BIOLOGICAL CHARACTERISTICS AND VIRAL SUSCEPTIBILITY OF A CANINE KIDNEY ADENOCARCINOMA CELL LINE (CKT)

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ABSTRACT

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By

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A cell line derived from a kidney adenocarcinoma of a 4-year-old dog was serially cultivated in a closed system for 85 subcultivations during a period of 2 years. The cells were epithelial-like in appearance, had an abnormal karyotype, grew in soft agar, and exhibited fluid transport activity, a function of secretory epithelium. Virus-like particles similar to C-type oncornaviruses were observed budding through and associated with the cell membrane. Growth of several viruses was supported in the cells with resulting cytopathic changes.

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A THESIS

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Dedicated to my wife

Cheryl

and my father and mother

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The *in vitro* cultivation of cells with stable characteristics is a valuable tool in biological and medical research. Biochemistry, microbiology, cancer research, and other fields of study have benefited from the use of cell cultures.

Relatively few canine cell lines have been established and described (2,35,48,68,74). A cell line derived from normal dog kidney (MDCK) was established by Madin and Darby in 1958 (74). This line has been widely used in animal virology (35). Rosanoff (68) established a testicular tumor cell line, and Kasza (48) established cell lines from a melanoma and a thyroid adenocarcinoma. The latter 2 lines had chromosome alterations (62) similar to those described by Adams *et al.* (2) in cells derived from a canine venereal tumor. Miles *et al.* (58) reported similar findings in cells derived from canine lymphosarcomas. Type-C virus particles have been associated with the canine venereal tumor cell line (72).

The purpose of the present study was to describe the cultural characteristics and viral susceptibility of a cell line derived from a kidney adenocarcinoma of a 4-year-old dog.

REVIEW OF THE LITERATURE

Development of Cell Culture

Claude Bernard (9), in 1878, emphasized the importance of the internal environment upon the regulatory activity of living tissue. To attempt to study cells and how they affect or are affected by the environment, he felt it was essential that cells be isolated in an artificial system thereby avoiding the influence of the host. The first steps made in this direction were made by von Recklinghausen (64) in 1866 and Wilhelm Roux (70) in 1885, who were able to keep cells and tissues alive *in vitro*. However, actual growth of cells was not obtained. Beebe and Ewing (7), in 1906, using canine serum as a medium, cultivated a canine lymphosarcoma, and Harrison (42), in 1907, using nerve fibers, actually demonstrated cellular growth by embedding tissue fragments in clotted lymph.

Considerable enthusiasm was generated by Harrison's work, but as a result of frequent bacterial and fungal contamination, interest was soon lost. Carrel, a surgeon and Nobel Laureate, in 1913 applied the technique of asepsis introduced to surgery. Carrel's discovery in 1913 (18) that embryonic tissue extracts contain growth promoting substances made it possible for the first time to propagate animal cells indefinitely. He cultivated chicken fibroblasts using chicken plasma as a nutrient, and was able to keep them alive for over 34 years. In

spite of the success of Carrel, tissue culture work was primarily confined to bacteriologists and surgeons who were familiar with the rigid demands of asepsis.

Early cell culture work employed nutritive media derived almost exclusively from the organism and consisted of blood plasma, serum, other body fluids and exudates, and extracts of tissues and organs. The complexity and variability of these naturally occurring materials made it difficult to use them in experiments designed to determine nutritional requirements. It was not until the advent of antibiotics in 1941 and the development of more refined mediums (5,41,60,82,85) that tissue cultures became more widely used. In recent years chemically defined mediums of considerable complexity have been developed (14,24,63,80).

Earle (26), in 1943, described one of the first cell strains to be established in continuous culture. The cells were derived from normal areolar and adipose tissue of a mouse (L Strain). During the 1950's numerous cell lines from human and animal origins were established. Gey *et al.* (37) established a stable cell line (HeLa) derived from a human cervical carcinoma in 1952. Other cell lines derived from human tumors were described by Fjelde (31), Berman and Stulberg (8), and Eagle *et al.* (23). Hull *et al.* (45) established a cell line from a Walker rat carcinoma in 1954.

Advances in tissue culture have greatly accelerated progress in virology. In 1913, Steinhardt *et al.* (76) demonstrated that rabbit cornea in plasma maintained the activity of vaccinia virus for more than a month. Twenty-two years later, Rivers and Ward (66) carried out

vaccinia vaccinations using a strain of virus which had been passaged for several years *in vitro* without intercurrent passages in animals. Sabin and co-workers (71), in 1936, were able to grow poliomyelitis virus in primary human embryonic neural tissue but not in other tissue. In 1949 another major development occurred when Enders *et al.* (28) grew the Lansing strain of poliomyelitis virus in human embryonic tissue of non-neural origin. This finding, which marked the beginning of modern virology, came at an opportune period in history. It was by this time that the hazards of cell culture contamination had been reduced due to the application of antibiotics.

Characteristics of Normal Cell Cultures

The development of improved methods for culturing animal cells *in vitro* has greatly increased the usefulness of such cells. Microbiology, pharmacology, pathology, immunology, genetics and biochemistry are a few fields which have been advanced by cultivation of cells *in vitro*.

Tissues and organs are dissociated into cell suspensions by the action of proteolytic enzymes such as trypsin (67). When inoculated into a flask, the cells that grow out are called primary cells. The monolayer thus formed will usually consist of fibroblast-like cells and epithelial-like cells (19). Repeated subcultivation of a primary cell culture usually causes selection for a certain cell type or types which become predominant. These cells appear unaltered in morphologic and growth properties. This diploid cell strain, as it is now called, must be transferred at a relatively high cell density to initiate growth in subcultures, and the cells have a limited life span, i.e., they cannot be subcultured indefinitely. For example, WI-38, a human diploid cell

strain useful in isolating human viruses, has a finite lifespan of approximately 50 population doublings (74).

The behavior of cells in organisms and cell cultures suggests that normal cells are inherently able to regulate their growth. This ability is manifested *in vitro* as postconfluence inhibition of cell division (25). The various kinds of intercellular interactions which could trigger postconfluence inhibition of cell division fall into 3 categories which are not mutually exclusive.

1. Metabolic cell-cell contact. This hypothesis states that there is a direct contact of metabolically active cells. An inhibitory substance is then transferred from one cell to another via membrane junctions, without traversing the external medium (79).

2. Mechanical cell-cell contact. This hypothesis proposes that inhibition may be caused by changes in cell shape, changes in area exposed to the medium or mechanical restraints upon cell movement (56).

3. Local diffusion gradient. Inhibition, as stated in this hypothesis, is caused by the establishment of a local diffusion gradient. Local concentrations of an inhibitor or local depletion of a substance necessary for cell division (or both) would trigger the mechanism for postconfluence inhibition (65). The "wound healing" phenomenon as described by Martz and Steinburg (56) provides evidence against a uniformly distributed inhibitor. Wound healing is initiated when a small patch of cells is scraped away from a stationary phase monolayer. Almost all cells near the edge of the scrape divide within a few days, but very few cells away from the scrape divide during this period. Thus, a very high mitotic rate and a very low mitotic rate exist side

by side in the same culture, which cannot be explained with a uniformly distributed inhibitor.

Occasionally during the multiplication of a diploid strain a few cells may exhibit altered morphology, increased growth rate, and increased growth efficiency. The clone derived from such cells has unlimited life and constitutes a permanent cell line.

Characteristics of Transformed Cell Cultures

Cell lines repeatedly transferred undergo changes due to the emergence and selection of variants. These cell lines occasionally are neoplastic and are said to have undergone a "transformation" to the neoplastic state (33). Cells may be transformed *in vivo* or *in vitro*. Histologic indications of *in vivo* transformation are: dysplasia, anaplasia, hyperplasia, increased nuclear-cytoplasmic ratio, and mitotic aberrations (75). Malignant neoplasms show varying degrees of invasiveness. The ultimate invasive potential is the metastatic or secondary growth (22).

