Miniature Cell Culture Systems for Use in Field Isolation of Viruses: Cell Stability and Sensitivity to Selected Laboratory-Propagated Viruses During Storage in Miniatrized Environments

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Cell cultures were miniaturized in 1-dr vials or in capillary tubes (100 by 3 mm) and examined for their capacity to maintain normal gross morphology and sensitivity to infection by viruses during storage under a variety of conditions. The purpose of these studies was to determine the feasibility of using such cultures in the field during epidemiological investigations. From the results of this work it was concluded that such a feasibility exists.

Epidemiological investigations on virus diseases, especially in isolated regions, have usually involved the collection and freezing of specimens and later transport to the main laboratory for purposes of virus isolation. The low titers and lability of many viruses in clinical specimens make it desirable to have sensitive tissue cultures in the field to propagate their infectivity to high titer before it decays. We report here on the morphological stability and virus sensitivity of several cell lines under conditions which incorporate the simplicity of operation and limitation of laboratory space demanded of much field work in the area of public health.

MATERIALS AND METHODS

Cells. The tissue bank of the Naval Medical School, Bethesda, Md., provided early passages of cells from the kidneys (HEK), lungs (HEL), and skin and muscle (HESM) of human embryos. We also utilized one of the established lines of human embryonic lung (WI-38) from the Wistar Institute, Philadelphia, Pa. In addition, early passages of cells from the kidneys of African green monkeys (MK) as well as continuously passaged HeLa cells were used. The embryonic cells were usually grown in Eagles minimal essential medium (MEM) plus 10% fetal calf serum (FCS) and maintained in Leibovitz medium L-15 (L-15) plus 1 or 2% FCS. MK cells were usually grown in Melnick's medium plus 5% FCS and HeLa cells in the medium of Hackett and McClain plus 10% FCS.

Viruses. Rhinovirus strain HGP was obtained from the U.S. Naval Medical Research Unit no. 4 at Great Lakes, Ill. It was passaged to high titer in a number of the human embryonic cell lines before use. Parainfluenza virus type 3 and poliovirus type 2 were similarly passaged from reference stocks maintained in liquid nitrogen.

Cell propagation in 1-dr vials. Uniform suspensions of cells (approximately 5 x 10⁶/ml) in growth medium were added in 0.5-ml amounts to sterile screw-cap 1-dr vials (Kimble Glass Co.). All vials were then positioned in a portable two-unit "honeycomb" circular rack shown in Fig. 1 capable of holding 504 vials in the space of a conventional roller drum. This and the −60 C freezer unit shown in Fig. 2 were designed for field work with miniature cell cultures. Additional developments on this equipment are in progress. After static incubation at 36 C overnight, the racks were rolled until confluent monolayers were formed. Each vial was then drained of fluid and completely filled (4 ml) with maintenance medium. Storage at room temperature was carried out with the vials upright between partitions in covered cardboard boxes.

Infection of cells propagated in 1-dr vials. Cells were drained of storage medium and inoculated in replicate with 0.5 ml of virus diluted usually with L-15 plus 1% FCS. The vials were then replaced in the circular rack and either rolled or kept static during incubation at 33...
or 36 C. Comparable uninfected cultures were maintained as controls.

Cell propagation in capillary tubes. Sterile capillary tubes (100 by 3 mm, Aloë Scientific Co.) were each dipped into a uniform suspension of cells (approximately $2 \times 10^6$/ml) in growth medium and approximately 0.2 ml incorporated by capillary flow. This volume formed a fluid column approximately 60 mm in length. After centering the column, one end of the tube was sealed with plastic putty (Critoseal, Aloë Scientific Co.). Groups of tubes were then positioned in a row between two strips of transparent library tape attached above the fluid column at the sealed end. This tape also served as a surface for recording the contents of each capillary. Incubation was carried out overnight at 36 C to achieve confluent monolayers. Medium changes were made by piercing the sealed end of the tube with a sterile 21-gauge needle and applying air pressure through a syringe to expel the fluid. Fresh medium was then added through the same opening by the reverse technique.

Infection of cells propagated in capillary tubes. Virus diluted in appropriate medium was inoculated in replicate onto cells in the manner described for making fluid changes. Static incubation was then continued at 36 C. Infective fluids were collected for virus assay by expelling them from each capillary into a sterile dilution tube with the aid of a syringe and needle as described.

RESULTS

The deterioration observed in normal gross morphology of each cell line in 1-dr vials during storage at room temperature is shown in Table 1.

