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In vitro oncogenicity of a virus isolate from sheep and cattle afflicted with weak calf and lamb diseases

Cyril Charles Jannke
The University of Montana

IN VITRO ONCOGENICITY OF A VIRUS ISOLATE FROM SHEEP AND CATTLE

AFFLICTED WITH WEAK CALF AND LAMB DISEASE
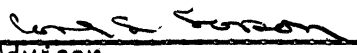
By

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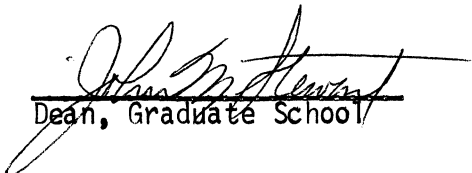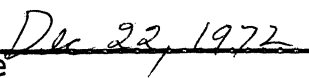Master of Science

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DEDICATION

With sadness, but fond rememberance, I dedicate this thesis and
all my work to two people who died during the course of my study. I
hope that the contributions made by this work will in some way reflect
their foresight, dedication and persistance. Each played a major role
in the resolution of the problems presented here, though this may not
be readily apparent. They have passed on, but will live in my memory
and fond rememberances. I dedicate this work to Helen E. Jannke and
Kenneth L. Kautz.

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Chapter 1

INTRODUCTION

Bovine and sheep cells infected with a virus suspected as the
etiologic agent of the disorder known as "Weak Calf and Lamb Disease"
transformed in cell cultures in vitro. This report describes the
morphologic conditions of the initial fibroblastic infection, the
process of cell transformation, and the characterization of transformed
cells. The properties of these transformed cells are compared to the
established criteria for classification of transformed cells and are
discussed to characterize further the virus associated with the dis-
eased lambs and calves. The physical properties and cell cytopathic
effect (CPE) of the isolated virus on primary bovine and ovine cell
lines has been reported (21).

The following discussion will be concerned with:

1. The growth of animal cells in culture.

2. Induction of transformation in cultured cells.

3. Cellular alteration in transformed cells.

4. Theory of cell-viral associations in transformation.

5. Viral association with tumorogenesis.

1

THE GROWTH OF ANIMAL CELLS IN CULTURE

Eukaryotic Cell Growth Conditions and Mitosis

The *in vitro* environment provided by tissue culture systems must meet the fastidious requirements of cells (34). The optimal temperature for *in vitro* growth of mammalian and avian cell growth is $37.5^{\circ}C$. The optimal pH range for cells in culture is 7.2 to 7.4.

The generation cycle of a single cell has been separated into four stages. These have been assigned the letters M, G_1, S and G_2.

1. Mitosis (M) is the division stage during which alignment and separation of chromosomes and daughter cell formation takes place. It lasts about 30 to 60 minutes.

2. The first gap stage (G_1) represents a reorganization stage after cell division. It varies in length of time.

3. The synthesis phase (S) lasts about 4 to 6 hours. During this time synthesis of DNA occurs.

4. The second gap phase (G_2) is the stage of preparation for mitosis, during which chromosome formation occurs. This stage lasts about 4 hours.

The total cell cycle varies from 8 hours to several days depending upon the growth rate of the cell.

Cells grown in culture are classified into two morphologic catagories. The epitheloid cells are rounded or equi-sided. The fibroblastic cells are spindle-shaped. These catagories do not refer to the histologic classification of the cell type but serve to emphasize a consistant morphologic property of cells in culture.

There are three states of cell growth found in cell cultures. The criteria used to define these depend on the longevity and growth properties of the cells. The states are; 1) primary culture, 2) stabilized culture, and 3) transformed culture.

Primary Cell Culture

Primary cell cultures are obtained by disaggregation of animal tissues. Initially, the primary cell culture consists of several histologic cell types such as lymphocytes, macrophages, fibroblasts, epithelial cells, and the specialized cells of organs. The in vitro life span of some of these cells is only a few days. The fibroblastic and epitheloid cells, of mixed histologic-cell type, survive beyond this period. In general, their in vitro life span is less than several months, or no more than 5 to 7 passages (34). After this time, the cells cannot be maintained. Cells in primary culture have several distinct properties distinguishable from the other two types of cell cultures. These properties are:

1. Usually, cells have a normal complement of chromosomes, i.e. diploid.

2. Primary cells preserve the properties of the in vivo parent cells.

3. The stability of the primary line varies with time.

4. The generation time of primary cells is generally from 32-72 hours.

5. The cells exhibit contact inhibition when grown in monolayer.

6. Fibroblastic cells grow in a line of common orientation.

7. The cells other than leukocytes must attach to a surface to divide.

8. The cells must be seeded above a critical concentration in order to survive.

9. Under defined medium conditions one morphologic type of cell grows consistently.

Stable Cell Cultures

Stable cells are altered primary cells that have survived beyond the limited culture period of normal primary cells. Stable cell lines have the general properties of:

1. Indefinite passage and survival.

2. Cell lines consist of one histologic or morphologic cell type.

3. Cells may be euploid or aneuploid.

4. Cells are often different from the parent primary cell.

5. Cells generally exhibit contact inhibition; they may stack, but not clone.

6. Cells must be seeded above a critical concentration.

7. Fibroblasts grow in line oriented patterns.

8. The cell form is stable, i.e., fibroblastic or epitheloid.

9. The cell generation time is 12-20 hours.

10. The cells do not grow well in suspension or soft agar.

Transformed and Tumor Cell Culture

Transformed and tumor cells exhibit similar in vitro culture

properties, which were summarized by Enders in 1965 (10). These
properties include:

1. Altered growth pattern, i.e., loss of contact inhibition,
occurrence of satellite colony formation.

2. Altered morphology.

3. Increased growth rate.

4. Increased persistence in serial cultures.

5. Altered metabolism.

6. Chromosomal abnormalities.

7. Reduced capacity to support multiplication of infectious
virus.

8. Increased resistance to reinfection with the transforming
agent as well as with certain other viruses.

9. Emergence of new cellular antigenic components.

10. Capacity to form neoplasms.

11. Ability to grow in a soft agar matrix, i.e., in
suspension (45).

12. Increased cloning efficiency (26, 34, 45).

Transformed and tumor cells are altered primary or stable cells
in a state of uncontrolled growth; a state which may be induced by
several oncogenic agents.

INDUCTION OF CELL TRANSFORMATION

The term transformation, as used by eukaryologists, describes
an in vitro state of cell growth induced by exposure of cells to a

foreign stimulus. This stimulus alters normal cell physiologic and cultural properties, which results in development of transformed cells. Transforming agents include 3,4-benzpyrine, 6,4-fluoronitroquinoline-N-oxide, N-nitromethylurea, X-irradiation, mycoplasma and viruses (10, 34). The biochemical transforming mechanism of action of these agents is unknown.

"Filterable" agents have been implicated in the transformation of cells and in tumorogenesis since early 1900, when Rous and Shope found that tumors developing in vivo could be passed from host to host by inoculation of tumor cell filtrates (11). The particles were identified as viruses or neoplastic mycoplasmas during the 1950's and 1960's. Since then many other viable agents have been associated with neoplastic disorders.

Viruses and mycoplasma often cause confusion since they may infect the cell culture simultaneously and also may produce the same effects individually on a cell culture (12, 27). Mycoplasma appear to have the capacity to induce transformation of stabilized cell lines in culture. The cells transformed by mycoplasma have morphologic and cultural characteristics similar to cells transformed by oncogenic viruses (27). Mycoplasma can be recovered in the initial stages of infection and transformation, but not in the final stage of transformation. Recently questions were raised regarding the ability of mycoplasma to cause transformation of cells or tumorogenesis (35).

The in vitro transformation of cells has served as an investigational model for the understanding of in vivo tumorogenesis. The growth patterns and physiologic properties of transformed and tumor

cells have been found to be similar. Not all cells which are neoplastic in vivo have been adapted to in vitro culture (47). Some transformed cell lines have been transplanted to isologous, syngeneic, or immuno-logically suppressed animals (1, 11). Several viruses which are oncogenic in vivo are capable of transforming cells in vitro (11). Several of the "non-oncogenic" viruses transform cells in vitro but do not cause tumorogenesis in normal host animals (11, 40). These vari-ables in the relationship of viral oncogenesis in vivo and transform-ation in vitro make a definite correlation between tumorogenesis and transformation difficult. Studies of the association of the viral oncogene with the genome of the eukaryotic cell have been made to de-termine the cause of viral transformation and tumorogenesis (2, 13, 19, 37, 43, 46).

Oncogenic Viruses

The oncogenic viruses are divided into two groups based on the nucleic acid possessed by the virus, i.e., riboviruses with RNA and the deoxyriboviruses with DNA.

Oncogenic Riboviruses. The oncogenic riboviruses are classi-fied as leukoviruses. All these viruses normally produce neoplastic disease in susceptible hosts, though they vary in their expression in different animals. They may cause infection when acting singly (Avian leukosis) or in combination with other viruses of the same subgroup (Rous sarcoma). The leukoviruses are divided into subgroups according to the host involved. These subgroups include avian leukoviruses, murine leukemia viruses, and murine mammary tumor virus. These viruses

are characterized by the presence of single stranded RNA, viron matura-
tion at the cytoplasmic membrane, and production of latent infections
in host animals. Many of the leukoviruses have a specialized enzyme
which catalyses the synthesis of complimentary DNA from the viral RNA.
This enzyme is the RNA-dependent DNA polymerase or reverse transcriptase
enzyme. Complimentary DNA is synthesized in latent and lytic infec-
tions caused by the leukoviruses (41). The complimentary DNA is be-
lieved to associate with cellular DNA and cause transformation of the
cells. Particles morphologically similar to leukovirus are suspected
as being the etiologic agents of leukemias of guinea pigs, cats,
cattle and man (11, 33). Transformation in cell culture has been
demonstrated by two non-oncogenic ("slow") riboviruses (39, 40).
These two viruses of sheep, Visna virus and progressive pneumonia virus
cause progressive nerve demyelination and respiratory disease respect-
ively, but do not cause tumorogenesis in the natural host. RNA-depen-
dent DNA polymerase is associated with these virons. It has not been
established whether a complimentary -DNA-viral oncogene integrates with
the cellular DNA, or if it activates a cellular oncogene (2, 43, 46).
Synthesis of cellular DNA, but not of protein, is required for cell
transformation. Division of cells is believed to be responsible for
the maintenance of the cellular DNA-associated DNA-provirus in progeny
cells.

Oncogenic Deoxyriboviruses. In vivo oncogenesis is a property
of three groups of deoxyriboviruses: poxviruses, adenoviruses, and
papovaviruses. The poxviruses produce benign tumors such as the

epitheliomas induced by fowl pox viruses, histiocytomas due to the

Yaba monkey viruses, and the fibromas of rabbits caused by myxoma

viruses. The papilloma subgroup of the papovaviruses are either onco-

genic or passenger viruses (no disease) in vivo. Simian virus 40

(SV40) either produced benign tumors or causes no infection in the

host animal, the Rhesus monkey (11). Polyoma virus of the papovaviruses

produces sarcomas and parotid tumors in newborn mice. The adenoviruses

produce respiratory infections in a range of natural hosts and are

oncogenic for mice and hamsters (5, 6, 11).

In vitro cell transformation occurs with the cotton tail rabbit

fibroma virus (poxvirus), most of the adenoviruses (human Adeno-12, 18

and 31, and bovine type 3), and with polyoma and SV40 (papovaviruses).

Transformation of cells in culture by oncogenic deoxyriboviruses

requires synthesis of cellular DNA and cell division (11, 13, 19).

This was shown by the use of chemical inhibitors which allowed the

integration of viral DNA with cellular DNA, but not replication of the

virus. Use of the DNA-mRNA transcriptase inhibitor, arabinosyl cytosine

(ARA-C), demonstrated that SV40 DNA integrated with Chinese hamster

cell DNA in oncogenically permissive and non-permissive cells. The

SV40 DNA was covalently linked with the cellular DNA (19). Fox and

Levine demonstrated that the greatest proportion of oncogenically

permissive cells initiated DNA synthesis within 24 hours after adsorp-

tion with SV40 (13). The oncogene of the deoxyriboviruses is thought

to be composed of less than 1/2 of the total viral genome (11).

Generally, the oncogenic deoxyriboviruses are replicated and

released from infected or transformed cells. In "viral free" transformed

cells, infectious virus can be recovered by use of Mitomycin C or cyclohexamid, by amino acid starvation or by cellular fusion with normal cells (22).

CELLULAR ALTERATION BY VIRAL INFECTION AND TRANSFORMATION

Changes in Cell Antigens

Some antigens found in transformed and tumor cells are differ= ent from those in normal cells. These antigens are associated with the presence of viral infection. The antigens are classified into four general catagories; Viral antigens (V antigens), Tumor=Specific Trans= plantation Antigen (TSTA or Surface antigen), Tumor antigen (T anti= gen), and miscellaneous viral associated antigens (Fiber antigen, C antigen and D antigen of adenoviruses).

Viral antigens. The viral (V) antigens are associated with the viron, generally with the viral capsomeres. Major viral groups have dissimilar V antigens, but within subgroups of adenovirus, avian leukosis and murine leukemia viruses there are common antigens. The V antigens are synthesized in cells transformed by avian and murine leukosis viruses, polyoma and SV40 viruses. V antigen is demonstra= ble by the following methods: complement fixation, hemagglutination inhibition, neutralization, agar diffusion, coprecipitation of radio= actively=labeled antigens, and fluorescent antibody techniques (11, 17, 37). The V antigen is heat stable at 56°C for 30 minutes and is sedimentable by ultracentrifugation (41).

Tumor Specific Transplantation Antigens (TSTA). These mem=
brane associated antigens were demonstrated to be viral directed by
immunizing adult hamsters with polyoma virus and later challenging
with isologous, polyoma-transformed, "virus free" cells. Tumors in=
duced by polyoma virus in newborn hamsters could not be passed into
immunized animals except when inoculated with an immunoparalysing
number of cells (11). The immunity was transferred by lymphocyte
transplantation. The viral specific TSTA are demonstrable by fluores=
cent antibody fixation, immune serum cytotoxicity by lysis of infected
cells, and complement fixation.

TSTA are viral specific in histologically different tumor
cells induced in different hosts (11). TSTA are produced in tumor
cells infected with the adenoviruses (31, 45), polyoma, SV40, Shope
papilloma, Rous arcoma virus (Schmidt-Ruppin Strain), and murine
leukoviruses (11). Synthesis of TSTA has been demonstrated only in
cell-transforming viral infections.

Tumor antigens. A non-viron-associated cellular antigen
called the T or tumor antigen was described by Heubner in 1967 (11).
This antigen is soluble and heat sensitive (41) and is detected by the
complement fixation test and by nuclear fluorescence in the fluores=
cent antibody test. The T antigen is produced by cells infected with
adenoviruses 11 and 18, SV40, Shope papilloma, polyoma and the avian
and murine lueokoviruses. The antigen is synthesized in infected
cells prior to viral DNA synthesis and in both viral-producing and
viral-free transformed cells.

Chromosome Abnormalities

Chromosome abnormalities caused by viral infections have been observed in in vitro cultures (8, 10, 14, 28, 30, 32). The abnormalities were chromosomal fracturing, dicentric formation, subdiploidy or hyperploidy. The chromosomal alterations were inconsistent in cell transformation or tumorogenesis (8). Cells resistant to superinfection by polyoma virus have fewer chromosomes than those which are susceptible. Resistance to superinfection may depend on the state of the cell genome (8).

Viruses causing chromosome abnormalities in vivo are rubella (32), Herpes virus hominis, herpes zoster, rubeola, and the yellow fever virus (10). Chromosomal aberrations have been observed in cells transformed in vitro by SV40, polyoma, adenovirus 12, Rous sarcoma virus, and Epstein-Barr virus (10, 14, 30). The chromosomal aberrations included random breakage, complete shattering, deletion and production of extra chromosomes. The release of lysozomes in infected cells is believed to cause chromosomal abnromalities (10).

Cell Membrane Alterations

Several investigators reported that the membranes of oncogenic cells, recently trypsinized cells, and normal dividing cells are similar in certain respects. These similarities include: a decreased adhesiveness for growth surfaces, an increased porosity manifested by leaking of small molecular weight molecules and an uncovered membrane surface determinant group which binds Wheat germ agglutinin (WGA) and Concanavalin A (4, 7, 49). Warren suggests that transformation may be a shift or slight loss of control of sequence in the M phase of the

generation cycle of a cell. The membrane of the resultant transformed cell is maintained in a state similar to that of a dividing cell (49). It has not been established whether the viral oncogene induced the membrane changes or whether the changes occur before the oncogene is expressed.