There are several criteria for the determination of neoplastic transformation *in vitro*. The 3 most important are production of a malignant neoplasm *in vivo* by inoculation of cells into a syngeneic host, growth in soft agar, and immortality. Not all criteria for transformation need be met; some transformed cell lines do not produce neoplasms when inoculated *in vivo* (22). Normal cells in tissue culture fail to divide when suspended in fluid or semisolid medium. Apparently they require attachment and extension on a solid substratum in order to divide (52). Virtually without exception all transformed cells that grow in soft agar will produce neoplasms when inoculated into a suitable

host (22). Neoplastic transformed cells may also exhibit different morphologic and growth characteristics when compared with normal cells *in vitro*. Examples are loss of postconfluence inhibition of cell division and increased saturation density (22). This relates only to fibroblast-like cells grown *in vitro*; transformed epithelial-like cells exhibit no morphologic alterations in many instances.

A number of vegetable proteins (lectins) such as wheat germ agglutinin, concanavalin A (con A), and phytohemagglutinin bind *in vitro* to carbohydrates on the surface of transformed cells but not normal cells. These polyvalent lectins will agglutinate the transformed cells *in vitro*. When mixed with normal cells no agglutination occurs (47). Normal cells treated with trypsin, a protease, will agglutinate with con A (46). Therefore, it is assumed that untransformed cells contain the same or similar sites on the cell surface as transformed cells but in a cryptic form. Transformed cells can be restored to normal growth characteristics by covering the agglutinin sites with monovalent con A obtained by treatment with trypsin (16).

Transformed cells may also show an altered karyotype. A culture that has acquired abnormal genetic characteristics but cannot produce tumors in animals is referred to as having undergone genetic transformation (33).

Chromosome Analysis

The relationship between chromosomal alteration and change from normal somatic cells into cancer cells is an important area of cancer research. While many investigators believe that chromosomal

alteration is only a secondary event in tumor induction, others believe it plays an important role.

Boveri (11), in 1914, assumed that abnormal mitosis, i.e., irregular distribution of chromosomes frequently found in tumor cells, produced the potentiality for unrestricted cell proliferation. This hypothesis was suported by Makino *et al.* (54) and others.

In general, some deviations from normal somatic cells are found in the chromosome complement of cells in primary and transplantable tumors. In most tumor types, the site and type of alteration in karyotype differ from one tumor to another (87), while in others, e.g., the canine venereal tumor (54), certain characteristic karyological changes are exhibited. On the other hand, in the majority of mouse leukemias produced with leukemogenic viruses, diploid karyotypes are retained (87). Takayama (81) and Makino (53) have suggested that each tumor population is characterized by the possession of a stemline karyotype deviating from that of normal cells. In their opinion, the genetic character of a tumor population is closely correlated with the chromosome constitution of its stemline cells. The karyotype of stemline lineage, however, is not always stable, and the stem cells are easily replaced by more vigorous cell types resulting in changes in stemline karyotype.

The canine venereal sarcoma exhibits a similarity in stemline karyotype. Various researchers have observed a modal number of 59 chromosomes, of which 16 or 17 are metacentric or submetacentric (2,6,54,72,81). This represents a wide deviation from the normal dog karyotype of 78 chromosomes with all the autosomes being acrocentric

(44). The consistency of similar chromosomal alterations in canine venereal sarcomas has led some investigators to postulate that this tumor type originated from a single dog (53,54,81). However, this "Adam and Eve" hypothesis has been questioned since similar alterations have been observed in cell cultures of canine lymphosarcomas (58), a melanoma, and a thyroid adenocarcinoma (62).

Miles *et al.* (58) suggested that centric fusion, a process whereby 2 acrocentric or telocentric chromosomes fuse to form a single metacentric chromosome, was a possible mechanism to explain the increase of metacentric and submetacentric chromosomes. That centric fusion does occur was substantiated by Allerdice and co-workers (3) working with mouse cell lines.

Species of Origin Determinations

Numerous methods have been developed for determining the species of origin of cell cultures. Some of these methods are hemagglutination (12), mixed agglutination (21), cytotoxic-antibody dye-exclusion (39), immunofluorescence (78), chromosome analysis (20), and isoenzyme characterization (59). The confirmation of species of origin is important as it is not uncommon for laboratory contamination of one cell type with another to occur. Franks *et al.* (32), utilizing the mixed agglutination test, found 29 cell lines contaminated with cells of other species. The cytotoxic-antibody dye-exclusion test (39) showed 3 cell types thought to have originated from porcine, chicken and monkey tissues to actually be human cells. Immunofluorescence, employed by Simpson and Stulberg (73), found 2 out of 26 cell lines to contain cells of species other than originally designated.

Chromosomal analysis of mammalian cell lines has also been used as an aid in the identification of the species of origin (20). Clausen and Syverton observed that the chromosomes of 10 altered lines with the supposed species of origin of cynomolgus monkey, laboratory rabbit, porcine, and bovine resembled human chromosomes. They also observed that 4 lines of supposed human origin and one of monkey origin possessed chromosome complements resembling the L-strain of mouse origin. These results were corroborated by immunologic experiments of Brand and Syverton (13) and was consistent with the virus susceptibility and cell morphology.

It is also possible that a cell line of one species may contaminate and overgrow a different cell line of the homologous species. For example, Gartler (34) observed that 20 human cell lines, presumably of Caucasian and Negro origins, contained a variant of glucose-6 phosphate dehydrogenase (G6PD). He suggested that these were contaminated with HeLa cells since this variant of G6PD occurred in only 30% of the Negro race and never in Caucasians. Isoenzyme electrophoresis (59) confirmed Gartler's experimental results and was consistent with his diagnosis. These findings indicate the importance of proper intraspecies as well as interspecies identification of cells.

Oncornaviruses Associated with Cell Cultures

Considerable attention in cancer research has been directed to the finding of C-type, RNA containing virus particles in cell lines under continuous culture (4,15,29,30,35,43,49,57,72,86). These virions are enveloped, approximately 100 nm in diameter, and appear spherical to pleomorphic. Assembly of the virion is at the cytoplasmic membrane

through which it buds. The core of C-type virions may be electron dense or electron-lucent (Figure 1). C-type particles contain 2 major antigens, an envelope antigen and an internal or group specific (gs) antigen. Two distinct gs antigens are found in the same protein chain; gs 1, which is species specific and does not cross react with viruses of other species, and gs 3, which is common to all mammalian viruses (38).

Other oncornaviruses, classified by morphology, are A- and B-type particles (Figure 1). Type A particles have an electron-lucent core surrounded by an electron dense area. The nucleocapsid is centrally located within a single membrane and the complete virion has the appearance of a "doughnut." It is approximately 90 to 120 nm in diameter, found in great numbers in the Golgi area, and may form inclusion bodies visible in the light microscope (10). The significance of A-type particles is as yet unknown. Type B particles differ from the C-type particles of leukosis viruses by their dense eccentric core and 2 envelopes which are separated by an electron-lucent space (17). Type B particles are variable in size, range from 90 to 150 nm in diameter, and occasionally reach 200 nm. The envelope surrounding the core is apparently derived from the endoplasmic reticulum, while the envelope of the virion is formed from elements of the cytoplasmic membrane (17). An example of B-type particles is the mouse mammary tumor virus which is transmitted through the milk of infected mothers (51).

Ellermann and Bang (27), in 1908, discovered the first tumor virus which causes leukemias in chickens. In 1911, Rous (69) described the cell-free transmission of a tumor-producing agent. This discovery demonstrated that solid tumors, in addition to leukemias, could be

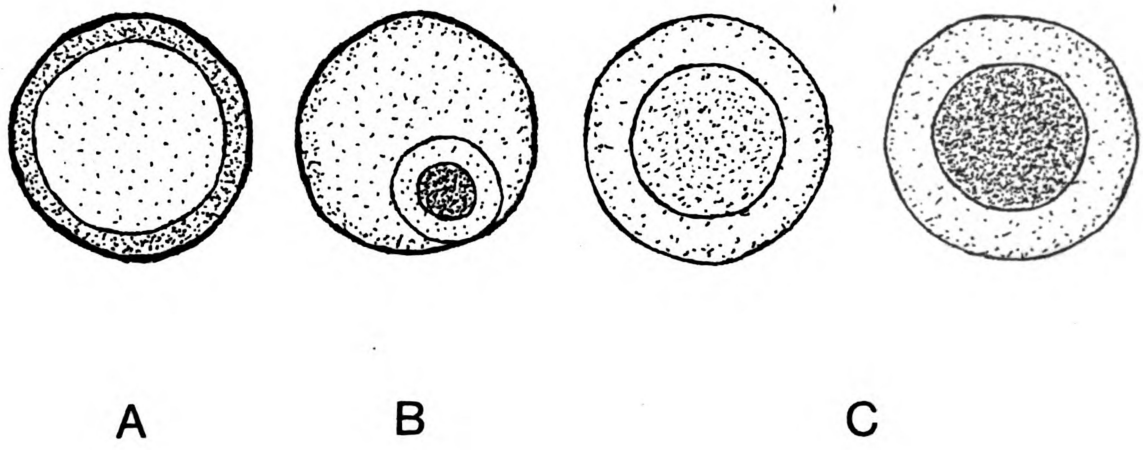

Figure 1. Fine structure of type A, B and C virus particles.

caused by viruses. Manaker and Groupé (55), in 1956, observed that Rous Sarcoma virus caused cells in culture to grow into localized foci. This observation served as a starting point for the development of a tissue culture assay for the virus (83).

