BIOLOGICAL CHARACTERISTICS AND VIRAL SUSCEPTIBILITY OF A RAT EMBRYONIC SKIN CELL LINE (RESI)

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ABSTRACT

BIOLOGICAL CHARACTERISTICS AND VIRAL SUSCEPTIBILITY

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By

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A cell line derived from normal skin of fetuses obtained from a female Sprague-Dawley strain rat has been established and grown for 3 years through serial cultivation. The cell line consists predominantly of fibroblastic-like cells and an abnormal heteroploid karotype with a modal number of 40 chromosomes was observed. Subcutaneous inoculation of weanling rats with viable cells produced tumors 2 weeks after inoculation. Histopathologic examination revealed the tumor type as fibrosarcoma-like. The cell line supported the growth of several human and animal viruses accompanied by degenerative changes leading to death of the cells.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

In recent years the development of cell culture techniques suitable for use in microbiological studies in general, and in virology and cancer research in particular, have contributed significantly to our understanding of disease processes.

Disadvantages encountered in the use of primary cell cultures as opposed to serially propagated cells for diagnostic virology and vaccine production have been extensively discussed and generally acknowledged (53,57,62,77).

Because of their rapid growth and ease of maintenance in a variety of cultural media, the establishment of numerous cell lines has provided useful living systems for various branches of biological and medical research. Such cell lines have originated from both malignant and non-malignant tissues. One of the important discoveries in this field was the capacity of some cell lines of nonmalignant origin to produce neoplasms (29,30,83,103,115). This suggests that the so-called "normal" lines, although established from nonmalignant tissues, have undergone changes during the period of cultivation *in vitro*.

To date, numerous established rat tumor continuous lines have been described (6,97,109,123,133). Most of these lines originated from tumors of endocrine tissues and maintained their organ-specific function during serial propagation *in vitro*. In contrast, relatively few lines derived from normal rat tissues have been described.

The present study was undertaken to characterize in detail a rat embryonic skin cell line designated RESI, which was established from normal skin obtained from fetuses of a female Sprague-Dawley strain rat.

REVIEW OF THE LITERATURE

Establishment and Characteristics of Cell Lines

Early Cell Cultivation

The cultivation of cells apart from their source was first accomplished by Harrison (48) who in 1907 observed living, developing nerve fiber in a plasma clot preparation. This technique was both effective and simple and, unlike earlier methods (70,100,126), it made the observation of developing cells a distinct possibility. Fell and Robison in 1929 (34) were the pioneers in organ culture. Their work with avian embryonic femora and limb-buds demonstrated that it was possible to keep animal organs alive outside the body for considerable lengths of time in different types of media. The more refined methods developed through the efforts of Enders, Parker, Earle and associates (27,31,89) made it possible to establish and maintain cells on a solid surface using a fluid overlay, thus greatly increasing the application of cell culture.

Though long-term maintenance of tumor tissue cultures was achieved first in 1924 by Fischer (36), who maintained a culture of Ehrlich's mouse carcinoma for 13 years, it was not until 1936 that Gey and Gey (43) were able to establish a stable culture of human fibrochondro-myxosarcoma which they maintained for a number of years.

Tumor Cell Cultivation

After the development of synthetic nutrient media, the routine use of antibiotics and improved methods for harvesting monolayer cultures, the techniques of investigators in this field broadened substantially.

Gey and co-workers (42) in 1951 obtained the well-known HeLa strain from human cervical carcinoma. This was the first success in the establishment of a stable strain of human epithelial tumor cells in monolayer culture. Following this significant step, several other cell lines became available for diagnostic and research work. The majority of these cell strains were of human tumor origin, e.g., HEp-1 from planocellular cancer of the uterine cervix, HEp-2 from epidermoid cancer of the larynx and HS-1 from a sarcoma, all of which were isolated by Fjelde (37). The cell line designated HEp-3 was derived from an epidermoid carcinoma of the cheek (84). The Detroit-6 epithelial line described by Berman and Stulberg (7) was isolated from sternal bone marrow of a patient with carcinoma of the lung. KB strain derived from epidermoid carcinoma of the oral cavity was reported by Eagle *et al.* (26) in 1955.

Cell strains from glandular tumors were obtained with great difficulty. Among these were LAC and MAC-21 from pulmonary adenocarcinomas (5,10), L-16 from primary cancer of the liver (13), 558 from a mixed salivary gland tumor (124), and a series of strains from mammary tumors (67).

Recently, a hyperdiploid human cell line derived from a neuroblastoma was isolated and has undergone more than 85 subcultivations (86). An epithelial cell line of human origin from an omental metastasis of a rapidly spreading cervical carcinoma was established (121), and a permanent heteroploid human cell line derived from an adenocarcinoma of the throat was reported by Peterson *et al.* (94).

Numerous neoplastic cells of animal origin were also successfully cultivated in artificial media. Two porcine embryonal nephromas were successfully explanted in cell culture by Pirtle (95). Membrane-bound aggregates of dense particles varying in diameter from 45 nm to 65 nm were observed in the cytoplasm of degenerating cells, but the significance of these particles has not been determined. In 1959 Rosanoff (98) reported on a continuous line of malignant cells from a canine Sertoli cell tumor.

In 1954, a rat carcinoma cell line was established by Hull (58) from the tissue of a Walker rat carcinoma which had been maintained in adult Harlan-Wistar rats. The cells were highly malignant for suckling rats; 90% or more of the animals developed tumors when inoculated subcutaneously with 10^6 cells. A stable cell line derived from a rat embryonal nephroma was described by Babcock *et al.* (3) in 1967. Transplantation to young rats was possible upon the inoculation of 10^6 or more cells subcutaneously, or intraperitoneally, but such transplants often regressed spontaneously.

Normal Cell Cultivation

A number of lines of continuously growing normal human cells have been developed. Chang developed subcultures of cells from conjunctiva, liver, appendix, and kidney (11). Perry *et al.* (92) described a strain of human skin epithelium that had been isolated from the skin of a 65-year-old man. One year later, Henle *et al.* (52) reported 5 human cell lines derived from human embryonic intestine, liver, lung, skin and muscle explants, and from a liver biopsy. The early cultures revealed fibroblast and histiocyte-like cells but after 60 to 92 days of total cultivations a new type of cell appeared, which grew rapidly

and could be maintained thereafter in serial passage, whereas the earlier types of cells were lost. Meanwhile, a human epidermal cell line derived from normal human skin was established by Wheeler *et al.* (131). The cells had growth and morphologic characteristics of cancer cells.

Human nasal cells and amnion cells were propagated in continuous cultivation by Jordan (61) and Hayflick (50), respectively. Hsu *et al.* (54) established a normal human skin cell line in 1960.

There are also many cell strains derived from normal animal tissues. Strain L, one of the first cell strains to be established in continuous culture, was derived from normal subcutaneous areolar and adipose tissue of a 100-day-old male C_3H/AN mouse by Earle (28) in 1940 and clone 929 was established by the capillary technique for single cell isolation from the 95th subculture generation of the parent strain L by Sanford *et al.* (104) in 1948. Evans *et al.* (33) reported the cultivation of a strain of cells from the liver of a 2-day-old male mouse in 1952.

Three strains of rabbit fibroblastic cells were established from adult testes, embryonic skin-muscle and adult skeletal muscles by Heff *et al.* (46). One of these cell lines designated RM3-73 required folic acid, nicotinamide, pantothenic acid, pyridoxal, riboflavin, and thiamine for continuous proliferation on a medium containing 2% dialyzed horse serum. Drew (23) and Parker *et al.* (88) also reported established epithelial cells from normal kidney of adult rabbits. In addition to these, Westwood *et al.* (128,129) described the establishment of cell lines from tissues of rabbits, monkeys and man, and drew particular attention to a type of cellular transformation which occurred in several of their strains.

An epithelial cell strain derived from calf kidney, which could be maintained *in vitro*, was isolated by Brion *et al.* (9) in 1957. Meanwhile, serial passage cultures of embryonic bovine lung, muscle, skin and kidney were described (45,127). The cells of each line were predominantly of epithelial type in young cultures and became more spindle-shaped in older cultures. Two other kidney cell lines derived from normal adult bovine and ovine were reported by Madin and Darby (76) in 1958.

A cell line derived from the kidney of a normal adult African Green monkey was reported by Yasumura *et al.* (134) in 1963. This was designated as the Vero cell line which since has been employed extensively in virus replication and other studies. The BHK-21 cell line was derived from the kidneys of 5 unsexed, 1-day-old baby hamsters in 1962 by Macpherson *et al.* (74) and the BHK-21 (C-13) was initiated by single-cell isolation from the parent line of BHK-21 (73). A normal canine kidney cell line was established by Madin *et al.* and characterized by Gaush *et al.* (41). More recently, Kasza (65) established a normal swine kidney cell line designated SK-6.

Only a few cell lines derived from normal rat tissues have been reported. In 1953 Goldblatt *et al.* (44) obtained 2 strains of fibroblastic-like cells originating from fragments of myocardium of a 5-day-old Slonaker-Addis rat. This particular strain of rat was known to develop tumors but cultured cells rarely showed neoplastic changes. This happened only after repeated exposure of the cultures to an atmosphere of nitrogen and did not occur in the control cultures of fibroblasts from the same rat heart grown continuously during the same period without exposure to nitrogen.

Cell Lines with Special Characteristics

In 1966 Tanigaki *et al.* (122) described several human leukemia cell lines which were initiated by Moore *et al.* (85) from peripheral leukocytes obtained from leukemia patients. These possessed immunoglobulin production capacity. In 1968, a new *in vitro* hormone-synthesizing cell system, line BEWO, derived from human gestational chorio-carcinoma, was permanently established by Pattillo *et al.* (91). A high degree of synthesis of functional hormone was maintained for 18 months in culture.

A thyroid adenocarcinoma cell line and a canine melanoma cell line have been under cultivation for more than $2\frac{3}{4}$ years and have been transferred more than 110 times (71). The thyroid cell cultures were epithelioid and arranged in a characteristic syncytial pattern but without colloid production. The melanoma cell line consisted of melanoblasts with spindle or polygonal shapes in a spongelike pattern and maintained the capacity to produce melanin.

Many studies on rat neoplastic cell cultures have been reported. Some were associated with differentiated functions which were characteristics of the tissue from which the cells were derived. Yasumura *et al.* (133) applied the single cell plating technique and developed 4 cloned cell lines of murine origin that performed organ-specific functions after being serially cultured for prolonged periods. These strains included steroid-secreting Leydig cells from a mouse testicle tumor, pigment-forming melanoma cells from a mouse melanoma, and 2 hormone-secreting strains from a rat pituitary tumor. One of the cell lines from the pituitary tumor secreted a substance similar to adrenocorticotropic hormone. A similar report concerned 3 clonal cell strains established from a transplantable rat pituitary tumor by the methods of

alternate culture and animal passage (123). These cells secreted growth hormone.