Sheppard et al. stated that cell DNA synthesis within 30 hours of viral infection was necessary but not sufficient for the membrane changes that exposed the WGA receptor sites (37).

THEORY OF VIRAL-CELL ASSOCIATION AND ONCOGENESIS

The association of oncogenic viral nucleic acid with cellular DNA has been demonstrated with deoxyriboviruses (13, 19, 51) and postulated for complimentary DNA of riboviruses (41, 42).

Synthesis of cellular DNA is a requirement for the integration of deoxyriboviral DNA. The influence of deoxyriboviral DNA on cellular mechanisms leading to the induction of transformation is still unknown.

Several theories have been proposed to explain the mechanisms of action of riboviral-complimentary DNA (41, 43, 46). Temin states that oncogenic nuclear DNA is transcribed to form an oncogenic messenger RNA which passes between cells. The intercellular messenger RNA (oncogenic ribovirus) enters another cell and is transcribed to form a complimentary DNA oncogene. The oncogenic DNA is termed the provirus (41, 43).

Todaro and Heubner propose that the DNA code for a complete oncogenic virus is contained in the DNA of all cells. Different areas of the chromosome may contain information for the formation of a

complete oncogenic viron. These areas may be transcribed individually or collectively, resulting in the production of the subunits or a completed infectious particle (46).

VIRAL ASSOCIATION WITH TUMOROGENESIS

Burkitt's Lymphoma and Epstein-Barr Virus

Burkitt's Lymphoma, described in humans in 1958, is a lymphoblastic proliferation involving all body organs, except the spleen and peripheral lymph nodes. In 1964, Epstein et al. isolated a herpes-like virus from these tumor cells in suspension culture. Since then the herpes-like virus (Epstein-Barr virus) has been isolated from other cells of both infected and normal individuals. The virus causes neural disorders in hamsters and old world monkeys, and in vitro transformation of human lymphocytes (28). Steward suggests that the ubiquitious distribution of the virus among normal individuals hampers early in vitro detection of in vivo disease. Increased viral levels may cause immune paralysis and proliferating tumor cells. A correlation is drawn between the distribution and pathogenesis of Epstein-Barr virus and avian and murine leukoviruses. The Burkitt lymphoma cells produce V antigens and TSTA specific for the Epstein-Barr virus.

The herpes viruses have been implicated as the etiologic agents in other proliferative disorders such as infectious mononucleosis, Marek's disease (avian lymphomatosis), frog renal carcinoma and Hodgkin's Disease (9).

Weak Calf and Lamb Disease

A viral agent isolated from animals having weak calf and lamb disease is oncogenic _in vitro_. _In vivo_ the virus causes immune suppression rather than cell proliferation (21). The role of pregnancy in the establishment of the disease is not completely understood. This report describes _in vitro_ viral transformation and relates this to the _in vivo_ non-oncogenic disease.

Chapter 2

METHODS AND MATERIALS

PROCESSING AND HANDLING OF CELL CULTURES

Types and Sources of Cells

Two types of cells were used in this study; primary sheep and bovine cell lines and stabilized lamb and fetal calf lines. Tissues for primary cell culture were kidney, salivary gland and testes. These were from fetuses, newborn, young and mature animals. The tissues were obtained from Daley's Processing Plant, Jack Ward, DVM, Gene Taylor, DVM, the Cook Sheep Ranch, the Cusker Ranch, and the Bitteroot Stock Farm.

Processing of Animal Organs and Tissues

Organ or tissue samples were obtained from normal and clinic= ally infected animals. The samples were placed in a sterile plastic bag (Whirl Pak, Griffith Laboratories) with 20 to 50 ml of Minimal Essential Medium (MEM) plus 10% fetal calf serum (FCS) culture medium. Samples were processed immediately or after storage at 4°C for 12 to 24 hours. Outer protective material was removed from the tissue, in a plastic petri dish (Falcon Plastics), with sterile surgical scissors. The tissue was washed with one rinse of Pd (pH 7.2). The desired area of the sample was transferred to a clean petri dish. The tissue was minced until no piece was larger than 1 mm^3 and transferred to a

trypsinizing flask (shop made). The tissue chunks were washed with one Pd (calcium and magnesium deficient PBS, Table 1) rinse and 30 ml of 0.25% trypsin in Pd (Difco 1:250, Table 2) was added to the flask. A magnetic stirring bar was added, and the flask was placed on a magnetic stirrer for 10 minutes. Then the cell-trypsin suspension was decanted. This trypsinizing procedure was repeated six times. The third through the sixth trypsinizations were saved for cell recovery by decanting the cell suspension through a gauze-covered funnel into a 500 ml centrifuge bottle (Bellco Glass). The centrifuge bottle, containing 50 ml of culture medium, had previously been placed in a ice bath.

The cells were recovered by centrifugation of the bottles at 1000 rpm (international Centrifuge, Size 2, number H9070) for 30 minutes. The supernatant medium was removed from the cell pellet by suction. The cells were resuspended in fresh culture medium at a dilution ratio of 1 ml cells: 50 ml medium. Five ml of this suspension was placed in a 250 ml plastic culture flask (Falcon Plastics, #3024) containing 10 ml of fresh culture medium. The pH of the medium was adjusted with 5% CO_2 and the flasks were cultured at 37°C for 72 hours.

Growth and Maintenance of Cell Cultures

Cells were cultured with several different growth media. The types of media used were Minimal Essential Medium (MEM), Medium-199 (M-199), and NCTC 135 (Gibco). Fetal calf serum was added at concentrations of 5%, 10%, 15% and 20% (Gibco, Difco, Microbiological Associates). The culture medium was prepared according to the

Table 1

Chemical Composition of Pd

Phosphate Buffered Saline, deficient (Pd)

NaCl . . . . 8.0 gm

KCl . . . . 0.2 gm

$Na_2HPO_4 \cdot 7H_2O$ . . . . 2.16 gm

KH_2PO_4 . . . . 0.2 gm

Glass distilled water . . . . 1.0 liter

Sterilize by autoclaving at $121^\circ C$ for 20 minutes

Table 2

Chemical Composition of Trypsin Solutions

Trypsin for Tissues and Monolayers

Solution A

NaCl . . . . 8.0 gm

KCl . . . . 0.4 gm

Glucose (dextrose) . . . . 1.0 gm

Trypsin:

Tissue . . . . 2.5 gm

Monolayer . . . . 1.5 gm

Glass distilled water . . . . 1.0 liter

Solution B

NaHCO$_3$ . . . . 0.58 gm

Versine (EDTA) . . . . 0.20 gm

Procedure:

Mix solution A for 45 minutes with about 1/4 of the NaHCO$_3$

Then add part B, and mix for an additional 15 minutes or

until the solution is clear.

Filter the solution sterilly

Store at -70°C

Thaw trypsin only once

formulations in Table 3.

The medium was changed every 3-4 days or as the medium acidity increased, shown by the yellow color of the indicator dye. The cells were passed when they grew to a monolayer, the maximum cell-density level in a single cell layer. The cells were removed from the growing surface (passing) by removing the growth medium, rinsing twice with Pd and incubating with 1.0 to 1.5 ml of 0.15% monolayer trypsin at 37°C for 10 minutes. Ten ml of fresh culture medium was added to the flask to neutralize the trypsin. The cell suspension was removed from the flask and centrifuged in a 15 ml round-bottomed culture tube at 900 rpm for 10 minutes. The supernatant medium was removed, and the cell pellet was resuspended in 5 ml of fresh culture medium by a forced pipetting action. The cells were diluted with culture medium to a stock concentration of about 1 x 10^6 cells per ml. One ml of the cell stock was added to new culture flasks (Falcon flasks 30 ml #3012, or 250 ml size) and fresh culture medium was added at approximately 5-10% of the flask volume. The pH of the medium was adjusted with 5% CO_2 and the flasks were incubated at 37°C for 12 hours before disturbing.

Detection and Inhibition of Mycoplasma in Cell Cultures

Many in vitro cell cultures may be infected with undetected mycoplasma from in vivo or in vitro sources (12, 27). Ushijima and Koostra attempted to culture mycoplasma from virus-infected primary fibroblasts and transformed cell cultures (47).

Table 3

Culture Media Formulations

Minimal Essential Growth Medium

MEM . . . . 9.6 gm

Glass Distilled Water . . . . 1.0 liter

Serum, inactivated at 56°C for 30 minutes . % concentration

NaHCO$_3$ . . . . 1.5 gm

Antibiotics * . . . . 1.0 ml/final
medium volume

Growth Medium 199 (M-199)

M-199 . . . . 9.9 gm

Glass Distilled Water . . . . 1.0 liter

Serum, inactivated at 56°C for 30 minutes . % concentration

NaHCO$_3$ . . . . 1.5 gm

1-Glutamine . . . . 0.1 gm

Antibiotics*. . . . . 1.0 ml/final
medium volume

Growth Medium NCTC 135

NCTC 135 . . . . 9.4 gm

Glass Distilled Water . . . . 1.0 liter

Serum, inactivated at 56°C for 30 minutes . % concentration

NaHCO$_3$ . . . . 1.5 gm

1-Glutamine . . . . 0.1 gm

Antibiotics* . . . . 1.0 ml/final
medium volume

* Antibiotic Stock Solution (1000X): Penicillin G . . 1 x 10^7 units,
   Streptomycin sulfate .. 1 x 10^7 mg., Glass Distilled Water . .
   100.0 ml. Store at -4°C. Shelf life about 6 months.

Cell cultures were treated once every 4 months for 72 hours with a stock solution of mycoplasma antibiotics: kanamycin (200ug/ 1.00 ml medium), neomycin (100ug/1.00 ml medium), and tetracycline-HCl (10ug/1.00 ml medium).

A study was made to determine if the mycoplasma antibiotics inhibited formation of the viral-caused CPE in normal calf testes primary cells (PCt-4). Ten ml of the 390K-pool stock virus was incubated with 0.1 ml of the stock mycoplasma antibiotics for 24 hours at 37°C. The PCt-4 cells growing on Leighton tube glass slides (Bellco Glass) were infected with the treated virus for 2 hours at 37°C. The infected cells were cultured in MEM plus 10% FCS containing 1.0 ml antibiotics per 100 ml medium for 48 hours at 37°C. The antibiotic medium was replaced by fresh MEM plus 10% FCS. Uninfected PCt-4 cells were used as controls. The cells were cultured for 14 days. They were fixed in Bouin's fixative (Table 4), stained with H & E (Table 5) and examined microscopically for the presence of eosinophilic nuclear inclusions (21).

Storage of Cells

Transformed, stable and primary cell lines were periodically frozen for storage. Trypsinized monolayer cells were diluted to concentrations from 5×10^5 to 5×10^6 cells per ml of MEM plus 10% FCS. Dimethyl Sulfoxide (DMSO, J.T. Baker Analysed) was added as 10% of the medium v/v. One ml of the cell-freezing suspension was placed in a sterile 2.0 ml ampule (Van Waters and Rogers). The neck of the ampule was sealed, and the ampule was wrapped in one layer of paper toweling

Table 4

Fixative Formulations

Bouin's Fixative

        Picric acid, saturated aqueous . . . . 75.0 ml

        Formalin, 100% aqueous . . . . 25.0 ml

        Glacial acetic acid . . . . 5.0 ml

            Wash in 50% to 70% ethanol with a few drops of
            saturated $LiCO_4$.

Carnoy's Fluid Fixative

        Glacial acetic acid . . . . 10.0 ml

        Ethanol, absolute . . . . 60.0 ml

        Chloroform . . . . 30.0 ml

Chromosome fixative

        Glacial acetic acid . . . . 10.0 ml

        Methanol, absolute . . . . 30.0 ml

            Prepare fresh weekly,

            Store at $4^{o}C$.

Methylene Blue Staining Fixative

        Glacial acetic acid . . . . 5.0 ml

        Ethanol, 95% or absolute . . . . 95.0 ml

Other Fixatives

        Methanol or ethanol, absolute.

        Acetone, dry.

Table 5

Stain Formulations

Acridine Orange

    Acridine Orange Stock Solution (life span 7 days)

        Acridine Orange . . . . 0.1 gm

        Distilled water . . . . 100.0 ml

        Tween 80 or Photo flo . . . . 0.2 ml

    Acridine Orange Working Solution(0.01% in buffer, pH 6.4)

        Acridine Orange Stock Solution . . . . 5.0 ml

        McIlvane's Buffer, pH 6.4 . . . . 45.0 ml

    McIlvane's Buffer pH 6.4 (shelf life 7 days at 4°C)

        Na_2HPO_4 . . . . 19.66 gm

        Citric acid . . . . 6.46 gm

        Distilled water to . . . . 1000 ml

Erlich's Hematoxylin

    Hematoxylin . . . . 2.0 gm

    Ammonia alum = $Al_2(SO_4)_3(NH_4)_2SO_4 \cdot 24H_2O$ . . . . . . 3.0 gm

    Alcohol, Methanol or Ethanol . . . . 100.0 ml

    Glycerol . . . . 100.0 ml

    Distilled water . . . . 100.0 ml

    Sodium acetate . . . . 2.4 gm

    Glacial acetic acid . . . . 10.0 ml

Eosin Y (Store in tightly capped bottle)

    Eosin Y . . . . 1.0 gm

    70% Ethanol . . . . 100.0 ml

Table 5 (continued)

Giemsa Stain

Siemsa Stock Solution . . . . 5.0 ml

Phosphate buffer pH 6.4 . . . . 5.0 ml

Distilled water . . . . 40.0 ml

Methyl Green-Pyronin Y

Methyl Green * . . . . 0.52 gm

Pyronin Y . . . . 0.10 gm

Distilled water (heat to 100°C) . . . . .100.0 ml

Place water in a 250 ml flask. Heat to boiling. Add dyes
and stopper for 5 to 7 days. Filter before use. Store in
amber glass, glass toppered.

* Extract methyl green with chloroform until the extracting
solution is blue instead of lavender.

Differentiating Solution

Tertiary butyl alcohol . . . . 30.0 ml

Absolute alcohol, Ethanol . . . . 10.0 ml

Methylene Blue

Methylene Blue . . . . 200.0 mg

Distilled water . . . . 100.0 ml

Dilute 1:4 with distilled water or Pd before use.
Filter before use.

Trypan blue

Trypan blue . . . . 200.0 mg

Pd or PBS . . . . 100.0 ml

Filter before use, may be diluted 1:2 with Pd.

and one layer of aluminum foil. It was labelled and placed in a
-70°C freezer (Revco).

Frozen cells were recovered by thawing at 37°C, washing twice
with MEM plus 10% FCS and seeding into a 30 ml culture flask. The cells
were incubated at 37°C for 72 hours before disturbing.

GROWTH AND MAINTENANCE OF CELLS IN CULTURE

In Vitro Infection of Primary Cells

Primary cell lines were maintained in culture for 14 days
(1-2 passages) to ascertain if the cells were naturally infected.
Infection was detected by the presence of nuclear inclusion bodies (21).
Culture medium was removed from uninfected submonolayer cultures and
0.5 to 1.0 ml of virus stock was added. The virus was adsorbed to the
cells for 1 to 2 hours at 37°C. Fresh culture medium was added and the
cells were cultured at 37°C. Viable cultures were examined for CPE on
an inverted phase microscope (Zeiss) at magnifications of 63X and
100X.

The cells were passed when they reached monolayer until the
"crisis stage" of infection (15). At this stage, passage was unsuc-
cessful until epitheloid or transformed cells repopulated the cultures.

Primary and Stabilized Cells used for Transformation

Viral-infected primary fibroblasts from calf kidney, salivary
gland, testes, sheep salivary gland and testes were used in transform-
ation experiments. Stabilized cell lines of embryonic bovine kidney,
trachea and lamb kidney (Micro. Assoc.) were infected, but did not
survive due to serum toxicity (47).

Fibroblastic cells from infected and normal animals were continuously passed in culture until the cells transformed or died. Most of the cell cultures were passed bimonthly, but one normal fetal calf salivary gland line (2FCSg) was passed twice in six months. The medium of the 2FCSg line was changed every 4 weeks.

Induction of Calf Primary Cell Transformation by
Stock Virus Pools

Groups of primary calf kidney (PCK-1) and salivary gland (PCSg-1) cultures were arranged to study the process of cell transformation by in vitro infection with virus pools, calf 390 kidney (390K-pool) and lamb salivary gland (OSg-4 pool) (21). Groups 1 through 4 were infected with 0.5 ml of the virus pools diluted 1:2 with MEM and 10% FCS (Table 6). Cells in groups 5 and 6 were adsorbed with 390K pool virus neutralized by incubation at 37°C for 2 hours with immune cow 1125 serum diluted 1:2. Groups 7 and 8 were uninfected control cultures. Groups 2, 4, 6 and 8 were cultured with NCTC-135 plus 20% FCS (Table 3) until the crisis stage of infection was reached. They were then cultured on MEM plus 10% FCS. Groups 1, 3, 5 and 7 were cultured with MEM plus 5% FCS.

