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ESTABLISHMENT AND CHARACTERIZATION OF A CELL LINE FROM THE AMERICAN OPOSSUM *(DIDELPHYS VIRGINIANA)*

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SUMMARY

A permanent tissue-cultured cell line (designated OK) has been established from kidney tissue of an adult American opossum. The OK line has been characterized with respect to morphology, chromosome constitution, tissue-culture requirements, and attainable mitotic arrest. The cells are epithelial-like with a stable nondiploid chromosomal modal number of 23. Cells grown in Eagle's minimal essential medium with 10% fetal bovine serum have a mean doubling time of 18 hr. The cell line OK is potentially useful for the isolation and purification of the mammalian X chromosome because of the size differential between the smaller X's and the larger autosomes.

Key words: Didelphys virginiana; opossum cell line; X chromosome.

INTRODUCTION

There has been considerable recent interest in the separation and purification of mammalian chromosomes as an approach to the study of chromosome structure and function (1,2). A cell line with a karyotype of low chromosome number and maximal morphological heterogeneity would facilitate the separation of purified populations of individual chromosomes for biological and biochemical study. Currently we are undertaking the isolation and characterization of the mammalian X chromosome as part of an investigation of the molecular mechanism of X chromosome inactivation (3).

One approach to chromosome isolation has been the use of gradient centrifugation to separate different size classes of chromosomes. Sucrose gradient centrifugation, for example, has been used to separate chromosomes that differ in size and DNA content by approximately a factor of two (4). The availability of a cell line derived from a mammal with a very small X chromosome would be useful for the preparation of purified X chromosomes for our investigation of X chromosome properties.

A number of marsupial species have X chromosomes sufficiently smaller than the autosomes so that the gradient separation of the X chromosomes might be possible if suitable cell lines were available (5). For this purpose, the American opossum has a favorable karyotype, and considerable cytogenetic information is available (6–8). We report here the establishment, maintenance and propagation in vitro of a cell line derived from kidney tissue of the American opossum. (This cell line has been sent to the American Type Tissue Registry.) In this cell line, the X chromosomes are considerably smaller than the autosomes.

MATERIALS AND METHODS

Media and nomenclature. The following media and buffers were obtained from Grand Island Biological Co., Grand Island, New York: Hanks' balanced salt (BSS); Eagle's minimal essential medium (MEM); Dulbecco's modified MEM (DBM); and Ham's F-10 (Ham's). Fetal bovine serum (FBS) was obtained from Irvine Scientific. Each lot was heat-inactivated and checked for toxicity, growth support, and microbiological and mycoplasmal contamination before use. Throughout this paper, the concentration of serum used is indicated after the medium designation; e.g. Ham's 20 is Ham's F-10 with 20% FBS. Unless

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otherwise indicated, all media are supplemented with 0.03% L-glutamine, 100 U per ml penicillin and 100 μg per ml streptomycin.

Establishment of cell line. Opossum cells were initiated in culture in July, 1975, from kidney tissue removed from an adult female with young in pouch. The animal was purchased from Thompson Wild Animal Farm, Clewiston, Florida. An excised kidney was rinsed three times in BSS containing penicillin (60 U per ml), streptomycin (100 μg per ml) and mycostatin (100 U per ml), minced and dissociated in 0.2% trypsin in BSS plus the above antibiotics for 10 min at 37° C. Tissues were allowed to settle for a few minutes; the crude supernate was discarded, and the remaining tissue reincubated with fresh trypsin-antibiotic solution for 10 min. The cells were transferred to MEM 10 plus 0.1% bactopeptone, penicillin (100 U per ml) and streptomycin (100 μg per ml), and cultivated in 100-mm dishes (Falcon) in a water-saturated atmosphere of 5% CO_2 in air at 37° C.

After 11 days of culture, a confluent cell sheet was obtained. The cultures were trypsinized (0.2% trypsin in BSS) and subcultured. Thereafter, cells were passaged by splitting 1:2 or 1:3 at 3-day to 10-day intervals up to the seventh passage. At passage 8, cell growth slowed considerably and most cells degenerated and detached from the dishes. A population of cells survived and later formed many small colonies. The surviving cell type was propagated to produce the cell line designated in this lab as OK.