By the injection of N-nitrosomethyl urea, rat glial tumors were induced and propagated in cultures by Benda *et al.* (6). Among 5 morphological distinct cell strains obtained, one clone contained S-100 protein, which was a unique feature of the brain of vertebrates. Shin *et al.* (109) reported that a stable, clonal, monolayer culture had been established from a transplantable tumor of testicular interstitial cells of the rat. The original culture contained 2 distinct cell types, epithelial and fibroblastic. The epithelial clone isolated from this culture secreted a high level of steroid hormones, was nearly diploid and was functionally stable through extended serial subcultures. The fibroblastic clone showed no steroidogenic capacity.

With single-cell plating and cloning procedures, a clonal strain of rat hepatoma cells which performed a liner-specific function in culture, was established by Richardson *et al.* (97). The cells synthesized rat serum albumin and secreted the protein into the culture medium. The cells have been grown in culture for 15 months and have shown no evidence of loss of specific function.

Application of Cell Cultures in Virology and Other Fields

Application of Cell Lines in Virology

Many of the cell lines described have come into service and are widely used in the cultivation of viruses, the study of metabolic and immunological processes, and cyto- and histochemical characteristics and other investigations.

In 1936 Sabin *et al.* (102) first succeeded in growing poliomyelitis virus in primary human embryonic neural tissue, but not in cultures of

lung, kidney, liver, or spleen tissues. Their findings emphasized the limited affinity of the virus of poliomyelitis. The first successful cultivation of poliomyelitis virus with accompanying cytopathic effects in human primary embryonic cells not of neural origin was achieved by Enders *et al.* (32) 13 years later.

In 1952 Dulbecco (24) introduced a new technique, the plaque assay system, for quantitative studies in animal virology. He demonstrated plaque formation by western equine encephalomyelitis virus in monolayers of chicken embryonic fibroblasts.

The usefulness of stable strains of normal and malignant cells in continuous cultures for the study of viruses has been reported in numerous papers. The adaptation of poliomyelitis virus on stable cell lines was developed through the efforts of Scherer *et al.* (108). They propagated poliomyelitis virus in fibroblastic cells of monkey testicle origin. Following this they demonstrated the usefulness of the HeLa cell line for quantitative studies of poliomyelitis virus and its use in serum neutralization tests to measure antibodies to poliomyelitis virus (107). Chang (11) and Swin *et al.* (120) also reported establishment of human epithelial and fibroblastic cells which were susceptible to poliomyelitis virus and several other human viruses.

The L strain cells were susceptible to infection with the virus of Rous Sarcoma (105) and to pseudorabies and herpes simplex (106). Type A inclusion bodies occurred with the latter two. Field *et al.* (35) described a plaque assay utilizing a stable line of murine embryonic cells (80) for the enumeration of mouse salivary gland virus. McClain and Hackett (79) described growth curves for vesicular stomatitis virus in a variety of monolayer systems, and Bachrach *et al.* (4) reported its growth in monolayer cultures of guinea pig kidney.

Madin *et al.* (75) used monolayer cultures of bovine kidney for the isolation of infectious bovine rhinotracheitis virus. In 1957 Warren *et al.* (125) found that vaccinia and vesicular stomatitis virus (New Jersey and Indiana strains) multiplied in bovine embryonic cell cultures and caused a characteristic cellular destruction. Adenoviruses, herpes simplex, B virus and Semlike Forest virus multiplied initially with characteristic cytopathic effects but could only be serially transferred a few passages.

In 1967 Kasza and Griesemer (64) demonstrated the cytopathic effect of more than 30 viruses on established canine thyroid adenocarcinoma and canine melanoma cell lines (71). Later, Kasza (63) isolated a reovirus from a pig by using different primary cell cultures and cell lines. The results indicated that the canine thyroid adenocarcinoma cell line was more susceptible and thus more useful than primary and established swine kidney cell lines.

A clonal line of swine kidney cells, PS(Y-15), used for the assay of Japanese encephalitis virus was described by Inoue *et al.* (59) in 1964. They found that plaques produced on a line of a wild type of PS cells were irregular in size and had irregular margins, whereas plaques produced on the stable PS cells were larger and uniform in size with regular margins. In 1969 Shadduck *et al.* (110) applied cytochemical and immunofluorescent techniques to study the effects of a pathogenic porcine adenovirus in primary and established swine kidney cell cultures. Their results indicated that the cytopathic effect was divided into 3 phases, and that there was no significant difference between the cell cultures. More recently, Kasza *et al.* (65) suggested that the use of swine kidney cell line SK-6 provided a valuable assay system for the study of swine fever and SV_{40} viruses.

The production of virus vaccines resulted in the establishment of numerous cell strains. In 1962 Hayflick *et al.* (49) used a human fetal diploid cell strain for the preparation of poliomyelitis virus vaccine. Chanock *et al.* (12) also produced a vaccine for the prevention of type 4 adenovirus infection by employing human diploid cells. It was pointed out that continuously cultured tissue cells afforded numerous potential advantages for the propagation of viruses to be used in vaccines (15).

Application of Cell Cultures in Other Fields

The development of cell cultures has contributed greatly in various fields. Sloboda *et al.* (114) analyzed cytologically the S91 mouse melanoma cell line. Their data indicated that control S91 cells contained neither melanin nor the enzyme dihydroxyphenylalanine (dopa) oxidase and the various treatments did not induce melanogenesis in these cells. However, cells exposed to high concentrations of L-dopa contained many granules of melanin which they suggested were phagocytized from the incubation medium.

In 1967 Garg *et al.* (38) described histochemical and electron microscopic studies of dog kidney cells in the early stages of infection with infectious canine hepatitis virus. They found that specific viral granules, which they called initial bodies, were in the nuclei of dog kidney cells as early as 10 hours after infection. These initial bodies had a homogeneously staining matrix with inner granules in the center and at the periphery. These granules resisted and were not affected by deoxyribonuclease.

Kasza *et al.* (64) successfully grew *Toxoplasma gondii* and *Histoplasma capsulatum* on established canine thyroid adenocarcinoma and canine melanoma cell lines.

Numerous cell strains (6,97,109,123,133) have been employed in studies of endrocrinology. Some of these cultures are believed to simulate, to an extensive degree, the physiological behavior of the normal glands *in vivo*. These cells provided a valuable alternative to the more conventional methods of endocrinology and raised doubts about the widely held idea of dedifferentiation in cultures.

Stone *et al.* (116) applied human cell strains for the study of cytogenetic effects of cyclamates, and there has been an interesting attempt to use cultures of various human neoplasms in the selection and study of substances with antineoplastic properties (25,60) and also in the study of aging (51).

More recently, Marble *et al.* (78) cultured *Anaplasma marginale* in a rabbit bone marrow cell strain and maintained it through several passages.

Typing of Cell Lines

Immunological Techniques

Mixed Hemagglutination Test. Based on the common antigen to both tissue cells and red cells of the same species, the mixed agglutination reaction for recognition of the species origin of cells in culture was developed by Coombs *et al.* (16,17). The reaction involves the formation of mixed clumps between tissue cells and indicator red cells if the 2 cell types possess a common antigen.

By employing the mixed agglutination test, Coombs *et al.* (18,19) in 1961 reported that ERK/1, a strain derived from rabbit embryonic kidney, and one of 3 strains of cells which were from pig or rabbit kidney, were contaminated with human cells. One year later, Brand *et al.* (8) found 17 transformed and 14 non-transformed established cell

strains derived from various animal species were either of mouse or human origin.

Immunofluorescence. In 1961 Stulberg *et al.* (117) described the development of an immunofluorescent method for rapid and specific identification of cultured mammalian cells. They conjugated guinea pig anti-cellular globulin fractions with fluorescein isothiocyanate. Dispersed cells were prepared from monolayer cultures of frozen cells and the resulting wet cell suspensions were treated with the labeled antibodies. Their results suggested that hemagglutination and fluorescence may be detecting different groups of antibodies since specific fluorescence was obtained in the absence of hemagglutination. By means of this technique, Simpson and Stulberg (113) showed that 2 of 46 cell lines contained cells of species other than originally designated.

Complement Fixation Test. In 1958 Coriell *et al.* (20) reported the results of antigenic analysis of various cell lines by means of a complement fixation test. In their studies, rabbit immune sera were prepared by injecting pure cultures of normal and malignant cells. A modified Kolmer technique with overnight fixation, and the use of 2 optimal units of antigen as determined by block titration with homologous antiserum, was employed. The results of these tests indicated extensive sharing of common antigens by long-term tissue culture cell lines and even between cells from different species. They concluded that the possible reasons might be due to cell, virus or bacterial contamination, loss of species specificity by the cell and increase of the component essential for the free living state upon prolonged multiplication *in vitro*.

Cytotoxic Antibody Test. McAllister *et al.* (80) showed that a cytotoxic antibody test was useful for immunologic studies of established cell lines. Later, Landy *et al.* (66) and Tuttle *et al.* (125) modified the cytotoxic antibody test and used such to provide a quick and simple method for the species identification of cultured cell strains. In this technique, the death of 50% or more of the cells, as shown by trypan blue uptake, was considered as evidence of antiserum cytotoxicity. The results with 21 cell lines of human and animal origin showed that all reacted specifically with antisera prepared against homologous but not against heterologous cell types.

Isoenzyme Characterization

The development of isoenzyme techniques (135) and the recognition of genetically determined enzyme polymorphisms opened the possibility of utilizing these as stable genetic markers for the detection of intraspecific contamination of cell cultures. Yasin (132) and Harris (47) showed that the incidence of isoenzyme polymorphisms in several different species was quite high and the number ascertainable by electrophoresis for any particular species was potentially large. Thus they suggested that isoenzyme studies might be employed for marking and identifying cell populations which had arisen from different individuals within the species.

In 1967 Gartler (39,40) found that most human heteroploid cell lines contained the same variant form of glucose-6-phosphate dehydrogenase (G-6-PD). He suggested that many of the heteroploid human cell lines developed by investigators might be derivatives of HeLa cells through contamination because of the occurrence of G-6-PD type A and phosphoglucomutase (PGM) type 1 isoenzyme in all of the permanent cell

strains he examined. HeLa cells were of Negro origin and the type A variant of G-6-PD was found in less than 30% of the Negro population and never in Caucasians. Later, Peterson *et al.* (93) used sucrose agar gel and cellulose acetate electrophoretic media for the determination of G-6-PD isoenzyme types. They found that the A and B type of G-6-PD occurred in human diploid cultured cell populations in accordance with the racial origin of the donor, while certain other heteroploid cell lines were found to possess A type G-6-PD irrespective of donor race.