Cell cultures were passed until the infected cells reached the crisis stage of infection. At each passage cells were grown on tissue culture slides (Labtek, Miles Labs.) and stained and examined for CPE.

When epitheloid cell outgrowth occurred, after the crisis stage, the PCSg-3 bottles from groups 2, 4 and 8 were passed. The rest of the cultures were maintained without passage. All cultures

were cultured a maximum of 90 days.

Table 6

Induction of Calf Primary Cell Transformation with
Stock Virus Pools

Group Number	Cell Type	Treatment	Passage-Trypsinization Interval
1	PCK-1 PCSg-1	Infected with 390K-pool	14 days
2	PCK-1 PCSg-1	Infected with 390K-pool	7 days
3	PCK-1 PCSg-1	Infected with OSg-4 pool	14 days
4	PCK-1 PCSg-1	Infected with OSg-4 pool	7 days
5	PCK-1 PCSg-1	Infected with 390K-pool neutralized with 1125 serum	14 days
6	PCK-1 PCSg-1	Infected with 390K-pool neutralized with 1125 serum	7 days
7	PCK-1 PCSg-1	Uninfected control	14 days
8	PCK-1 PCSg-1	Uninfected control	7 days

Cell Growth on Glass Slides

Cells were grown on glass slides in Leighton Tubes (Bellco Glass) or slide culture chambers (Labtek, Miles Laboratories). The cells were usually seeded at a concentration of 1×10^5/ ml of medium. The medium was changed every 5 days. The culture chambers were placed in a carbon dioxide culture box in the 37°C incubator. The box was gassed with 5% CO_2 for 1-2 minutes after the box door was sealed.

Cell Growth in Suspensions of Soft Agar

Preparation of cells. When cells grown in 250 ml Falcon flasks reached monolayer, they were trypsinized. The cells were washed, centrifuged, and resuspended in 5 ml of MEM plus 10% FCS. Large clumps of cells were allowed to settle out of the supernatant by allowing the tube to stand for 1 to 2 minutes. Four ml of the supernatant medium containing single cells was removed and placed in a new culture tube. The cells were counted on a hemocytometer (American Optical). Concentration of the single cell suspensions was adjusted to 1×10^3 cells/ml. One drop of the cell suspension was mixed with one drop of 0.2% Trypan blue (Allied Chemical) in Pd. The mixture was allowed to stand for 1-2 minutes before counting the cells. Viable cells did not stain with Trypan blue (18, 44). Percent viability was expressed as the number of live cells per 100 cells counted.

Preparation of Growth Medium. Two times normal concentration (2X) of 1.0% and 0.6% Ionagar (Oxoid #2, Consolidated Laboratories) in glass distilled water were dissolved by autoclaving for 15 minutes at $121^\circ C$. The hot agar was filtered through a 1.2 micron Millipore filter pad (25mm microfilter, Millipore Filter Corporation). During the filtering process, the agar was dispensed into 10 ml aliquots in culture tubes. The tubes of agar were stored at $4^\circ C$ until needed. Soft-agar culture media, prepared at 2X normal culture concentration included MEM, M-199 and NCTC-135. The media was prepared as MEM plus 0%, 5%, 10%, 15%, 20% FCS, M-199 plus 10% FCS and NCTC-135 plus 20% FCS (Table 3). These 2X media were mixed with the 2X agar at a dilution of 1:2 at $45^\circ C$.

Suspension of the cells. One ml of 1 x 10^3 cells/ml of media was added to 1 ml of 0.3% agar in medium at 45°C. The final cell dilution was 5 x 10^2 per ml of 0.3% agar in medium.

Placement of the cells and agar in growing chambers. Transformed sheep salivary gland, OSg-12, calf kidney, 390K-18, and normal calf primary kidney PCK-4 cells were grown in soft agar in 96 well Microtiter culture plates (Falcon Plastics, Microtest II #3040).

Experiment 1. Each cell line was grown under three soft agar conditions. Eight rows of 12 wells each were organized as follows: the wells of three rows contained 0.05 ml of the soft agar-cell suspension (25 cells per well) and 0.10 ml of NCTC plus 20% FCS, a second set of three rows contained 0.05 ml of 0.5% agar medium pad, 0.05 ml of the 0.3% agar cell suspension and 0.05 ml of medium, and a set of two rows contained 0.1 ml of 0.5% agar pad and 0.05 ml of 0.3% agar cell suspension. Each layer of agar was allowed to solidify for 4 minutes at 27°C before addition of more material.

Experiment 2. 390K-20 transformed cells were used in a soft agar growth medium experiment. Cells were suspended and grown in agar and MEM containing 0%, 5%, 10%, 15% and 20% FCS. Duplicate rows of 8 wells each contained 0.05 ml of cell-agar suspensions (25 cells per well) and 0.10 ml of MEM with the respective FCS concentrations.

Experiment 3. OSg-12 and PCK-4 were grown in 1.5 ml of 0.3% suspension agar over 4.0 ml of 0.5% agar pad in petri dishes. The final cell concentration in the suspension agar was 1.0 x 10^4 cells per ml. MEM plus 10% FCS was used as the growth medium. The 0.3% suspension agar was overlayed with 3.0 ml of medium.

All soft-agar plates and dishes were placed in the CO_2 box in the $37^{\circ}C$ incubator. The plates were inspected for cell growth at 3, 7 and 14 days by microscopic inspection.

Recovery and outgrowth of cells from soft agar. Single and colony cells growing in soft agar for 14 days were recovered in fluid-medium cell culture. The agar from six wells was placed in a 30 ml Falcon flask. Duplicate flasks were made for each cell line. The cells were released from the 0.3% agar matrix by forced pipetting action. Three ml of MEM plus 10% FCS was added to each flask. The pH was adjusted with 5% CO_2 and the flasks were placed in a $37^{\circ}C$ incubator. After 7 days the cultures were inspected for cell growth. Representative photomicrographs were taken of out-growing cells.

MORPHOLOGIC STUDIES OF CELLS IN CULTURE

Examination of Viable Cells

Viable cultures were monitored by microscopic inspection with a Zeiss Inverted Phase Contrast Microscope. The cells were observed at total magnifications of 60X and 100X with white light and at 100X with phase contrast incident light. Photographic recordings of growing cells were made with a Zeiss-Ikon 35 mm camera back attachment for the microscope. Pictures were recorded on Kodak Tri-X black and white film (ASA 400). Exposures were made between 1/2 and 1 second with a heat barrier and green contrast filter in the light path. The light was adjusted to maximum intensity.

Fixation and Staining of Cells

Cells were cultured on microscope slides for fixation and staining in order to observe the culture and intracellular detail. The medium was removed from the slide cultures, and the slides were rinsed in non-sterile Pd (pH 7.2). The cells were fixed and stained as follows:

Hemotoxylin and Eosin Y staining. The cells were fixed in Bouin's fixative (Table 4) for at least 1 hour. The slides were then transferred to 80% ethanol for at least 2 hours to remove the excess Bouin's fixative. The cells were stained with this procedure:

1. Stain in Erlich's Hemotoxylin (Table 5)   30 minutes

2. Rinse in tap water   2 dips

3. Destain with 1% HCl in 70% ethanol   2 dips

4. Develop in Lithium Carbonate   2 minutes

5. Rinse twice in distilled water   30 seconds each

6. Counterstain with 1% Eosin Y in 70% ethanol   5-10 minutes

7. Remove excess eosin by dipping in 2 baths of 95% ethanol and 2 baths of 100% ethanol   3 dips each bath

8. Clear in two baths of xylene   30 seconds 1st 5-10 minutes, 2nd

9. Remove from the xylene and mount with a clean #1 coverslip (25 mm x 20 mm) and Permount Mounting media

10. Dry slide at $27^{\circ}C$ for 12 hours before examination

Normal-fetal-calf salivary gland fibroblasts (2FCSg-4) and primary calf salivary gland transformed by sheep virus stock (PCSgSV-4) were grown on duplicate two-chambered tissue culture slides in MEM plus 10% FCS for 3 days. The slides were removed from culture and washed

with Pd. One slide was stained with Acridine Orange and the
other with Methyl Green-Pyronin Y by the methods of Schiffer (36) and
Taft (24).

Acridine Orange staining (A/O)

1.	Fix cells in absolute methanol	3 minutes
2.	Remove slides and air dry	
3.	Hydrate in 80%, 70%, 50% ethyl alcohol and water	5 dips each
4.	Dip in 1% acetic acid in water	4 dips
5.	Place in distilled water	2 minutes
6.	Place in McIlvane's buffer, pH 6.4 (Table 5)	5 minutes
7.	Stain in Acridine Orange solution pH 6.4 (Table 5)	2 minutes
8.	Destain in McIlvane's buffer	2 minutes
9.	Blot slides dry	
10.	Wet mount with fresh McIlvane's buffer and #1 coverslip	

The cells stained with acridine orange were observed on a
Zeiss microscope with a mercury-arc Ultra-Violet light source. The
passable light excitation range was adjusted to between 330-500 mu
with excitation filters. The ocular barrier filters were adjusted to
pass A/O light of excitation in the range of 470 to 650 mu.

Methyl Green Pryronin staining (MGP)

1.	Fix slides in Carnoy's fixative (Table 4) without prior drying	10 minutes
2.	Hydrate cells with 95%, 75%, 50% ethanol and water	5 dips each
3.	Stain slides with MGP (Table 5)	10 minutes

4. Rinse slides twice in water   1 minute each

5. Blot slides dry

6. Differentiate slides in differen-
   tiation solution   1-2 minutes

7. Dehydrate in fresh differentiation
   solution   4-5 minutes

8. Clear twice in xylene   10 minutes each

9. Mount with permount and #1 coverslip

10. Dry at 27°C   12 hours

11. Examine cells under white light

Karyogram Analyses of Cells

Chromosome studies were made of normal primary fibroblastic,
infected primary fibroblastic and transformed cells. The cell lines
included; calf kidney and salivary gland, sheep salivary gland and
testes.

Chromosome preparations were made from cell cultures according
to Ushijima's modified method (47):

1. Cells were cultured in MEM plus 10% FCS to one-half mono-

   layers in 30 ml falcon flasks.

2. Stock colchicine (Calbiochem, 10-25 mg/ml medium) was

   added to each flask, which was then cultured for 4 hours

   at 37°C.

3. The culture medium was removed, and the cells were washed

   twice with Pd. One ml of 0.15% Trypsin (without versine)

   in Pd was added to each flask.

4. The flasks were cultured for 1 minute at $37^{\circ}C$. Five ml of MEM plus 10% FCS was added, the cell suspension was removed, and the cells were centrifuged in 15 ml conical centrifuge tubes at 900 rpm for 5 minutes.

5. The cells were resuspended in hypotonic Pd (20% 1X Pd in distilled water) and incubated for 10 minutes at $27^{\circ}C$. One drop of fresh fixative was added (Table 4). The cells were again centrifuged and the supernatant was discarded.

6. The cells were resuspended in 2.5 ml of fixative and incubated for 20 minutes at $4^{\circ}C$. The cells were centrifuged and the supernatant was discarded.

7. One ml of fixative was added to the cell button, the cells were incubated for 5 minutes at $27^{\circ}C$, and recentrifuged at 400 rpm for 5 minutes. The supernantant was discarded.

8. Six drops of fresh fixative were added to the tubes. The cells were resuspended, dropped onto a precleaned, water-moistened glass slide, and allowed to dry at $27^{\circ}C$.

9. The preparations were stained in Giemsa stain (Table 5) for 15-30 minutes.

10. The preparations were washed with water and air dryed.

11. The slides were dipped in absolute ethanol and then cleared in xylene for 10 minutes.

12. The slides were mounted with Permount and a #1 coverslip, and allowed to dry for 12 hours at $27^{\circ}C$.

The chromosome spreads were examined on a Zeiss microscope at 1000X. Representative spreads were photographed with a microscope

35 mm camera back with Panatomic-X film at one exposure slower than indicated by the light meter. The negatives were developed for 15 minutes in Microdol-X diluted 1:3. Prints were made on Kodabromide F-4 paper. Xerox copies of the chromosome prints were cut out for karyogram analysis. The chromosomes were arranged according to the scheme of Gustavsson (16).

Chapter 3

RESULTS

MORPHOLOGY OF PRIMARY AND TRANSFORMED CELLS IN CULTURE

Viable Stock Culture Cells

Primary cells grew as small spindle-shaped fibroblasts with a
nuclear-cytoplasmic ratio of 1:4 and non-uniform nucleoli. Epitheloid
cells did not grow in these cultures after the first passage. The
fibroblasts grew radially in a line-oriented fashion to form a conflu-
ent monolayer (Figure 1). The cells demonstrated contact inhibition
and were passable only by the trypsinization process. Exposure to
trypsin for 10 or more minutes was required to remove more than 40% of
the attached cells. The attachment rate of the primary cells in MEM
plus 10% FCS (MEM/10) varied from 60% to over 90% depending upon the
cell type involved.

Uninfected primary cells were grown in culture for 8-10 weeks
or 5-10 passages. At this time, the submonolayer cell growth began to
decrease. The cells then vacuolated, died and peeled off the growing
surface in sheets.

Cells infected in vivo or in vitro showed increased growth and
post-trypsinization attachment rates when compared to uninfected cells
(21). The majority of the fibroblastic cells of infected cultures
entered a crisis stage on the third to sixth passage at 10 to 16 weeks
of age. Transformed cells were observed among these fibroblasts as
small epitheloid cells forming colonies. The transformed cells were

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derived from fewer than 1% of the fibroblasts of the pre-crisis stage culture. Four or five foci of transformed cells started growing at the same time.
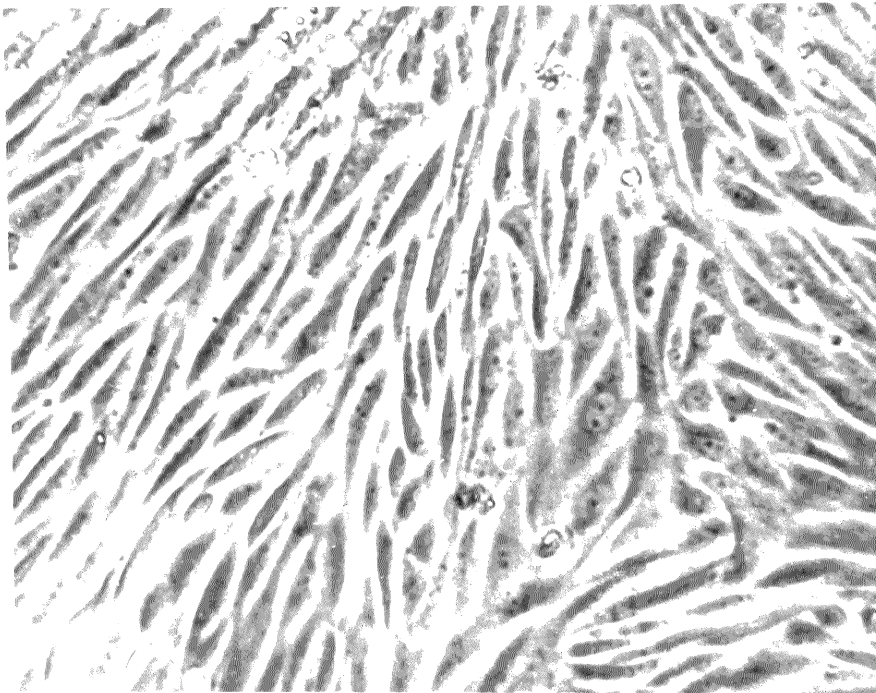
Figure 1

Uninfected Calf Kidney Fibroblasts. 1st passage, 14 day old primary culture. Note line-of-orientation growth. Phase contrast. X160