Until 1951, leukemia viruses had not been demonstrated in animals other than chickens. Gross (40), in that year, succeeded in passing mouse leukemia from one mouse to another with cell-free extracts.

In 1973 Lieber *et al.* (49), using the supernatant reverse transcriptase assay, examined 31 cell lines of various species from the American Type Culture Collection. Readily detectable amounts of oncornaviruses were demonstrated in 1 pig, 2 Chinese hamster, and 3 rat cell lines. A fluorescent antibody technique was utilized by Ferrer *et al.* (30) to detect C-type particles in cell lines derived from leukemic cows. The electron microscope was used by Armstrong *et al.* (4) to detect C-type particles in pig cell lines, and by Sapp and Adams (72) in a canine venereal tumor cell line.

Burger and Noronha (15) purified infectious C-type particles from a cell line derived from a leukemic cat. When high concentrations of such particles were inoculated into young dogs, a disease similar to that seen in the cat was produced. Furthermore, the feline gs antigen was detectable in the dog cells.

Lowry *et al.* (50) studied the activation of murine leukemia virus (MLV) in cell lines from embryos of the high leukemic AKR mouse. Two cell lines were negative for MLV as determined by extensive testing for infectivity, viral antigens, morphologic particles, and reverse transcriptase activity. However, on rare occasions, a spontaneous

activation estimated at a frequency of 10^{-8} or 10^{-9} would occur. An exposure of these cells to 5-iododeoxyuridine (IDU) or 5-bromodeoxyuridine (BDU) induced as many as 0.5% of the cells to produce MLV, an increase of about 10^{6} over the spontaneous rate. Aaronson *et al.* (1) exposed 13 non-virus producing clones of the mouse BALB/c embryo line to BDU, which induced the production of C-type virus particles.

A further modification of the above technique involved the use of dimethyl sulfoxide (DMSO) (77). Dimethyl sulfoxide was added to a human tumor cell line previously activated by IDU and a tenfold increase in observable virus particles occurred over treatment with IDU alone.

A recent hypothesis proposed by Todaro and Huebner (84) states that vertebrates contain the genetic information for producing a C-type virus in an unexpressed form in every somatic and germ cell. Furthermore, they suggest that this genetic information has been part of the genetic makeup of cells since early in evolution and can persist for hundreds of generations in cell culture without production of virus. The endogenous "virogene" (genes for production of virus) and the "oncogene" (part of the virogene, responsible for transformation to a tumor cell) are maintained in an unexpressed form by unknown repressors in normal cells. Derepression of the repressor substance would allow expression of the genes. Vertical transmission by the germ cells is the usual mode of transmission. The work previously described of Lowry *et al.* and Aaronson *et al.* (50,1) with DNA analogs have added substance to the oncogene hypothesis since nonproducing clones have been induced to form C-type particles.

An alternate hypothesis put forth by Temin (82) states that RNA tumor virus replication is via a DNA intermediate which can be transmitted in cells as DNA. The DNA intermediate, called a provirus, is a template for the production of progeny viral RNA. This protovirus hypothesis is consistent with the oncogene hypothesis in the contention that the tumor cell has regions of DNA specifying synthesis of RNA that leads to transformation, but this DNA is created by successive RNA to DNA to RNA information transfers. The provirus is not present in germ cells but is generated as the animal develops. Thus only a small fraction of somatic cells would contain all the genes necessary for C-type virus production.

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ARTICLE

BIOLOGICAL CHARACTERISTICS AND VIRAL SUSCEPTIBILITY OF A CANINE KIDNEY ADENOCARCINOMA CELL LINE (CKT)

By

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(Manuscript to be submitted to the American Journal of Veterinary Research)

SUMMARY

A cell line derived from an adenocarcinoma of a kidney from a 4-year-old dog was serially cultivated in a closed system for 85 subcultivations during a period of 2 years. The cells had an abnormal karyotype and exhibited fluid transport activity, a function of secretory epithelium. Virus-like particles similar to C-type oncornaviruses were observed budding through and associated with the cell membrane. Growth of several viruses was supported in the cells with resulting cytopathic changes.

INTRODUCTION

The *in vitro* cultivation of cells with stable characteristics is a valuable tool in biological and medical research. Biochemistry, microbiology, cancer research, and other fields of study have benefited from the use of cell cultures.

Relatively few canine cell lines have been established and described (1,5,7,20,22). A cell line derived from normal dog kidney (MDCK) was established by Madin and Darby in 1958 (22). This line has been widely used in animal virology (3,5). Rosanoff (20) established a testicular tumor cell line, and Kasza (7) established cell lines from a melanoma and a thyroid adenocarcinoma. The latter 2 lines had chromosome alterations (17) similar to those described by Adams *et al.* (1) in cells derived from a canine venereal tumor. Miles *et al.* (15) reported similar findings in cells derived from canine lymphosarcomas. Type-C

virus particles have been associated with the canine venereal tumor cell line (21).

The present study describes the characterization and viral susceptibility of a cell line derived from a kidney adenocarcinoma of a 4-year-old dog.

MATERIALS AND METHODS

Origin and establishment of the CKT cell line. The CKT cell line was established by one of us (AWR) from a tumorous kidney of a male 4-year-old Miniature Poodle. The cells were dispersed by overnight trypsinization (19) (ca. 16 hours) and cultured in a closed system using Eagle's minimum essential medium with Earle's base supplemented with 0.5% lactalbumin hydrolysate, 1 mM sodium pyruvate and nonessential amino acids (modified EMEM) with 10% bovine fetal serum (BFS). Potassium penicillin G, streptomycin sulfate, and polymyxin B sulfate were added at the rate of 150 units/ml, 150 µg/ml and 75 units/ml, respectively.

The cells were subcultured at 1-week intervals at a split ratio of 1 to 3 bottles for the first 5 subcultures. The subculture interval was extended at the sixth subculture to 5 weeks due to a reduced growth rate, and the split ratio was decreased to 1 to 1. Medium was replaced at 1-week intervals. By the 9th subculture, the growth rate had increased and the split ratio was extended to 1 to 3. Thereafter, the cells were routinely subcultured at 7- to 10-day intervals without a change of medium. Cells were preserved in liquid nitrogen at the 15th and 50th subculture. The methods used for subculturing and preserving cells were as described elsewhere (19).

Glass and plastic ware. Water dilution bottles (Kimble, Toledo, Ohio) fitted with plastic screw caps were used for stock cultures of cells, and 1-milliliter glass ampules (Weaton Glass Company, Millville, New Jersey) were used for the freezing and storage of cells. Disposable 30-ml plastic flasks (Falcon Plastics, Los Angeles, California) were used for growth rate, growth efficiency, and medium evaluation. Disposable 16 x 15 mm plastic petri dishes (Falcon) were used for soft agar suspension cultures.

Growth rate. The 22nd subculture and the 70th subculture were used in the growth rate study. Five-milliliter aliquots of cell suspensions containing 5.5×10^4 cells/ml (22nd subculture) and 5.0×10^4 cells/ml (70th subculture) were seeded into disposable plastic flasks and incubated at 36 C. Quadruplicate counts of 2 flasks at each subculture level were made in a hemacytometer at 12-hour intervals for the first 4 days and thereafter at 24-hour intervals. The growth medium was replaced at 4-day intervals, and the experiment was terminated when the number of cells decreased or detached from the flask.

Growth efficiency. The growth efficiency determination was performed on the 70th subculture: 5.0 ml cell suspensions of 2.2×10^5, 1.1×10^5, 5.5×10^4, 2.2×10^4, 1.1×10^3 cells/ml were seeded into disposable plastic flasks. Two flasks for each dilution were employed and medium was replaced at 4-day intervals. The time required for confluent monolayers to form was recorded.