Montes *et al.* (82) examined the G-6-PD and lactate dehydrogenase (LDH) patterns of 86 animal cell lines by using starch gel electrophoresis. They pointed out the significance of the isoenzyme technique in the characterization and identification of animal cell cultures from a variety of species, and they constructed a "fingerprint" identification chart for ready identification of animal cells from 20 of 22 different taxonomic groups.

Chromosome Analysis

On the basis of the different characteristic modal number of chromosomes in various species, Levan (68) employed chromosome analysis to identify some human tumor and normal cell strains. He found that 2 of the normal cell types had near triploid stemline numbers and contained structural chromosome rearrangements indicating that the serial growth *in vitro* had induced modification of their chromosome complement. The chromosome appearance was similar to those of neoplastic cells. Hsu *et al.* (56) studied 5 cell strains of murine origin. They found 2 strains, MC3 and CF1-C9 from bone marrow of mice, which appeared to be contaminated with L strain cells. Later, Clausen *et al.* (14) made a comparison of the chromosome number and morphology in 28 established cell lines; 14

of these lines were transformed, and the rest were not. In no instance was the chromosome morphology of the transformed cells similar to that of non-transformed cells of primary cell cultures of the supposed species of origin. Five cell lines reported from rabbit, 2 from monkey, 1 each from cow and swine, were similar in chromosome morphology to human cells. One supposedly from monkey and 4 from human were, in morphology, similar to mouse. Defendi *et al.* (21) found that 4 cell lines, 3 of supposedly human origin and 1 of rabbit origin, were actually of mouse origin.

Preservation of Cell Cultures

The establishment of numerous strains of mammalian cells created many problems with regard to their maintenance. In 1954 Scherer and Hoogasian (111) studied various methods of preserving cell strains and observed that either fast or slow freezing in media with 20 to 30% glycerol, storage at -70 C, and rapid thawing were optimal conditions for the preservation of HeLa cells.

In a review of the mechanics of freezing (81), it was pointed out that cell damage might be minimized if non-toxic concentrations of water binder were allowed to pass through cell membranes over temperature ranges at which intracellular crystallization and dehydration denaturation usually occur. This principle was applied by Stulberg *et al.* (118). They described a procedure of slow freezing in 5% glycerol medium, storage at -70 C, and rapid thawing as an efficient and practical means of preserving both epithelial and fibroblast-like human cell strains.

One year later, dimethyl sulfoxide had come into use replacing glycerol. Lovelock *et al.* (72) pointed out that its effects are

equivalent, if not superior, to those of glycerol. Ashwood-Smith (1,2) and Dougherty (22) showed that it permeated cells more rapidly and thus provided higher recoveries of certain cell types, particularly fibroblasts and lymphocytes. Lindsey and Chow (69) found that it was a satisfactory method for preserving bovine embryonic kidney cells.

In 1962 Stulberg *et al.* (119) described a method involving a controlled-rate slow freeze apparatus, and the use of liquid nitrogen for storage. They showed that various strains possessed varying viability and freezing characteristics, and that such differences between cell strains provided additional means for cell characterization.

Silver *et al.* (112) showed that shortwave diathermy offered no advantage over agitation in a 37 C water bath for thawing small masses of frozen tissues and cells, but appeared to be more effective in the recovery of viable cells from large pieces of tissue such as canine and human kidney.

MATERIALS AND METHODS

Materials

Cell Cultures

The cell strain being characterized is of rat embryonic skin origin. It was established by Mr. A. W. Roberts (96) at the Clinical Microbiology Laboratory at Michigan State University for use in clinical virology. This cell line had been designated as RESI and had undergone 140 successive subcultures. The cell line was initiated from the skin taken from 7 15- to 20-day-old fetuses obtained by cesarean section from a female Sprague-Dawley strain rat.

Other cell cultures used in this study are listed in Table 1.

Media

The various media used for the propagation of the cell cultures are also summarized in Table 1. Hanks' LHY which consisted of Hanks' balanced salt solution supplemented with 0.5% lactalbumin hydrolysate,[a] 0.05% Tc yeastolate[a] and 10% lamb serum was prepared in the laboratory. All others were obtained as a prepared powder from Grand Island Biological Company (GIBCO).[b] Sera used in the media were also obtained from GIBCO and inactivated at 56 C for 30 minutes prior to use. All

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[a]Difco Laboratories, Detroit, Michigan 48232.

[b]GIBCO, Grand Island Biological Company, 3175 Staley Road, Grand Island, N.Y. 14072.

Table 1. Cell cultures and media used for cultivation

Cell Cultures	Media		Normal Split Ratio
	Growth	Maintenance	
RESI (rat embryonic skin)	Hanks' LHY + 10% lamb serum	Hanks' LHY + 2% lamb serum	1:3
Primary rat embryonic skin	Hanks' LHY + 10% lamb serum	Hanks' LHY + 2% lamb serum	NA*
Bovine embryonic kidney (BEK)	Hanks' LHY + 10% lamb serum	Hanks' LHY + 2% lamb serum	1:2
PK-15 (pig kidney cell line)	Hanks' LHY + 10% lamb serum	Hanks' LHY + 2% lamb serum	1:3
Vero (African Green monkey kidney)	Hanks' LHY + 10% lamb serum	Hanks' LHY + 2% lamb serum	1:3
HEp-2 (human epidermoid carcinoma)	M-199 + 10% fetal calf serum	M-199 + 2% fetal calf serum	1:3
Primary guinea pig kidney (GPK)	Modified EMEM** + 10% fetal calf serum	Modified EMEM + 2% fetal calf serum	NA
Equine kidney (EK)	Modified EMEM + 10% fetal calf serum	Modified EMEM + 2% fetal calf serum	1:3
Primary mouse embryo (ME)	Modified EMEM + 10% fetal calf serum	Modified EMEM + 2% fetal calf serum	NA
BHK-21 (baby hamster kidney)	Modified EMEM + 10% fetal calf serum	Modified EMEM + 2% fetal calf serum	1:3
CKT*** (dog kidney adenocarcinoma)	Modified EMEM + 10% fetal calf serum	Modified EMEM + 2% fetal calf serum	1:3

*Not applicable.

**Eagle's Minimum Essential Medium, supplemented with 0.5% lactalbumin hydrolysate, 1 mM sodium pyruvate and nonessential amino acids.

***A. Wayne Roberts and G. R. Carter, Clinical Microbiology Laboratory, Michigan State University.

media were prepared with triple glass distilled water and sterilized by positive pressure filtration through a 0.22 μ membrane filter.[c] The media were collected in sterile 500-ml screw-cap bottles and stored at 4 C. Prior to filtration, the following antibiotics[d] were added: potassium penicillin G, 150 units per ml; streptomycin sulfate, 150 μg per ml; and polymyxin B sulfate, 75 units per ml.

Additional media used for the purpose of determining cell-media compatibility were Basal Medium (Eagles) (BME) with Earle's base and modified McCoy 5-A, both obtained from GIBCO.

Trypsin Solution (0.25%)

The trypsin solution was prepared from 1:250 DIFCO trypsin in a final concentration of 0.25% by weight using Hanks' BSS as the diluent. Antibiotics and sterilization were the same as described for the media; storage was at -20 C.

Versene Trypsin Solution

The versene-trypsin stock solution (10x) was prepared from disodium ethylenediaminetetraacetate (EDTA) and 1:250 DIFCO trypsin in a final concentration of 0.2% versene and 0.5% trypsin by weight, using Hanks' BSS as the diluent. Antibiotics as previously described were added at the rate of 10x normal. The solution was sterilized by filtration and stored at -20 C in 50-ml amounts. For use, 1 part of the stock solution was diluted with 9 parts triple distilled water.

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[c]Millipore Corporation, Bedford, Massachusetts 01730.

[d]Pfizer, Laboratories Division, 6460 West Cortland Street, Chicago, Illinois 60635.

Fluorescent Antibody Conjugates

Immunofluorescence was one method employed to determine RESI cell susceptibility with certain viruses. The conjugates used and their source are listed in Table 2.

Glass and Plastic Ware

All glassware employed in this study was hard borosilicate.[e] Water dilution bottles with plastic screw caps were used for stock cultures of cells and 16 x 150 mm test tubes with silicone rubber stoppers[f] were used for viral susceptibility tests. Disposable 30-ml plastic flasks[g] were used for growth efficiency, growth rate, and media evaluation. One milliliter glass ampoules[h] were used for the freezing and storage of cells and viruses.

Laboratory Animals

A New Zealand White rabbit was used for the production of anti-rat hyperimmune serum and suckling mice and chicken embryos were used in the propagation of some of the viruses. Weanling rats and weanling hamsters were used to test for the capacity of the RESI cells to produce neoplasms.

Viruses

Viruses employed in this study are listed in Table 3.

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[e]Kimble Products, Owens-Illinois, Toledo, Ohio.

[f]The West Company, Phoenixville, Pennsylvania.

[g]Falcon Plastics, Division of B-D Laboratories, Inc.

[h]Weaton Glass Company, Millville, New Jersey.

Table 2. Fluorescent antibody conjugates

FA Conjugate	Source
Infectious bovine rhinotracheitis	NADL*
Bovine viral diarrhea	NADL
Hog cholera	NADL
Canine distemper	BioTec Laboratories**
Canine herpesvirus	BioTec Laboratories
Canine SV_5	BioTec Laboratories
Transmissible gastroenteritis	Dr. J. W. Black***
Anti-rabbit globulin	The Sylvana Company†

*National Animal Disease Laboratory, Ames, Iowa.

**BioTec Laboratories, Inc., 9456 Marshall Drive, Lenexa, Kansas 66215.

***Kentucky Department of Agriculture, Hopkinsville, Kentucky 42240.

†The Sylvana Company, 22 East Willow Street, Milburn, New Jersey 07041.

Table 3. Viruses

Virus	Strain or Designation	Contributor	Stock virus prepared in	Titer ($TCID_{50}$/0.1 ml)
Human Viruses				
Adenovirus type 1	AD-71*	Dr. M. Becker**	HEp-2	5.0***
Poliovirus type 2	Sabin	Dr. M. Becker	Vero	7.5
Herpes simplex	MacIntyre	Dr. M. Becker	Vero	6.5
Influenza B	---*	Dr. M. Becker	Vero	2.5
Coxsakievirus A_4	72-394(MDPH)	Dr. M. Becker	Suckling mice	6.5(LD_{50}/0.1ml)
Coxsakievirus A_{16}	72-399(MDPH)	Dr. M. Becker	Vero	5.5
Coxsakievirus B_2	---*	Dr. M. Becker	Vero	5.5
Coxsakievirus B_5	---*	Dr. M. Becker	Vero	7.5
Coxsakievirus B_6	---	Dr. W. Mack†	Vero	5.5
Echovirus 4	Pesascek	Dr. M. Becker	Vero	3.5
Echovirus 9	Hill	Dr. M. Becker	Vero	4.5
Animal Viruses				
Bovine adenovirus type 3	WBRI	Mr. P. Stuart††	BEK	2.5
Infectious bovine rhinotracheitis (IBR)	---	Pitman Moore	BEK	6.0

Table 3 (cont'd.)

