The WIRL-3 Rat Liver Cell Lines and Their Transformed Derivatives

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SUMMARY
Two epithelial cell lines were established from the liver of a weanling rat. The cells retained their epithelial morphology after transformation by simian virus 40. They also transformed spontaneously and grew to higher cell densities than the parent cultures or grew in suspension. The "normal" and transformed cell lines have some but not all the characteristics associated with hepatocytes. They have glucose 6-phosphatase activity, secrete serum globulin, and, in confluent monolayers, show tight cell junctions and intercellular canaliculi resembling bile canaliculi. On the other hand, the cells have low levels of inducible tyrosine aminotransferase, no detectable δ-aminolevulinic acid synthetase and aryl hydrocarbon hydroxylase activity that is inducible with benz(a)anthracene but not phenobarbital.

INTRODUCTION
In recent years several cell lines derived from normal rat liver by different techniques have been described (4-6, 9, 11, 15, 20, 21). These vary in the degree to which they express liver-specific properties such as inducible TATF activity and the synthesis and secretion of serum proteins, but none of the cell lines exhibits all the functional characteristics of normal hepatocytes. This diversity of properties of liver cell cultures, which has also been observed in hepatoma cell lines, was recently discussed by Potter (17) in a review of a "Workshop in Liver Cell Culture."

MATERIALS AND METHODS

Culture Media
WIRL-3 cells were grown in modified BME containing twice the standard concentration of vitamins and amino acids and 10% FCS (Flow Laboratories, Rockville, Md). During the initial isolation procedures, the medium was supplemented with 0.006% ferric nitrate and contained 0.45% rather than 0.1% glucose (supplemented BME). These supplements were eliminated from the medium once the cell lines had been established. For the first few subcultures, the WIRL-3 cells were dispersed with 0.125% trypsin in Ca++- and Mg+++-free phosphate-buffered saline. Thereafter, a solution of 0.025% trypsin and 0.02% EDTA was used.

For storage, cells were suspended in BME containing FCS and 10% glycerol or dimethyl sulfoxide and frozen at -70° in a Revco refrigerator or at -196° in liquid nitrogen.

Initiation of Liver Cell Cultures
The primary cell cultures, designated WIRL-3, were initiated according to a method similar to that described by Coon (4-6). The liver from a 4-week-old randomly bred male Wistar rat was finely minced and suspended in a solution of Ca++- and Mg+++ -free Hanks' balanced salt solution containing 0.1% trypsin (Flow Laboratories), 0.1% collagenase (twice crystallized; Worthington Biochemical Corp., Freehold, N. J.), and 1% FCS. The suspension was shaken on a Dubnoff shaking incubator at 37° for 60 min and centrifuged at 700 rpm. The upper half of the pellet, which contained mainly blood cells and cell debris, was discarded. The cells in the lower half were washed twice, resuspended in modified BME and plated in Falcon plastic Petri plates at a density of 1.5 x 10⁴ cells per sq cm. The plates were incubated at 36.5° in a humidified atmosphere continuously flushed with 5% CO₂ in air. Most of the cell suspension did not attach to
the surface of the plates and after 24 hr, the original medium containing the unattached cells was removed. The cells adhering to the surface were rinsed gently and refed with fresh medium.

Chromosome Analyses

Cells were grown in roller bottles and for 1 to 3 days during the early stationary phase of growth the medium was collected daily and replaced (150 ml medium per bottle). Pooled media (300 to 450 ml) were concentrated by ultra-filtration to approximately 20 ml, dialyzed against 0.9% NaCl solution, and further concentrated to 0.2 to 0.4 ml with an Amicon 8 MC microultrafiltration system. The concentrated medium was analyzed by double diffusion in agarose using Cordis immunodiffusion cells I or II (Cordis Laboratories, Miami, Fla.) and by immunoelectrophoresis using a Gelman immunoelectrophoresis apparatus (Gelman Instrument Corp., Ann Arbor, Mich.). Rabbit antisera against whole rat serum proteins and against whole bovine serum proteins were obtained from Miles Laboratories, Inc., Kankakee, Ill. There was no immunological cross-reactivity between the rabbit antisera against rat serum and FCS or bovine serum.

Tests for Tumorigenicity of Cell Lines

To test for tumorigenicity in rats, newborn Wistar or DA rats were given inoculations of 0.1 ml of cell suspension either i.p. or in the intrascapular region s.c. The rats were examined for tumor growth weekly for a minimum of 6 months. To test for tumorigenicity in the hamster cheek pouch, 5- to 6-week-old Syrian hamsters (Lakeview Hamster Colony, Newfield, N. J.) were anesthetized with Nembutal and 0.1 ml of cell suspension was inoculated just under the epithelial layer of each pouch. One day prior to cell inoculation and twice weekly thereafter, the hamsters were given inoculations i.m. of 2.5 mg cortisone acetate. At weekly intervals the hamsters were anesthetized and the pouches were examined for tumor growth.