Evaluation of growth and maintenance mediums. The mediums used for the growth and maintenance evaluation were Eagle's minimum essential

medium (EMEM), modified EMEM, Earles' basal medium (BME), BME diploid, medium 199 (M-199), McCoy 5A, and Hanks' balanced salt solution supplemented with 0.5% lactalbumin hydrolysate and 0.05% TC yeastolate (LHY Hanks'). The mediums were obtained commercially (International Scientific, Cary, Illinois) as a prepared powder except for LHY Hanks' which was prepared in the laboratory. Growth mediums contained 10% BFS and maintenance mediums contained 2% BFS. Antibiotics as described earlier were employed. Mediums were added to disposable plastic flasks in 4.5 ml amounts. Subsequently, 0.5 ml of a cell suspension of 6.0×10^5 cells/ml was added. Five flasks were employed for each medium, and the mediums were replaced at 4-day intervals. When a monolayer was formed, the growth mediums were replaced with maintenance mediums in 3 of the flasks; the growth mediums in the remaining 2 flasks were left unchanged. Observations were made as to the period of time cells retained their healthy appearance. Degeneration was the criterion used in the determination.

Chromosome analysis. Chromosome analysis was performed on the 17th, 42nd, and 65th subculture of CKT cells. The method as described by Merchant *et al.* (14) was used to obtain metaphase preparations which were stained with a modified Giemsa stain (18).

Viral susceptibility. The 65th subculture and several subsequent subcultures of CKT cells were used for viral susceptibility testing. Cell cultures employed for the propagation of the stock viruses were pig kidney [PEN (1)], African green monkey kidney (Vero), canine kidney (MDCK), human cervical carcinoma (HeLa), equine kidney (EK), and bovine

kidney (BK). Avian viruses were cultivated in chicken embryos (CE). The viruses tested and the host systems used for their cultivation are tabulated (Table 1). Cell cultures were initiated as described elsewhere (19); the medium used was the same as previously described for CKT cells.

Stock viruses were diluted 1:10. Two tubes of CKT cells were used for each virus and the inoculum was 0.2 ml/tube. The cells were incubated at 36 C and observed each day for cytopathologic effects (CPE). When CPE was evident (2+ or greater) or at 7 days postinoculation, cells and medium were frozen, thawed and reinoculated into CKT cells. At least 3 passages were made with each virus. Cytopathologic effects and fluorescence microscopy, where applicable, were the criteria used for determining infectivity.

Viruses that did not produce CPE in CKT cells were inoculated back into the original host systems to determine if they had grown without producing observable effects. Viruses that propagated in CKT cells were comparatively titrated in the original host systems used for propagating the viruses and in CKT cells.

Culturing of cells in soft agar. The method of Macpherson (10) was used with a slight modification, the only change being the use of 20% BFS with modified EMEM. Disposable plastic petri dishes were seeded with 5.5×10^4 cells/petri dish. Controls employed were the MDCK cell line and a rat embryonic skin cell line (RESI) (8). The cultures were incubated at 36 C in an atmosphere of 5% CO_2 and air at 80% humidity. They were examined at 12 days and 18 days after seeding.

Table 1. Viruses

Virus	Cell culture or animal source of stock virus	Viral titer ($TCID_{50}$/ml except where indicated)
ANIMAL VIRUSES		
Pseudorabies	PEN (1)	5.0
Vesicular stomatitis (Indiana)	PEN (1)	8.0
Hog cholera	PEN (1)	4.5
Equine rhinopneumonitis (A-163)	EK	3.5
Eastern equine encephalomyelitis	Vero	7.5
Western equine encephalomyelitis	Vero	5.5
Equine cytomegalovirus (82-A)	EK	3.0
Equine adenovirus	BK	2.5
Bovine adenovirus type 3 (WBRI)	BK	4.0
Infectious bovine rhinotracheitis	BK	5.5
Bovine virus diarrhea	BK	5.0
Canine herpesvirus	MDCK	1.5
Canine adenovirus type 2 (Manhattan)	MDCK	7.0
Canine SV5 (958)	MDCK	7.0
AVIAN VIRUSES		
Newcastle disease virus (Roakin)	CE (AC)*	6.5 (LD_{50}/ml)
Infectious laryngotracheitis	CE (CAM)	4.5 (IE_{50}/ml)
Fowl pox	CE (CAM)**	6.0 (ID_{50}/ml)
HUMAN VIRUSES		
Adenovirus type 1 (AD-71)	Vero	5.5
Poliovirus type 2 (Sabin)	Vero	8.5
Herpes simplex (MacIntyre)	HeLa	5.5
Coxsackievirus A16	Vero	4.5
Coxsackievirus B_2	Vero	6.5

Table 1 (continued)

Virus	Cell culture or animal source of stock virus	Viral titer ($TCID_{50}$/ml except where indicated)
Coxsackievirus B_5	Vero	6.5
Coxsackievirus B_6	Vero	5.5
Echovirus 9 (Hill)	Vero	4.5
Reovirus 1	HeLa	4.5
Reovirus 2	HeLa	3.5

Data are expressed as reciprocal of log of virus dilution.

*Allantoic cavity.

**Chorioallantoic membrane.

Tumorigenic capabilities. The 67th subculture of CKT cells was used to evaluate tumorigenicity *in vivo*. The cells were washed 3 times and resuspended in Hanks' balanced salt solution (BSS). Three 5-week-old mixed breed dogs (littermates) were separately inoculated with 25×10^6 cells subcutaneously (SQ), intraperitoneally (IP), and intramuscularly (IM). One additional littermate was inoculated IP with 50×10^6 cells and IM with 25×10^6 cells. Six weanling Syrian hamsters were inoculated with 1×10^6 cells; 3 were inoculated IP and 3 were inoculated via the cheek pouch.

The dogs were housed in concrete runs and fed a standard diet. The hamsters were maintained in standard cages (3 per cage). Immunosuppressants were not administered. The dogs and hamsters were sacrificed 150 days after inoculation.

Species of origin determination. Hyperimmune anti-canine serum was prepared by inoculating a New Zealand White rabbit with normal primary canine kidney cells. The inoculum, consisting of 2.0 ml of an emulsion of cell suspension (10^6 cells/ml) and equal parts of Freund's complete adjuvant (Difco, Detroit, Michigan) was administered subcutaneously at 4 sites on the rabbit's back. Twelve days later, 1 ml of the cell suspension without adjuvant was injected intravenously. The rabbit was bled 13 days later and the serum was collected and inactivated for 20 minutes at 60 C.

Confirmation of species of origin of the CKT cells was determined by employing the immunofluorescence technique of Stulberg *et al.* (25) with slight modifications, and the cytotoxic-antibody dye-exclusion test as described by Greene *et al.* (6). The immunofluorescence

technique was modified by using the antiserum described above in an indirect test rather than the direct test as described. Antirabbit globulin conjugate was obtained commercially (Sylvana, Millburn, New Jersey) and used at a 1:4 dilution. Vero cells were employed as a control in the immunofluorescence test. Vero and MDCK cells were used as controls in the cytotoxicity test.

Sterility tests. The 24th and 73rd subcultures were checked for contaminating microorganisms. Sabouraud's dextrose agar, blood agar, and mycoplasma broth and plates were inoculated with antibiotic free CKT cell suspensions. Filtered CKT fluid from the 84th subculture was inoculated into MDCK cells and underwent 2 blind passages. The 81st subculture of CKT cells was used for electron microscopic examination. The procedure used was similar to that described elsewhere (23). Grids were examined in an HU-11 Hitachi electron microscope at 75 KV.

RESULTS

The CKT cells were derived from a tumorous kidney diagnosed histopathologically as an adenocarcinoma (Figures 1 and 2). They were fibroblastic in appearance during the first 5 subcultures. At the 6th subculture the growth rate of the cells diminished considerably. A few areas of epithelial cells were observed at the 7th subculture, and by the 9th subculture the cells were entirely epithelial-like in appearance (Figure 3). "Blebs" or "domes", raised areas of cells one cell layer thick, were observed as early as the 18th subculture and remained evident through 85 subcultures during a period of 2 years (Figure 4).