Virus	Strain or Designation	Contributor	Stock virus prepared in	Titer ($TCID_{50}$/0.1 ml)
Bovine viral diarrhea (BVD)	NADL	NADL	BEK	3.0
Equine rhino-pneumonitis	A-163	Dr. J. T. Bryan†††	EK	3.5
Equine encephalo-myelitis, Eastern (EEE)	---	Pitman Moore	Vero	6.5
Equine encephalo-myelitis, Western (WEE)	---	Pitman Moore	Vero	6.0
Horse cyto-megalovirus	82-A	ATCC$^{\phi}$	EK	3.0
Vesicular stomatitis	Indiana	ATCC	Chicken embryo (CE)	5.0(LD_{50}/0.1ml)
Hog cholera (HCV)	MSU isolate	MSU$^{\phi\phi}$	Pk-15	5.0(PFU/0.1ml)
Pseudorabies	---	NADL	Pk-15	6.5
Transmissible gastroenteritis (TGE)	MSU isolate	MSU	Pig	unknown
Canine herpes	MSU isolate	MSU	CKT	2.5
Canine distemper	Rockborn	Cornell$^{\phi\phi\phi}$	CKT	unknown
Canine SV_5	958	Cornell	CKT	5.0
Canine adeno-virus type 2	Manhattan (Cornell isolate)	Cornell	CKT	6.5

Table 3 (cont'd.)

Virus	Strain or Designation	Contributor	Stock virus prepared in	Titer ($TCID_{50}$/0.1 ml)
Mouse adenovirus	FL	ATCC	Primary mouse kidney	5.25
Mouse hepatitis	MHV 3 (originally Craig)	ATCC	Mice	unknown
Pneumonia virus of mice	#15	ATCC	BHK-21	HA 1:256, mouse RBC
Encephalo-myocarditis	EMC	ATCC	Mice (I.C.)ΔΔ	7.8 (LD_{50}/ 0.03ml)
Salivary gland virus (mouse)	Smith MSGV	ATCC	Primary mouse kidney	4.5
K-virus	Kilham	ATCC	Mice (I.C.)	7.0 (LD_{50}/ 0.1ml)
Guinea pig herpesvirus	LK 40	ATCC	Primary guinea pig kidney	4.0
Rat virus	Kilham	ATCC	Rat embryonic cells	5.0
Avian Viruses				
Infectious bronchitis (IBV)	IBV-42 (#5 Vero)	Dr. C. H. Cunningham[Δ]	Vero	4.0
Newcastle disease	Roakin	Dr. C. H. Cunningham	CE	6.0 (LD_{50}/ 0.1ml)

Table 3 (cont'd.)

Virus	Strain or Designation	Contributor	Stock virus prepared in	Titer ($TCID_{50}$/0.1 ml)
Infectious laryngo-tracheitis (ILT)	MSU isolate	MSU	CE	4.0 (LD_{50}/ 0.1ml)
Fowl pox	MSU isolate	MSU	CE	5.0 (LD_{50}/ 0.1ml)

*Obtained through Center for Disease Control by Dr. Becker.

**Michigan Department of Public Health (MDPH).

***Reciprocal of log of viral dilution.

†Department of Microbiology and Public Health, Michigan State University.

††The Central Veterinary Laboratory, Ministry of Agriculture, Fisheries and Food, New Haw, Weybridge, Surrey, England.

†††University of Kentucky.

ϕAmerican type culture collection.

ϕϕClinical Microbiology Laboratory, Michigan State University.

ϕϕϕVeterinary Virus Research Institute, Cornell University.

ΔDepartment of Microbiology and Public Health, Michigan State University.

ΔΔIntracerebral inoculation.

Methods

General Cell Culture Procedures

Preparation of Primary Cells. All tissues for primary cells were obtained from normal animals which had been exsanguinated. Tissues were removed aseptically and placed in sterile beakers containing Hanks' BSS, prewarmed to 37 C to prevent drying. They were then removed and placed in sterile petri dishes where they were minced finely with scissors. The minced tissues were placed in sterile trypsinizing flasks[i] with Teflon coated magnetic stirrers[j] and approximately 50 ml of Hanks' BSS per gm of tissue was added. The flasks were stirred on electromagnets[j] for approximately 2 minutes. Tissues were allowed to settle and the supernatant fluids were discarded. This step was repeated twice after which approximately 50 ml of 0.25% trypsin solution per gm of tissue was added. Flasks were then placed on electromagnets and trypsinization was carried out at 4 C overnight (approximately 16 hours). Following trypsinization, cell suspensions were passed through 4 layers of sterile gauze to remove untrypsinized large fragments. Suspensions were then poured into 50-ml conical centrifuge tubes[k] and centrifuged at approximately 600 rpm (250 xg) for 10 minutes in a horizontal swinging-head.

The supernatant fluids were discarded and the resulting packed cells seeded into appropriate growth media (Table 1) at the rate of

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[i] Kimble Products, Toledo, Ohio 43601.

[j] Scientific Products, Evanston, Illinois.

[k] Corning Glass Works, Parkersburg, West Virginia.

1.5×10^6 cells per ml. Cell suspensions were then added to water dilution bottles, flasks and tubes depending on the need. Amounts added were 15 ml, 5 ml and 1 ml, respectively. All were incubated at 36 C in a stationary position. Tubes were placed in a test tube rack[1] with a 5° slope. After 24 hours' incubation, the media were decanted and fresh growth media were added.

Maintenance and Subculture of Cells

After monolayers were formed, growth media were replaced with essentially the same media except that the serum content was lowered to 2% (see Table 2). Cells were refed with such media at 4-day intervals or when considerable lowering of the pH was evident.

All cells were routinely subcultured at 7- to 12-day intervals and at other times when appropriate. Maintenance media were removed and the cells were rinsed for a few seconds with a few milliliters of versene-trypsin solution. Additional versene-trypsin solution was added in an amount equal to that used for the growth and maintenance media. The cells were then incubated at 36 C for 3 to 10 minutes, and shaken occasionally until the cells detached from the surface. Cell suspensions were poured into 50-ml conical centrifuge tubes and centrifuged as described earlier. The supernatant fluids were discarded and 3 ml of growth media were added to the packed cells. The cells were aspirated with 5-ml pipettes equipped with rubber bulbs and the resulting suspensions were added to additional growth media in order to obtain the desired split-ratio (see Table 1).

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[1]Arthur H. Thomas Company, Philadelphia, Pennsylvania.

Evaluation of RESI Cells

Growth Rate. The 150th subculture was used for the growth rate study. Methods used were similar to those previously described (65). RESI cell suspension containing 9.0×10^4 cells per ml was added to 40 disposable tissue culture flasks at a rate of 5 ml per flask. Cell counts were made at intervals of 12 hours during the first 8 days and at 24 hours for an additional 5 days. Cells from 2 flasks were counted each time and the average number was calculated. Dispersion and collection of the cells were as described earlier for subculturing. Four separate counts were made in a hemacytometer and the average number of cells was taken.

Growth Efficiency. The 148th passage was used for the growth efficiency study. The procedures followed were similar to those previously described (65). Cell concentrations of 6.25×10^3, 1.25×10^4, 2.5×10^4, 5.0×10^4, 1.0×10^5 and 2.0×10^5 cells per ml were seeded in 5-ml amounts into disposable plastic flasks. Two flasks for each concentration were used. Growth media were replaced every 5 days and the time required for confluent monolayers to form was recorded.

Maintenance Test. The 152nd subculture was used for the maintenance test. The cell suspension was distributed to 4 disposable plastic flasks in 5-ml amounts. The concentration of cells was 2.5×10^5 cells per ml. Two of the flasks were refed with maintenance media as soon as confluent monolayers were formed and thereafter at 5-day intervals. Media in the other 2 flasks were not changed and remained the same throughout the experiment. Observations were made as to how well cells retained their healthy appearance.

Sterility Test. For the sterility test, the 150th passage was used. The cell sheet was rinsed with an antibiotic-free growth medium and then removed by scraping with a rubber policeman. The cells were washed twice with antibiotic-free growth medium and resuspended at the normal split ratio. After developing confluent monolayers, the cells and medium were collected and inoculated onto blood agar, Sabouraud dextrose agar and mycoplasma media. Cells were also checked for possible viral contaminants by electron microscopy.

Cell Preservation

Storage of Cells. The 149th subculture was preserved by freezing. Cells were collected and diluted with the freeze medium to approximately 2.5×10^6 per ml. The freeze medium consisted of the growth medium with 2x normal concentration of serum and with dimethyl sulfoxide (DMSO) at a final concentration of 10%. The cell suspension was distributed in 1-ml amounts to ampoules which were subsequently sealed by direct flame. Proper sealing was checked by placing the ampoules in a beaker containing a crystal violet solution. This was then placed in a desiccator jar and a vacuum was pulled. When the vacuum was released the dye entered unsealed ampoules which were discarded. Ampoules were placed at -20 C for 16 hours after which some were removed and placed at -70 C and in liquid nitrogen.

Thawing of Frozen Cell Suspension and Initiation of Cell Growth

Frozen cells were checked for viability at intervals of 30 days. The ampoules were removed from the freezer or liquid nitrogen and rapidly thawed in a 37 C water bath. The thawed cell suspensions were immediately seeded to water dilution bottles containing 15 ml of prewarmed growth medium. Dye exclusion tests were employed for determining

viability of the frozen cell suspensions. One milliliter of each cell suspension was mixed with 1 ml of 0.4% trypan blue. Stained and unstained cells were enumerated by 4 separate counts in a hemacytometer. The results were expressed as the percentage of viable cells per ml. The water dilution bottles containing the remainder of the cells were incubated at 36 C and the rate of outgrowth was observed.

Media Evaluation

The 164th subculture was employed for media evaluation. According to the methods for subculturing, the cells were resuspended in different growth media, Hanks' LHY, EMEM, modified EMEM, M-199, BME, and McCoy 5A. The concentration was 1.5×10^5 cells per ml. Daily microscopic observations were made, and the time required for the development of confluent monolayers was recorded.