The studies described below were performed on cells passaged between tissue-cultured generations 9 to 55. After passage 16, the cell line was regularly propagated in MEM 10. Cells were detached using 0.05% trypsin, 0.03% EDTA in phosphate-buffered saline, pH 7.4. OK cells lacked mycoplasma when tested at passage 18 using electron microscopy (9) and at passage 37 using 33258 Hoechst fluorescent microscopy (10).

Opossum cells were considered fully adapted to tissue culture after passage 15. At this time, cultures were transferred to various media including MEM 20, DBM 20, Ham's 10 and Ham's 20 (Fig. 1,2).

Mitotic arrest. Dose-response curves were determined, by the following procedure, for colchicine (Calbiochem), Colcemid (Calbiochem) and Velban (Lilly). Twenty-four hr after subculturing at a density of 1.0×10^6 cells per 25-cm^2 flask, cultures in log phase were fed fresh media containing various concentrations of the mitotic-arresting agents. Duplicate cultures were harvested after 6 hr for mitotic-index determinations. Maximal arrest doses were used for further comparisons in which replicate cultures were exposed to each compound and harvested at 2-hr intervals (Fig. 4).

Cell morphology. Opossum cells grown under standard culture conditions were studied by using phase-contrast and bright-field microscopy of living and fixed monolayers. Cells were grown for 24 to 120 hr in plastic culture dishes and on albumin-coated microscope slides. Additional study of nuclear morphology was made from direct squash preparations stained with acetic orcein.

For transmission electron microscopy (TEM), a confluent culture at passage 43 was fixed at 37° C in situ in a culture flask with glutaraldehyde-formaldehyde fixative (11). Cells were post-fixed in 1% OsO_4 in 0.1 M sodium cacodylate buffer at pH 7.2. A uranyl-acetate rinse was used to increase contrast (12). Sections were stained with lead citrate to increase contrast further (13) and examined in a Philips 301 electron microscope.

For scanning electron microscopy (SEM), cells at passages 9, 12, 19, 43, 46 and 49 were fixed in Karnovsky's fixative (11) and then postfixed in OsO_4 in situ in culture dishes. After dehydration in ethanol, the cells were critical-point dried, coated with about 100 Å of gold and examined in an ETEC Autoscan.

Chromosome analysis. Standard methods for air-dried slide preparation were used for karyotype evaluation. Velban (8.8 μg per ml, 3.5 hr) was used to obtain an optimal mitotic index with minimal chromosome contraction. Optimal chromosome spreading with minimal chromatid separation was obtained with a hypotonic treatment of 0.05 M KCl, 20 min at room temperature, and fixation in 3:1 ethanol:glacial acetic acid for 15 min. Several air-dried slides were prepared for determinations of chromosome distributions at several passages. The major chromosomes modes were calculated from a minimum of 100 cells per passage. Air-dried slides from later passages were C-banded following the procedure of Hsu (14). A number of preparations were made without an arresting agent or hypotonic pretreatment for observations on cells in anaphase.

EXPERIMENTAL RESULTS

Establishment

Successful adaptation of opossum cells to tissue-culture conditions involved a pronounced alteration in growth characteristics at passage 8.

Two weeks after the cessation of growth of most cells at this passage, colonies of surviving cells were obtained with markedly different morphology than that of the preceding population. The new cell strain had a faster growth rate than the preceding cell line and initially was subcultured 1:2 upon reaching confluency (every 4 to 7 days). Population-doubling times on MEM continued to decrease with continued passage (33 to 48 hr at P12, 16 to 33 hr at P30, 12 to 27 hr at P50). During the first few passages following adaptation (P9 to P12), the cells became much more susceptible to trypsinization and were easily released by 5 to 10 min of trypsin-EDTA treatment. After passage 16, bactopeptone was discontinued as a supplement in the medium (see below). Since then, fetal bovine serum has been maintained at 10% for routine passage.