Transformation by SV40

One-day-old monolayer cultures were fed with medium containing SV40 (strain RH 911) diluted to the appropriate concentration and were subcultured weekly thereafter. Coverslip preparations were stained indirectly for SV40 T antigen, using hamster anti-T serum (Flow Laboratories) and fluorescein isothiocyanate-conjugated goat anti-hamster globulin (Antibodies, Inc., Davis, Calif.).

RESULTS

Establishment of Cell Lines. Most of the primary WIRL-3 cells that attached to the plates initially appeared to be fibroblasts. However, on Day 8 patches of cuboidal epithelial-like cells, separate from other cells on the plates, were seen. These patches were ‘‘ring isolated’’ with silicone-coated glass cylinders, and the cells were trypsined and transferred to 60-mm plates. After 4 weeks of incubation and 6 changes of medium, 1 of these subcultures, designated WIRL-3C, showed isolated colonies of epithelial-like cells and matrices of large, nondividing, fibroblast-like cells. The epithelial cells from 1 colony were again transferred after ring isolation, and the subculture was designated WIRL-3B.

The 2 cell lines, WIRL-3C and WIRL-3B, were refed regularly, subcultured by trypsinization 4 to 5 times at 8- to 12-day intervals, and thereafter generally showed similar
Rat Liver Cell Lines

morphological and growth characteristics. Cells of both lines were frozen at various passage levels and the descriptions that follow represent observations made on repeated long-term cultivations, using early passage cells reconstituted from the freezer and carried subsequently in these laboratories.

Characteristics of Line WIRL-3C. For approximately 20 subcultures at split ratios of 1:4 or 1:6 weekly, the cells of line WIRL-3C retain an epithelial morphology (Fig. 1a). The cells grow in islands that eventually coalesce. Binucleate cells are frequent and there are an average of 2.4 nucleoli per cell, with about one-half the cells having 2 nucleoli. Several points about WIRL-3C cells at this stage are apparent from the growth curve shown in Chart 1a: (a) following subcultivation, there is a loss of more than 50% of the cells as a result of their failure to attach to the surface of the culture vessels; (b) the population doubling time during logarithmic growth is 19 to 22 hr; and (c) the rate of cell multiplication levels off when the cell density reaches approximately $6 \times 10^4$ cells per sq cm, but growth can be reinitiated by feeding the cultures with fresh medium.

There is a change in the growth pattern of the WIRL-3C cells after about the 20th subculture. One first observes small, discrete areas of densely packed cells that are apparently no longer sensitive to contact inhibition of cell division (Fig. 1b). The frequency of mitotic figures and of binucleated and multinucleated cells is increased; the nuclei show a heterogeneous morphology and the average number of nucleoli per cell is increased. With further subculture, cells in these areas of rapid division frequently lose their epithelial morphology.

The growth curve of WIRL-3C cells at this stage (Passage 30) shows that, as with the lower passage cells, there is a large loss of cells on Day 1 (Chart 1b). However, most of the cells that do attach to the surface of the plates probably do replicate, since a colony-forming efficiency of 20 to 30% is observed when $10^5$ or $10^6$ cells are plated in 60-mm plates. After the cells recover from the trauma of subcultivation, growth proceeds exponentially until a density of 15 to $20 \times 10^4$ cells per sq cm is reached (Chart 1b), a density 2 to 3 times that of the lower passage cells. Once a monolayer is formed, the further increase in cell number is due primarily to the contact-insensitive cells growing as discrete patches throughout the monolayer (Fig. 1b).

After about 40 passages, WIRL-3C sublines may go in either of 2 directions. In one, a continuous cell line is established which consists of a heterogeneous mixture of epithelioid and fibroblast-like cells (Fig. 1c). Alternatively, foci of cells in the areas of high density become necrotic and degenerate, individual epithelial cells enlarge but fail to divide, and the combination of the 2 phenomena leads to the eventual death of the subline.

Transformation of WIRL-3 Cells by SV40. Seventh passage WIRL-3B and WIRL-3C cells were infected with SV40 at a multiplicity of 40 plaque-forming units/cell. No
SV40 T antigen-positive cells were seen for 2 weeks. By the 3rd week, however, islands of small, densely packed cells that were T antigen positive became evident throughout the monolayers. Within a week, the SV40-transformed cells had overgrown the "normal" cells and all cells in the cultures of the 2 lines contained T antigen. Passage of the SV40-infected WIRL-3C cells was discontinued.

