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THE ORIGIN AND CHARACTERISTICS OF A PIG KIDNEY CELL STRAIN, LLC-PK$_1$

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SUMMARY

A stable epithelial-like pig kidney cell strain has been established. This strain has been carried through more than 300 serial passages, has remained free of microbial and viral contaminants, and has retained a near diploid number of chromosomes. Attempts to produce tumors with these cells in immunosuppressed laboratory animals have been uniformly negative. The cells have grown rapidly in monolayer cultures with a split ratio of 1 to 15 at weekly intervals, but have failed to proliferate in suspension cultures. A subline adapted to growth on serum-free medium 199 has been carried through 145 passages on this medium. Several unusual morphologic features have been observed in these cultures including three-dimensional "domelike" structures. These cells have been found susceptible to some viruses and have been especially useful for viruses of domestic animals. LLC-PK$_1$ cells have produced significant levels of plasminogen activator.

Key words: pig kidney cells; dome-like structures; chemically defined media; plasminogen activator; virus susceptibility.

INTRODUCTION

Trypsin-dispersed cells prepared from the kidneys of domestic pigs grow well in tissue culture and can readily be established as permanent cell strains. At least six such strains have been described, or mentioned, in the literature (1-6). Most of these reports deal with virus susceptibility. Several pig kidney strains have been developed in our laboratory, and one of these, LLC-PK$_1$, is described in detail in this report. This strain has some unique characteristics which may be of interest to investigators in several areas of research.

MATERIALS AND METHODS

Cell culture preparation and origin of LLC-PK$_1$. The kidneys from a 17-lb juvenile male Hampshire pig were aseptically removed after exsanguination of the donor animal. Both kidneys were minced, pooled together, and trypsinized by the procedure described by Younger (7). A 1-to-400 suspension was made of the final cell pack in medium 199 containing 10% horse serum (HS) and 100 units penicillin and 100 μg streptomycin per ml. This was used as the inoculum for 16-oz bottle cultures. By 4 days of incubation at 37°C, the cultures were essentially confluent, and were refed with medium 199 containing 5% HS and antibiotics. The first subculture was made on the 6th day by stripping the cells from the glass with 0.1% Armour's crystalline trypsin prepared in Earle's balanced salt solution. Antibiotics were discontinued at this time. Passages were made at approximately weekly intervals, and the split ratio was gradually increased until it became routine at 1 to 15. A total of 88 consecutive passages were made before the cell strain was frozen and stored in liquid nitrogen. It has been in and out of our frozen cell bank on numerous occasions over the 17 years since its origin in 1958. The highest passage to which these cells have been advanced is 318.

Media, sera and cell strains. Medium 199 (M199) was prepared in our laboratories, essentially according to the formula given by Morton in her review article (8). Hanks' balanced salt solution was used, and the following slight modifications were made: Niacin 0/0.75, inositol 0.05/0.50, Tween 80, 50/20.0 and ATP 2.0/10.0 (Lilly/Morton, mg/l). Other media were purchased from commercial sources. HS was obtained from our own horses; fetal bovine serum (FBS) was purchased. The Y-15 cells were obtained from Dr. Benjamin Sweet, and PK$_{15}$ was

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TABLE 1

TUMORIGENICITY TESTS WITH LLC-PK$_1$ CELLS

Cell Passage	Animals (Nos.)	Cell Inoculum	Inoc. Site	Immuno-suppression
176	Rats[a] (14)	3×10^5	Brain	X-ray and Cortisone
80	Hamsters[b] (10)	10^6	Pouches	Cortisone
146	Hamsters[b] (9)	10^6	Pouches	Cortisone
176	Hamsters[b] (9)	3×10^5	Pouches	Cortisone
199	Hamsters[b] (6)	9.75×10^5	Pouches	Antilymphocyte Serum
176	Hamsters[c] (24)	3×10^5	S. C.	None

[a]Twenty-one days old.
[b]Weanlings.
[c]Neonates.

purchased from the ATCC. LLC-PK$_2$ and LLC-PK$_3$ were developed by the same procedure described for LLC-PK$_1$. LLC-PK$_2$ was initiated from the kidney cortex tissue from one donor, while LLC-PK$_3$ was from the medulla of the kidney of another donor. Both donors were 14-day-old male Hampshire pigs. A modified or altered subline of LLC-PK$_1$, LLC-PK$_{1A}$, was obtained from Mr. Paul Kelley.