**Table 1. Morphological stability of six cell lines in 1-dr vials during storage at room temperature**

| Cell line | Storage | Interval (days) | Cell morphology* |
|-----------|---------|-----------------|------------------|
| HESM      | L-15 + 1% FCS | 29              | 4+               |
| HESM      | L-15 + 1% FCS | 54              | 3+               |
| HESM      | L-15 + 1% FCS | 88              | 1+               |
| HEL       | L-15 + 1% FCS | 27              | 4+               |
| HEL       | L-15 + 1% FCS | 51              | 2+               |
| HEL       | L-15 + 1% FCS | 95              | 2+               |
| HEK       | L-15 + 2% FCS | 15              | 4+               |
| HEK       | L-15 + 2% FCS | 28              | 2+               |
| HEK       | L-15 + 2% FCS | 51              | 2+               |
| WI-38     | L-15 + 2% FCS | 15              | 4+               |
| WI-38     | L-15 + 2% FCS | 28              | 2+               |
| WI-38     | L-15 + 2% FCS | 51              | 2+               |
| MK        | L-15 + 2% FCS | 15              | 4+               |
| MK        | L-15 + 2% FCS | 28              | 3+               |
| MK        | L-15 + 2% FCS | 51              | 2+               |
| HeLa      | L-15 + 2% FCS | 7               | 3+               |
| HeLa      | L-15 + 2% FCS | 21              | 0                |

*4+, Cells confluent and normal in gross morphology; 3+, cells not confluent but normal in gross morphology; 2+, cells not confluent and abnormal in gross morphology; 1+, cells show severe degeneration and many have detached; 0, cells show complete degeneration and most or all have detached.
TABLE 2. Capacity of five cell lines to replicate after morphological deterioration during storage in 1-dr vials at room temperature and 36 C

| Cell line | Storage | Cell morphology | Per cent positive |
|-----------|---------|-----------------|-------------------|
| HESM L-15 + 1% FCS | Room 88 | 1+ | 0/15 | 0 |
| HESM MEM + 3% FCS | Room 88 | 1+ | 0/28 | 0 |
| HESM L-15 + 1% FCS | 36 C 88 | 1+ | 5/20 | 25 |
| HESM MEM + 3% FCS | 36 C 88 | 1+ | 3/13 | 23 |
| HEL L-15 + 2% FCS | Room 51 | 2+ | 3/14 | 21 |
| HEK L-15 + 2% FCS | Room 28 | 2+ | 8/8 | 100 |
| WI-38 L-15 + 2% FCS | Room 28 | 2+ | 8/8 | 100 |
| MK L-15 + 2% FCS | Room 51 | 2+ | 13/14 | 93 |

a 4+, Cells not confluent but normal in gross morphology; 2+, cells not confluent and abnormal in gross morphology; 1+, cells show severe degeneration and many have detached.

b Followed by change to fresh growth medium and incubation at 36 C; ratio of number of vials positive to number of vials tested.

All vials contained fully confluent monolayers showing excellent gross morphology at the start of storage. HESM cells showed no deterioration after 1 month at room temperature. Even after 2 months the cells still attached showed normal morphology. By 3 months, severe deterioration had occurred, and most of the cells were detached from the glass surface. The morphological stability of HEL cells treated similarly was nearly as good, and even though HEK, WI-38, and MK cells were not as stable in this respect, they still exhibited fair morphology after 1 month of storage. HeLa cells, however, deteriorated rather rapidly under these conditions; within 1 week some detachment had already occurred.

In addition to these findings, other work demonstrated that pretreatment of the glass surface of the 1-dr vials with Calgon, metasilicate, concentrated mineral acids, sodium bicarbonate, or steam before washing did not lead to improved cell stability when they were used later in experiments similar to that described. Secondly, comparable results were noted with each cell line when replicate vials were in part retained at the laboratory, whereas the remainder were air-shipped to Pearl Harbor, Hawaii, and later returned after a storage period at room temperature.

The capacity of certain cell lines in the 1-dr vials to replicate after morphological deterioration became evident during storage at room temperature or 36 C was also determined. Each vial was drained of fluid, and 0.5 ml of fresh growth medium was added, followed by continued rolling incubation at 36 C. Observations were made