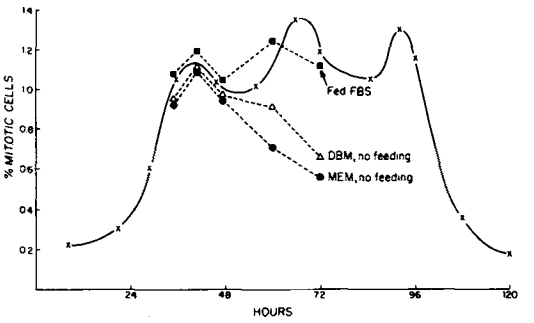

FIG. 2. Mitotic indices of OK cultures. ×——×, Growth curve from two experiments with initial density of 1.0×10^6 cells per 25 cm²; two flasks for each point; medium (8 ml MEM 20) replacement every 24 hr. Four-thousand cells were counted for each time point. ●——●, Duplicate cultures fed MEM 20 at 24 hr only. △——△, Duplicate cultures fed DBM 20 at 24 hr only. ■——■, Duplicate cultures fed MEM 20 at 24 hr and then supplemented with FBS (1.2 ml to 5 ml) at 24 hr.

Cell Culture

To determine optimal conditions for culture and to obtain preliminary data on growth characteristics and cell cycle dynamics, the following experiments were carried out.

Effect of different media and fetal bovine serum concentration. Growth curves (Fig. 1) for cells grown on MEM and Ham's were similar with a 24-hr lag period followed by rapid growth. However, cells grown in Ham's stopped growth after reaching confluency on day 2, whereas cells grown in MEM and Dulbecco's modified MEM continued growth for an additional 2 days. In all experiments, slightly higher cell densities were realized with 20% as opposed to 10% FBS. The addition of bactopeptone had no detectable effect on growth characteristics. Minimal essential medium with a serum concentration of 10% was considered appropriate for subsequent study and routine serial passage.

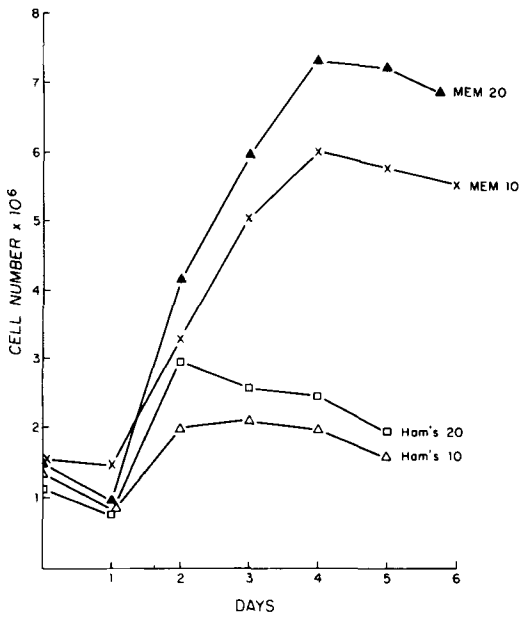

FIG. 1. Growth curves for OK cells grown on various media. Average number of cells from four 25-cm² flasks determined for each point. Data from cells from passages 41 through 45. The numbers after the medium abbreviations refer to the serum concentration.

Effect of feeding frequency. The mitotic index in logarithmically growing cultures increased to a maximum of about 1.4 and remained above 1.0 throughout the active growth phase in cultures fed at regular intervals (Fig. 2). Cultures typically responded to feeding with a sharp increase in the number of mitoses about 12 hr after the addition of fresh medium. Comparing the mitotic indices in cultures deprived of fresh medium with those fed fetal bovine serum alone suggests that continued replenishment of serum (at least every 24 hr) is necessary for optimal growth of OK cells (*data not shown*).