Quantitative growth studies. Replicate cultures were planted in T15 flasks as described by Evans and co-workers (9), and nuclear counts were performed by the method of Sanford and co-workers (10). Procedures for growth of cells in suspension cultures were published previously (11).

Karyology. Chromosome preparations were made either by the method of Tjio and Puck (12) or by that of Moorhead and Nowell (13). Direct counts were made under oil immersion at a magnification of ×1125 from cells which were still intact. More precise counts were obtained from photomicrographs.

Tumorgenicity studies. These tests were performed as indicated in Table 1. The tests on 21-day-old white rats were done by the method of Sigel and co-workers (14). In the hamster cheek-pouch studies the animals were treated with 5.0 mg cortisone at the time of cell inoculation, and 5 additional doses were given during the next 24 days. The cells contained in 0.1 ml of medium were inoculated into both cheek pouches. Hamsters immunosuppressed with rabbit antihamster lymphocyte serum (Microbiological Associates) were given 0.25 ml on 2 consecutive days prior to cell inoculation, on the day of inoculation, and at 3- to 4-day intervals during 24-days post cell inoculation. Neonatal hamsters were inoculated subcutaneously in the nape of the neck.

Virology. An influenza A strain (1134) and a B strain (1760), isolated in human cell cultures, were obtained from Dr. William G. Mogabgab. These strains were passed 8 and 12 times in primary rhesus monkey kidney cells (pRMK) prior to passage in LLC-PK$_1$ cells. All other influenza viruses were originally egg-passaged strains, carried in our laboratories, which had been adapted to growth in pRMK cells. Swine parvovirus was obtained from Dr. D. L. Croghan. Hemagglutination (HA) assays for parvovirus were done in tubes with 0.5 ml of virus dilution, and 0.5 ml of 0.4% guinea-pig red blood cells. M199 was used as the diluent in these tests. The tubes were incubated for 2 to 4 hr at room temperature before the tests were read out. $TCID_{50}$ (Tissue culture infective dose) determinations and HA assays were done 7 days after virus inoculation.

Plasminogen activator. For production of plasminogen activator (PA) cultures were grown to confluency with serum-containing medium, and then refed with M199 only. Samples of the medium were taken periodically for PA assay. The assays were done by the fibrin clot lysis method of Astrup (15) and expressed as CTA units per ml (Committee on Thrombolytic Agents).

Liquid nitrogen preservation and tests for contamination. Cultures in the advanced logarithmic growth phase were used for freeze-preservation. The monolayer was washed with M199, and then stripped from the flask by treatment with 0.2% crystalline trypsin solution. Following two rinses with M199, 1 to 3×10^6 cells were put into glass-sealed ampoules in 1.0 ml M199 containing 3% FBS and 10% filtered glycerol. The ampoules were held in the vapor phase of liquid nitrogen for 30 min, and then submerged for storage. The subline adapted to serum-free M199 was handled in like manner, except that the serum was omitted. For recovery, ampoules were thawed quickly in a 37°C water bath, and the cells were transferred to 10 ml M199 with 3% FBS and 2% filtered