The transformed cells of calf and sheep kidney, salivary gland and testes appeared morphologically similar. The cells had a reduced nuclear-cytoplasmic ratio of 1:1, and two to four uniform nucleoli. In areas of extreme cell-crowding, the nuclei of adjacent cells appeared to be separated only by the cytoplasmic membranes. Transformed

cells were passed by trypsinization for 2 minutes, cell layer scraping
or placement of spent culture medium in clean flasks. The attachment
rate of all transformed cells was above 90% in MEM/10. Many cells
grew in conical stacks as rounded cells on top of a flattened basal
cell layer (Figure 2). The height of the conical stack was about one-
half of the colony diameter. When the conical stack was about 1 mm
high, the center area detached, producing a crater-like colony. New
cells immediately began to repopulate the center of the crater.
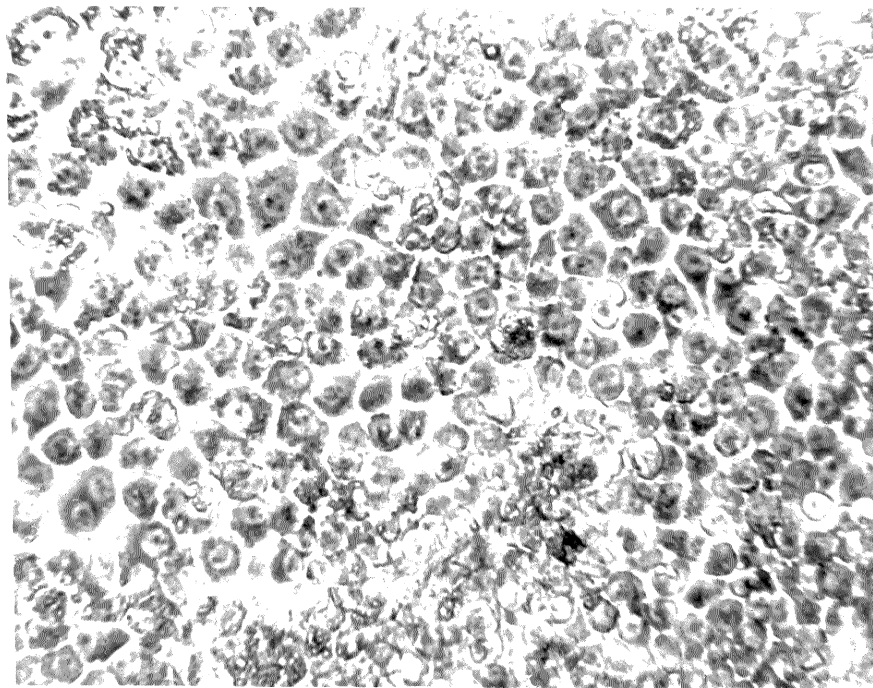
Figure 2

Transformed Calf Kidney Cells. 18th passage, 7 day old cul-
ture. Note random growth pattern and stacked cells. Hemotox-
ylin stain. Phase contrast. X160

Transformed cells showed no permanent reversion of cell morph-
ology to the parent fibroblastic form when seeded at low cell concen-
trations or when held in continuous culture for over 6 months without
passage. One percent of the cells at monolayer were morphologically
abnormal, appearing as giant or polykaryocytic cells. The transformed
cells did not exhibit contact inhibition, and grew in multilayered
sheets or stacks. Dying cells did not vacuolate or enter a crisis
stage but "rounded up" as individual cells and looked like dried peas.
Basal layer cells were normal while upper layer cells were dying.

Stained Cells

Hemotoxylin and Eosin staining. Uninfected primary and stable
calf and sheep cells stained with H & E showed normal nuclear basophilia
and cytoplasmic eosinophilia. Nucleoli numbered from 1 to 6 per cell
and stained dark blue. There were no nuclear or cytoplasmic inclus-
ions. Cytoplasmic vacuolization was evident as the cells grew older
and cell growth slowed.

Infected fibroblasts showed increased cell enlargement, debris,
polykaryocytosis, cytoplasmic vacuolization and eosinophilia, and
eosinophilic nuclear inclusions.

Transformed cells appeared as uniform polygonal cells with 2 to
4 nucleoli. The intensity of cytoplasmic eosinophilia was greater than
that of the parent fibroblasts. The nucleus was slightly more granu-
lar but contained no eosinophilic inclusion bodies. Eosinophilic
cytoplasmic inclusions were formed in some cells as the culture medium
became exhausted. Cells in suspension or in stacks appeared as dark

blue spheres. Two to five percent of the cells were observed in the metaphase stage of mitosis. A "mosaic tile pattern" was observed in colonies of fixed cells.

Acridine orange staining. Normal fetal calf salivary gland fibroblasts (2FCSg-4), infected calf kidney fibroblasts (PCK-4), transformed calf salivary gland and kidney (PCSgSV-4 and 390K-12) were stained with acridine orange. The cytoplasmic area of all of these cells fluoresced red under excitation by U.V. light. The nuclear area fluoresced yellow-green. Dividing cells had bright red cytoplasm and bright yellow chromosomal material.

The cytoplasmic staining of the 2FCSg-4 cells was not uniform. Non-staining cytoplasmic areas appeared as black holes. The nuclei stained dull yellow-green with a few bright areas that appeared to be clumped nucleic acid material. The nucleoli fluoresced dull orange. Shading of red fluorescence was seen overlapping the margin of the nucleus in dying cells.

The infected PCK-4 cells had more of the yellow-green clumped material than did the 2FCSg-4 cells. The cytoplasm did not stain uniformly red. Holes were observed in the cytoplasmic staining. A large amount of red and yellow-green debris was present.

The PCSgSV-4 and 390K-12 transformed cells were about one-half the size of the fibroblastic cells. Non-dividing transformed cells in all layers of stacked colonies appeared the same. The cytoplasm stained a uniform bright red. The nuclei had no clumped nuclear material as was observed in the fibroblastic cells. Some of the nuclei did not stain at all, and left dark green holes in the cytoplasm. The

nucleoli fluoresced bright orange-red. Aggregation of nucleic acid material formed yellow rings around the nucleoli. Representative photomicrographs of the acridine orange stained cells are shown in figures 3 and 4.
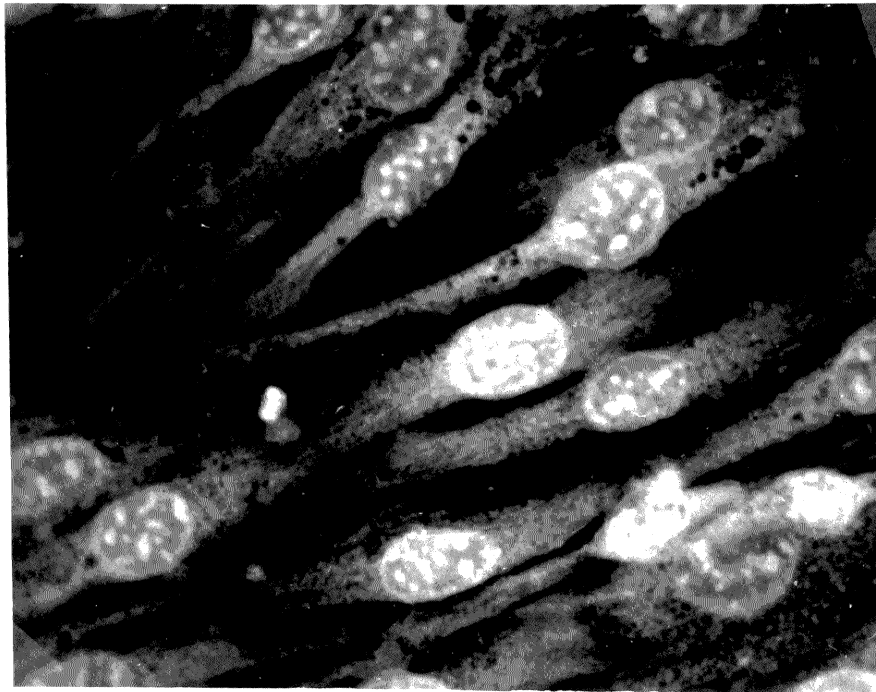
Figure 3

Uninfected Sheep Salivary Gland Fibroblasts. 4th passage, 7 day old primary culture. Acridine orange stain. X 400
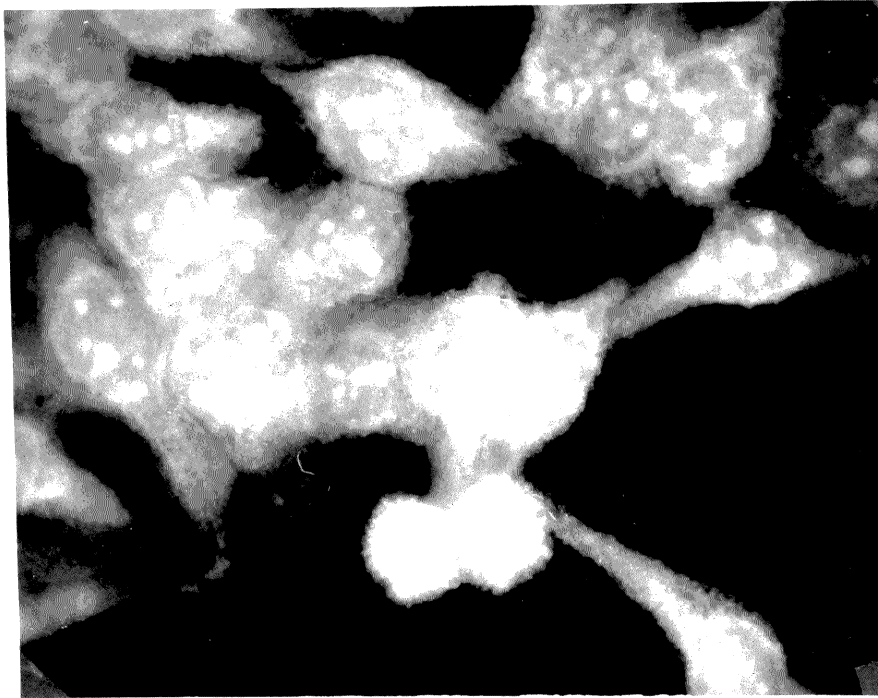
Figure 4

Transformed Sheep Salivary Gland Cells. 12th passage, 7 day old culture. Note small amount of cytoplasm and nucleoli uniformity. Acridine orange stain. X400

Methyl Green-Pyronin Y staining. Normal fetal calf salivary gland fibroblasts (2FCSg-4) and transformed calf salivary gland cells (PCSgSV-4) were stained with Methyl Green-Pyronin Y stain to detect differences in nucleic acid synthesis. The 2FCSg-4 fibroblasts stained poorly. The nuclei of the cells stained light blue-green, and the cytoplasm stained light pink. Nucleoli did not stain. The nuclear area of dividing cells stained deep blue-green and the cytoplasm stained intensly red in a diffuse area near the nuclear area.

The PCSgSV-4 transformed cells stained more intensly than the normal cells. The nuclei were medium blue-green in color. The nucleoli were evident as blue intranuclear bodies. The cytoplasm stained medium red. A ring of bright red color was observed around the nuclear membrane in most of the cells. Dividing transformed cells appeared similar to dividing fibroblasts.

GROWTH PROPERTIES OF PRIMARY AND TRANSFORMED CELLS

Media Requirements

The optimal medium and fetal calf serum concentration for the growth of primary and transformed cells was determined by media trials. Primary calf kidney (PCK-4), transformed calf kidney (390K-18), and transformed sheep salivary gland (OSg-12) cells were seeded into duplicate 30 ml Falcon flasks at concentrations of 1×10^1, 1×10^2, 1×10^3 or 1×10^4 cells per flask. Three ml of media was added as MEM plus 5% FCS (MEM/5) or NCTC 135 plus 20% FCS (NCTC/20). The pH of the media was adjusted to 7.2 and the bottles were cultured for 21 days at $37^\circ C$. The media was changed at 7 day intervals. The results are outlined in Table 7.

The attachment rate of cells seeded in NCTC/20 was about 90%. At 21 days about 50% of the colonies contained more than 20% polykaryocytic or giant cells. The growth of the cells ceased after 3 weeks in culture. The attachment rate of cells cultured in MEM/5 was about 70%. The cells in the colonies appeared to be uniform. About 1% polykaryocytic or giant cells were present. The cells continued to grow after 4 weeks of culture.

Table 7

Transformed Cell Plating Efficiency in Fluid
Medium Culture

Cell line -	OSg-12		390K-18		PCK-4	
Medium -	MEM/5	NCTC/20	MEM/5	NCTC/20	MEM/5	NCTC/20
cells/flask						
10^1	+	+	+	+	-	-
10^2	+	+	+	+	-	-
10^3	+	+	+	+	-	-
10^4	+	+	+	+	(+)	(+)

+   good to excellent colony formation

(+) poor cell growth, less than 100 colonies/flask

-   no cell growth

In the second media trial, 390K-18 cells were placed in dupli-
cate Falcon flasks at a concentration of 1 x 10^3 cells per flask.
Three ml of media was added to each of the duplicate flasks as either
MEM/5, MEM/10, MEM/15 or MEM/20. The media was changed at 7 day in-
tervals during the 21 day culture period. Table 8 outlines the results
of this trial, which demonstrated the effect of different FCS concen-
trations on the growth of transformed cells.

Cells grown in MEM/10 and MEM/15 had a high cell seed re-
covery rate, a good growth rate, and uniform cells were present in the
colonies. Cells continued to divide on top of the basal cell layer
after 21 days. The uniformity and growth of the cells in MEM/5 was
similar to that of the cells in MEM/10 and MEM/15. The cells in MEM/5

had a lower cell seed recovery rate and slower colony growth rate. The cells in MEM/20 had the highest cell seed recovery rate, but stopped growing after 21 days. Entire colonies of "abnormal" cells, such as polykaryous, giant or distented cells were observed throughout the MEM/20 culture. More than 60% of the colonies contained abnormal cells.

Based on the results of these experiments, stock cell lines were seeded in MEM plus 10% FCS, and established cell cultures maintained on MEM plus 5% FCS.

Table 8

Effect of Varying Serum Concentration on Cell
Growth on 390K-18 Cells

	Medium/Serum Concentration				
	MEM/0	MEM/5	MEM/10	MEM/15	MEM/20
Cell attachment rate*	0	70%	85%	85%	90%
Cell growth	-	S	G	E	G
Cell Morphology	-	U	U	U	A

* Approximated attachment rate 24 hours after seeding

S = slow growth rate as compared to MEM/10 stock cultures

G = good growth rate compared to stock cultures

E = excellent growth rate compared to stock cultures

U = uniform throughout colony

A = abnormal cells constitute colony

Plating Efficiency of Cells

The first media trial also served as a plating efficiency experiment. Table 7 shows that the minimal seeding concentration of PCK cells is greater than 1×10^4 cells per 30 ml flask or greater than 3.3×10^3 cells per ml of medium. The PCK-4 cells were passed three times. Most of the parent fibroblasts died, but slender, vacuolated fibroblastic cells which never re-established the original morphologic form or confluent monolayers of the parent fibroblasts were formed. This morphologic type was the last cell type seen in dying uninfected primary kidney cell cultures.

The minimal seeding concentration for the transformed cells, OSg-12 and 390K-18, is less than 1×10^1 cells per 30 ml Falcon flask, or less than 3.3 cells per ml of culture fluid (Table 7).

In a second trial, 390K-12 cells were passed twice in 4 days. On the sixth day, the cells were trypsinized, washed and resuspended to a final concentration of 5×10^2 cells per ml of media. Viable counts were made with 0.2% Trypan blue. Two drops or 0.1 ml of the cell-seeding suspension were placed in the center of the growing surface of a 30 ml Falcon flask (40-70 cells per flask) with a dispensing pipette (shop made). Ten flasks were seeded in this manner. The cells in each flask were observed as single cells and counted on the inverted Zeiss microscope at 60X and 100X magnifications. After the total cell count of each flask was recorded, 3.0 ml of NCTC 135/20 was added (Table 9). The pH of the flasks was adjusted with 5% CO_2, and the flasks were placed in a 37°C incubator. The cells were inspected every 4 days with the inverted microscope. The NCTC/20 was changed

Table 9

Plating Efficiency of 390K-15 Transformed Cells

Bottle Number	Initial Number of cells seeded*	Final Number of Clones	Microscope Clone count	
1	44	27	31	
2	68	33	33	
3	57	30	35	
4	46	15	16	
5	45	27	27	
6	44	26	28	
7	54	25	25	
8	41	21	21	
9	41	23	24	
10	44	--**	--**	
	440	227	243	Totals
	---	51.6%	55.2%	Plating Efficiency

* Viability counts indicated that 100/100 cells were viable

** Flask medium turned basic on the second day and killed the cells. This bottle was not included in the calculations.

every 10 days during the first 20 days of culture. For the second 20 days of culture, the medium was changed to MEM/5. After the 40th day of culture, the cell colonies were gently washed twice with Pd. The cells were fixed for 15 minutes with 5% glacial acetic acid in 95% ethanol. The fixative was removed, and the cell colonies were stained for 5 minutes with 0.5% Methylene blue in Pd diluted 1:4 with fresh Pd. The stained colonies were washed twice (1 minute each) with PBS, pH 7.2. A grid was drawn on the underside of the growing surface of the flasks to aid in location of uncounted colonies. The colonies were counted by gross inspection and by use of a 6X stereo microscope. Colonies less than 1.0 mm in diamter were not counted.

