CYTOLOGICAL ABERRATIONS IN CULTURES OF “NORMAL” MONKEY KIDNEY EPITHELIAL CELLS*

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Profound changes are known to occur in the morphology of mammalian cells adapted to grow in culture (1–5). The cells often lose their characteristic appearance and usually can no longer be identified with their original tissue source. Berman et al. (5) recently pointed out that the conditions of in vitro growth enable morphological change to take place that is never expressed in vivo, and they suggest that “factors operate in vivo to suppress or control the expression of morphologic potentialities which can be observed in vitro.” Conversely, it would be of interest to know what factors are involved in bringing about the many types of morphologically aberrant cells which characterize cultures derived from various normal and malignant tissues.

The present study on the adjustment and selection of cells leading to in vitro growth was carried out on short term primary cultures of monkey kidney epithelial cells. The results obtained emphasize the high frequency of nuclear aberrations which occur even in such short term cultures. In particular, the paper illustrates the magnitude of cellular diversity that exists in such primary cultures of monkey kidney cells, the only approved source material for viral vaccines used in human beings.

Material and Methods

Tissue Cultures.—Suspensions of rhesus kidney cells (6) were diluted to 350,000 cells per ml. Amounts of 1 ml. were seeded into Leighton tubes (containing removable 22 × 11 mm. coverslips) and 6 ml. into 2 ounce prescription bottles. Three groups of cells were cultured as follows:—

Group A: Cells were seeded in serum-containing medium M-H (0.5 per cent lactalbumin hydrolysate–2 per cent calf serum in Hanks’ salt solution) which has been used extensively for monkey kidney cells (7). The cultures were replenished with fresh M-H medium after 4 days and at 48 hour intervals thereafter.

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**Group B:** Cells were seeded in synthetic medium SM-2 (8, 9). The cultures were replenished with fresh SM-2 after 4 days and at 48 hour intervals thereafter.

**Group C:** Cells were seeded in synthetic medium SM-2. After 4 days the cultures were replenished with SM-2 minus glycine and glucose, a growth-limiting medium for the kidney cells in culture (8, 9). The cells were left in the deficient medium for 48 hours then replenished with complete SM-2.

During the course of this study 15 different cell preparations were examined. Two suspensions were selected for the detailed study reported here. Suspension I represents the more typical culture response. The cultures derived from suspension II showed an exaggerated degree of cellular aberrations. This series represents the maximum range of heterogeneity found, and was chosen to emphasize the range in variability possible from experiment to experiment.

**Cytological Preparations:** The cells were fixed in 10 per cent neutral formalin, alcohol-acetic acid (3:1) or methyl alcohol. The collodion-strip technique (10) was used to remove the cells from the 2 ounce bottles and to fix them to glass slides as described by Reissig et al. (11). Such cells and the cells grown directly on removable coverslips were stained with either hematoxylin-eosin, the May Grünwald-Giemsa stain, or the Feulgen reaction counterstained with fast green.

**Mitotic Activity:** For each experimental period 2,000 cells were counted from areas selected at random in both coverslips and strip preparations. The mitotic activity is expressed as the percentage of cells found in mitosis.

**Mitotic Aberrations:** Abnormal metaphase and anaphase mitotic figures were scored from Feulgen-fast green and May Grünwald-Giemsa stained preparations. Counts were made of 200 metaphase and anaphase figures on each coverslip (7 cultures from each of the three groups in suspensions I and II). Multipolar spindles, anomalous spindles with scattered chromosomes, lagging or precocious movement of chromosomes, chromosome fragments, sticky chromosomes, and chromosome bridges were counted.

**Other Abnormalities:** Non-mitotic cellular aberrations such as giant nuclei, binucleate and multinucleate cells, nuclear fragmentation or blebbing, micronuclei, nuclear inclusions, and phagocytosis were studied and counted on Feulgen-fast green and hematoxylin-eosin preparations. Counts were made of 2,000 cells per culture (5 cultures from each of the three groups in suspensions I and II).

**OBSERVATIONS**

**Mitotic Activity in Response to Nutrient Replenishment:**

It has been shown that the mitotic activity of freshly seeded, monkey kidney cells grown in serum-containing medium M-H reaches a peak during the 3rd to 4th day (12), and then rapidly declines if the medium is not changed. Cells grown in synthetic medium (SM-2) reach their peak of mitotic activity 4 to 5 days after seeding. The present study follows the mitotic activity of cultures during two consecutive medium changes, comparing the cultures grown in serum-containing medium to those grown in synthetic medium SM-2, and to cultures subjected to a glycine- and glucose-deficient medium.