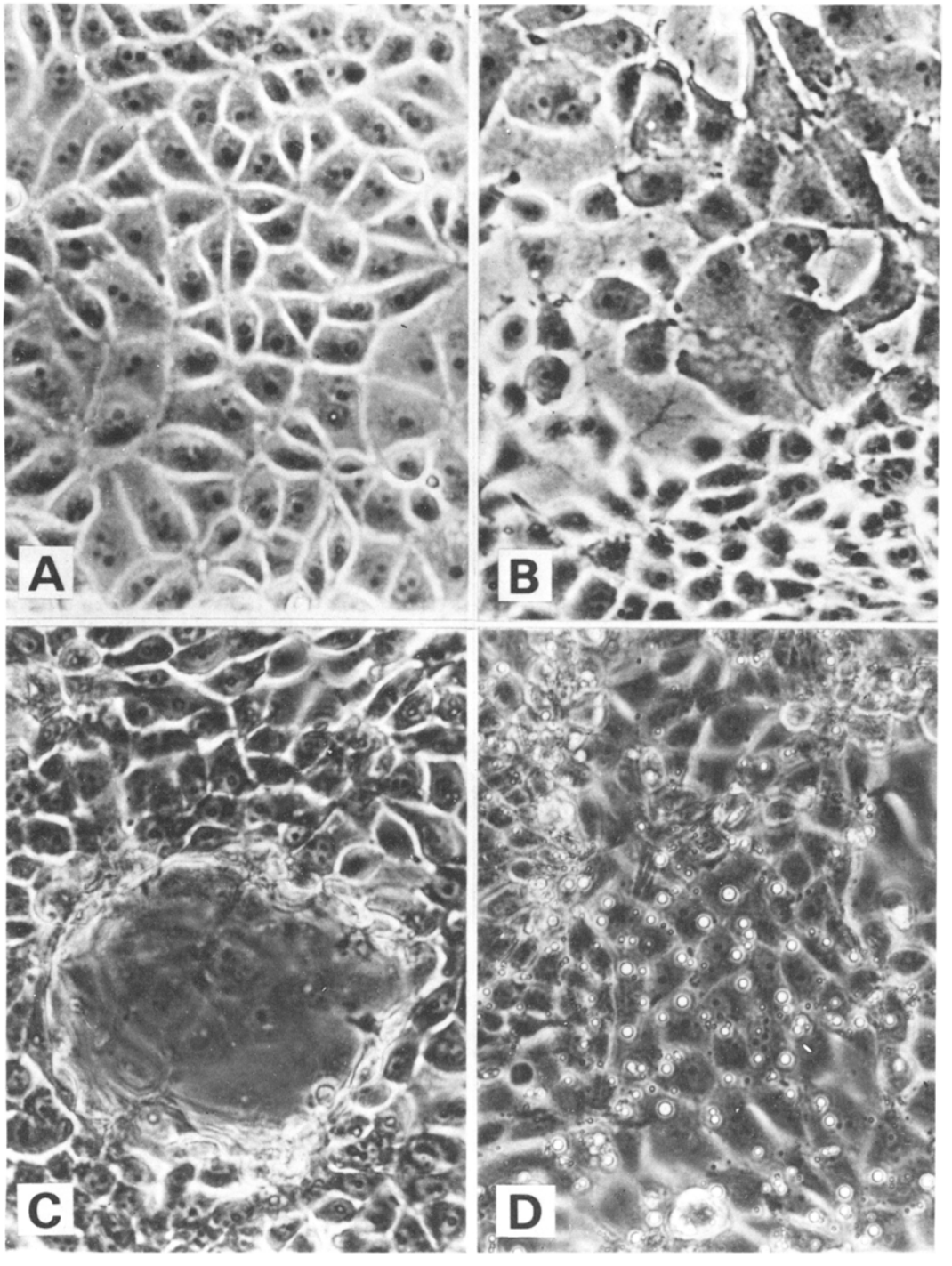

FIG. 2. Photomicrographs illustrating the morphology and variations observed in the growth pattern of LLC-PK_1 cells. *A*, Normal or predominant growth pattern. *B*, Large irregular shaped cells seen in isolated areas where cell sheet appears to be pulling apart. *C*, Three-dimensional "dome-shaped" structures (focus on cells in monolayer). *D*, Circular bodies attached to cell surface which appear in cultures on serum-free, or horse serum fortified media. Magnification on the negatives was ×94 before uniform enlargement of each photomicrograph.

glycerol. After one wash in M199 with 3% FBS, the cells were resuspended in growth medium and planted in culture.

Tests for mycoplasma were done on 0.5 ml of spent medium, or on cell suspensions at the time of freezing. This sample was inoculated into 7.0 ml of Eaton agent broth medium and incubated for 4 or 5 days at 37°C. Two subsequent blind subcultures were made in the broth medium, and to Eaton agar plates. Plates were observed for 7 days. In addition, 0.1 ml of the cell medium was planted on an agar plate and incubated anaerobically for 7 days. Test for other microbial agents included inoculation of sodium thioglycollate and Sabouraud's broth, with incubation at both 37°C and room temperature for 2 weeks.