Chromosome Study

The chromosome study was performed by a modified technique developed from that of Rothfels *et al.* (99) and Patil *et al.* (90). The 70th, 154th and 171st subcultures were used for the chromosome study. Three-day-old cells in water dilution bottles were treated with a growth medium containing 6 µg colchicine per ml and incubated at 36 C for 3 hours. The medium was decanted and the cells were detached from the glass with versene trypsin solution. The cell suspension was poured into a conical centrifuge tube and centrifuged as described earlier. The supernatant fluid was carefully removed and 10 ml of prewarmed (37 C) 1% sodium citrate was added. The cells were gently but thoroughly resuspended and then incubated for 10 minutes at room temperature (25 C). This suspension was then centrifuged and all but approximately 0.3 ml of the supernatant fluid was removed. The cells were resuspended in this amount and 5 ml

of freshly prepared cold Carnoy's fixative (1 part glacial acetic acid; 3 parts reagent grade methanol) was added. Without bubbling air through the fixative, the suspension was mixed thoroughly with a 5-ml pipette. The mixture was allowed to stand at room temperature for 5 minutes after which it was centrifuged lightly. The supernatant fluid was discarded and fresh cold Carnoy's fixative was added and mixed. This mixture was allowed to stand at room temperature for 8 minutes. This fixation was repeated a third time and allowed to stand for 10 minutes. After the third change of Carnoy's fixative, the cells were centrifuged and all but 0.5 ml of the fixative was removed. Cells were resuspended in this amount. Using a Pasteur pipette, 1 or 2 drops of the cell suspension were dropped from a height of 5 to 6 inches onto a clean microscope slide freshly dipped in 70% methanol. The slide was immediately and quickly passed through a flame to ignite the methanol. When the flame had burned out the slide was exposed to a gentle stream of air to remove remaining water. Slides were stained with a modified Giemsa's stain (90), pH 9, 10 minutes at room temperature (25 C). Stained spreads were washed in cold running tap water for 1 minute and air dried. The chromosome numbers were counted under a light microscope using 45x and 100x objectives with a 10x ocular.

Neoplastic Properties of Cells

The 166th subculture was used to determine RESI neoplastic properties. The inoculum of 0.2 ml contained 1.0×10^6 cells in saline solution and was administered to weanling rats and weanling hamsters. Two rats were inoculated subcutaneously and 2 were inoculated intraperitoneally. The 2 hamsters were inoculated via the cheek pouch. Control rats and hamsters were inoculated with primary RES cells, cells derived

from a canine kidney adenocarcinoma (CKT), and saline solution only. The number of cells and the amount of inocula were the same as for the RESI cells. The rats and hamsters were observed daily and killed 4 weeks postinoculation.

Cell Typing

Preparation of Anti-Rat Hyperimmune Serum. Primary rat embryonic skin cells were cultured as described earlier. After monolayers were formed, the cells were rinsed with PBS (pH 7.5) solution and removed from the glass with a rubber policeman. The cells were washed in 2 additional changes of PBS solution. Cell counts were made and the cells, amounting to 6.5×10^7 cells, were suspended in 6 ml of distilled water. Of this, 1 ml was mixed with an equal part of Freund's complete adjuvant. A New Zealand White rabbit was injected subcutaneously at 4 sites on the back, using 0.5 ml of the emulsion at each site. The rabbit received a booster injection intravenously with 1 ml of the cell suspension without adjuvant 2 weeks later. Two more injections of 2 ml of cell suspension were administered intravenously at weekly intervals. The rabbit was bled 2 weeks after the last injection. The serum was collected and inactivated at 56 C for 30 minutes. The titer of the serum was determined by a hemagglutination test using rat red blood cells.

Immunofluorescent Technique

Species antigens of the RESI cell line were detected by a modified technique described by Stulberg *et al.* (117). The 156th subculture of cells was used in the determination. The Vero cell line and primary rat embryonic skin cells were used for controls. The cells of

monolayer cultures were dispersed with versene trypsin solution and the resulting cell suspensions were washed 3 times with PBS solution pH 7.0. Each cell suspension was brought to approximately a 2% concentration by volume in PBS solution. Two-tenths milliliter of each cell suspension and 0.2 ml of a mixed suspension of RESI cells and Vero cells were mixed separately with equal volumes of the anti-rat hyperimmune rabbit serum. The mixtures were placed in serologic tubes and placed in a 37 C shaker water bath. They were agitated moderately for 30 minutes after which the suspensions were washed with 3 changes of PBS solution. Following the last wash all but approximately 0.5 ml of the supernatant fluids were decanted, and the cells were resuspended. Two-tenths milliliter of a 1:4 dilution of fluorescein labeled anti-rabbit globulin was added to each tube which was again agitated for 30 minutes. Stained suspensions were washed with 3 changes of PBS solution and the cells were resuspended as described above. One drop of each suspension was placed on fluoro-slides[m] and covered with #1 coverslips. They were examined with a Zeiss fluorescence microscope using BG-12 and UG-2 exciter filters (Schott) and OG-4 and GG-4 barrier filters (Schott).

Viral Susceptibility Spectrum

The 151st subculture and several subsequent subcultures were used for viral susceptibility tests. Cells were cultivated in 16 x 150 mm culture tubes with silicone rubber stoppers. When monolayers were formed the growth media were replaced with maintenance media. Two tubes were used for each viral suspension and the inoculum was 0.2 ml per tube. The viral suspensions were stock preparations diluted 1:10.

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[m]Aloe Scientific, St. Louis, Missouri.

The cultures were observed daily under a light microscope for cytopathic effect (CPE). The cells and media were collected and frozen when sufficient CPE developed (2+ or greater) or at 7 days postinoculation. At least 3 subcultures were made with each virus. Viruses that did not cause CPE were examined by other techniques following the 3rd subculture. These techniques were immunofluorescence, inclusion body staining and inoculation back into the original host system (Table 2). Giemsa's stain was used in staining for inclusion bodies. Those viruses that grew were subsequently titrated on the RESI cell line and in the original host system used for preparing the stock viruses in order to determine RESI cell sensitivity.

RESULTS

General Features of RESI Cells

The RESI cell line was ordinarily transferred once a week at a 1:3 split ratio with a resulting confluent monolayer developing on the 3rd day. The cultural pattern was fibroblastic (Figures 1 and 2) throughout the cultivation. The cells were spindle-shaped with central nuclei which were rounded or slightly elongated, and contained 2 to 7 nucleoli. The cell sheet was firmly attached to the glass and 3 to 5 minutes were required to separate the cells when subculturing.

Growth Rate

The growth rate is shown graphically in Figure 3. The seeding rate was 0.9×10^5 cells per ml. Cellular proliferation was slow for the first 12 hours after seeding, but by the end of 24 hours the cell population had approximately doubled. The cell number had tripled at near 48 hours, and at 72 hours a confluent monolayer was formed with the cell population having approximately quadrupled. Cellular proliferation increased after the first medium change and the cells continued to replicate until the 9th day, after which a slight decrease in cell population occurred. At the end of the 9th day, the number of cells had increased approximately 16 times that of the original concentration.

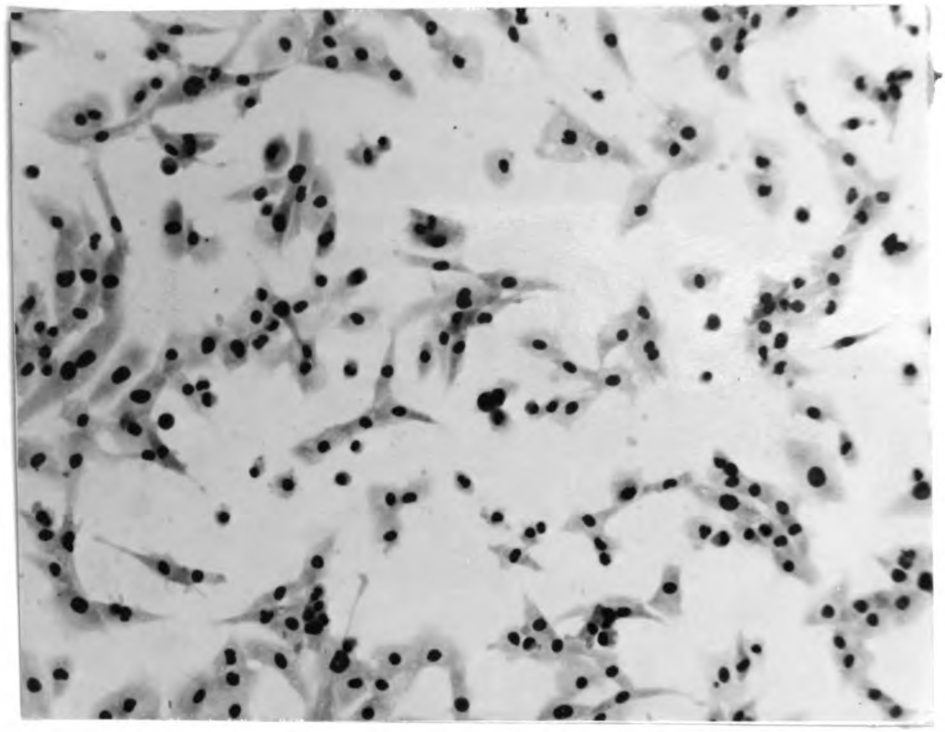

Figure 1. RESI cell culture, 166th passage, 12-hour-old culture, Giemsa's stain; x 130.

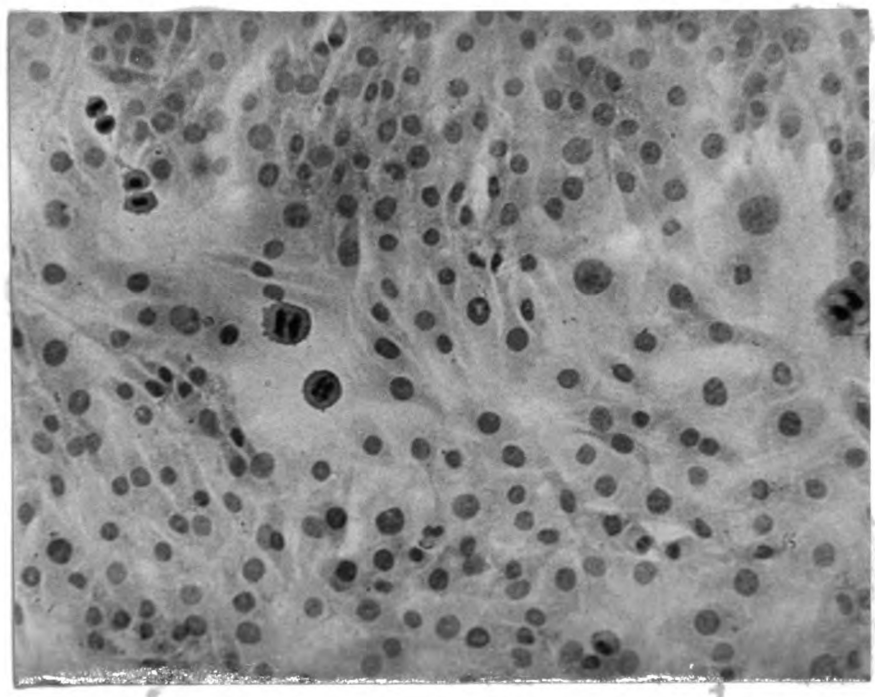

Figure 2. RESI cell culture, 166th passage, 3-day-old culture. Note mitotic figures. Giemsa's stain; x 208.