TABLE 3. Morphological stability of five cell lines in capillary tubes during storage at room temperature and 36 C

| Cell line | Storage | Cell morphologya |
|-----------|---------|-----------------|
| HESM MEM + 10% FCS | Room 7 | 4+ |
| HESM MEM + 10% FCS | Room 14 | 1+ |
| HEK L-15 + 2% FCS | Room 7 | 4+ |
| HEK L-15 + 2% FCS | Room 39 | 2+ |
| HEK L-15 + 2% FCS | 36 C 39 | 2+ |
| HEK MEM + 10% FCS | Room 7 | 4+ |
| HEK MEM + 10% FCS | Room 39 | 2+ |
| WI-38 MEM + 10% FCS | Room 4 | 4+ |
| WI-38 MEM + 10% FCS | Room 18 | 1+ |
| MK MEM + 10% FCS | Room 7 | 4+ |
| MK MEM + 10% FCS | Room 14 | 1+ |
| HeLa MEM + 10% FCS | Room 4 | 4+ |
| HeLa MEM + 10% FCS | Room 18 | 0 |

a 4+, Cells confluent and normal in gross morphology; 3+, cells not confluent but normal in gross morphology; 2+, cells not confluent and abnormal in gross morphology; 1+, cells show severe degeneration and many have detached; 0, cells show complete degeneration and most or all have detached.

b Open end of each capillary under water during storage.
periodically for the outgrowth of new cells coupled with metabolically induced shifts in pH. The results in Table 2 demonstrate that replication was possible even after periods where extensive morphological deterioration was present. HESM cells stored at 36°C, but not at room temperature, retained the capacity for outgrowth even after approximately 3 months, at which time the cells showed severe degeneration and many had detached from the glass surface. Results were comparable with cells stored either in L-15 plus 1% FCS or MEM plus 3% FCS.

The deterioration observed in normal gross morphology during storage of the cell lines after outgrowth in replicate capillary tubes is summarized in Table 3. Each tube contained an essentially confluent cell monolayer exhibiting normal gross morphology at the beginning of storage. Within 2 weeks all were severely degenerated; however, the human embryonic cells did retain good to excellent morphology for approximately 1 week under these conditions.

Additional work gave results which indicated that cells degenerated most rapidly when both ends of the capillary were sealed with putty and least rapidly when the one open end was kept under water to prevent internal evaporation. Medium changes made before storage or pretreatment of the capillaries with Calgon, metasilicate, or serum before use contributed little or nothing to prolonging normal cell morphology after outgrowth.

Table 4. Sensitivity of five cell lines in 1-dr vials to virus infection after storage at room temperature

| Cell line | Storage | Interval (days) | Infecting cell line | Yield<sup>a</sup> (TCID<sub>50</sub>/0.5 ml) from titrations in |
|-----------|---------|-----------------|---------------------|----------------------------------|
|           | Medium  |                 |                     | Fresh cells<sup>b</sup> | Stored cells<sup>c</sup> |
| HESM      | L-15 + 1% FCS | 30              | Rhinovirus          | 10<sup>3</sup> | 10<sup>3</sup> |
| HESM      | L-15 + 1% FCS | 30              | Poliovirus          | 10<sup>3</sup> | 10<sup>3</sup> |
| HESM      | L-15 + 1% FCS | 30              | Parainfluenza       | 10<sup>3</sup> | 10<sup>3</sup> |
| HEL       | L-15 + 2% FCS | 15              | Rhinovirus          | 10<sup>2</sup> | 10<sup>2</sup> |
| HEL       | L-15 + 2% FCS | 51              | Rhinovirus          | <10<sup>4</sup> | 10<sup>2</sup> |
| HEK       | L-15 + 2% FCS | 15              | Rhinovirus          | 10<sup>2</sup> | 10<sup>2</sup> |
| HEK       | L-15 + 2% FCS | 28              | Rhinovirus          | 10<sup>2</sup> | 10<sup>2</sup> |
| WI-38     | L-15 + 2% FCS | 15              | Rhinovirus          | 10<sup>2</sup> | 10<sup>2</sup> |
| WI-38     | L-15 + 2% FCS | 28              | Rhinovirus          | 10<sup>2</sup> | 10<sup>2</sup> |
| WI-38     | L-15 + 2% FCS | 51              | Rhinovirus          | 10<sup>2</sup> | 10<sup>2</sup> |
| MK        | L-15 + 2% FCS | 15              | Rhinovirus          | 10<sup>2</sup> | 10<sup>2</sup> |
| MK        | L-15 + 2% FCS | 28              | Rhinovirus          | 10<sup>2</sup> | 10<sup>2</sup> |
| MK        | L-15 + 2% FCS | 51              | Rhinovirus          | 10<sup>2</sup> | 10<sup>2</sup> |

<sup>a</sup> From approximately 10<sup>3</sup> TCID<sub>50</sub> infecting dose.

<sup>b</sup> The number of tissue culture infective doses to the 50% level.

<sup>c</sup> Confluent cell sheets established immediately before use.

<sup>d</sup> Confluent cell sheets stored about 1 month at room temperature before use.