RESULTS

Growth studies. LLC-PK$_1$ is a relatively fast-growing strain. Over-all generation time during the growth period was about 30 hr, but during the logarithmic growth phase the generation time was 7.5 to 9.0 hr. A representative growth curve is seen in Fig. 1. An inoculum of 10^4 cells per cm^2 of growth area provided confluent cultures of near maximum population density in 7 days when medium was renewed at 48-hr intervals. The maximum population density in F75 flasks, for example, was 3 to 4 $\times$ 10^7 cells. Cultures could be initiated with much smaller inocula (as low as 50 to 100 cells per cm^2), but longer growth periods were required to reach confluency.

Numerous attempts were made to grow LLC-PK$_1$ cells in agitated fluid suspension cultures under a variety of conditions and medium formulations. Cell survival was noted for as long as 20 to 30 days, but no significant growth occurred at any

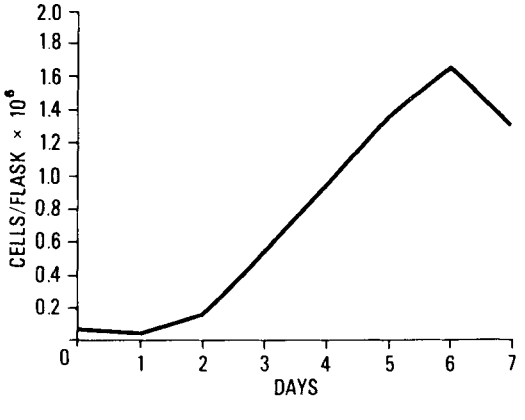

FIG. 1. Plot of the growth of LLC-PK$_1$ cells in T-15 flasks on medium 199 with 5% fetal bovine serum.

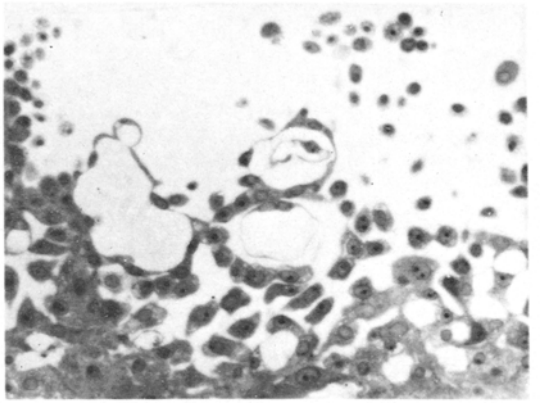

FIG. 3. One-micron-thick section of a culture of epoxy embedded LLC-PK$_1$ cells cut perpendicular to the plane of the cell sheet. Magnification was $\times$430 before enlargement. The scattered individual cells in the upper portion are loose cells caught in the embedding medium.

time. However, the altered subline, LLC-PK$_{1A}$, grew well in suspension culture attaining population densities as high as 10^7 cells per ml.

Morphology. These cells are epithelial-like, with rather large round-to-slightly-oval-shaped nuclei. Most nuclei contain one to three nucleoli. Multinucleated cells are rare, but occasional binucleated cells may be observed. These features are evident in Fig. 3. The usual pattern of cell growth is seen in Figure 2*A* along with variations which occur as depicted in 2*B*-*D*. The large irregular-shaped cells in 2*B* appear in some cultures in areas where the cells seem to be pulling away from each other. The ring-like structures seen in Figs. 2*C*, and 3 are of interest as similar structures have been described (16,17) in other types of cultures. These rings actually are three-dimensional and the term "domes" used by McGrath and Blair (17) is more descriptive. When viewed at $\times$100 magnification, the entire "dome" cannot be brought into focus in a single plane of focus. In Fig. 2*C* the focus is on the cells in the monolayer. The center, or top of the "dome," can be observed by raising the objective lens 10 to 15 microns above the plane of focus of the monolayer. The diameter of the "domes" may vary from 100 to 1000 microns, and their number from a few per culture to hundreds. Under time-lapse cinematography, the "domes" are extremely active. On several occasions small numbers of cells were subcultured from isolated areas in cultures showing each of the three morphologic features demonstrated in Fig. 2*A*-*C*. This was done by placing a small steel cylinder (5mm diameter) over the area, and collecting the cells by trypsin treatment of the area