The WIRL-3B/SV cells are epithelioid but are smaller than the parent cells and have more nucleoli and a larger nuclear:cytoplasmic ratio. The cultures contain the multinucleated cells and lobulated and fragmented nuclei typical of SV40-transformed cells (Fig. 2). Although the cells do not shed infectious SV40, virus is recovered after hybridization with African green monkey kidney cells (P. Swetly, personal communication). WIRL-3B/SV cells grow rapidly and can be subcultured at a ratio of 1:10 or 1:20 every 4 to 5 days. The growth curve of Passage 29 cells shown in Chart 2 indicates a doubling time of 12 to 14.5 hr, about one-third less than that of nontransformed cells. Growth plateaus when the cultures reach a density of 40 x 10^4 cells per sq cm, but with a change of medium the cell density increases 3-fold within 48 hr.

**Derivation of Line WIRL-3BS.** One subline of WIRL-3B, derived from cells frozen at Passage 17 and subsequently reconstituted, underwent a unique pattern of spontaneous transformation. At about the 25th passage of this subline, some cells in discrete areas in the monolayers showed intense basophilia and a nuclear morphology that was markedly different from that of the normal cells (Fig. 3). The altered cells grew in a densely packed arrangement and in some instances seemed to be growing in multilayers. The subline continued to show a mixed pattern of contact-inhibited and contact-insensitive cell growth for another 20 passages when subcultivation was discontinued. Prior to this, however, a suspension culture, which was designated WIRL-3BS, was initiated by vigorously shaking the culture vessel and detaching cell aggregates from the areas of increased cell density. These were transferred to an Erlenmeyer flask containing medium in an air:liquid ratio of 5:1, and the flask was incubated on a rotary shaker. The cells immediately began to grow in suspension and were subcultured initially at a split ratio of 1:3, subsequently at a ratio of 1:10 or 1:20, and after 1 year, at a ratio of 1:100 weekly.

WIRL-3BS cells grow in tightly packed clumps which may become as large as 0.3 mm diameter and are difficult to disaggregate into single cells with either trypsin, collagenase, or hyaluronidase. The clumps can be pelleted, fixed, and sectioned to show the epithelial morphology of the individual cells and their tubule-like growth patterns (Fig. 4a).

**Electron Microscopy.** The various cell lines showed no clear differences in their ultrastructures. The cells were thin and elongated soon after seeding but were isometric and epithelial-like when the cultures reached confluency (Fig. 5a). Thereafter, lysosome-like bodies, autophagosomes, and residual bodies became abundant. Glycogen particles were absent throughout all stages of growth. At confluency, cells developed an extensive network of rough and smooth endoplasmic reticulum; the rough portions were frequently dilated and filled with an electron-dense material. At this time, there was also evidence of cell differentiation in that cell contacts became extensive and intercellular canaliculi with microvilli and junctional complexes were numerous (Fig. 5a). This was also observed in clumps of WIRL-3BS cells (Fig. 5b).

**Chromosome Analyses.** When analyzed at Passage 31, the median chromosome number of the WIRL-3C cell line was 42 (91% of 33 metaphase cells analyzed). Cells of the transformed lines, WIRL-3B/SV and WIRL-3BS, had 38 to 45 chromosomes with 55 to 60% of the 26 metaphase cells analyzed from each cell line having 42 chromosomes.

**Biochemical Analyses.** The TATF activity of WIRL-3 cells was low and increased slightly, if at all, when the cells
Rat Liver Cell Lines were pretreated with dexamethasone before assaying (Table 1). Both the constitutive and induced levels of TATF were much lower than those found in the rat hepatoma cell line, HTC (19). The levels of glucose 6-phosphatase activity in both the WIRL-3C and WIRL-3B/SV cells were similar to those found in HTC cells (Table 2). The levels of constitutive aryl hydrocarbon hydroxylase activity in WIRL-3C cells were similar to those found in hamster embryo cell cultures (7); treatment of the cells with benzo(a)pyrene, but not with phenobarbital, induced at least a 3-fold increase in activity (Table 3). The activity of δ-aminolevulinic acid synthetase in WIRL-3 cells, as measured by the formation of porphyrin, was very low and not inducible by allylisopropylacetamide. Synthesis of porphyrin from δ-aminolevulinic acid was below detectable levels.

**Secretion of Serum Proteins.** The results of a typical immunodiffusion experiment, using concentrated culture media from WIRL-3 cells, are shown in Fig. 6. A clear precipitation line was found between rabbit antiserum against rat serum (inner well) and an antigen in medium from cultures of WIRL-3B cells (Outer Wells 3 and 5). This precipitation line did not fuse with the precipitation band given by rat albumin (strong lines in Wells 1, 2, and 6) but did fuse with the precipitation line of another serum protein(s).