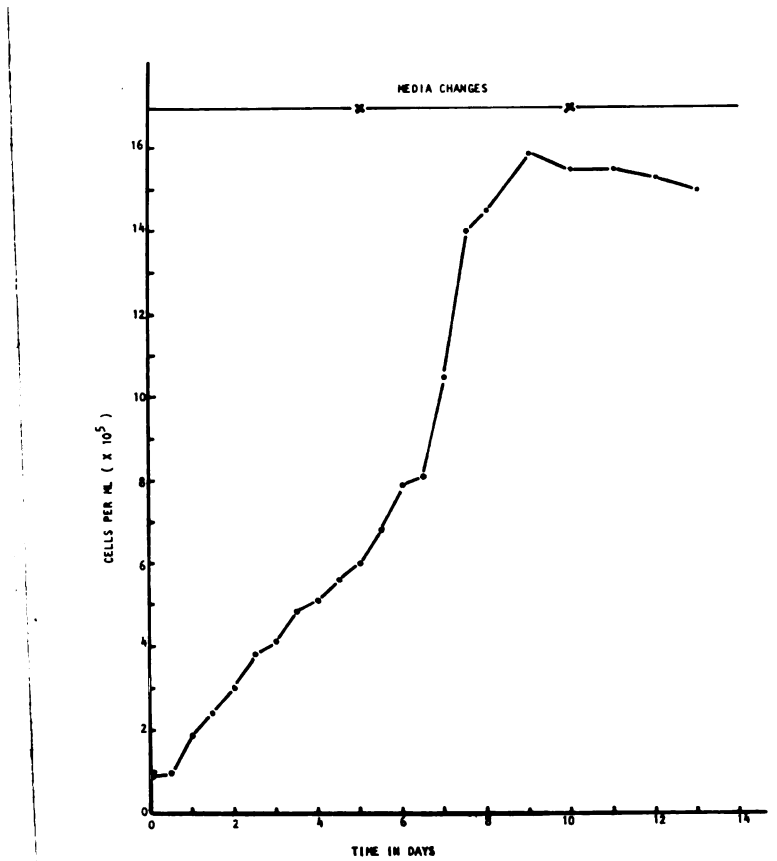

Figure 3. Growth curve of RESI cell line with media change.

Growth Efficiency

The growth efficiency is shown graphically in Figure 4. When the seeding rate was 6.5×10^3 cells per ml, a confluent monolayer developed in 12 days. At a seeding rate of 1.0×10^5 cells and 2.0×10^5 cells per ml, the cell sheet became confluent on the 3rd day and 2nd day, respectively.

Sterility Test

The cell cultures were free of fungi, bacteria, mycoplasmas and viruses when examined by appropriate methods. Examination by electron microscopy did not reveal any organisms in the cells.

Media Evaluation

The results of media evaluation are summarized in Table 4. Confluent monolayers were formed in 3 days with cells growth in Hanks' LHY, modified EMEM and McCoy 5A media. In EMEM medium, 6 days were required for the formation of a confluent monolayer, and in BME and M-199 media 12 days were required.

Table 4. Days required for the formation of confluent monolayers in different media

Medium	Hanks' LHY	Modified EMEM	McCoy 5A	EMEM	BME	M-199
Day	3	3	3	6	12	12

Cell Preservation

The results of tests for the viability of frozen cells are presented in Table 5. The cells survived at -70 C and in liquid nitrogen

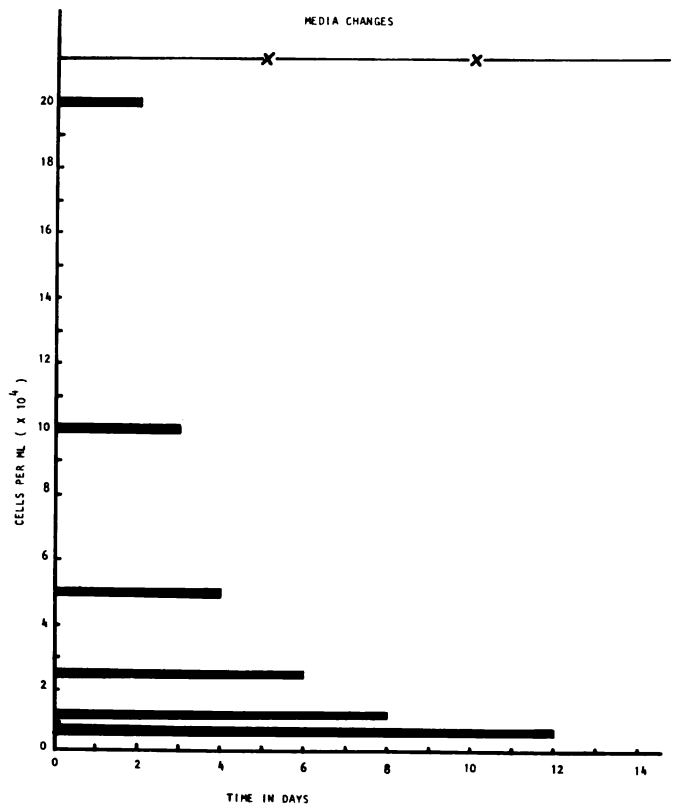

Figure 4. Histogram indicating time required for development of confluent monolayers at various seeding rates.

for at least 4 months. The percentage of viable cells was essentially the same at 1 month and at 4 months for cells stored in liquid nitrogen. Cells stored at -70 C appeared to be damaged most within the first 2 months' period of storage, and storage at -20 C was totally ineffective for preserving the cells. The 69th subculture had been preserved in liquid nitrogen for 3 years; 63% of the cells were viable when tested.[n]

Table 5. Storage at -20 C, -70 C and in liquid nitrogen of RESI cell line

Time in Storage	Dye Exclusion Test: Percentage of Viable Cells at		
	-20 C	-70 C	Liquid Nitrogen
30 days	0	60 (4 days)*	87 (3 days)
60 days	0	47 (9 days)	84.5 (3 days)
90 days	-	50 (9 days)	82 (3 days)
120 days	-	49.5 (9 days)	81 (3 days)
3 years	-	-	63 (4 days)

*Days required for confluent monolayer.

Maintenance Test

The cells could be maintained for 12 days without a change of medium. Degeneration began on the 13th day and the cells completely detached from the glass on the 17th day after seeding. When the growth medium was replaced with the maintenance medium and refed at 5-day intervals, cells could be maintained for 21 days. Cell degeneration occurred on the 22nd day and the cells completely detached from the glass on the 26th day.

[n]Preserved by Mr. A. Wayne Roberts.

Cell Typing

The interpretation of the staining reactions was based entirely on the presence or absence of peripheral fluorescence of the cells. Specific fluorescence was observed with RESI and RES cells, but not with Vero cells. Figure 5 shows the specific staining of RESI cells in a mixture of RESI and Vero cells.

Chromosome Study

The RESI cells were heteroploid with chromosome numbers ranging from subdiploid to tetraploid. Four hundred metaphase cells of the 154th subculture were observed. The greatest number of metaphase figures was clustered in a narrow range of 38 to 42 chromosomes. The modal chromosome number was a subdiploid 40 (19.75% of cell population) (Figure 6), rather than the diploid number of 42 (13.75% of cell population). No consistent marker chromosome was observed although in approximately 12% of the cells a large metacentric chromosome was present (Figures 7 and 8). The 2 largest pairs of chromosomes were subtelocentric and the medium-sized chromosomes were all metacentric and represented approximately 40% of the total chromosome number. The chromosome patterns of the 70th and 171st subcultures were similar to that of the 154th subculture except slightly different proportions of the modal and diploid numbers were evident. In the 70th subculture 16.5% of the cells had a modal number of 40 and 15.3% of the cells contained the diploid number. Examination of the 171st subculture showed that 25% of cells had a modal number of 40 chromosomes and 15% of cells contained 42. The large metacentric chromosome was represented in approximately 12% of cells of both the 70th and 171st subcultures.

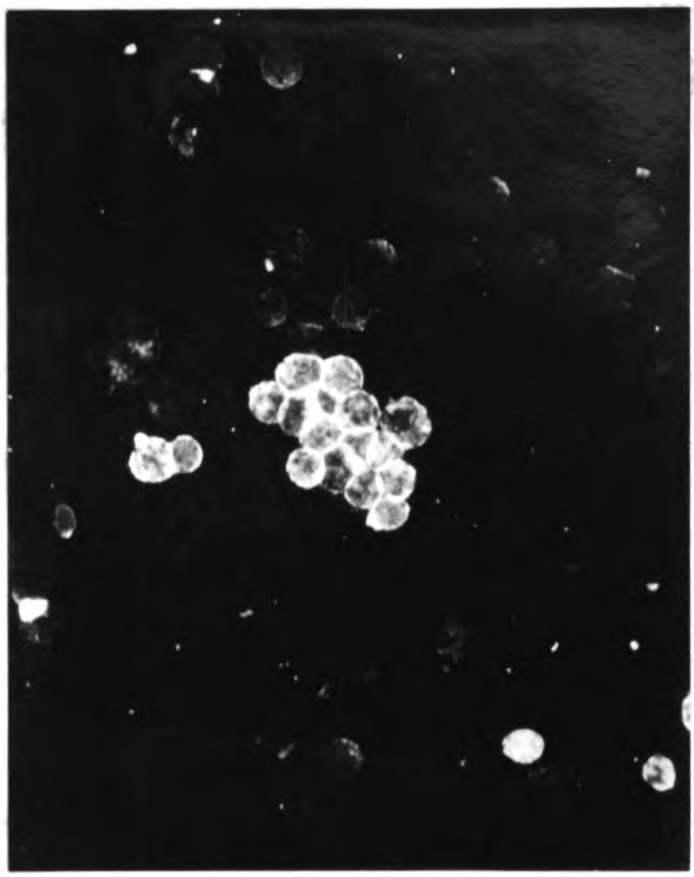

Figure 5. Mixture of RESI cells and Vero cells stained with anti-rabbit globulin conjugate, showing specific reaction with RESI cells; x 400.