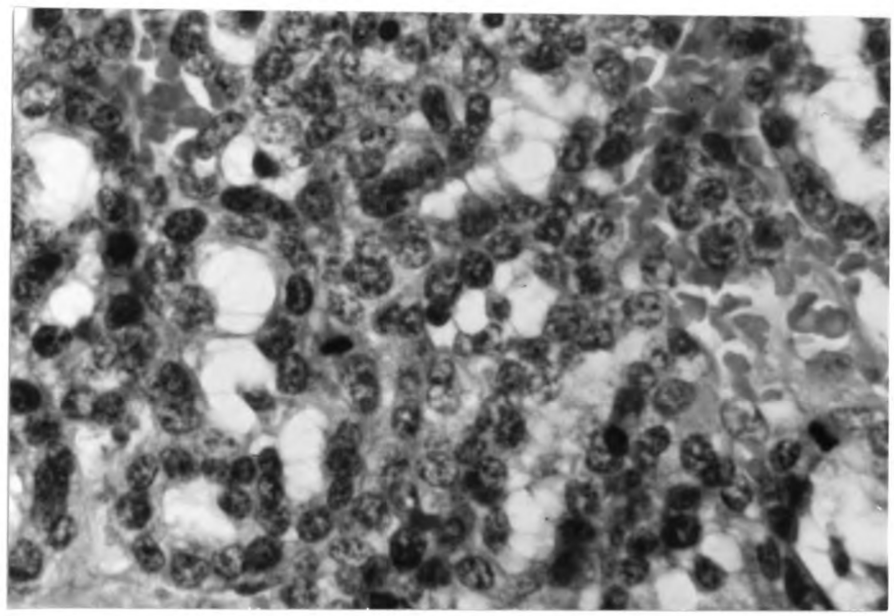


Figure 1. Section of adenocarcinoma. Notice the tubule formation (arrow). Hematoxylin and eosin stain; X 1280.

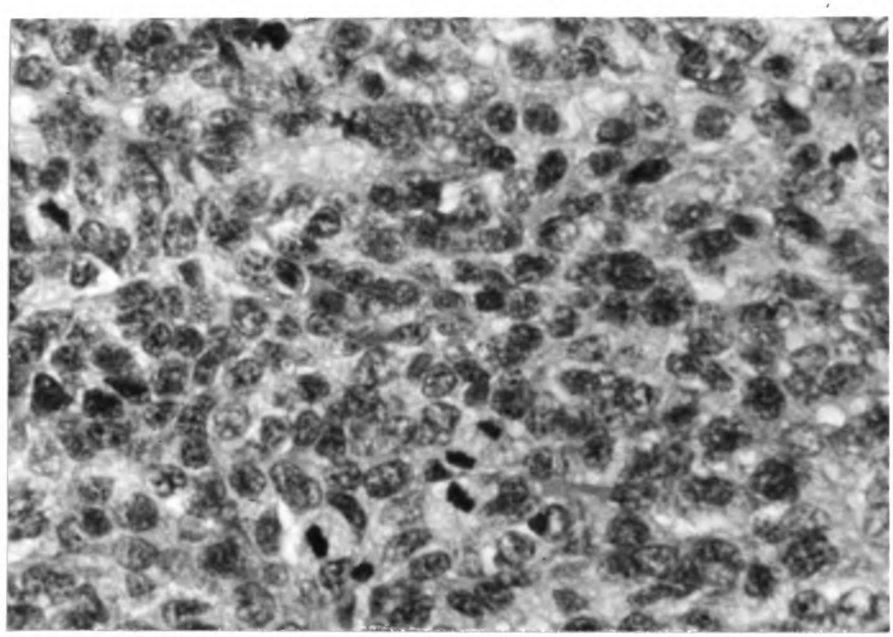


Figure 2. Section of adenocarcinoma. Notice the high mitotic activity (arrows). Hematoxylin and eosin stain; X 1280.

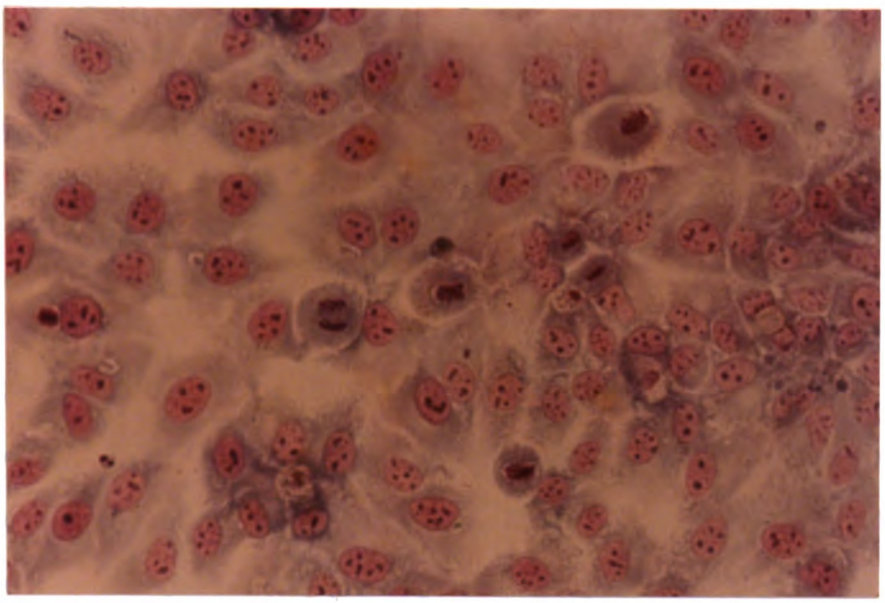


Figure 3. Monolayer of CKT cells. Giemsa's stain; X 640.

Figure 4. Monolayer of CKT cells exhibiting "bleb" or dome formation. Unstained; X 123.

These blebs attained a size up to 4 mm in diameter and were visible macroscopically.

The growth rate of the CKT cell line is shown graphically (Figure 5). The mean doubling time for exponential growth was determined using the equation:

$$N_t = 2^{kt}No$$

where No is the population size at a certain time, N_t is its size at a subsequent time, t, and k is the exponential growth rate constant, defined as the number of doublings per unit time. The mean doubling time is the reciprocal of the exponential growth rate, which was 36 hours for the 70th subculture and 45 hours for the 22nd subculture. The cells detached from the surface at a stationary density of 3.0×10^5 cells/cm^2 during the 70th subculture, and reached a stationary density of 3.8×10^5 cells/cm^2 during the 22nd subculture before the cell number decreased.

A monolayer was formed within 2 days in the growth efficiency test (Figure 6) when the cells were seeded at a concentration of 2.2×10^5 cells/ml, and 8 days when the cell concentration was 1.1×10^4 cells/ml. When seeded at 1.1×10^3 cells/ml, 22 days were required for a monolayer to form.

The results of CKT growth in selected mediums are presented (Table 2). Modified EMEM, EMEM, McCoy 5A, and LHY Hanks' supported the growth of CKT cells; other mediums did not.

The chromosome complement of CKT cells at 3 subculture levels is shown graphically (Figure 7). Fifty metaphase figures were counted for

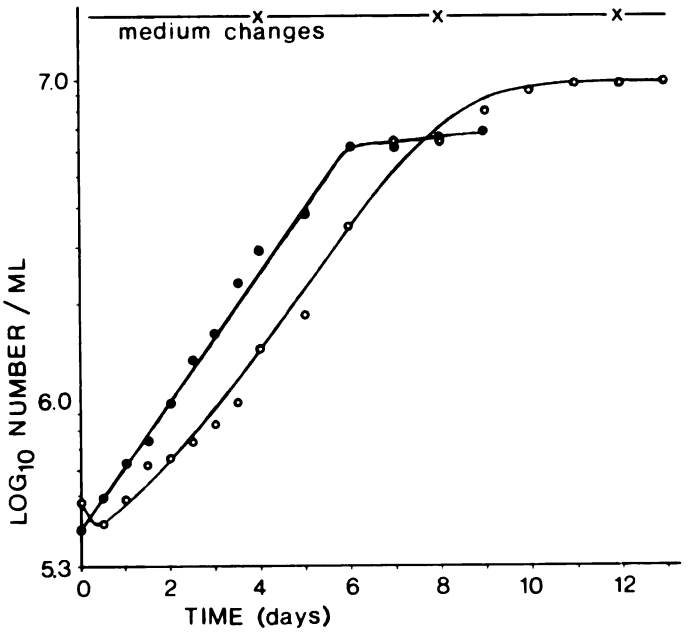


Figure 5. Growth curves of CKT cells at the 22nd (open circles) and the 70th (closed circles) subcultures.