TABLE 2

DISTRIBUTION OF CHROMOSOME NUMBERS IN LLC-PK$_1$ CELLS AT VARIOUS PASSAGE LEVELS

Passage Level	Number of cells with chromosome counts of						
	<36	36	37	38	39	40	>40
88	4	8	21	33	17	11	4
90	0	1	8	31	2	42	10
146	5	8	11	42	13	13	1
148	2	5	9	22	5	3	0
156	2	3	18	58	7	1	10
172	1	3	19	51	9	12	3
227	0	6	13	26	4	1	4
305	0	0	4	30	11	14	17

inside of the cylinder. In every instance these colonies, on further cultivation, produced all three patterns of growth regardless of the type from which they originated.

An unusual morphological property of this strain is seen in Fig. 2*D*. When cultures are grown on HS-containing medium, or on serum-free M199, many small circular bodies or "globules" appear attached to the cell surface. This does not occur when FBS is included in the medium. Time-lapse studies suggested that these "globules" might be pieces of the cell membrane pinched off during periods of high activity. If so, then they reattached firmly to the cell surface.

Karyology. The normal diploid domestic pig cell contains 38 chromosomes, which according to McFee (18) has 24 metacentrics, submetacentrics or subtelocentrics, and 12 acrocentrics or telocentrics in the autosomal constituency. The sex chromosomes, in the case of LLC-PK$_1$, are an X and Y, to complete the complement of 38 chromosomes. The results of chromosome counts made on various passage levels of LLC-PK$_1$ cells are seen in Table 2. Most of the counts were in the 36 to 40 range. From passage 88 through 305 in Table 2, the percentage of cells containing 36 to 40 chromosomes remained fairly uniform at about 90%. For passages 148 through 305 listed in Table 2, 50 to 66% of the counts within the group were 38, while the number of cells with 38 chromosomes was somewhat lower in the 88th to 148th passages.

How much these variations in counts of two chromosomes (plus or minus), from the normal of 38, represented the true chromosome numbers of these cells, and how much was due to technical error was not certain. In some instances the presence or absence of one or two chromosomes was confirmed from photomicrographs of mitotic cells, and from idiograms. Gains or losses occurred in all four groups of chromosomes (13), but most frequently involved the group of six pairs of teleocentric chromosomes. The X and Y chromosomes were identified in most preparations and showed no abnormality. When extra chromosomes were observed, they were similar in size and shape to the other, or normal, chromosomes of the cell. No marker chromosome was recognized.

Adaption to chemically defined medium. These cells were adapted to growth on serum-free, chemically defined M199. The 172nd passage of the parent strain, grown on M199 with 3% HS, was used to initiate this series of passages on M199 only. Large inocula of about 10^6 cells per flask were used to initiate these cultures. In the early passages the interval between subcultures was 2 to 3 weeks, and splits were made at a 1 to 2 ratio. As adaption to serum-free medium improved, subcultures were made at 7- to 10-day intervals, and the split ratio was increased to 1 to 3. Subpassages were facilitated by washing the trypsinized cells twice with M199 before planting. Cultures were refed three times weekly. Mitotic cells were readily observed in these cultures. A total of 145 passages have been made to date on serum-free M199.

Following adaption to M199, attempts were made to grow the cells on other chemically defined media. Cells from the 42nd passage were subcultured to Waymouth's media MAB 87/3 (19) and MD 705/1 (20). The cultures were carried through five serial passages without any impairment of growth. Waymouth's medium MB 752/1 (21), however, failed to support growth. Eagle's MEM with Earle's salt solution (22) and Morrison and Jenkin's HI-WO5 (23) also failed to support growth.