Effect of inoculation density. Fig. 3 illustrates that the rate of cell growth is a function of cell density and rises to a maximum of about 6×10^6 cells per 25-cm² flask. When larger numbers of cells were plated, growth declined gradually due to cell crowding. Based on these data, an inoculation density of 1.0 to 1.5×10^6 cells per flask was adopted for further cell characterization.

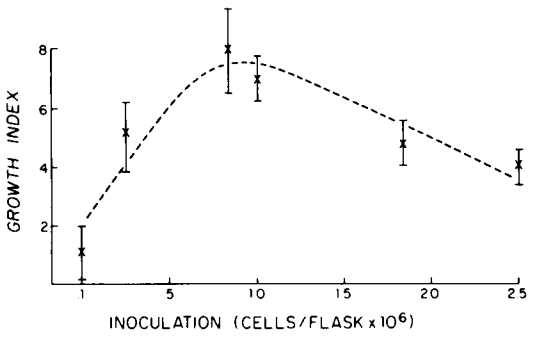

FIG. 3. Growth of OK cultures at different inoculation densities. Each point represents the combined mean ± S.E. from four 25-cm² flasks. Growth index is the number of cells per flask at 24 hr divided by the number of cells per flask at 96 hr. The medium (MEM 20) was changed every 24 hr.

Mitotic Arrest

A comparison of Velban, Colcemid and colchicine revealed maximal mitotic arrest in log-phase cultures at concentrations of 0.8, 5.9 and 2.5 μg per ml, respectively. Fig. 4 presents a time-course experiment using these concentrations. The arresting agents tested exhibited marked differences in the initial rate of accumulation and in the maximum number of accumulated metaphase cells.

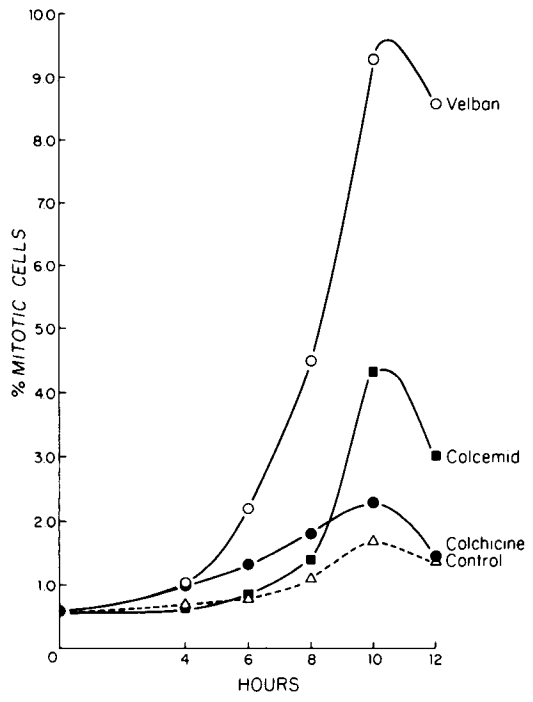

FIG. 4. Arrest of mitosis by Velban (0.8 μg per ml), Colcemid (5.0 μg per ml) and colchicine (2.5 μg per ml).

Colchicine and Velban produced equivalent numbers of metaphase cells at 4 hr treatment and were equally useful as mitotic arrestants for karyotype studies. However, Velban proved far more effective than Colcemid or colchicine for obtaining large numbers of metaphase cells for chromosome isolation experiments. A mitotic index of 7.0 to 9.0 was obtained routinely with 9-hr Velban block using 0.5 to 1.0 μg per ml in log-phase cultures grown on MEM. This dosage produced maximal arrest with only small numbers of metaphases having undesirable chromosomal characteristics (about 1% of the metaphase cells exhibited either extremely contracted chromosomes or premature centromere separation).