Two representative flasks were prepared for photographic recording of colony growth. The flasks were filled with PBS and placed on a labeled photographic mask over a lighted background. Two 200 watt flood lamps were used for indirect lighting of the lettering on the mask. The cells and mask were photographed with a 35 mm camera (Pentax). The photograph of the flask colonies is shown in figure 5.

The second plating efficiency experiment was designed to demonstrate the plating efficiency of single transformed cells. The results are shown in Table 8. Microscopic inspection of the 390K-15 cell colonies showed that the majority of the cell clones were larger than 1 mm and could be observed by the unaided eye. No colonies smaller than 0.5 mm formed, indicating that there was no cell detachment and secondary colony growth. Table 9 shows the initial cell viability (100%) and seeding counts, the final clone number, and the cell plating efficiency of 55.2%. Flask #10 was excluded from the calculations

since a gas leak allowed the media to become basic and the cells died on the second day of the experiment. Control kidney cells were not used as they would not survive in a seeding concentration of less than 1×10^4 cells per flask.

CALF 390 KIDNEY CELL PLATING EFFICIENCY

390K-18
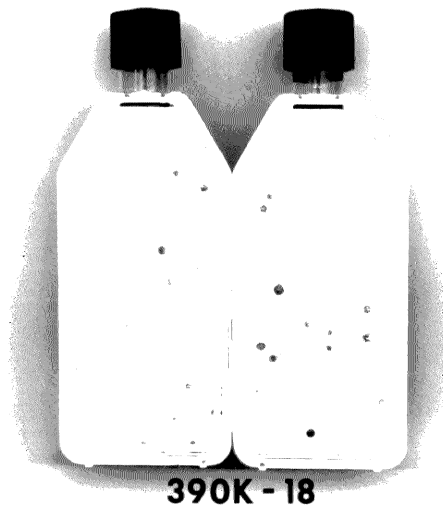
Figure 5

Plating Efficiency of Transformed Calf Kidney Cells. 18th passage, 40 day old cultures. Dark spots are cokines of cells. Metheylene blue stain. Approximately 1/2 actual size.

Sattellite Colony Formation

Observations of the cloning ability of transformed cells suggested that viable cells detached from the clones and reattached at distant sites. Transformed calf kidney cells. 390K-15 and 390KT-4 (transformed cells passed by scraping colonies off the growing surface).

embryonic bovine kidney, FBK-15, and sheep transformed salivary gland,
OSg-14, were used to test satellite colony formation. One ml of M-199/
10 containing 1×10^4 cells was placed at the bottom of a 30 ml Falcon
flask which was placed in an upright position. Two ml of fresh M-199/
10 was added to each flask. The flasks were carefully lowered to a
15° slanted position with the cap of one flask resting on the side of
another. The front miniscus of the media was marked on the underside
of the slanted culture flask. The flasks were cultured in the slanted
position for 24 hours at $37^o C$. After the 24 hour cell attachment period,
the media was renewed and the flasks lowered to a normal horizontal
position, in which the media covered the entire flask growing surface.
The culture media was changed every 3 days during the 21 day culture
period.

The clones were examined by microscopy. They were photographed
by photomicroscopy and by back light macrophotography with a 35 mm
camera (Figure 6). FBK-45 showed contact inhibited fibroblasts. The
white areas on this flask were scratches on the outer surface. The
monolayer was not apparent to the unaided eye. 390K-15 showed conflu-
ent layers of transformed cells. 390K-15S, 390KT-4 and 390KT-45 show-
ed the satellite colony formation by cells detached from the initial
seed area behind the black line. Colonies arose from single and
clumped cells. Non-uniform cell stacking occured in the initial seed
area of the two slanted flasks, which indicated colony formation on
top of the basal cell layer.
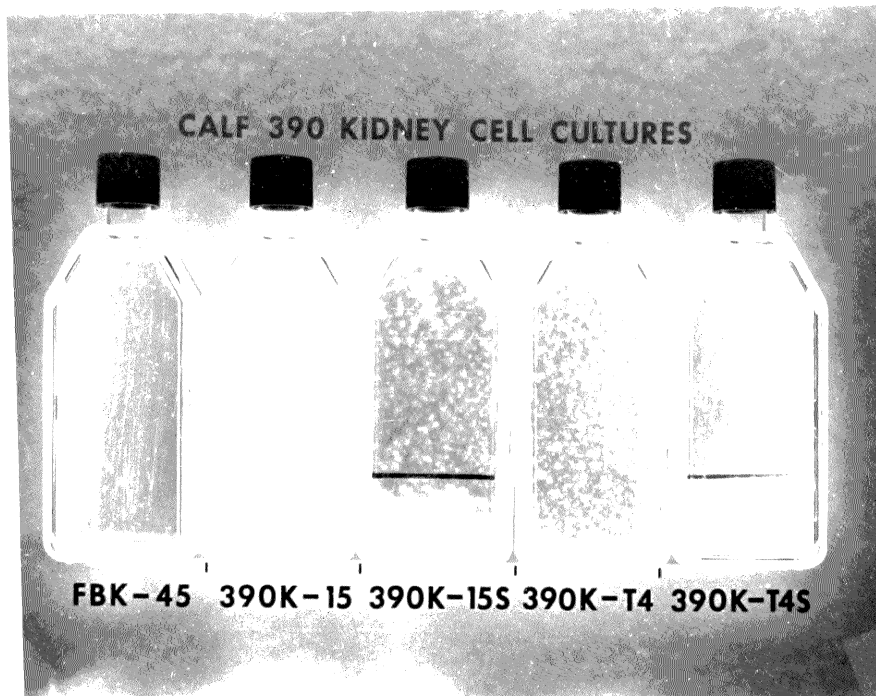
Figure 6

Satellite Colony Formation by Transformed Calf Kidney Cells.
a. FBK-45 is a stable fetal bovine kidney cell monolayer, 45 passages.
b. 390K-15 is a trilayer culture of transformed calf kidney cells, 15 passages.
c. 390K-15S is a slanted flask culture of 390K-15. Satellite colonies are seen as white spots above the miniscus line.
d. 390K-T4 is an incomplete, multilayer culture of transformed calf kidney cells, 4 passages. This cell line was passed by growth surface scraping.
e. 390K-T4S is a slanted flask culture of 390K-T4. The satellite colonies are seen as white spots above the miniscus line, 4 passages.
All cultures are 21 days old. Approximately 1/2 actual size.

Transformed Cell Growth in Hamster and Immune Cow Serum

390K-16 cells were adapted to growth in hamster serum. Hamster serum (HS) was obtained from pooled bleedings of adult white hamsters. The serum was inactivated for 30 minutes at $56^{\circ}C$. The cells were cul- tured in M-199 with varying amounts of FCS and HS. The total serum concentration was maintained at 10% of the medium volume. The 390K-16 cells were seeded at 5 x 10^5 cells per 30 ml Falcon flask in 2% HS, 8% FCS. The cells were grown for four days, passed and grown in 2% HS, 8% FCS for another four days. The serum was then changed to 4% HS, 6% FCS for four days of culture. The cells were passed and reseeded in 4% HS, 6% FCS for four more days. This procedure was repeated, in- creasing the HS concentration every eight days.

One flask was seeded with 5 x 10^5 390K-16 cells. The cells were grown for 48 hours on 10% FCS and then changed to M-199/10% HS. The culture was passed twice in three weeks.

390K-16 cells were grown in the presence of immune serum from cattle (21). The cells were seeded at 5 x 10^5 cells per 30 ml Falcon flask in M-199/10 and cultured for 48 hours at $37^{\circ}C$. Fresh M-199/10 and 1% inactivated cow serum was added to the cultures. The cow serum was selected from immune cows 1125, 888, and 774 and from non-immune cow 9150. The cells were cultured for three weeks in the presence of immune serum.

The 390K-16 transformed cells being adapted to growth in ham- ster serum survived for 20 days, but soon stopped dividing and died. The cells showed increasing vacuolization as the culture time and HS concentration were increased. The cells did not change appreciably

in size or shape.

The 390K-16 cells grown in 10% FCS and then in 10% HS survived 21 days for two passages before dying in the same manner as the gradually adapted cells.

The 390K-16 cells grown in the presence of immune cow serum 1125, 888, and 774 and non-immune cow serum 9150 survived in culture for 21 days. They showed little vacuolization or cell death. The division rate was slightly decreased, but the cells appeared morphologically and culturally similar to normal stock seeding cells.

SPECIFIC PHYSIOLOGIC PROPERTIES OF CELLS

Cells Grown in Soft Agar with NCTC-135 plus 20% FCS

OSg-12, 390K-18 and PCK-4 were grown in soft agar suspension in microtiter tissue culture plates. The first experiment was designed to test whether both normal and transformed cells could grow in suspension in 0.3% soft agar. Single cells of each of the above cell lines were cultured in the wells of sets of rows. The single cells of OSg-12 formed multicellular clumps in the 0.3% agar with 0.10 ml of NCTC/20. All of the other wells of all plates contained non-growing single-seed cells.

The OSg-12 colonies in 12 wells were counted. The cell cloning efficiency in soft agar was calculated by dividing the average number of colonies per well X 100, by the number of seed cells (25 cells per well). The results are summarized in Table 10.

Table 10

Soft Agar Cloning Efficiency of Transformed Cells
(Medium NCTC-135 plus 10% FCS)

Cell type	Total Colony count (12 wells)	Average Colonies per well * suspended	all colonies	Plating efficiency Suspended colonies	Total colonies
OSg-12	282** 252	21	23.5	84.0%	94.0%
390K-18	0	0	0	0	0
PCK-4	0	0	0	0	0

* each well was seeded with 25 cells in 0.05 ml suspension agar

** wells contained 0.05 ml of 0.3% agar cell suspension and 0.10 ml of NCTC/20

The OSg-12 plating efficiency was 98%. A few seed cells formed attached colonies. These colonies had the characteristic cell morphology of the stock cells, without multi-layering. 390K-18 and PCK-4 cells did not grow in the soft agar. Representative photomicrographs of cell colonies and single cells are shown in figures 7 and 8.

Recovery of cells from soft agar suspension. The OSg-12 cells remained viable and were recovered in liquid medium culture. A photomicrograph of the colony outgrowth is shown in figure 9.

The 390K-12 and PCK-4 cells remained as single cells, entrapped in the agar. Microscopic inspection of these cells showed that they were dead.
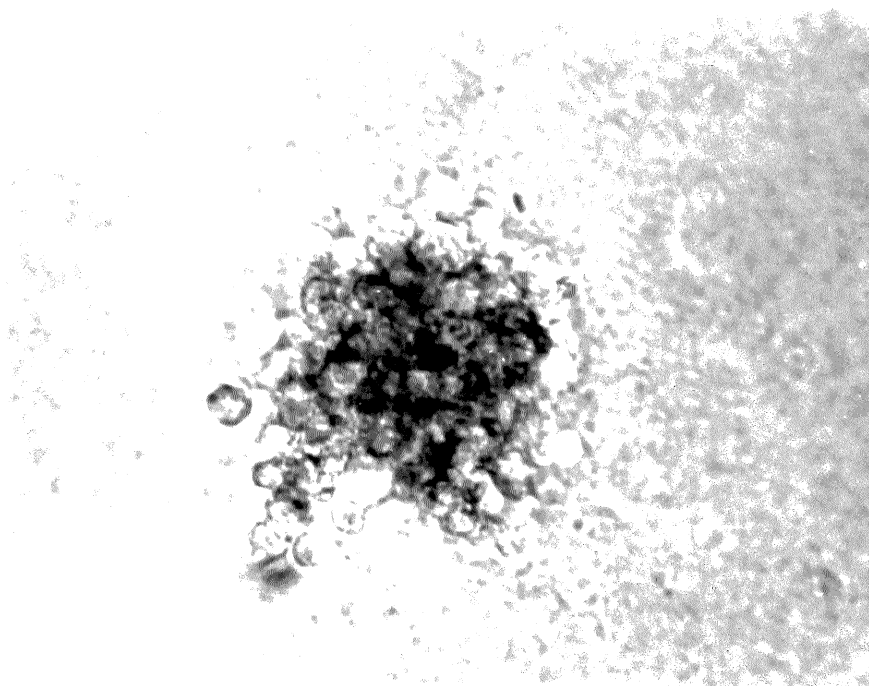
Figure 7

Growth of Transformed Sheep Salivary Gland Cells in 0.3%
Agar. 12th passage, 21 day old culture. The clump of cells
arose from a single cell. Phase contrast. X160
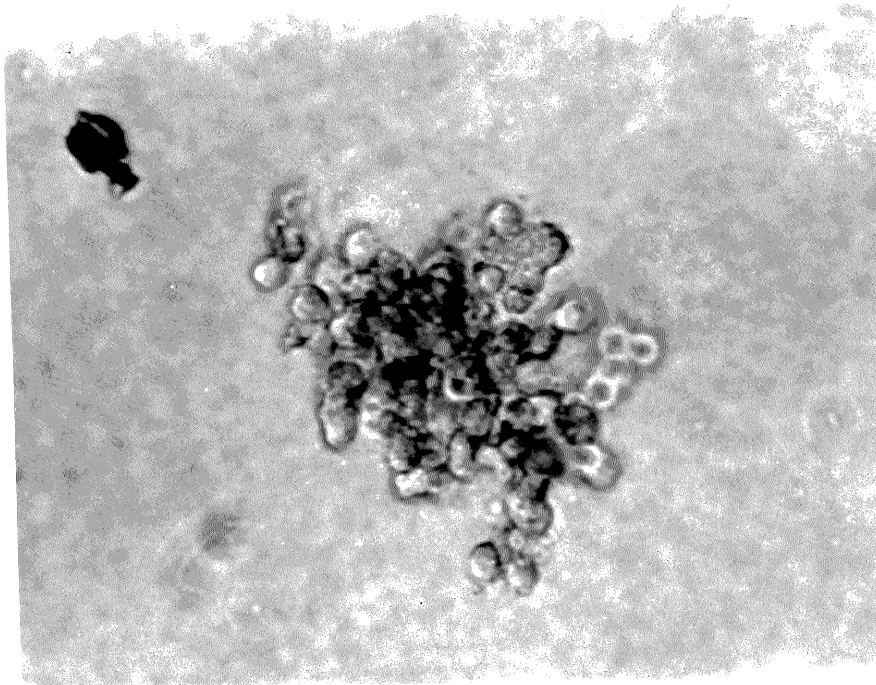
Figure 8

Growth of Transformed Calf Kidney Cells in 0.3% agar.
18th passage, 21 day old culture. The clump of cells
arose from a single cell. Phase contrast. X160
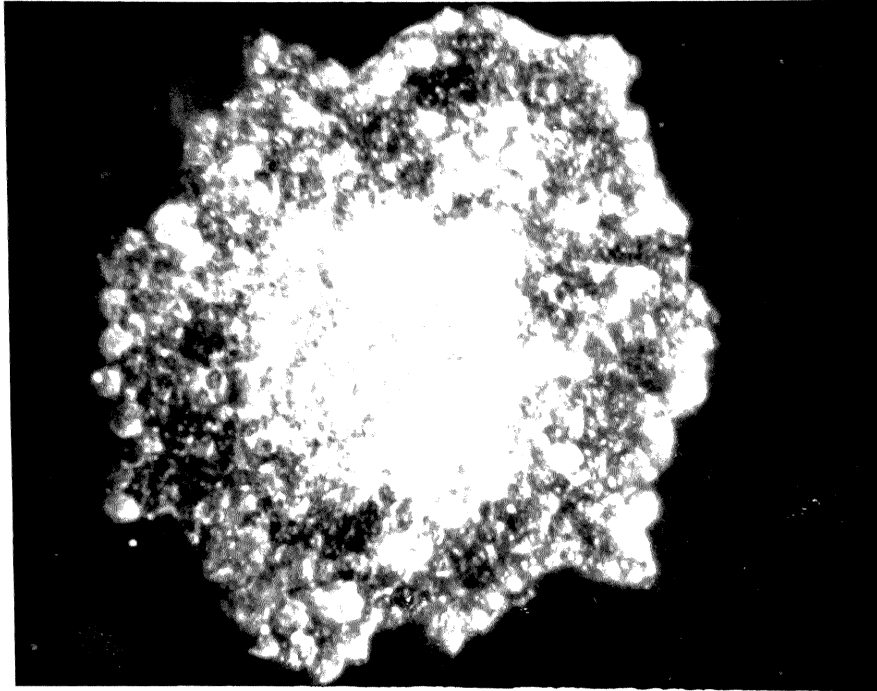
Figure 9

Outgrowth of a Transformed Sheep Salivary Gland Cell
Colony from 0.3% Agar. 12th passage, 7 day old culture.
The central white area is the agar colony. Outgrowing
basal layer cells appear as dark circles. Stacked
cells appear as white circles. Phase contrast. X100

Growth medium test in soft agar. 390K-20 cells (25 cells

seeded per well) were used in a growth medium test in soft agar be-

cause they had not grown in the NCTC/20. Table 11 shows the cell col-

ony counts after 14 days of culture in 0.3% agar. Figure 10 shows

a colony of 390K-20 cells growing in soft agar with MEM/15.