Limited nutritional studies were undertaken with LLC-PK$_1$ cells adapted to M199. It was

found that growth was inhibited if Tween 80 was omitted from the medium, but that growth was unaffected by omission of the nucleotides. Amino-acid analysis of 72-hr spent medium from confluent cultures indicated that tryptophan, methionine, serine, alanine and hydroxyproline were not utilized, and that the greatest depletions occurred in the following amino acids: asparagine, arginine, glutamic acid, glutamine, proline, cystine-cysteine, and isoleucine. The remaining nine amino acids were utilized at intermediate levels. The pattern of amino-acid utilization varied slightly in younger cultures undergoing mitosis. Glucose dropped from 100 to 25 mg% in 72 hr.

Tumorigenicity studies. These studies in immunosuppressed rats and hamsters were done as outlined in Table 1. All failed to provide evidence of the tumorigenicity, or heterotransplantability, of LLC-PK$_1$ cells. A few hamsters developed small nodules a few days after inoculation, but these quickly disappeared. Many of the animals were observed for over a year.

Virus sensitivity. The susceptibility of LLC-PK$_1$ cells to the viruses studied are indicated in Table 3. The studies with coxsackievirus B5, poliovirus type I and swine influenza were previously reported (24). Yields with influenza A (1134) and B (1760) were 10^3 $TCID_{50}$ per 0.5 ml as determined by cytopathic end points. Strains PR301, Lee B and swine influenza produced 10^4 to 10^5 $TCID_{50}$ per 0.5 ml. Best growth of parvovirus occurred when the virus was inoculated at the time of cell planting, or within 1 or 2 days after planting. Confluent cultures failed to support growth of this virus. Yields from LLC-PK$_1$ cells were $10^{2.5}$ to $10^{3.0}$ per 0.5 ml, while the Y-15 pig kidney control cells produced 4.5 to 5 logs of virus per 0.5 ml. A rather nondescript cytopathic effect was observed which consisted mainly of cell sloughing. Hemagglutinin titers of culture fluids ranged from 1 to 16 to 1 to 256, and HA activity was detected in culture fluids at one log dilution beyond the cytopathic end points.

TABLE 3

VIRUS SUSCEPTIBILITY OF LLC-PK$_1$ CELLS

Viruses	Growth
Coxsackie B5	+
Poliovirus I	—
Swine Influenza	+
Influenza A (1134)	+
Influenza PR301	+
Influenza Lee B	+
Influenza Jap 305	—
Influenza PR8A	—
Influenza GL1739	—
Swine Parvovirus	+
Swine TGE[a]	+
NDV[a]	+
BVD[a]	+
Pseudorabies[a]	+

[a]These data very kindly furnished by Dr. Charles Gale.

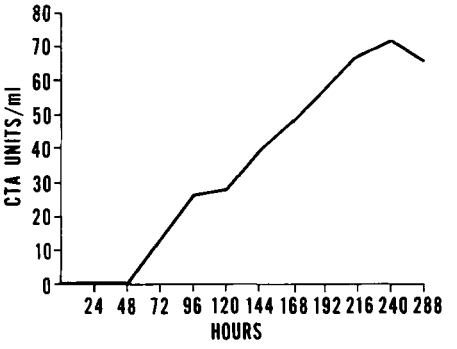

FIG. 4. Typical plot of plasminogen activator yield from cultures of LLC-PK$_1$ cells.

Plasminogen activator studies. In a survey of numerous cell cultures, both primary and continuous cell strains, for presence of PA, strain LLC-PK$_1$ was found to be the highest producer of this enzyme. Most cultures produced no measurable PA. One of seven monkey kidney cell strains, LLC-MK$_2$ (25), produced PA in the order of 2 to 5 CTA units per ml, and traces were found in Maitland cultures of primary monkey kidney tissue. Some plantings of primary human embryo kidney cells produced PA. Yields from strain LLC-PK$_1$ in these initial tests, however, were in the order of 50 or more CTA units per ml. The concentration of PA in the supernatant culture medium increased with time as seen in Fig. 4. Several sublines of LLC-PK$_1$ have been developed, one of which, LLC-PK$_1$ (LP$_{100}$), produced even higher levels of PA than did the parent strain.

PA production by LLC-PK$_1$, as indicated, was a rather unique characteristic of this strain of pig kidney cells, and yields have remained high through more than 300 passages. On numerous occasions primary monolayer cultures of pig kidney cells were tested for PA production but these were uniformly negative. Two other pig kidney strains developed in our laboratories, LLC-PK$_2$ and LLC-PK$_3$, also failed to produce PA at any time through 100 serial passages. Another high

passage pig kidney strain, Y-15 (2), did not produce PA, but the PK_{15} strain did show some activity.