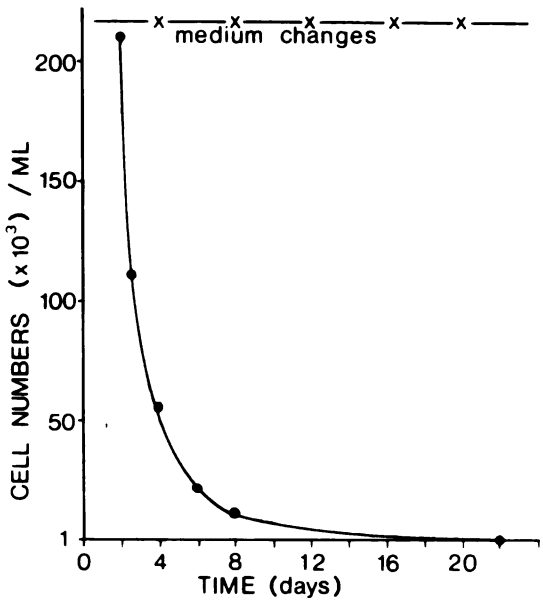


Figure 6. Growth efficiency of CKT cells. Curve indicates the time required for development of confluent monolayers at various seeding rates.

Table 2. Evaluation of mediums

	Monolayer formation, medium supplemented with 10% BFS (days)	Degeneration of monolayer (days)	
		2% BFS*	10% BFS**
Modified EMEM	3	11	11
EMEM	3	12	7
BME	none	--	--
BME Diploid	none	--	--
M-199	none	--	--
LHY Hanks'	3	12	8
McCoy 5A	3	8	8

*After formation of a monolayer serum content was changed to 2% and replenished every 4 days.

**Medium not replenished after a monolayer formed.

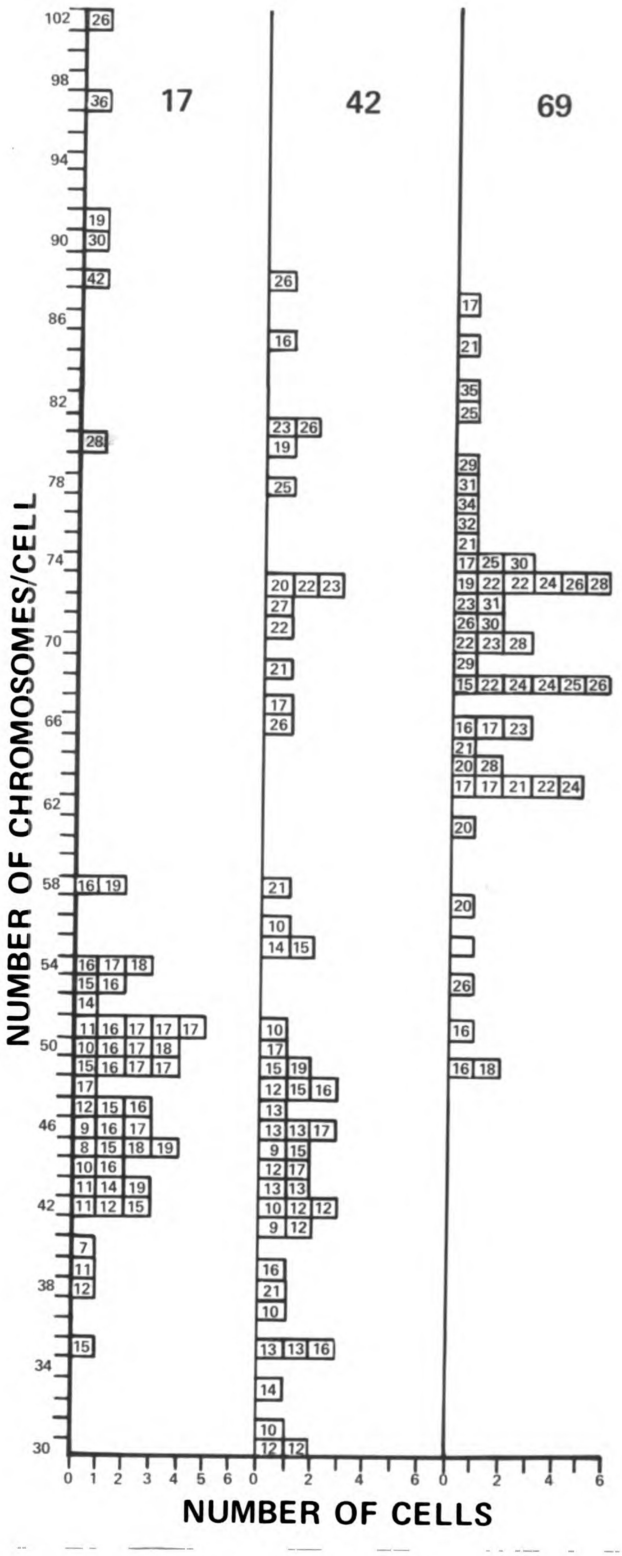


Figure 7. Chromosomal patterns of CKT cells at the 17th, 42nd and 69th subcultures. Number in box represents the number of metacentric or submetacentric chromosomes.

each subculture. Most of the cells of the 22nd subculture contained chromosomes clustered in a range of 42 to 54 chromosomes per cell, whereas chromosomes of the 42nd subculture were spread over a wider range. The 69th subculture contained chromosomes which were again clustered, but in a higher range of 63 to 74 chromosomes per cell. A high frequency of metacentric and submetacentric chromosomes were observed at all passage levels (Figure 8). The karyotype of CKT cells differed markedly from that of normal dog cells, which have a diploid number of 78 chromosomes per cell with only the X and Y being metacentric (Figure 9).

Of 27 viruses tested, the CKT cell line was susceptible to 8 (Table 3). The viral CPE produced in CKT cells was similar to that produced in cell cultures normally used to propagate the viruses, except for pseudorabies virus, which produced extensive syncytia resulting in extremely large polykaryocytes (Figure 10). These polykaryocytes contained more than a hundred nuclei in some instances. Viruses that grew in CKT cells did so, in most instances, to an extent comparable with their propagation in the original host systems (Table 3).

The CKT cells were not adequate for the initial propagation of the other 20 viruses tested; equine rhinopneumonitis was adaptable. Eastern equine encephalomyelitis (EEE) produced CPE initially but not in subsequent passages or when dilute inocula were used. No evidence of infection was observed when these viruses were inoculated back into the original host system.

Cell aggregates in soft agar were not observed immediately after seeding. Examination of cells at 12 days revealed that approximately

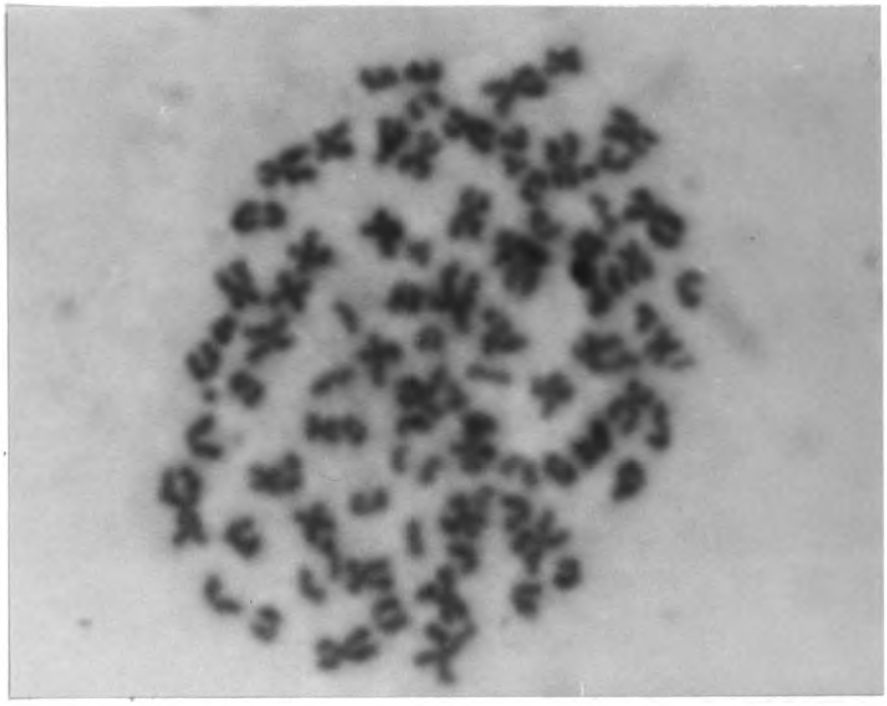


Figure 8. Chromosomes of CKT cells. Notice the large number of metacentric and submetacentric chromosomes. Giemsa's stain; X 3,200.