Table 5. Sensitivity of three cell lines to virus infection before and after outgrowth in capillary tubes

| Cell line | Origin for use | State when infected | Infecting cell line | Yield<sup>e</sup> (TCID<sub>50</sub>/0.5 ml) from titrations in |
|-----------|----------------|---------------------|---------------------|----------------------------------|
| HESM      | Freezer | Suspension | Rhinovirus | 10<sup>4</sup> |
| HESM      | Freezer | Suspension | Poliovirus | 10<sup>4</sup> |
| WI-38     | Freezer | Suspension | Rhinovirus | 10<sup>4</sup> |
| WI-38     | Freezer | Monolayer | Rhinovirus | 10<sup>4</sup> |
| WI-38     | Passage | Suspension | Rhinovirus | 10<sup>4</sup> |
| WI-38     | Passage | Monolayer | Rhinovirus | 10<sup>4</sup> |
| WI-38     | Passage | Suspension | Poliovirus | 10<sup>4</sup> |
| WI-38     | Passage | Monolayer | Poliovirus | 10<sup>4</sup> |
| MK        | Passage | Suspension | Rhinovirus | 10<sup>4</sup> |
| MK        | Passage | Monolayer | Rhinovirus | 10<sup>4</sup> |
| MK        | Passage | Suspension | Poliovirus | 10<sup>4</sup> |
| MK        | Passage | Monolayer | Poliovirus | 10<sup>4</sup> |

<sup>e</sup> From approximately 10<sup>4</sup> TCID<sub>50</sub> infecting dose.

<sup>f</sup> The number of tissue culture infective doses to the 50% level.
An alternative to storing cells at ambient temperature in capillary tubes before use is to maintain them in the frozen state until needed. Therefore, the cells line above were resuspended in normal growth medium plus 10% dimethyl sulfoxide, frozen (1 C/min) by using a Linde model BF 3B liquid nitrogen freezer controller, and stored in a Linde model LR 35-9 refrigerator. At various times these cells were thawed rapidly, diluted in additional medium, centrifuged, and drained of fluid. They were then resuspended in fresh growth medium and put into capillary tubes in the manner and concentration described above. This was done as rapidly as possible to prevent changes in the pH of the medium. Under these conditions, all cell lines achieved full confluency within 24 hr of incubation at 36 C in the capillaries. Their normal gross morphology did not deteriorate during subsequent storage at either room temperature or 36 C more rapidly than comparable lines passaged without freezing.

The capacity of the cells to replicate virus after various storage periods at room temperature in 1-dr vials or capillary tubes was next determined. Such cultures in 1-dr vials were drained of fluid and infected with approximately 10^2 tissue culture infective doses (TCID50) of rhinovirus, poliovirus, and parainfluenza virus in the manner described. After a 5-day incubation period the fluid was collected from each culture and assayed for infectivity by using comparable cells freshly prepared in 1-dr vials or, in some instances, after a storage period at room temperature of 1 month. The results are given in Table 4, and, with those in Table 1, the results show that the stored lines were capable of synthesizing each virus even after moderate deterioration in cell morphology occurred during storage. Virus assays were not influenced significantly by the age of the cells used.

Capillary tube cultures derived from frozen or passaged sources were infected with approximately 10^4 TCID50 of the above viruses soon after achieving confluency. In addition, similar cells also were infected at the time of passage into each capillary. This was done by incorporating approximately 0.1 ml of cell suspension (approximately 4 X 10^6/ml) into each capillary and then adding a similar volume of diluted virus. These additions were made without permitting the leakage of cells into the virus reservoir nor the introduction of an air bubble between the two solutions. The fluid column was centered, sealed, and mixed as described above. Incubation at 36 C was then continued for 5 days, and the fluid from each tube was assayed for infectivity in a fashion similar to that used previously. The results shown in Table 5 give evidence that capillary cell cultures, as well as those in 1-dr vials, were capable of a degree of virus synthesis comparable to that obtained under conventional conditions.

**DISCUSSION**

The major objective of this study has been to determine the feasibility of utilizing, under simplified conditions, cell cultures in miniaturized environments for field work on the epidemiology of virus diseases. Cultures in conventional containers have been used previously by Melnick (1) and others for a similar purpose. We have shown that human embryonic and monkey kidney cells were capable of retaining normal morphology, the capacity for replication, and susceptibility to infection by selected viruses after storage periods in 1-dr vials of 1 month or longer at room temperature. Similar cells in vials pretreated in the manner outlined by Rappaport and co-workers (3, 4, 5) and Nordinling et al. (2) were comparable in these respects to cultures in untreated vials.

The viruses also could be propagated by cells growing in capillary tubes. However, the poor morphological stability of such cultures required that they be infected without prior storage. Poor diffusion probably was responsible for this effect since a distinct pH gradient within the column of medium was usually established in capillary cultures incubated several days with one end of the tube open and the other sealed with putty.

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