Yields of PA were enhanced significantly by treatment of LLC-PK_1 cells (and other producing strains) with colchicine, the vinca alkaloids, various derivatives of both, and by some other antimitotic agents (26). Details of these studies will be described in additional reports from our laboratory.

Preservation by freezing and tests for contamination. LLC-PK_1 cells were recovered successfully on numerous occasions after liquid nitrogen freezing and storage. A 1.0 ml frozen sample containing 2 to 3 $\times$ 10^6 cells, after thawing and planting in an F75 plastic flask, provided a confluent culture in 5 to 7 days. The longest period of storage with recovery of viable cells, to date, was 7.5 years. The subline adapted to M199 without serum required longer recovery periods after low-temperature storage, but was consistently recovered. The longest period of storage tested thus far was 12 months.

This strain, like others maintained in our laboratory, was tested periodically for mycoplasma and other microbial contaminants. Mycoplasma were not detected. In view of the report describing "C" type virus particles in the PK_{15} cell strain (27), LLC-PK_1 cells were studied extensively by electron microscopy for the presence of these or other virus-like particles. None was detected.

Discussion

The principal purpose of this report was to describe the origin, development and special characteristic of a stable pig kidney cell strain, LLC-PK_1. In addition to the more thorough description of the cells and of their handling in the laboratory, several areas of research, in which these cells have been used to advantage, were mentioned. Complete details of the latter were not presented as such was beyond the scope of this communication. Most of these studies will be reported in papers to follow.

Morphologically, strain LLC-PK_1 cells were epithelial-like and displayed several unusual features described in the text. The three growth patterns seen in Fig. 2*A-C* did not appear to be genetically controlled, because clones derived from small numbers of cells picked out of areas of growth of each morphological type produced all three types on further cultivation. These, however, were not single cell clones. The type of growth seen in Fig. 2*B* was observed less frequently than the others, and may represent an early stage in the development of the ring structure. The ringlike structures were most interesting and have been studied in some detail. Although these structures are very prominent in this cell strain, we have observed them in another one of our kidney strains, LLC-MK_4, derived from squirrel monkey kidneys, and in a rat prostrate carcinoma strain, PT_1, obtained from Dr. Morris Pollard.

There was no evidence that LLC-PK_1 cells had undergone transformation or neoplastic change. The microscopic morphology of the cells was more like that of normal cells than that of neoplastic cells, and the chromosome number remained close to the diploid number. Chromosome stability in other strains of pig kidney cells was reported by Ruddle (28). The cells failed to proliferate in suspension cultures, and at least one author (29) relates such failure to the "unaltered" condition of cells. However, the "altered" subline, LLC-PK_{1A}, with a modal chromosome number of 65, grew well in suspension culture and to high cell densities. None of the attempts to produce tumors in immunosuppressed animals were successful.

LLC-PK_1 cells were adapted to growth on serum-free M199. To our knowledge, no other report has been made of sustained growth of cells through many serial passages on this medium, although it has been a widely used medium when supplemented with serum. This subline has been used for nutritional studies and for assay of various potential growth factors.

The virus studies were rather limited and did not reveal any outstanding utility of these cells for study of viruses infecting man. Of the influenza viruses the swine strain produced the highest titers in these cells. However, Dr. Charles Gale of our Veterinary Sciences Department found LLC-PK_1 cells to be highly susceptible to four viruses of importance to veterinary medicine (Table 3).

The production of significant levels of plasminogen activator was of particular interest to us. This enzyme converts plasminogen to plasmin, the enzyme which lyses fibrin clots. In this respect PA is identical to urokinase obtained from urine, which has been used clinically in the treatment of thromboembolic disease. Many factors were identified which influenced the production of PA, and these were used successfully to further enhance yields. These kidney cells, however, failed to produce renin, and no evidence of postagian-

dins was detected in spent medium.

Strain LLC-PK$_1$ was placed in the American Type Culture Collection where it is catalogued as strain ATCC No. CL 101. It is available from this source.

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