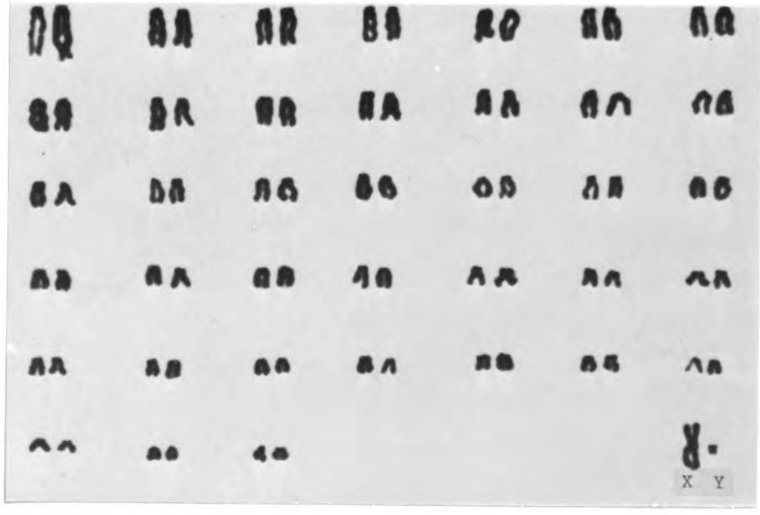
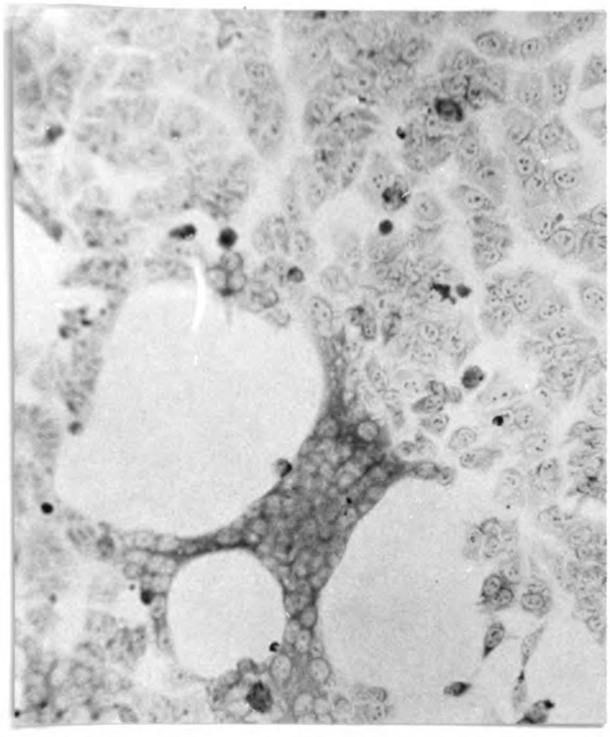
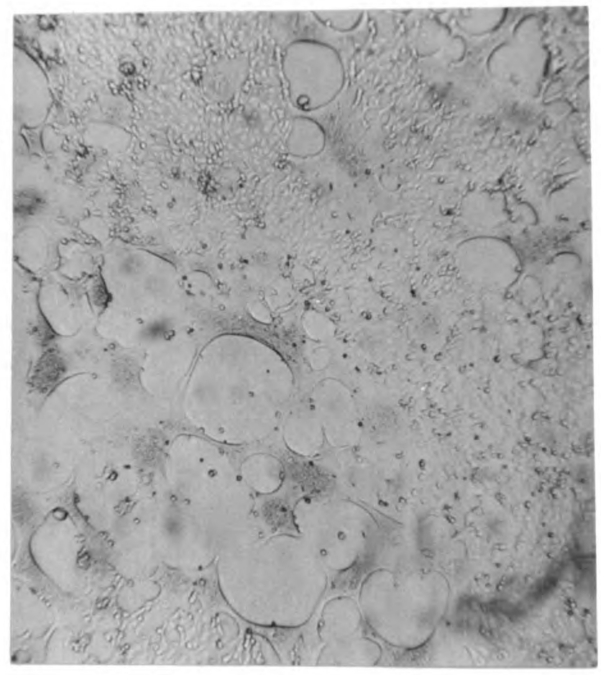


Figure 9. Normal karyotype of the male dog. From Hsu, T. C., and K. Benirschke. 1967. In An atlas of mammalian chromosomes. Springer-Verlag, NY.



a



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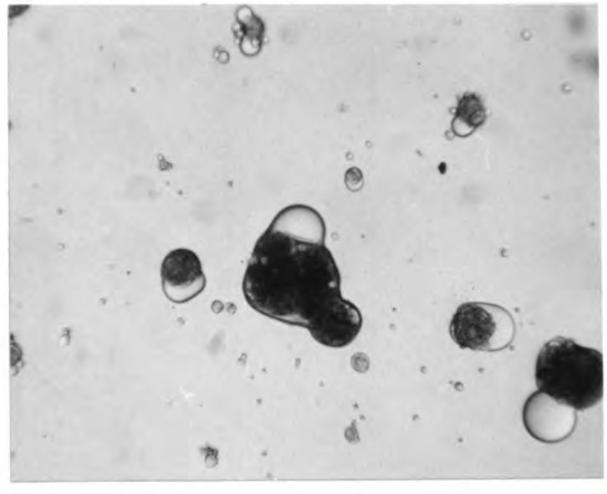

c

Figure 10. CKT cells infected with pseudorabies virus. (a and b) - Notice the extensive syncytial formation (arrows); (c) - Advanced infection. Polykaryocytes detached from the substratum with balloon-like appearance. (a) - Giemsa's stain; X 640, (b) - Unstained; X 112, (c) - Unstained; X 112.

Table 3. Comparative titrations of viruses in the CKT cell line and in the donor host systems

	Titration in CKT cell line ($TCID_{50}$/ml)	Titration in original host system ($TCID_{50}$/ml)
Pseudorabies	6.0	5.0
Vesicular stomatitis	8.0	8.0
Canine herpesvirus	3.5	1.5
Canine adenovirus type 2	4.0	7.0
Canine SV5	5.5	7.0
Herpes simplex	7.0	5.5
Reovirus 1	5.5	4.5
Reovirus 2	4.0	3.5

Data are expressed as reciprocal of log of virus dilution.

60% of the CKT cells had divided with 40% having too great a number to count (Figure 11). Cell division in the positive control (RESI) was 80% with most aggregates (70%) containing too many cells to count. In contrast, there were only a few aggregates of cells (5%) observed in the negative control (MDCK) and these only contained 2 and 4 cells.

The CKT cells were confirmed to be of canine origin. Peripheral fluorescence was observed with CKT cells but not with Vero cells. The results of cytotoxic-antibody dye-exclusion test are presented (Table 4).

Table 4. Results of cytotoxic-antibody dye-exclusion test for species identification

	Preinoculation serum (viability)	Anti-normal dog serum (viability)
MDCK	91%	0%
CKT	98%	1%
Vero	99%	100%

Contaminating bacteria, fungi, mycoplasma and cytopathic viruses were not observed. Electron microscopic observations of CKT cells revealed particles similar to type C viral particles budding through the cell membrane (Figure 12). These were also observed outside of the cell in association with the cell membrane (Figure 13). Sizes ranged from 90 nm to 130 nm in diameter.

No gross or histopathologic lesions were observed in dogs and hamsters inoculated with CKT cells.