Table 11

Soft Agar Medium Test with 390K-20 Cells

FCS Concentration (%)	Total Colony Count (16 wells)	Avg. # Colonies per well	Plating Efficiency (%)
0	0	0	0
5	7	0.44	1.8
10	57	3.6	14.4
15	122	7.6	30.4
20	153	9.6	38.5
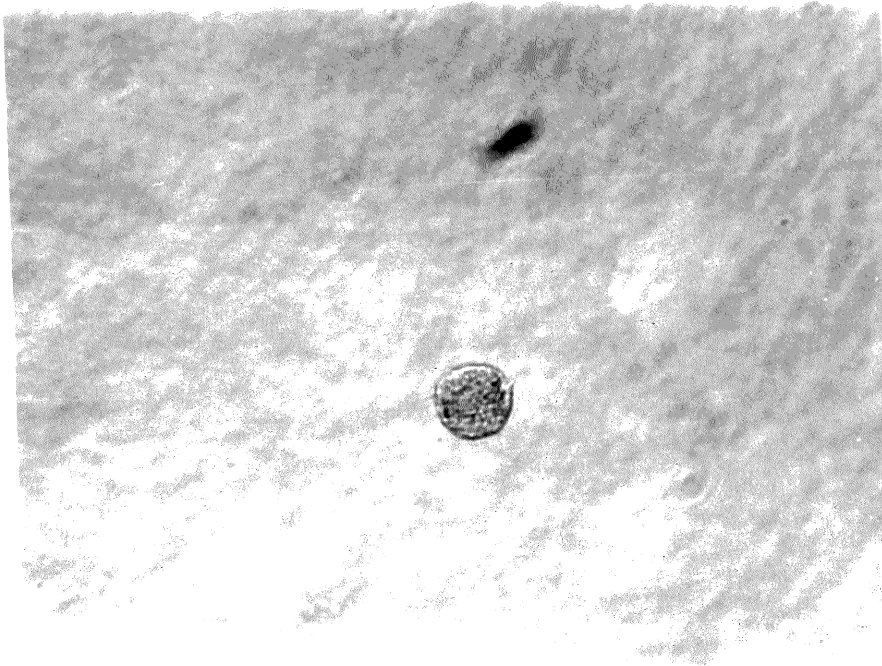
Figure 10

Growth of Uninfected Calf Kidney Fibroblast in 0.3% Agar.
4th passage, 21 day old culture. Note the dried pea appear-
ance of the dead, single seed cell. Phase contrast, X160

The plating efficiency of 390K-20 cells in MEM plus 0%, 5%,
10%, 15% and 20% FCS was 1.8%, 14.4%, 30.4% and 38.5% respectively.

The 390K-20 agar suspension colonies in MEM/15 were recovered
in liquid medium cultures. The outgrowth of the 390K-20 cells was
identical with that of the OSg-12 cells.

Petri dish growth of OSg-12 and PCK-4 cells. OSg-12 and PCK-4
cells seeded in 0.3% soft agar with MEM/10 were cultured for 14 days.
The OSg-12 cells formed clumps of suspended colony cells. The PCK-4

cells remained as single cells and gave no indication of growth or acid production.

Mitotic Index of Transformed Cells

The mitotic index (number of dividing cells per 1000 total cells) is used to indicate the growth rate of a cell culture (34). The mitotic indexes of 390K-25 and OSg-19 transformed cells were determined in the following experiment. Monolayer cultures grown in MEM/10 were trypsinized. Clumps of cells were allowed to settle out of suspension for 1-2 minutes. One ml of a suspension containing 1×10^4 cells per ml was added to each of the chambers of sixteen 2-well tissue culture slides (Lab-Tek, Miles Laboratories #2002). Cells of 390K-25 or OSg-19 were added to separate chambers of the same slide. One ml of fresh MEM/10 was added to each well and the slides were placed in the CO_2 incubator box at $37^{\circ}C$. The medium was replaced every 72 hours for 8 days.

Cells were prevented from further division at the metaphase stage of mitosis with colchicine (20). The colchicine was added to MEM/10 at a concentration of 20 ug per ml of medium. Five ml aliquots of the MEM/10 plus colchicine were placed in 15 ml culture tubes and stored at $-4^{\circ}C$ until needed. Duplicate slides were prepared for determination of the mitotic index at 1, 2, 3, 5, 7 and 9 days of culture. The culture medium was removed from the chambers of one slide and 1.5 ml of the MEM/10 with colchicine was added. The slides were incubated in the CO_2 box for 4 hours, fixed in Bouin's fixative, and stained with H & E.

Five hundred cells were counted in each of the colchicine-treated cultures at 160X magnification with a Zeiss light microscope. The cell counts were recorded by use of a hand counter (Clay-Adams). The untreated duplicate cultures were used to monitor normal growth of transformed cells. Cells in which mitosis had been stopped in metaphase by colchicine were rounded and raised above the basal cell layer. Colonies of less than 10 cells were not counted. Cells in all stages of mitosis were observed in the control cultures. The mitotic index was expressed as percentage of dividing cells per 500 cells counted. The mitotic index of the 390K-25 and OSg-19 cells increased from 6% to 28% during 6 days of culture. On the ninth day, the number of dying cells was so great that the detection of dividing cells was impossible. The results are recorded in Table 12.

Karyogram Analysis

The normal chromosome diploid complement of cattle is 58 autosomes and 2 sex chromosomes. The normal chromosome diploid complement of sheep is 52 autosomes and 2 sex chromosomes. The autosomes of sheep and cattle occur as autocentric chromosomes or horseshoe-shaped chromosomes. Male animals have one large X and one small Y (x-shaped) sex chromosome. Female animals have two large X sex chromosomes. Chromosome spreads from normal sheep and cattle tissue culture cells (testes, salivary gland and kidney) were made. A representative karyogram from normal sheep testes and calf kidney is shown in figures 11 and 12. Both spreads had the normal diploid complement of chromosomes. Chromosome spreads from infected fibroblasts of calf salivary gland are

Table 12

Mitotic Index of Transformed Cells

Days In Culture	Dividing Cell Count (500 normal cells)		Mitotic Index	
	390K=25	OSg=19	390K=25	OSg=19
1	33	59	6.6	10.8
2	750	57	15.0	10.4
3	84	83	16.8	16.6
5	140	80	28.0	16.0
7	100	125	20.0	25.0
9	NA	NA	NA	NA

NA = not available. cells were dying in culture

shown in figure 13. The karyogram appeared normal. Chromosome spreads from transformed sheep testes (OSt=10) and transformed calf kidney (390K=6) are shown in figures 14 and 15. The transformed cell spreads showed chromosomal centromeric translocation, chromosomal fragmentation, aneuploidy and polyploidy. Abnormalities varied between different spreads of transformed cell chromosomes.

Tumorogenesis in Mice and Hamsters

Two experiments were performed to test the tumorogensis of the virus in laboratory animals. Pooled 390K virus was inoculated into new-born Balb/C mice. 390K=18 and OSg=14 transformed cells were inoculated into the cheek pouch tissue of cortisone-treated weanling hamsters.

Inoculation of Balb/C mice with 390K-pool. Five one-day-old Balb/C mice were inoculated intraperitoneally with 0.1 ml of the

Figure 11

Karyogram of Normal Calf Kidney Fibroblast Chromosomes.
4th passage. 58 acrocentric autosomes and 2 metacentric
sex chromosomes are present. Giemsa stain. X400
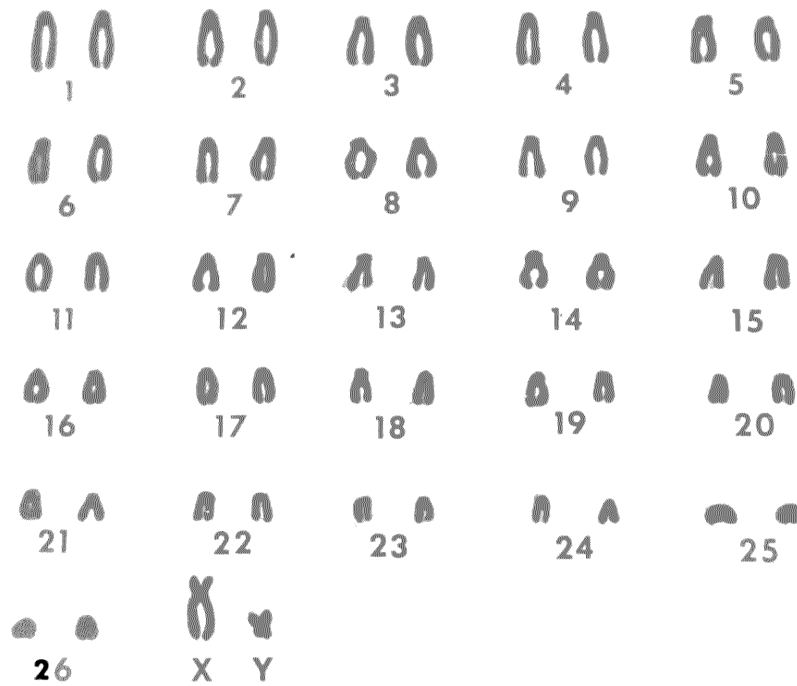
Figure 12

Karyogram of Normal Sheep Salivary Gland Fibroblast
Chromosomes. 4th passage. 54 acrocentric autosomes
and 2 metacentric sex chromosomes are present. Giemsa
stain. X400

Figure 13

Chromosome Spread of an Infected Calf Kidney Fibroblasts.
4th passage. 58 acrocentric autosomes and 2 metacentric
sex chromosomes are present. Giemsa stain. X400
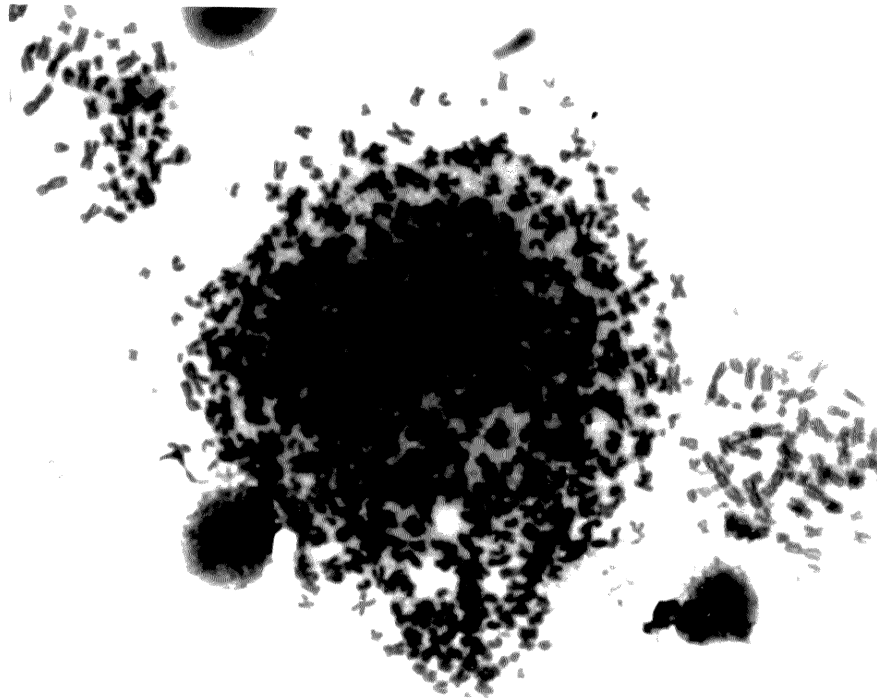
Figure 14

Chromosome Spread of Transformed Calf Kidney Cells.
6th passage. Chromosomes from 3 cells are shown.
Note the chromosomal polyploidy and centromeric trans-
location. Giemsa stain. X400
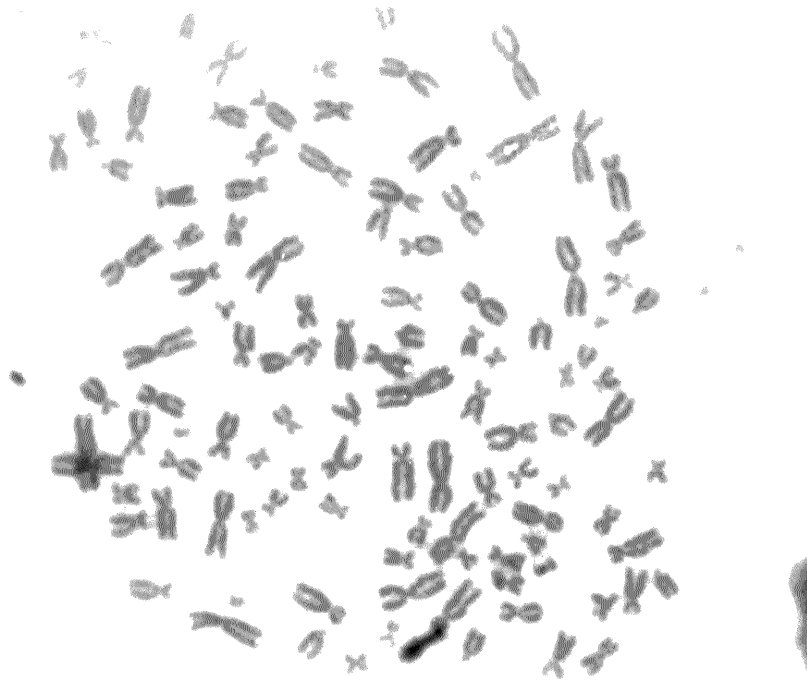
Figure 15

Chromosome Spread of Transformed Sheep Salivary Gland
Cells. 12th passage. Chromosomes of one cell cover
the entire field. Note the chromosomal polyploidy and
centromeric translocation. Giemsa stain. X400

390K-pool virus. Five uninoculated mice from the same litter were neg-

ative controls. The mice were observed for 42 days post-inoculation.

At the end of this period, palpitation of the infected mice gave no

indication of tumorogenesis or organ enlargment and the experiment was

terminated.

Inoculation of hamsters with transformed cells. Ten weanling

hamsters were inoculated subcutaneously with 0.1 ml of cortisone

acetate (UpJohn 25 mg/ml) twice weekly for three weeks. Twenty-four hours after the first injection of cortisone, each of five hamsters was inoculated in the right cheek pouch tissue with 1 x 10^5 390K-18 cells in MEM/5. Each of the other five hamsters was inoculated in the right cheek pouch tissue with 1 x 10^5 OSg-14 cells. The inoculation site was observed at 7 day intervals for signs of tumor growth.

The hamsters were sacrificed at 21 days post-inoculation. The right cheek pouch of each hamster was opened, inspected for tumor growth and compared to the left cheek pouch. There were no apparent foci of tumor growth in the five hamsters inoculated with 390K-18 cells. The liver, kidneys, spleen and lungs of these hamsters appeared normal. One hamster of the five inoculated with the OSg-14 cells had a tumor-like nodule distal to the site of cell inoculation. The internal organs of the five hamsters appeared normal.

The tumor-like nodule of the OSg-14 inoculated hamster appeared to be in the cheek pouch tissue. The nodule was hard and about 2mm in diameter. The nodule was removed, diced with surgical scissors, and cultured in MEM/10 in a 30 ml Falcon flask at 37°C. After 10 days of culture, fibroblastic and small epitheloid cells were observed "growing out" from the nodule chunks. The epitheloid cells grew in layers. The morphology of these cells was very similar to that of bovine and sheep transformed cells. At 15 days of culture, colonies of epitheloid cells were formed among senile fibroblastic cells by cell detachment and relocation from the original nodule colonies. The characterization of these cells is presently in progress.

Sheep Testes Feeder Layer Infection

Formation of infectious virus particles by transformed cells was demonstrated by infection of normal feeder layer cells. This experiment did not differentiate between cellular release of free virus or viral infection through membrane to membrane contact of adjacent cells.

390K-12 transformed cells (1×10^4 cells/ml) were attached and grown in MEM/5 on Leighton tube glass slides. Twenty-gour hours after the 390K-12 cells were seeded, 5×10^4 uninfected primary sheep testes cells (PSt-10) were added to each of the Leighton tube slide cultures without removal of the culture medium. Duplicate control cultures were seeded with 390K-12 or PSt-10 cells to monitor the growth properties of the isolated cell types. The Leighton tube cultures were incubated at $37^{\circ}C$ in the CO_2 incubator box. One set of slides was fixed in Bouin's fixative and stained with H & E at 2 weeks, and another set at 4 weeks of culture. The cells were examined at 400X and 1000X on a Zeiss microscope. Photomicrographs were taken of representative feeder layer and transformed cells (figure 16).