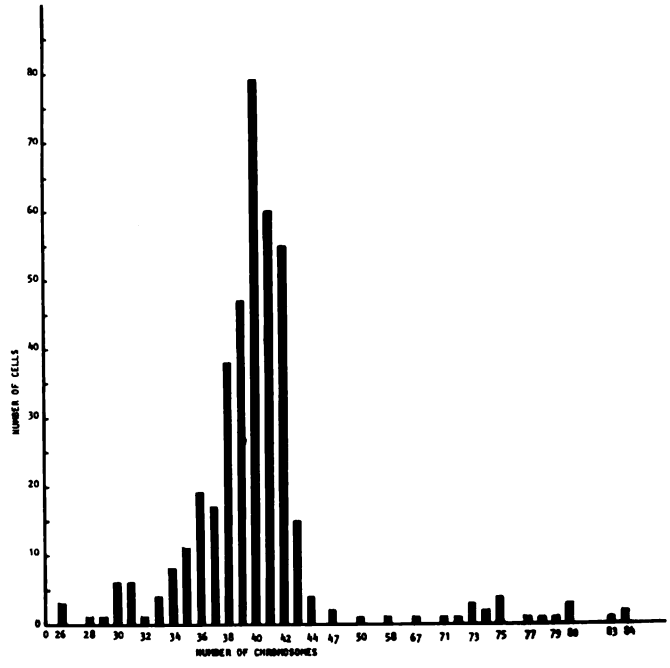

Figure 6. Histogram of the RESI cell line prepared from chromosome counts on 400 metaphase figures. Note the mixoploid population of the cells and the modal chromosome number of 40.

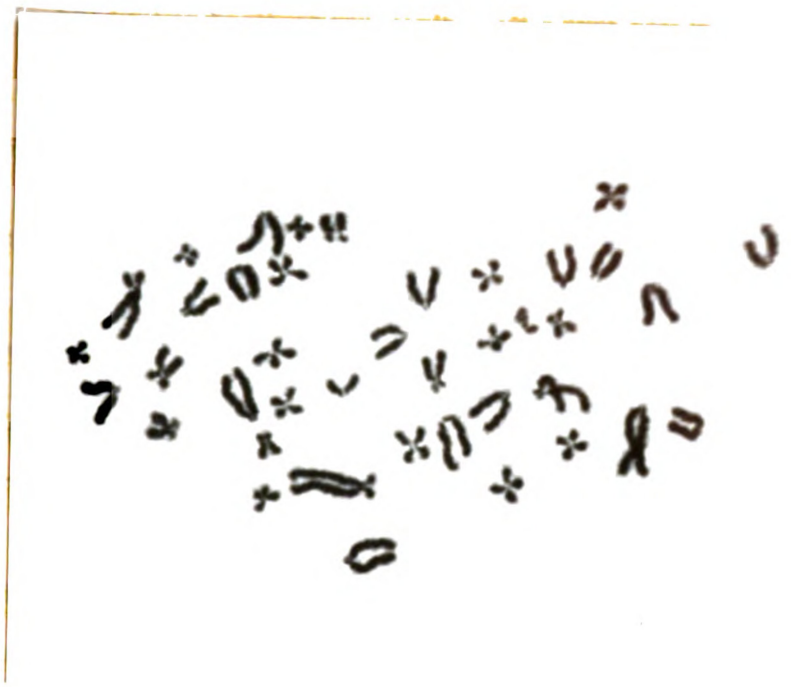

Figure 7. Metaphase figure with 40 chromosomes from the 154th subculture of the RESI cell line. Giemsa's stain; x 1300.

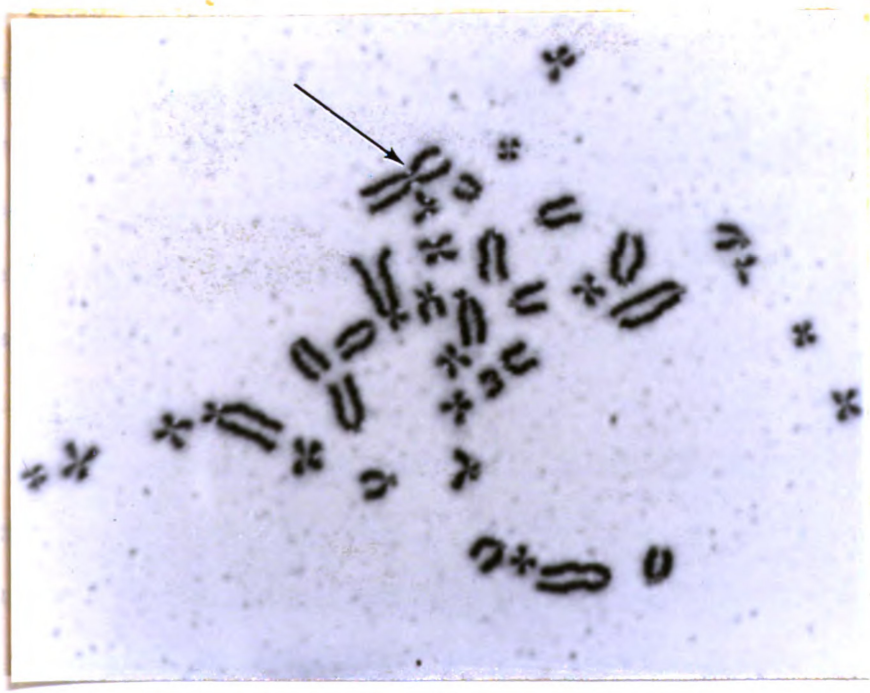

Figure 8. Metaphase figure with 37 chromosomes from the 154th subculture of the RESI cell line. Note large metacentric chromosome (arrow). Giemsa's stain; x 1300.

The percentages of cells containing the modal number of chromosomes in the 3 different cultural levels, and the percentages of cells containing the diploid number, are summarized in Table 6.

Table 6. Distribution of the cell population of 40 and 42 chromosomes in the 70th, 154th, and 171st subcultures

Subculture	Percent of 40 chromosomes in the RESI cell population	Percent of 42 chromosomes in the RESI cell population
70th	16.5	15.3
154th	19.75	13.75
171st	25	15

Neoplastic Properties

Rats inoculated subcutaneously with 1.0×10^6 cells developed nodules which became palpable in 2 weeks and continued to increase in size. At 3 weeks postinoculation they had reached a size of 1.0 x 1.5 cm and were removed for histopathologic examination. Tumors were considered to be fibrosarcoma-like (Figures 9 and 10). Tumor cells varied in the size and shape of the nuclei with hyperchromatism and irregular coarse chromatin. The tumor cells were grown *in vitro* for comparative studies. Relatively slight variations in size and shape of nuclei from those of RESI cells were observed (Figure 11). The development of confluent monolayers in secondary tumor cells required the same period as did the RESI cells. The chromosome pattern also revealed similarities to that of RESI cells. It was heteroploid with a modal number of 40 (34%) and a considerable number of diploid cells (14%). The occurrence of the large metacentric chromosome had increased considerably (38%).

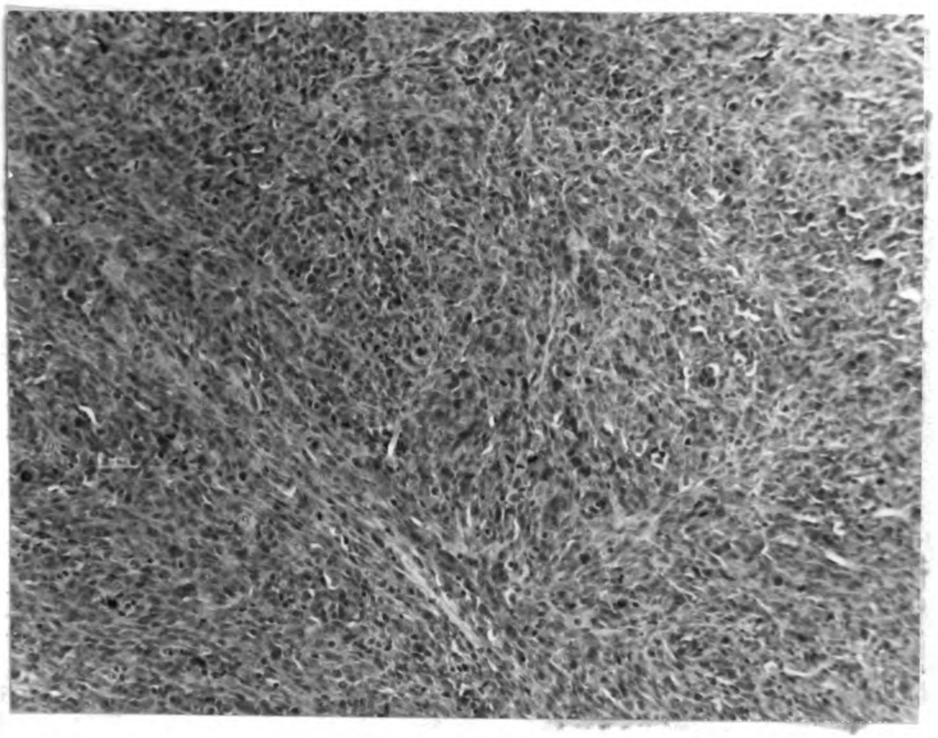

Figure 9. Section of fibrosarcoma-like tumor. Note the varied size and shape of the nuclei with hyperchromatism and irregular chromatin. Hematoxylin and eosin stain; x 130.

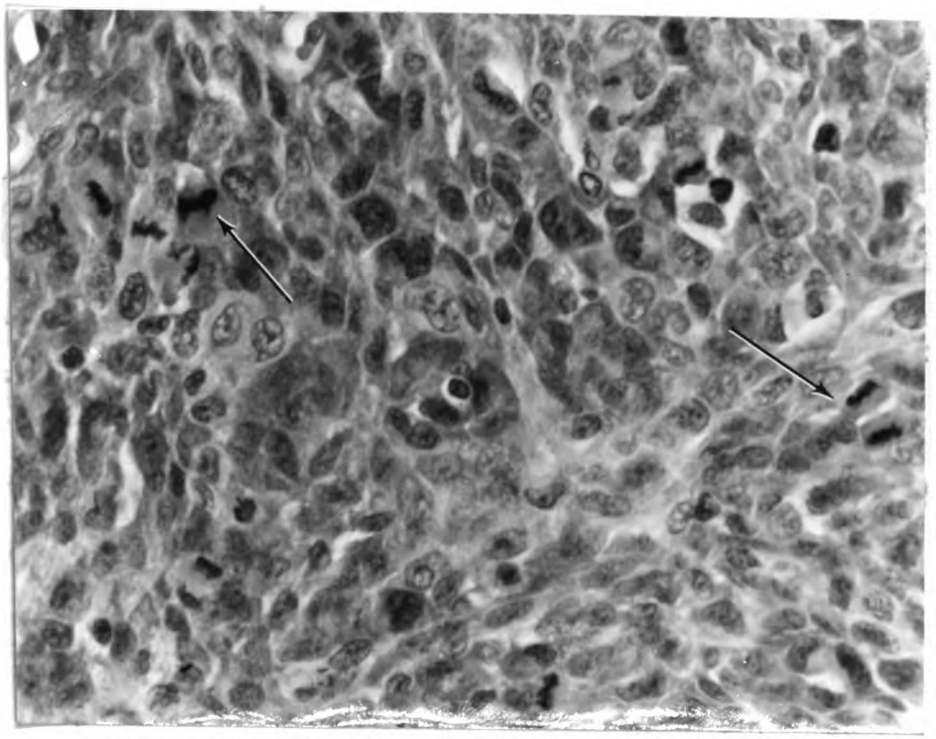

Figure 10. Section of fibrosarcoma-like tumor. Note the mitotic figures (arrow). Hematoxylin and eosin stain; x 520.