Cell Morphology

Cell morphology at or near confluency is epithelial as shown in Fig. 5*ab*. Cells are very flat, oval to polygonal, 20 to 40 μm in diameter, with occasional multinucleated cells up to 100 μm. Cell nuclei are usually oval with a distinct nuclear membrane visible with phase-contrast optics. Several large, dense nucleoli are visible in most cells. Mitotic figures appear normal in living and fixed monolayers. Mitotic cells are conspicuously rounded in Ham's F-10 cultures, and retraction filaments are readily observed as cells lift up off the culture surface in preparation for division. In contrast, many of the mitotic cells in MEM cultures remain flattened against the growth surface during division. An occasional anaphase bridge or lagging chromosome was the only mitotic abnormality observed in direct orcein squash preparations.

Ultrastructural examination revealed numerous desmosomes between adjacent cells in confluent cultures (Fig. 5*c*). Sparse, irregularly disposed microvilli cover the surfaces on many cells. In addition, some cells have cilia protruding from their surfaces. Careful examination of several hundred cells using TEM revealed no structures that could be considered virus particles.

The shape of OK cells is greatly influences by the density of the cultures. Cells not in contact with others appear pleomorphic and, in general, they are more compact than cells in contact with each other. Once confluency has been reached, most of the cells assume an extremely flat morphology. When confluent, cells exhibit little ruffling; cell surfaces are either smooth or sparsely covered with microvilli or blebs. Similar variation in cell surface elaborations of fibroblastic

cells have proved useful in identifying cells in different phases of the cell cycle (15).

Cells from early passages (i.e. P12) were conspicuously more compact and elongated in com-