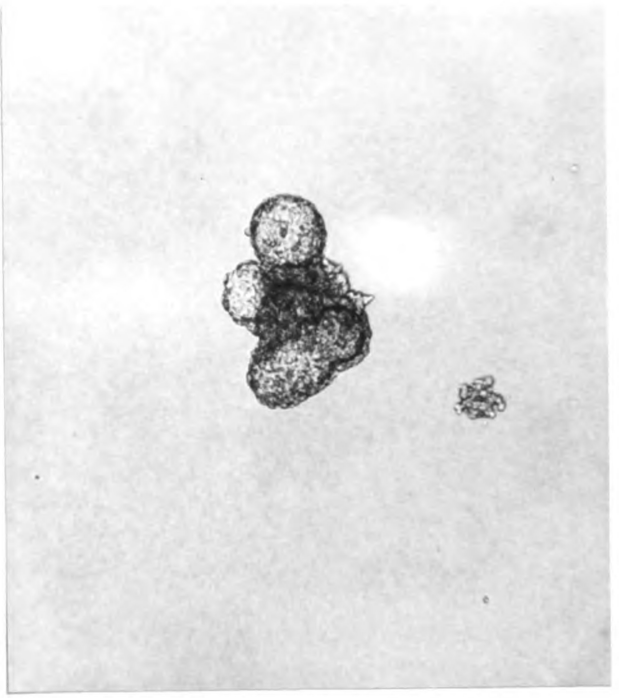

Figure 11. Growth of CKT cells in soft agar. Notice the vesicle formation due to fluid transport activity (arrow). Unstained; X 192.

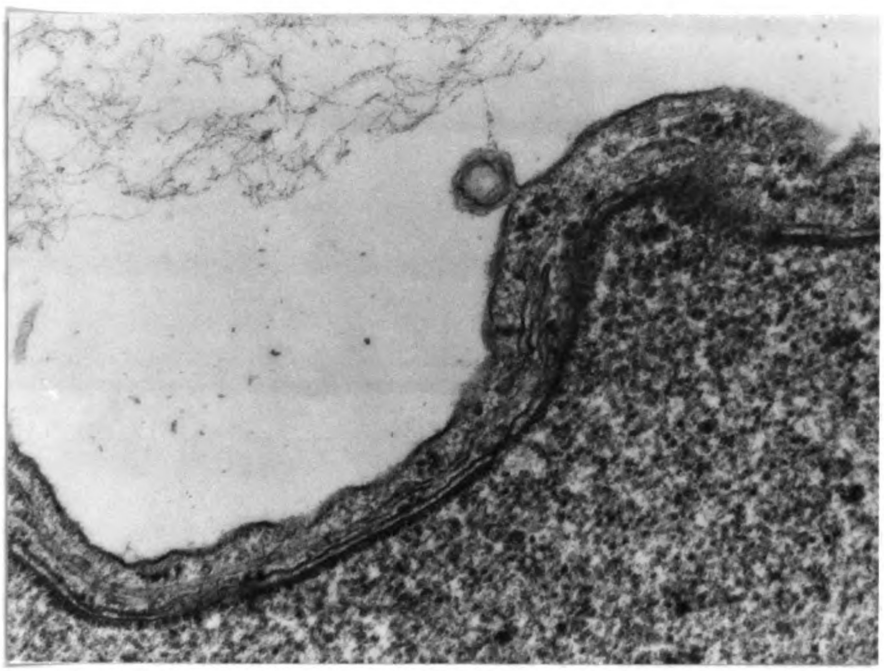

Figure 12. Electron micrograph showing a C-type virus particle budding from the cytoplasmic membrane (arrow). Uranyl acetate and lead citrate; X 52,500.

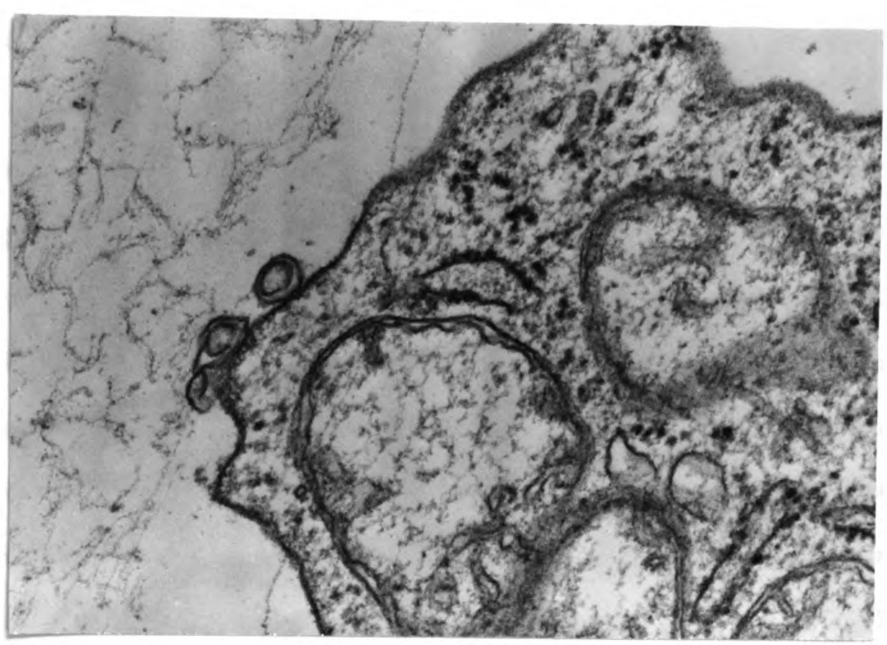

Figure 13. Electron micrograph showing C-type virus particles outside the cytoplasmic membrane (arrows). Uranyl acetate and lead citrate; X 55,500.

DISCUSSION

The "blebbing" effect observed in CKT cells has been described in other cell lines, viz., normal canine kidney (MDCK) (9), human breast carcinoma (24), and mouse mammary tumor (13). Bleb formation is probably a result of fluid transport activity as previously suggested (9) but does not reflect an organ specific function. Rather, it appears to be a cellular function of secretory epithelia which would indicate that other glandular tissue such as thyroid, adrenal, pancreas, and mucous membranes are potentially capable of forming blebs *in vitro*.

The CKT cells grew rapidly, had a high growth efficiency, and were capable of growth in several mediums. However, they could not be maintained for extended periods of time and usually detached from the surface within 2 weeks following the formation of a monolayer. Cellular growth was adversely affected by postconfluence inhibition as indicated by the low stationary density.

Chromosomes of CKT cells were markedly different from normal dog cells. There was a reduction of chromosomes with a corresponding increase in the number of metacentric and submetacentric chromosomes. This phenomenon has been described in canine venereal tumors (2,11, 12,16,26) and cell lines derived from tumors of dogs (1,15,17). It has also been reported in other animal species, particularly those that have a high number of acrocentric chromosomes per cell (15). A possible mechanism to explain the increase in metacentric and submetacentric chromosomes is centric fusion, a process whereby 2 acrocentric chromosomes fuse at the centromere to form a single chromosome (15).

The viral susceptibility spectrum of CKT cells was limited in that only 8 of 27 viruses were successfully propagated. As the results indicate, the CKT cell line offers no advantage over presently available cell lines in propagating these viruses. The effects observed with Eastern equine encephalomyelitis virus and Western equine encephalomyelitis virus was possibly due to an abortive infection, i.e., no virions or only incomplete virions were produced. The abnormally large syncytia produced by pseudorabies virus indicates that the cell membranes of infected CKT cells are easily fused. The transparent areas observed within the membrane of detached polykaryocytes may have been due to a damaged sodium-potassium pump as a result of membrane incorporated viral antigens. The nonfunctional pump would result in a loss of K^+ and a corresponding uptake of Na^+ and Cl^- causing an influx of water. This increase of water within the cell would result in swollen cells with a transparent appearance.

Growth of CKT cells in soft agar suspension indicates tumorigenic capabilities; virtually without exception, cells capable of growing in soft agar produce neoplasms upon inoculation into a suitable host (4). The fact that CKT cells did not produce tumors in the present study may have been due to several factors, e.g., the immunogenicity of CKT cells and the immune responsiveness of the animals employed.

The virus-like particles associated with CKT cells were similar to C-type oncornaviruses (27). Their presence did not result in any observable adverse effect.

The long term cultivation and cultural characteristics of CKT cells fulfill the criteria of an established cell line. The fact that

they were derived from cancerous tissue, have neoplastic properties, and release C-type viral particles may make them useful for cancer research. Physiologists may also find CKT cells useful as they exhibit fluid transport activity, a cellular function of secretory epithelia.

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