To confirm the suggestion that the protein(s) secreted by WIRL-3 cells is identical to a serum protein other than albumin, concentrated media were analyzed by immunoelectrophoresis. As shown in Fig. 7, a precipitation band was observed in the region of α₂-globulin. Thus, WIRL-3 cells do not secrete albumin but do secrete an α₂-globulin, probably α₂-haptoglobin or ceruloplasmin, which reacts with rabbit antiserum against whole rat serum. We also found that HTC hepatoma cells resemble WIRL-3 cells in the types of serum proteins secreted. Neither secretes albumin but both secrete an α₂-globulin.

**Tumorigenicity of WIRL-3 Cell Lines.** WIRL-3 cells are derived from noninbred Wistar rats but resemble DA rats in histocompatibility antigenicity, as determined by mixed hemagglutination (S. Hausman, personal communication). Therefore, they were tested for tumorigenicity in newborn rats of both strains and in the cheek pouch of cortisone-treated Syrian hamsters. Morphonologically transformed cell lines produced tumors after transplantation into the hamster cheek pouch. The largest tumors were about 10 sq mm, and most regressed.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Tyrosine aminotransferase activity of WIRL-3 cells</th>
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<tr>
<td>Cells</td>
<td>Experiment</td>
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<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>Rat Liver*</td>
<td></td>
</tr>
<tr>
<td>HTC</td>
<td>–</td>
</tr>
<tr>
<td>WIRL-3C</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
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<tr>
<td></td>
<td>2</td>
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<td>3</td>
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<tr>
<td>WIRL-3B/SV</td>
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* nmoles substrate used per min per mg protein.
* Male Sprague-Dawley rats (6 weeks old) were starved for 18 hr and decapitated. Livers were excised and homogenized (1:2) in 0.25 M sucrose. Whole homogenates were centrifuged (100,000 x g for 2 hr) and the supernatants were assayed.
* Cells were pretreated for 24 hr with 10⁻⁵ M dexamethasone phosphate.
* Cells were pretreated for 8.5 hr.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Glucose 6-phosphatase activity of WIRL-3 cells</th>
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<tr>
<td>Cells</td>
<td>Specific activity*</td>
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<tr>
<td>---------</td>
<td>-------------------</td>
</tr>
<tr>
<td>Rat liver</td>
<td>163</td>
</tr>
<tr>
<td>HTC</td>
<td>17.5</td>
</tr>
<tr>
<td>WIRL-3C</td>
<td>25.6, 30.3, 17.2, 18.0</td>
</tr>
<tr>
<td>WIRL-3B/SV</td>
<td>15.3</td>
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</tbody>
</table>

* nmoles P₁ liberated per min per mg protein.
* Values obtained in 4 separate experiments.

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<tr>
<th>Table 3</th>
<th>Aryl hydrocarbon hydroxylase activity of WIRL-3C cells</th>
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<tr>
<td>Experiment</td>
<td>Pretreatment</td>
</tr>
<tr>
<td>1</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>Benzanthracene</td>
</tr>
<tr>
<td></td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>Benzanthracene</td>
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<td></td>
<td>Phenobarbital</td>
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* One unit of enzyme activity catalyzes the production, during the incubation period, of an amount of fluorescence equivalent to that of 1 pmole of 3-hydroxybenzo(a)pyrene.
DISCUSSION

Those characteristics of the WIRL-3 cell lines that may be considered hepatocyte associated are the epithelial morphology, the tight cell junctions and intercellular canaliculi resembling bile canaliculi that are evident in confluent monolayers and cell clumps, the secretion of serum globulin, and the hepatoma-like tumors produced by transformed cell lines. Glucose 6-phosphatase activity, which is characteristic of liver and a few other tissues such as kidney, was clearly detectable in WIRL-3 cells, although the values were lower than those found in intact liver.

However, if WIRL-3 cells are derived from hepatocytes, then a considerable loss of differentiated properties must have occurred during either the initial isolation or the continuous growth in vitro, as indicated by other characteristics of the cells. No glycogen particles were seen. An enzyme normally found in liver, δ-aminolevulinic acid synthetase, was not detectable or inducible. Phenobarbital did not induce aryl hydrocarbon hydroxylase, although in vivo it does induce this enzyme in liver. Finally, the WIRL-3 cells have very low levels of inducible TATF, although the presence or absence of this enzyme probably cannot be considered a determinant of the liver cell origin of a particular cell line since individual clones of hepatoma cells in culture may show a wide range of TATF inducibility.

ACKNOWLEDGMENTS

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