接培養法にて1976年11月に樹立された．この細胞株の同定は光顕，電頭学的観察，染色体分析，更にはヌードマウスへのもどし移植により原腫瘍由来であることが既に立証されている．しかし，この細胞株の性状についてはいまだ十分に解明されていない．そこで今回著者は，いくつかの細胞種の混合する細胞集団である KU-2 細胞株よりクローン分離を試み，6つのクローンを得た．形態学上，それぞれに異質性が認められた．また，KU-2 細胞株の細胞周期に関する情報を得るために S 期同調培養法を行なった．S 期7〜8時間，M 期1〜2時間，G 期2時間，G 期6〜7時間とそれぞれ推定された．倍加時間（24時間〜26時間）より短い世代時間（16〜17時間）であったことは，過剰チミジンの細胞毒性によるものと思われた．一方，長期培養により，C型ウイルスゲノムの発見が容易になると想像されるので，KU-2 細胞株について，逆転写酵素である R-D polymerase 活性を測定することにより C 型ウイルスの検出を試みたが，結果は陰性で，ヒト癌細胞では一般に発見されることはないとの一般的見解と符合した．