The control cells appeared normal throughout the culture period. The feeder-layer-culture cells, 390K-12 and PSt-10, underwent several morphologic changes during the culture period. The 390K-12 cells at the periphery of the colony, in contact with the PSt-10 cells, became larger than the internal-colony cells. Nuclear granulation and cyto-plasmic vacuolization was more pronounced in the peripheral 390K-12 cells than in the internal-colony or control cells. There was no evidence of nuclear or cytoplasmic inclusion formation in any of the 390K-12 cells.
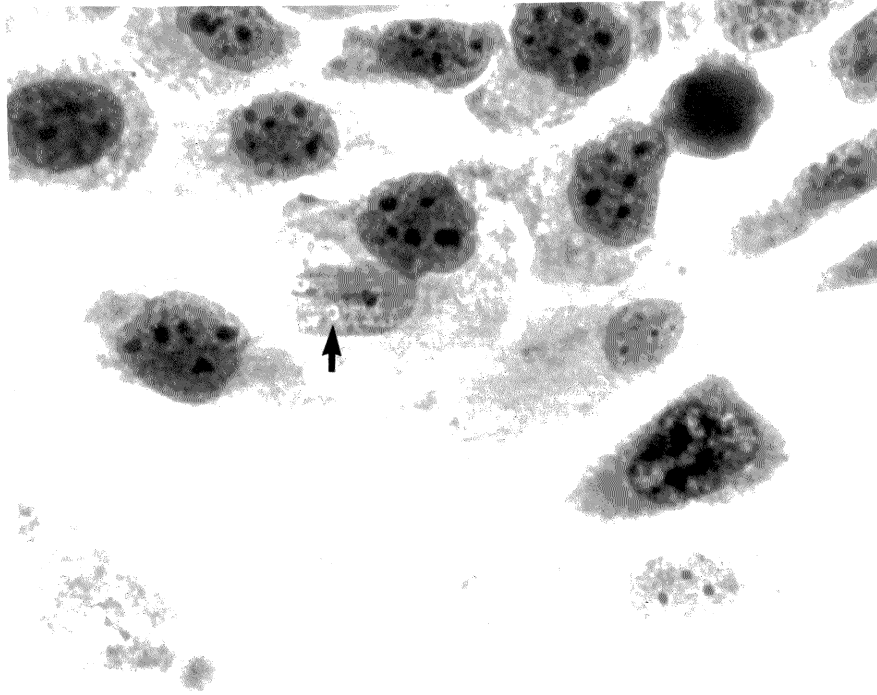
Figure 16

Normal Sheep Testes Fibroblast Feeder Layer Infection
by WCLD Virus from Transformed Calf Kidney Cells. 21
day old culture. The sheep fibroblasts are identifi-
able by their lightly staining nucleus and nuclear in-
clusions (arrows). An unidentified cell, in the middle
right area, has many nuclear inclusions and dense nuc-
lear staining. Hemotoxylin stain. X400

The CPE of PSt-10 cells of the feeder layer in contact with

the 390K-12 cells varied. The morphologic changes of the PSt-10 cells

included: giant cell formation, loss of the fibroblastic spindle

shape, swollen nuclei, cytoplasmic granulation, and nuclear eosino-

philic inclusion bodies. The nuclear inclusion body formation was most

evident in cells of the 4 week old cultures. The inclusions were

small but well pronouced, similar to those observed in the early

expression of this cytopathic effect in infected fibroblastic cells (21). CPE was less pronouced in Pst-10 cells that were distant from the 390K-12 colonies.

Superinfection of Transformed Cells

Attempts were made to infect OSg-12 transformed cells with 390K virus to induce the CPE characteristic of infected fibroblasts. Two x 10^5 OSg-12 cells were placed in each well of three 2-well tissue culture slides (Lab-Tek #2002). One and one-half ml of MEM/5 was added to each well. The slides were cultured in a CO_2 incubator box for 7 days at $37^\circ C$. The medium was removed and 0.1 ml of 390K pool virus was added to one well on each slide. One tenth of a ml of MEM/5 was added to the other well. The virus was adsorbed to the cells for 1 hour in the CO_2 incubator box at $37^\circ C$. One and one-half ml of fresh MEM/5 was added to all wells and the cells were cultured in the CO_2 box at $37^\circ C$. The medium was changed every 7 days. Slides were fixed in Bouin's fixative and stained with H & E at 14 and 21 days post-infection. The cells were examined with a Zeiss microscope (400X) for viral caused CPE.

The superinfected OSg-12 cells appeared morphologically identical to stock cultures of Osg-12. There was no indication of viral caused cellular CPE (cytoplasmic vacuolization or eosinophilic nuclear inclusions). Further evidence of species cross-infectivity by virus pools is discussed in this report and elsewhere (21).

Transformed Cell Agglutination by Immune Bovine Serum

Transformed bovine kidney cells (390K-11, 390K-14) were incu-
bated with bovine blood serum to test the cell specific agglutination
titers of immune and non-immune cattle. Brood cows who had previously
delivered a WCLD calf were considered immune. Non-immune animals were
infected calves and their dams.

Non-immune serum was collected from the WCLD infected 10 day
old calf 529, 4 week old calf 390, cow 774 (529 dam) at 10 days post-
parturition (774A serum) and cow 9150 (390 dam) at 4 weeks post-part-
urition. Immune serum was collected from cow 774 (774C) and cow 1125
at least 3 months after delivery of an infected calf. The pooled
1125/774 serum was used in immune serum therapy of infected calves.

Two day old calves 34 and 50 were inoculated with a 0.22 um
filtered 390K pool virus preparation; 1.0 ml intramuscularly, 1.0 ml
subcutaneously and 1.0 ml intranasal drip. Serum samples were collect-
ed at 1 day pre-inoculation and 7 and 21 days post-inoculation.

Whole blood from test animals was collected in sterile 10 ml
blood collection tubes by J.K. Ward, D.V.M. The blood was allowed to
clot for 1 hour and was centrifuged at 1500 rpm for 20 minutes. The
supernatant serum was removed by aspiration with a sterile Pasteur
pipette. Test sera and commercial FCS was inactivated at $56^{o}C$ for
30 minutes. The sera were stored in sterile 15 ml culture tubes at -4°
C until needed.

The cells were agglutinated in the wells of 96-well microtiter
plates. The sera were serially diluted in single rows of 11 wells.
The twelvth well of each row contained FCS diluted 1:2. The 12 wells

of the last row of each plate were Pd control wells. One drop of Pd
(0.05ml) was placed in each of the first 11 wells in rows A-G.with a
50 ul dispensing pipette. One drop (0.05 ml) of test serum was added
to the first well in rows A-G. The test sera were serially diluted
from 1:4 to 1:2048 with a microtiter 0.05 ml serial diluter in wells
2-11 of rows A-G. One drop (0.05 ml) of Pd was placed in each of the
wells of the 8th row (H). One drop (0.05 ml) of FCS diluted 1:2 was
added to the twelfth well of rows A-G.

Transformed cells (390K-11, 390K-14) and control F1 (human
amnion) stable cells were removed from the growing flask by trypsin-
ization. The cells were centrifuged and stored in fresh medium for 12
hours at 4°C. The cells were then washed 3 times with Pd and the cell
concentrations were adjusted with Pd to 5 x 10^5 cells/ml. One drop
(0.05 ml) of the cell suspension (1 x 10^4 cells/0.05 ml) was added to
each well of the microtiter plate. The plates were covered with micro-
titer sealing tape and incubated at 37°C for 60 minutes. The cells
were inspected on the inverted microscope at 60X magnification and then
transferred to 4°C for 12 hours. Sera dilutions with positive agglu-
tination titers clumped more than 10% of the cells in groups of great-
er than 3 cells.

The sera of non-immune cows had a maximum agglutination titer
of 1:32 for 390K cells. Sera of naturally infected calves had a max-
imum agglutination titer of 1:16, and synovial fluid had an agglutin-
ation titer of 0. Sera of experimentally infected calves had a rising
agglutination titer of 1 or 2 dilution units. Immune cows had agglutin-
ation titers from 1:128 to 1:256. Control F1 cell agglutination titers

ranged from 1:8 for infected calves and acute stage cows, to 1:32

in immune cows. FCS controls had agglutination titers from 0 to 1:2

(Table 13).

Table 13

Summary of 390K-11, 14 Cell Agglutination Titers
of Bovine Serum

Animal Number

Cell line Experimental Time	Cows				Calves		
	9150	774A	774C	1125/774	529/SF*	390	FCS
390K-11 10 min.	1:4	1:8	1:32	1:16	0/0	0	0
390K-11 60 min.	1:8	1:16	1:64	1:32	0/0	0	0
390K-11 24 hours	1:32	1:32	1:256	1:128	1:16/0	1:4	0

* 529/SF is 529 serum titer/ synovial fluid titer

Virus Treated Calf Serum Agglutination Titers

Cell line Experimental Time	34-1	34-2	34-3	50-1	50-2	50-3	FCS	774A	774C
390K-14 24 hours	1:4	1:4	1:16	1:16	1:32	1:32	1:2	1:16	1:64
	9150								
	1:4								

Induction of Cell Transformation

Transformed cells in most of the sheep and bovine cell cultures appeared to arise from fibroblastic cells without a transitional cell type. An experiment was performed to determine if transformation of bovine salivary gland and kidney cells could be induced and to demonstrate the progression of the cell forms from infected fibroblasts to transformed cells.

Uninfected salivary gland (PCSg-1) and kidney (PCK-1) fibroblastic cells were infected with 390K pool virus, OSg-4 pool virus and 390K pool virus neutralized as outlined earlier at 2 weeks of age. The cells were passed 1 week post-infection. The infected fibroblastic cells in submonolayers and confluent monolayers vacuolated after 2 weeks and were not passed until after the crisis stage of infection. The cells remained in this vacuolated stage for 2 weeks. During this time, the normal and neutralized virus treated cells appeared normal but were not passed. Slides of H & E stained cells showed nuclear inclusions in the infected cells but not in the normal or neutralized virus treated cells.

At 6-8 weeks post-infection, epitheloid cells began to grow in the submonolayer cultures of cells infected with 390K and OSg-4 pool virus. These cells began growing in two submonolayer salivary gland cultures treated with neutralized virus. The epitheloid cells did not grow in the viral infected monolayer cultures or in the uninfected control cultures at submonolayer or confluent monolayer.

At 9 weeks post-infection, only the epitheloid cells were growing in those cultures in which they had earlier appeared. The

epitheloid cells varied in size from 1/2 to 2X the size of normal fibro=
blasts. The cultures showed radial monolayer growth, some stacking
and simultaneous rapid cell growth and cell death. Cell cloning was
not evident. Less than 40% of the cells were removed from the growing
surface by 10 minutes of trypsinization. Control and infected cell
passage was resumed. The control cells did not recover from the
fourth passage (9 weeks of age). The epitheloid cells which were pass=
ed once gave rise to a foci of small epitheloid cells that appeared
identical to transformed cells. The PCSg-4 transformed cells (390K=
pool infected) were lost due to the high pH of the medium from flask
CO_2 leakage. The OSg-4 infected transformed cells were transferred to
new culture flasks. Stained preparations of these cells showed that
they were morphologically identical to other transformed cell lines.

The infected monolayer cultures still maintained their stab-
ilized state at the end of 12 weeks. The culture medium had to be
replaced every 4-5 days. The neutralized-virus-treated cells and
those cultures that had epitheloid and fibroblastic cells did not give
rise to transformed cells.

Representative photomicrographs of transforming cultures are
shown in figure 17. The results of this experiment are summarized in
Table 14.

Detection and Inhibition of Mycoplasma in Cell Cultures

Supernatant culture medium from newly infected fibroblastic
cells and transformed cells was cultured to detect the presence of
mycoplasma (47). These cultures were maintained for 4 weeks.
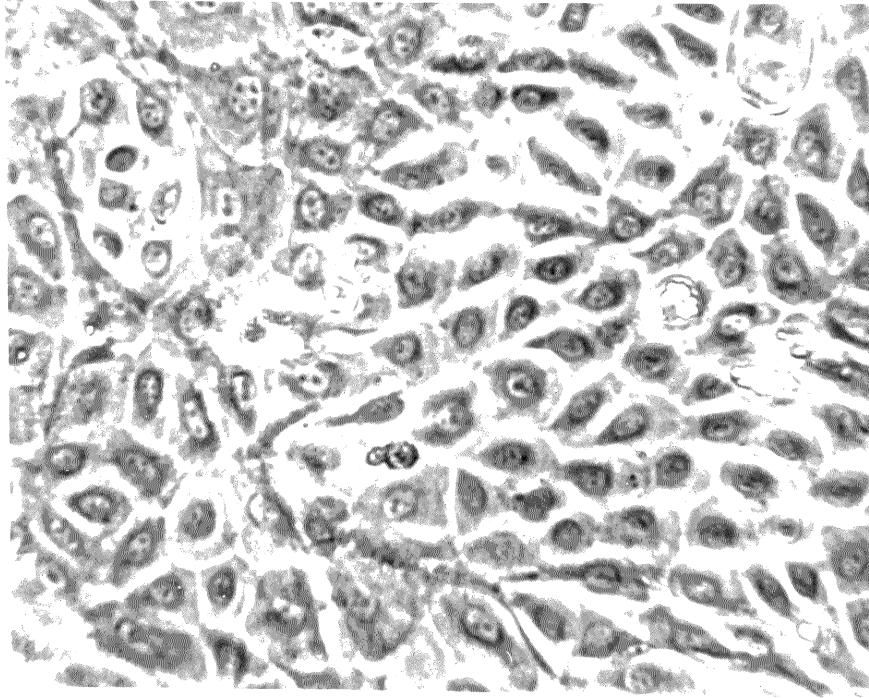
Figure 17

Transforming Calf Salivary Gland Epitheloid Cells
4th passage. Cells were infected with the sheep
OSg-4 WCLD virus pool. Note the random sizes and
shapes of the cells. 8 weeks post-infection.
Phase contrast. X160

Table 14

Results of Primary Calf Cell Transformation
by 390K and OSg-4 Virus Pools

Group Number	Cell Type	Original Fibroblast Death	Cell Outgrowth	Cell Transformation
1	PCK-2	-	maintained original monolayer	∞
	PCSg-2	∞		-
2	PCK-3	+	epitheloid and slender fibroblasts	-
	PCSg-4	+		(+)lost culture
3	PCK-2	-	maintained original monolayer	-
	PCSg-2	∞		∞
4	PCK-3	+	epitheloid	-
	PCSg-4	+	epitheloid	+
5	PCK-2	+	none	∞
	PCSg-2	+	none	∞
6	PCK-3	+	epitheloid and slender fibroblasts	-
	PCSg-3	+		∞
7	PCK-2	+	none	-
	PCSg-2	+	none	∞
8	PCK-3	+	slender fibroblasts	∞
	PCSg-4	+	none	∞

No indication of mycoplasma growth was observed. In cultures routinely treated with antibiotics specific for mycoplasma, no indication of cytopathic effect was attributable to mycoplasma (12).

Chapter 4

DISCUSSION

CELL MORPHOLOGY

Normal Cells

The uninfected primary fibroblasts, which did not survive for
more than 5 passages, did not demonstrate any CPE at the time they
entered the declining phase of growth. The declining phase of these
cells can then be classified as characteristic when compared to that
of other primary cell lines (34). The normal primary fibroblastic
cells exhibit the established characteristics of primary cells which
include: a normal, diploid number of chromosomes, contact inhibition,
growth along a common line-of-orientation, attachment to a surface in
order to divide, a critical seeding concentration greater than 1×10^4
cells per flask, a generation time of more than 36 hours, a low mitotic
index (about 1% of the total cells), little stacking of cells on basal
layer cells, maintenance of the fibroblastic shape and cell stability
for less than 5 passages or about 8-10 weeks.

Infected Cells

Fibroblasts infected in vivo or in vitro must be cultured for
at least 14 days before the presence of infectious virus can be detec-
ted. This requirement indicates that the virus should be classed as
a "slow acting" virus (21). The long period of viral expression and
unsuccessful attempts to increase the titer of extracellular virus

indicated either that the virus is weakly virulent for normal host cells or that the viral nucleic acid associates with the cellular DNA in a latent-like phase of infection. Association of viral nucleic acid with cellular DNA would account for the oncogenic and transformation potential of this slow-acting virus. The virus could be classified with either the oncogenic deoxyriboviruses or the oncogenic riboviruses.