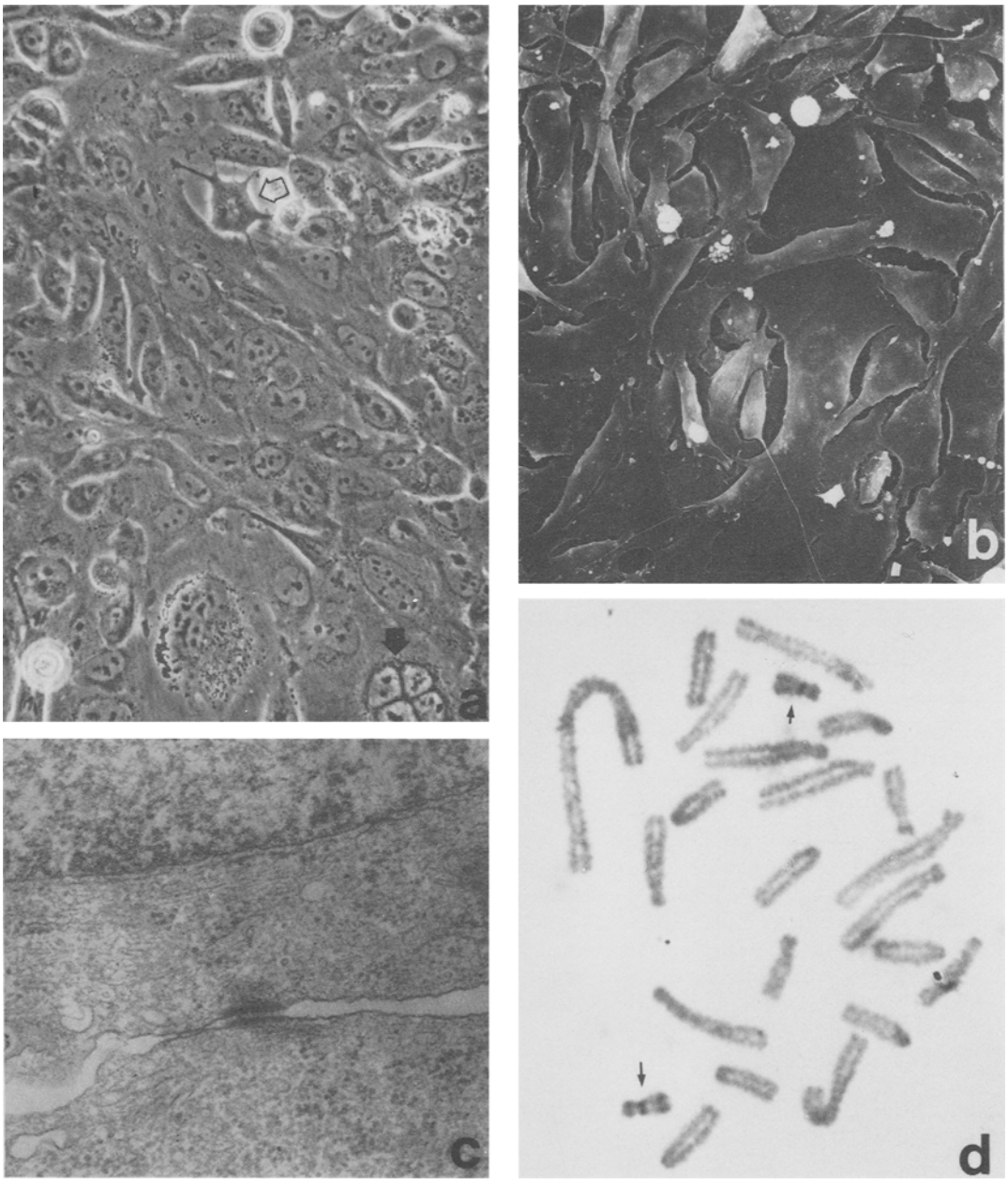

FIG. 5. *a*, Morphological aspects of OK cells grown on Ham's 20; passage 30, 72-hr culture. Mitotic cell (*open arrow*); multinucleated cell (*solid arrow*). ×300. *b*, Scanning electron micrograph of a culture of OK cells at passage 43. ×177. *c*, Transmission electron micrograph of a desmosome formed between cells in a stationary-phase culture at passage 43. These structures exhibit a localized increase in cytoplasmic density adjacent to the junction and a thin intermediate line in the intercellular space midway between the plasma membranes at the adjacent cells, characteristic of well formed desmosomes. ×46,750. *d*, Metaphase cell stained for constitutive heterochromatin (C-banding), illustrating the most frequent karyotype consisting of one large metacentric, a graded series of smaller, mostly acrocentric and telocentric chromosomes, and the two small submetacentric × chromosomes (*arrows*).

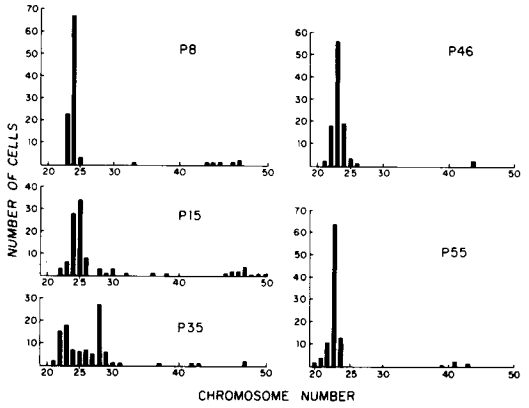

FIG. 6. Chromosome distributions at early and late passages.

parison to cells from late passages (i.e. P30). The morphological differences between early and late passages were associated with slower growth rate and lower saturation density achieved by these cells. Cells frozen at P9 and P12 and thawed after 2 months of storage retained a relatively elongated cell shape, slow growth and low growth density for at least six post-thaw subcultures.

Cell suspensions prepared from OK cells attach rapidly to plastic surfaces with 75% of the cells firmly attached after 20 min. The cells tend to grow in clumps until confluency is reached. Cells attach poorly to glass surfaces with only 20% of the cells firmly attached and growing in loose clusters after 24 hr.

Karyotype Assessment

Karyotype evolution in cell line OK at first followed a trend toward increasing aneuploidy (Fig. 6). At about passage 30, a broad, bimodal distribution in chromosome number was observed. By about passage 40, a relatively stable unimodal distribution was established. At this time, a distinctive large metacentric marker chromosome appeared. Currently, OK cells show a unimodel distribution with about 60% of the cells having 23 chromosomes and 30% having either 22 or 24 chromosomes. The marker chromosome is present in 95% of the cells. Although the normal diploid karyotype no longer is present in the cell line, nearly all cells retain the two X chromosomes as the smallest members of the complement (Fig. 5*d*). C-banding indicates that the X's of the cell line OK are similar to the X chromosome of the normal opossum karyotype (8). A C-banding analysis comparing the rearranged OK karyotype with the normal opossum karyotype has not yet been attempted.