Figure 11. Fibroblast-like cells of primary tumor culture (40 hours old). The cells are similar to the RESI cells seen in Figure 1. Giemsa's stain; x 130.

Figure 12. Cytopathic effect of pseudorabies virus in RESI cell line 20 hours postinoculation. Giemsa's stain; x 208.

Rats and hamsters were inoculated intraperitoneally and via cheek pouches, respectively, with RESI cells but did not develop tumors during a period of 6 weeks postinoculation. No tumors were observed in control rats and hamsters.

Viral Susceptibility Spectrum

Among 38 viruses tested, the RESI cell line exhibited a narrow susceptibility spectrum. Only 9 viruses, herpes simplex, pseudorabies, equine rhinopneumonitis, WEE, EEE, vesicular stomatitis, IBR, canine adenovirus type 2 and Kilham rat virus propagated in RESI cells. The majority of viruses examined failed to multiply. The susceptibility of the RESI cell line fell into 4 categories, as shown in Table 7.

Pseudorabies and herpes simplex multiplied readily causing rapidly destructive CPE within 12 hours postinoculation. Figure 11 illustrates the CPE caused by pseudorabies virus 20 hours postinoculation. No intranuclear inclusions were observed. Equine rhinopneumonitis virus, WEE and EEE, and IBR required 24 hours to cause visible cellular degeneration. Equine rhinopneumonitis virus had a very slow progressive cytopathic effect on the cells. WEE and EEE caused diffuse cellular degeneration with complete destruction of the cell sheet 5 days following the first visible signs of infection. The CPE caused by IBR subsequently cleared up and the cells appeared normal 4 days postinoculation. No viral replication could be demonstrated in subsequent passages.

Cellular degeneration was observed 5 days postinoculation with Kilham rat virus and the cell sheet was destroyed slowly. Canine adenovirus type 2 multiplied in this cell line but produced no visible CPE. The susceptibility was confirmed by the inoculation of RESI cell infected fluid back into the CKT cell line with resulting CPE characteristic of canine adenovirus type 2 three days postinoculation.

Table 7. The viral susceptibility of the RESI cell line

Viruses which propagated with characteristic CPE* and could be serially transferred	Viruses which propagated initially with characteristic CPE* but could not be serially transferred	Viruses which propagated without CPE but could be serially transferred	Viruses which failed to propagate	Viruses which failed to propagate (cont'd)
Human Viruses	Animal Viruses	Animal Viruses	Human Viruses	Animal Viruses (cont'd)
Herpes simplex	IBR	Canine adenovirus type 2	Adenovirus type 1	Canine SV_5
Animal Viruses			Poliovirus type 2	Canine distemper
WEE			Influenza B	Mouse adenovirus
EEE			Coxsakievirus A_4	Mouse hepatitis
Equine rhinopneumonitis			Coxsakievirus A_{16}	Pneumonia virus of mice
Vesicular stomatitis			Coxsakievirus B_2	Encephalomyocarditis
Pseudorabies			Coxsakievirus B_5	Salivary gland virus (mice)
Kilham rat virus			Coxsakievirus B_6	K virus**
			ECHOvirus 4	Guinea pig herpesvirus
			ECHOvirus 9	
			Animal Viruses	Avian Viruses
			Bovine adenovirus type 3	Infectious bronchitis
			BVD	Newcastle disease
			HCV	Infectious laryngotracheitis
			TGE	Fowl pox
			Horse cytomegalovirus	
			Canine herpes	

*CPE which was typical of a particular virus when grown in the original host cell.

**CPE was the only criterion used for determining infectivity.

The results of titrations of viruses grown in the RESI cell line and in the original host system for preparing the stock viruses are summarized in Table 8.

Table 8. Comparative titrations of viruses in RESI cell line and in the original host system

Virus	Titer $TCID_{50}$/0.1 ml	
	RESI cell line	Original Host
Herpes simplex	6.5*	6.5 (Vero cells)
Equine rhinopneumonitis	3.0	3.5 (EK cells)
WEE	6.5	6.5 (Vero cells)
EEE	5.0	6.0 (Vero cells)
Vesicular stomatitis	4.5	4.0** (chicken embryo 10-day-old)
Pseudorabies	5.5	6.5 (PK-15 cells)
Kilham rat virus	6.0	6.0 (rat embryo T.C.)

*Reciprocal of log of virus dilution.

**LD_{50}/0.1 ml.

DISCUSSION

A previously established cell line (RESI) derived from fetal rat skin was characterized. The cultural pattern had been fibroblastic-like throughout the serial cultivation and no change had been detected in its maintenance requirements, proliferative capacity, or cultural pattern. These characteristics, plus the long-term cultivation, fulfill the criteria of an established cell line. The 140th to 171st subcultures were used in the characterization. The 69th subculture that had been frozen in liquid nitrogen was also used for chromosome studies. The line has a very long life span in subcultures, and with adequate media changes maintains its healthy appearance for several weeks. Fully developed cultures could be produced in as few as 48 hours if 2.0×10^5 cells per ml were seeded. When relatively small numbers of cells were transferred, full development required 2 weeks. The cell line was able to propagate in several media. This might prove advantageous when an experimental design dictates the use of a prescribed medium.

An intensive study was conducted to demonstrate that the cell line was free of extraneous organisms. After 3 years of cultivation it remained free from contaminants detectable by inoculation of prescribed culture media. In addition, no contaminating agents were detected by electron microscopy. The cell line can be stored in liquid nitrogen and at -70 C. The advantages of using this cell line are that it is free from any detectable contaminants and that cultures can be

maintained for many weeks without significant degeneration. This allows a long observation period for experiments.

The cell line was found to be neoplastic to weanling Sprague-Dawley strain rats. Tumors were produced when a large number of viable cells were inoculated subcutaneously.

The chromosomal pattern was heteroploid lying in the hypotetraploid region with a modal number of 40 chromosomes. It is obvious from the results presented that the cell line did progressively, but slowly, change its chromosomal pattern from diploid to heteroploid in the course of *in vitro* cultivation, and apparently the stemline of the cell line gradually predominated in the cell population. No significant increase of cells containing the large metacentric marker chromosome was observed in cells of the 70th subculture and cells of the 171st subculture. It is unfortunate that earlier subcultures were not available for chromosome studies, thus making possible determination of the time at which transformation of the chromosomal pattern occurred. Primary cultures initiated from the tumor produced by RESI cells also had a modal number of 40. It was interesting to find a higher percentage of cells containing the large metacentric chromosome and additional new marker chromosomes. These phenomena were consistent with previous observations by Nichols *et al.* (87). They suggested that the possible explanation for such was that a large and a small metacentric chromosome had each broken in the area of the centromore and then reunited to form 2 phenotypically similar chromosomes. They postulated that carcinogenesis was initiated through primary chromosomal changes in the form of chromosomal breakage and/or point mutation and that those early changes which are significant are usually too small to be seen microscopically. Studies of the mechanisms of transformation in cell lines indicated

that the occurrence of mitotic aberrations was due to genotypic diversity (129). If these aberrations did not lead to serious losses in chromosomes, adaptation of cell cultures to *in vitro* condition might follow smoothly in the near-diploid population and transformation of cell morphology would not occur. Ruddle (101) also found no evidence that alteration of cell morphology was causally related to changes of chromosomal patterns. These observations were confirmed in the RESI cell line.

The capacity of the cell lines of non-malignant origin to produce tumors has been reported previously (29,30,83,103,115). It has long been known that neoplasms usually possess abnormal chromosome numbers and that all the fast-growing lines examined are mostly heteroploid. It has been speculated (55) that the transformation of chromosomal patterns may be a phenomenon of adaptation and selection within a cellular population. Goldblatt and Cameron (44) exposed cultures of rat fibroblasts to an atmosphere of pure nitrogen and obtained malignant conversion. They suggested that the usual method of bottle culture might produce a modified atmospheric condition, perhaps a partial anaerobiosis, and might stimulate such a transformation. If the malignant conversion and chromosomal changes are truly related, it also would mean that the metabolism of the heteroploid cells is more adapted to an anaerobic condition than that of diploid cells.

The RESI cells were susceptible to several viruses, especially some of the herpesvirus group, but the susceptibility spectrum was rather limited. Viruses that propagated did, in most instances, as well within RESI cells as they did in the host system used for growing the stock virus. Although IBR was initially capable of producing CPE, it failed to replicate and could not be recovered in subsequent transfers.

The initial CPE was typical of that observed in BEK cells but eventually cleared up and could not be detected after 96 hours. No CPE occurred when dilute inocula were used. The initial CPE was very characteristic and not typical of a cytotoxic reaction. It would seem that it was more likely due to an abortive infection, i.e., no viral particles or only noninfectious particles were produced. The phenomenon observed with IBR virus resembled the so-called Von Magnus effect.

This cell line may be useful in cancer research because of its tumorgenicity and, should this be so, additional cytological and enzymatic work should be undertaken.

SUMMARY

A rat embryonic skin cell line (RESI), established from normal skin obtained from fetuses of a female Sprague-Dawley strain rat was subcultured 140 times during a period of 3 years. The cultural pattern was fibroblastic-like throughout the cultivation. The chromosome complement of the cell line was heteroploid, and the modal number was 40. Cultures grew rapidly, could be maintained 12 days without a change of medium, and confluent monolayers developed 3 days post seeding of 1.0×10^5 cells per ml. The cell line grew compatibly in several media and could be preserved by freezing and quickly recovered as actively proliferating cells with no changes in the growth rate or cellular characteristics. The species origin was confirmed by an immunofluorescent technique. The cells possessed the ability to produce tumors when weanling rats were inoculated subcutaneously with 1.0×10^6 cells. The cell line was susceptible to several human and animal viruses.

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