The consistant occurrence of the previously mentioned CPE of infected cells indicates that a virus(es) is the probable etiologic agent of the Weak Calf and Lamb Disease (WCLD) (21). It also suggests that a singularly active agent(s) is responsible for the stabilization and transformation of the fibroblastic cells.

The stabilization of primary cultures by virus is not completely understood. There must be viral interaction with cellular components resulting in increased cytoplasmic eosinophilia. In some cultures, the infected cells grow faster than the normal cells. These cultures have large numbers of the eosinophilic cells. The eosinophilic fibro= blasts are always observed in infected cultures which transform, and are never observed in normal cultures. It is very proba ble that these cells are "hyperplastic" precursors of the transformed cells, similar to cells in stages of transformation as discussed by Montangier (29).

The outgrowth of the unstable epitheloid PCK-4 and PCSg-4 cells from infected fibroblasts is apparently an intermediate stage of cell transformation. These cells exhibit only some properties of primary cells and require trypsinization to initiate the final cell changes to transformation. If these cells are held as long term

cultures, they may transform without passage, but the increasing level of cell death makes this doubtful. The fact that the control cultures do not give rise to the epitheloid cell type indicates that this cell morphologic change is viral induced.

The outgrowth of transitional stage infected cells in cultures infected with neutralized virus is inconsistant with neutralizing anti= body theory. Bovine immune serum is known to inhibit nuclear inclusion body formation in virus-infected fibroblasts (21), and to reverse the progression of the disease in acutely infected lambs and calves (48). Since the cells were not cultured in the presence of antiserum, three explanations of the epitheloid cell outgrowth in these cultures exist. 1) The cells may have been cross contaminated from other cultures, but controls indicated that this probably did not happen. 2) The cells may have been infected with a non=detectable level of virus, but again, control cells did not indicate this. 3) The "neutralized" virus may have gained entry to the cells in the presence of the anti= body. This would indicate that the antibody is not specific for the outer lipid coat of the virus (21), but toward an internal viral antigen released by lysing cells during the progression of infection.

Transformed Cells

All transformed sheep and calf cells were remarkably similar in morphology, staining properties and culture characteristics. The reduction in the nuclear:cytoplasmic ratio was due to a decreased amount of cytoplasm, which is characteristic of established transform= ed cells.

Fibroblastic cells from sheep or calf sources may be transform-
ed by either sheep or calf virus stocks. It was observed that fibro-
blastic cultures would not transform in less than 3 passages. This
indicated that cells had to be actively growing in order for the trans-
formation to ake place, which is a characterisitc of cell transformation
(13, 29). The similarities of CPE formation in infected cells from
both species, mode of transformation of cells, and the cross infect-
ivity of these virus stocks indicated that the two stock viruses were
the same. Histologic examination of fixed tissue sections from diseased
and normal animals revealed no tumorogenesis in vivo (48). The virus
may act in a manner similar to Visna and Progressive Pneumonia virus of
sheep. These riboviruses are non-oncogenic in vivo, but are oncogenic
for hamster tissue in vitro. The WCLD virus may be oncogenic for ham-
sters and transferred by the transplantation of transformed cells.

The transformed cells from different cell lines meet the fol-
lowing criteria: loss of contact inhibition, altered cellular and
culture morphology, increased growth rate, increaed persistance in
serial cultures, altered metabolism, chromosomal abnormalities, in-
ability to support extensive multiplication of the infectious virus,
increased resistance to superinfection with the transforming agent,
ability to grow in the suspension of a soft agar matrix, increased
cloning efficiency and satellite colony formation, and cell transloca-
tion by supernatant medium.

The transformed cells gave rise to identical progeny cells
throughout the passage and self-seeding stages. The stability of the
transformed cells indicates that these cells are modified by a

transforming factor (viral oncogene) rather than "spontaneously trans-forming" due to a growth property of the cells, as observed with the BHK21 cell line (25).

Some colonies which arose from single cells appeared abnormal. The cell morphology ranged from enlarged epitheloid cells to poly-karyocytes in the same colony. Growth of abnormal transformed cells is a result of high serum concentration in the medium. More than 50% of the colonies in the 20% FCS medium test experiment were abnormal. Most of the abnormal cells resumed normal growth and morphology when the FCS concentration was changed to 5%. Cells from the same seeding stock grown in 10% FCS or 5% FCS gave rise to about 10% abnormal col-onies. These abnormal cells exist in stock cultures, but are prevented from forming large colonies by crowding of rapidly growing normal cells.

Acridine orange staining of transformed cells showed that all of the rounded cells seen on the basal monolayer were dividing cells. Time sequence observations of dividing transformed cells showed that all cells flattened on the growth surface or the basal cell layer af-ter the telophase stage of division (G_1 phase). Estimates of culture activity can be based on the number of rounded cells observed in cul-ture colonies. Dividing transformed cells were never observed in the supernatant medium of fluid cultures.

The high plating efficiency of the transformed cells suggests an increased hardiness and different character of these cells when compared to fibroblastic cells. Rapidly dividing colonies grew from single transformed cells. The morphologic stability of the transformed

cells was investigated by isolation of a colony derived from a single cell. The colony was broken up into cell clumps and single cells in culture flasks. The sibling seed cells gave rise to normal and abnormal colonies at about the same rate as in mixed low seed cultures or stock culture medium. This suggests that abnormal cell formation is due to non-uniform mitosis of transformed cells resulting in a variety of chromosomal aberrations.

SERUM REQUIREMENTS

Cells seeded in serum concentrations of 15% or more had high attachment and initial outgrowth rates; those seeded in serum concentrations of 5% or less had low attachment and slow outgrowth rates. The cells of cultures seeded for cell-medium testing or plating efficiency had a viability rate of 100%. Established cultures were grown in serum concentrations as low as 1.5% with no increased death of cells. This low serum containing medium is referred to as holding medium. The cell mitosis decreased as the serum concentration was decreased, but the cells that were attached remained viable with no morphologic changes. When the cells were passed, the serum concentration had to be increased to 10% FCS to retain attachment rates of greater than 80%. The attachment of cells to a culture surface requires a serum factor known as fetuin, which was not present in adequate amounts in medium with low concentrations of serum.

Serum Toxicity

The concentration of FCS used in the culture of transformed cells is important in establishing successful cultures. Inhibition

of normal cell growth and abnormal cell formation was noted in transformed cell cultures grown in NCTC-135/20 in fluid and soft agar cultures. The calf transformed cells (390K-series) were especially susceptible to the toxicity of this medium. The transformed sheep cells (OSg-series) showed some effects of toxicity, but were able to continue growing. MEM/20 had a lesser degree of toxicity for these cells.

Three explanations for the serum toxicity exist. The first is that the serum contained low levels of toxic substances. This suggests that there are varying levels of toxic substances in all commercial FCS. These toxic substances may be cell specific and not detected by the culture of quality control cell lines. At high concentrations of serum in culture medium, these substances were in sufficient quantity to modify the growth patterns of the transformed cells. The effects of toxicity observed with MEM/20 suggests that the toxic substance was contained in the serum and not in the NCTC-135.

The second explanation is that the osmolarity or other physical factors were altered in the high serum concentrations, such that normal cell growth was altered. This suggests that the transformed cells require specific physiologic culture conditions not required by other cells (47). The rapid growth of the cells may not allow cell adaptation to media with altered physical conditions. The specific interplay of transformed cells with the environment is not understood.

The third explanation is that abnormal cells, which did not survive at lower serum concentrations, survived in the highest concentrations. This is based on the inconsistancy of chromosomal replication of the transformed cells. The cells seeded in high serum concentrations

had a higher attachment rate than those seeded in low serum concentra-
tions. Since the cell population of the cultures was randomly selected,
some of the cells growing in high serum concentration may not have
survived in low serum concentrations. This survival factor might be
determined by the completeness of the cell genome. The abnormal cells
may not be able to synthesize a complete enzyme complement, and there-
fore require an enriched medium in order to survive.

Hamster and Immune Cow Serum

Transformed cells grown in inactivated adult hamster serum
showed effects of serum toxicity at concentrations above 6% in 4-7
days. The cells stopped dividing, became enlarged and granulated. Two
weeks after the addition of high HS concentrations the cell death rate
increased. The cells were passable, but the attachment rate was less
than 50%. After three weeks of culture, the cells were dead and the
cultures were discarded. The source of the serum toxicity is unknown,
but assumed to be incompatibility of transformed bovine cells with ham-
ster serum. HS growth adaptation might be accomplished by using medium
containing 1-2% hamster serum and 1% B-globulins (Cohn fraction IV)
or 0.5% lactalbumin hydrolysate (47). The medium would require added
fetuin for attachment of cells. The B-globulins provides the cell
growth factor that is lacking in low serum medium (47).

Transformed cells grew in 1% immune cow and 1% non-immune cow
serum for 3 weeks with no signs of serum toxicity. The cells were
able to tolerate this low level of immune serum and were not aggregated
in culture as in the immune serum agglutination tests. Cell-antibody

interaction did not alter the morphology of the cells. The antibody concentrations should be increased with the use of purified antibody to determine if there is any cell growth alteration by antibody-cell interaction. Purified antibody-cell interaction would detect the presence of in vitro cell-contained antigens similar to the antigenic determinants necessary for the production of immune serum in vivo. This interaction would demonstrate a definite relationship between the in vivo disease antigens and the antigens of transformed cells. Further investigation might reveal whether the cell antigens were TSTA or V antigens. Non-viable transformed cells might then be used for immunization of non-immune cattle.

PRESENCE OF THE VIRAL AGENT IN TRANSFORMED CELLS

Superinfection

Sheep transformed cells (OSg-12) were superinfected with 390K-pool stock virus. At 12 and 21 days post-infection, there was no cytopathic effect attributable to the presence of the virus. The cell morphology and growth properties of the cells were not changed. The conclusion is that the virus did not infect the cells. Since this virus has displayed a slow induction of cytopathic effect, it may be argued that the virus infected the cells, but cell growth was too rapid to allow viral expression. One of the cell types of infected fibroblastic cultures was a rapid growing "hyperplastic" fibroblast. This cell type often had larger and more extensive nuclear inclusion formation than other cells of the culture. Cell growth rate may not influence the CPE formation. The interpretation of a successful

infection must then be based on the formation of nuclear inclusions

in the cells (21). The absence of CPE formation in the superinfect-

ed transformed cells may be caused by: 1) "non-entry" of the virus,

2) entry and "non-uncoating" of the virus or 3) entry, uncoating and

"non-expression" of the viral genome. The absence of infection may be

caused by rapid cell proliferation during which the virus could not

be replicated or by prevention of superinfecting viral nucleic acid

intergration by an already present viral oncogene.

Viral Production by Transformed Cells

Transformed cells (390K-12) were grown with uninfected feeder

layer fibroblasts (PSt-10). The 390K-12 cells grew in colonies with

PSt-10 cells around the perimeter. The membranes of the two cell types

touched at the perimeter of the colony.

The morphological changes observed in the perimeter 390K-12

cells may be attributable to biochemical activity in the transformed

cell associated with the release of increased amounts of virus. Super-

natant fluid from transformed cell cultures does not contain detectable

levels of virus (21), but the potential to form infective virons is

present in these cells. This was demonstrated by passage of WCLD

virus from transformed cells to normal fibroblastic cells causing

nuclear inclusion body formation. The level of viral production may

be increased by "inducing agents" or by cell fusion, as was demonstra-

ted with other "virus free" cells (22, 40).

The level of viral caused CPE was greatest in the PSt-10

cells nearest to the transformed cell colonies, indicating that the

virus is passed through direct cell to cell contact. However, the

CPE observed in distant cells indicated that free infectious virus is released. The method of transformed cell passage of WCLD virus is un- determined. The production of viral associated CPE in feeder layer cells indicates that the original infecting agent is being produced in low quantities in transformed cells.

Chromosome Alterations

The chromosome complement of control and infected fibroblastic cells was normal. The virus did not influence chromosome replication until after the crisis stage was manifest in fibroblastic cultures. Karyogram analyses of transforming cells have not been completed. The transformed cells showed chromosomal abnormalities of polyploidy, aneu- ploidy and subdiploidy. Translocation of chromosomes was present in all of the transformed chromosome spreads. Gustavsson theorised that the chromosome translocation in cattle is a result of centromeric fus- ion of the largest and smallest autosomes (16). The WCLD virus trans- formed cell translocation and polyploidy was so irregular that the identities of translocated chromosomes could not be discerned y com- parison with normal karyograms. Chromosomal a hormalities in viral infected cells are viewed as a result of the viral infection or trans- formation, rather than the cause of the cell CPE or transformation (38). Though viral-cellular genome associations have been observed in lytic and transforming viral infections (13, 19, 37, 38), random chromosomal damage may be a result of viral-mediated lysozome re- lease (38). It is impossible to determine the method or results of translocation in the transformed cells of this experiment without

further information about the viral oncogene effect on chromosomal structure.

Chromosomal abnormalities of WCLD virus transformed cells may cause formation of abnormal transformed cells. It is doubtful that the inconsistant chromosomal abnormalities are the cause of the consistant state of transformation in the cells.

Immunologic Reactions

The transformed cell agglutination by immune cow serum indicates that there is a cellular antigen in the in vitro cells that is common to an antigen in in vivo infected animals. Preliminary studies indicate that the agglutinating antibodies might be cytolytic when complement is added to the system. The cytolytic antibody titers were not as high as the agglutinating antibody titers. The cytolytic antibody presence was noticed in immune and convalescent serum, but not in acute-stage infection serum. These results indicate that there are common TSTA present in the transformed cells and in infected animals which elicit a specific response in convalescent animals.

Transformed sheep cells were agglutinated by the bovine immune serum, and transformed bvine cells were agglutinated by immune sheep serum. The agglutination titers of these cross species agglutinations were not as high as the species specific agglutination titers. This might be explained by the lack of similar TSTA between different species. The agglutination of F1 (human amnionic fibroblast) cells indicated a low level of non-specific agglutination activity by the test serum.

Complement fixation experiments were performed to detect in-tracellular (T) and viral (V) antigens (21). These tests demonstrate that there are no complement=fixing antibodies in immune cow serum (888) specific for antigens in transformed sheep cells (Ot=35), 390K=pool virus or OSg=4 pool virus. The Herpesvirus hominis control re-action had a titer of 1:32 with immune rabbit serum. There are no complement=fixing antibodies associated with WCLD or the viral isolate.

Non=complement binding, immune reactive TSTA, T or V antigens may exist in infected or transformed cells (21, 47). Fluorescent antibody experiments were performed to detect these T or V antigens in transformed cells. The test system used immune bovine serum and rabbit anti=bovine=gamma globulin serum conjugated with fluorescein isothio=cyanate. The results are inconclusive, as the transformed and control cells both fluoresced to the same degree. The cells had been grown in FCS. Even though the cells were washed and the rabbit antiserum sup=posed to be specific for gamma globulin, there may have been some in-teraction between residual FCS and the rabbit antiserum. The experi=ment should be performed with specific antiserum from rabbits and transformed cells grown in medium with rabbit serum.

Tumorogenesis

Inoculation of stock 390K=pool virus into newborn mice did not result in tumor production. Immuno=suppressed mice might be used, but these results indicate that the virus is not tumorogenic for Balb/C mice. This is consistant with the results of in vitro viral infectivity tests of different cell lines. The virus did not infect F1, vero

(African green monkey kidney), human primary tonsil, goat testes or primary rabbit kidney cells. Positive viral infection was observed only in bovine and sheep cell lines.

Inoculation of transformed cells into cortisone-acetate treated hamsters resulted in tumor formation in one of the ten animals. The appearance of the epitheloid cells recovered from the nodule indicates that the nodule was a tumor, induced by in vivo release of the onco-genic WCLD virus from transformed sheep cells. Further investigation of this tumor cell line should include transplantation to new hamsters, karyogram analysis, soft agar growth, cell agglutination with immune bovine serum, and cell feeder layer infection to prove that the WCLD virus is oncogenic for hamsters.

Chapter 5

SUMMARY

A virus suspected as the etiologic agent of the disease known as "Weak Calf and Lamb Disease" has been shown to be oncogenic _in vitro_ for sheep and bovine cells. Studies indicate that the virus is specific for bovine and sheep cells and causes a slow _in vitro_ infection with minimal cell death. Continuous culture of the infected cells results in the outgrowth of transformed cells which meet the criteria necessary for classification as transformed cells. Transformed cells from sheep and bovine fibroblastic cell lines are stable in _in vitro_ culture. The cellular morphology and growth patterns of transformed sheep and bovine cells from three different tissues are very similar. The infecting virus is present in the transformed cells as demonstrated by feeder layer, superinfection and immunologic tests.

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