DISCUSSION

Investigations on X chromosome isolation should be aided by a well characterized, rapidly proliferating opossum cell line with a stable karyotype. This report describes the establishment of a continuous cell line from kidney tissue of a female American opossum. Evaluation of growth characteristics, cell morphology and chromosome constitution during long-term cultivation (55 passages) indicates that a rapidly proliferating, morphologically and karyologically stable cell line has evolved. Initial growth of cells consisted of a mixture of fibroblastic and epithelial cells which subsequently evolved into apparently pure cultures of epithelial-type cells after passage 8. This pronounced change to a faster-growing, epithelial-type cell seems to have been a transformation to a phase 3 permanent cell line (16). Viral particles were not apparent in late-passage cells. However, this does not rule out integrated or otherwise difficult-to-demonstrate viruses. The new cell line, designated OK, continued to change gradually in growth characteristics and karyology until it reached an apparently stable equilibrium at about passage 40. During this period of cell evolution, doubling time decreased markedly and saturation density increased to at least twice that of early-passage OK cells. They also acquired the ability to grow as multilayers when fed frequently with rich medium (MEM 20% FBS, medium changed every 12 to 24 hr).

The results of the present investigation suggest that near optimal growth of OK cells can be maintained using Eagle's minimal essential medium supplemented with 10% fetal bovine serum and serial passage every 48 hr at a split ratio of 1:3. Cells are seeded routinely at a density of 1.7 to 6.7 $\times 10^4$ cells per cm^2 and subbed after first reaching confluency (1.0 to 1.7 $\times 10^6$ cells per cm^2). Experiments using various medium components indicate that OK cells rapidly deplete a serum factor(s) and that frequent medium change or addition of serum (at least every 24 hr) is necessary for rapid proliferation. It also should be noted that Velban is far superior to Colcemid or colchicine in effecting mitotic arrest in OK cells (Fig. 4). Preliminary analysis of the cellular kinetics of late-passage cells indicates a mean cell cycle time of 16 hr during log growth in MEM 10 (*unpublished data*).

Ultrastructural examination of cell cultures confirmed the epithelial-like morphology of OK cells. As logarithmically growing cultures reached

confluency, cells became extremely flat and exhibited numerous well formed desmosomes. The relationship between cell morphology and cell density is interesting because of our desire to use selective shake-off techniques to obtain mitotic cells (17,18) for chromosome isolation studies. The more compact morphology of cells observed at lower densities suggests that mitotic cells might be dislodged more easily under these conditions. Some of our preliminary experiments indicate that this indeed is the case. While well rounded mitotic cells occur in cultures at higher densities, these cells may maintain contacts with adjacent cells, making them more difficult to remove. The presence of desmosomes between cells in denser culture also may influence the ease with which mitotic cells can be differentially dislodged. Desmosomes are known to persist through cell divisions in some tissues (19,20).

The in vitro development of aneuploidy in cell line OK was characterized by a gradual drift in modal chromosome number and apparently reached an equilibrium with cells of 22 to 24 chromosomes. This gradual replacement of diploid by nearly diploid cells contrasts with the classic course of events in aneuploid cell lines involving primary transformation from a diploid to a tetraploid population of cells described for placental as well as marsupial cell lines (21, 22). Normal diploid cells ($2n = 22$) are not present in cell line OK. However, chromosomal rearrangement is confined to the autosomes and the X chromosome remains the smallest element. Furthermore, the two X chromosomes in OK cells appear identical to the normal opossum X in size, morphology and C-banding characteristics. Our preliminary experiments on chromosome replication patterns have confirmed that the two X chromosomes in OK cells initiate replication asynchronously as reported for opossum lymphocytes in vitro (23).

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