The cultures grown in serum-containing medium (group A) showed a dense growth of cells 48 hours after the first replenishment (6-day-old cultures). The mitotic index started to decline at this time (Table I). Upon replenishment, the cells continued to divide, again showing a decline 48 hours later (8-day-old cultures).
The cultures grown in the synthetic medium SM-2 (group B) maintained a higher mitotic activity during the first 48-hour replenishment period (4 to 6 days, Table I). This is probably related, at least in part, to a slower depletion of nutrients since the cell population at the time of the 1st replenishment was lower than that in group A. At the time of the 2nd replenishment the cell populations appeared to be the same in groups A and B. Both groups continued to divide with group B still maintaining a higher mitotic index.

### TABLE I

**Mitotic Activity of Kidney Epithelial Cells (Rhesus) in Culture Grown in Different Nutrient Media**

Based on counts of 4,000 cells for each experimental period. Per cent mitosis represents the average of samples from two different cell suspensions.

<table>
<thead>
<tr>
<th>Hrs.</th>
<th>A* Mitosis</th>
<th>B† Mitosis</th>
<th>C§ Mitosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>per cent</td>
<td>per cent</td>
<td>per cent</td>
</tr>
<tr>
<td>1st replenishment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>3.2</td>
<td>3.6</td>
<td>3.3</td>
</tr>
<tr>
<td>12</td>
<td>2.6</td>
<td>3.4</td>
<td>3.2</td>
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<tr>
<td>20</td>
<td>2.9</td>
<td>2.4</td>
<td>—</td>
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<tr>
<td>24</td>
<td>3.0</td>
<td>4.4</td>
<td>1.6</td>
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<td>26</td>
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<tr>
<td>28</td>
<td>2.0</td>
<td>3.8</td>
<td>1.0</td>
</tr>
<tr>
<td>30</td>
<td>1.9</td>
<td>3.6</td>
<td>—</td>
</tr>
<tr>
<td>36</td>
<td>2.3</td>
<td>4.5</td>
<td>0.7</td>
</tr>
<tr>
<td>40</td>
<td>2.5</td>
<td>3.3</td>
<td>0.5</td>
</tr>
<tr>
<td>48</td>
<td>1.8</td>
<td>3.1</td>
<td>0.3</td>
</tr>
<tr>
<td>2nd replenishment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>2.2</td>
<td>2.6</td>
<td>1.6</td>
</tr>
<tr>
<td>15</td>
<td>2.0</td>
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<td>18</td>
<td>2.1</td>
<td>2.9</td>
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<td>2.5</td>
<td>3.2</td>
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<td>2.8</td>
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</tr>
<tr>
<td>48</td>
<td>1.0</td>
<td>1.4</td>
<td>3.3</td>
</tr>
</tbody>
</table>

* Group A. The cells were seeded in serum-containing medium M-H. After 4 days the cultures were replenished with fresh M-H at 48 hour intervals.
† Group B. The cells were seeded in synthetic medium SM-2. After 4 days the cultures were replenished with fresh SM-2 at 48 hour intervals.
§ Group C. The cells were seeded in synthetic medium SM-2. After 4 days the cultures were replenished (1st replenishment) with a growth-limiting medium (SM-2 minus glycine and glucose). After 48 hours in the deficient medium, the cultures were replenished with complete SM-2.
This suggests that although the cells seeded and grown in synthetic medium are slower in adapting to in vitro growth, once adjusted, they maintain a viability as good as, or better than, cells in serum-containing medium.

The cultures subjected to the deficient medium (group C) showed a sharp decline in mitotic activity within 24 hours (Table I). Only an occasional cell in mitosis was found after 40 to 48 hours in this medium. This interference with the mitotic activity of cultures essentially at the peak of their growth phase was not accompanied by extensive cell death nor loss of viability. When these cells were replenished with complete SM-2, a burst of mitotic activity occurred 20 to 30 hours later (Table I).

Mitotic Aberrations:

The frequency and types of mitotic abnormalities found in groups A and B were generally independent of the medium used and the mitotic index of the cultures. The mitotic activity within these two groups varied from 1 to 4.6 per cent, yet the percentage of mitotic aberrations per 200 dividing cells was fairly uniform (Table II, suspension I). The cultures of group C, however, contained a higher percentage of multipolar mitosis. This was not directly correlated to the increased mitotic activity some of these cultures attained (Table I). For example a culture with a mitotic activity of 2.0 per cent had 5 per cent multipolar mitoses whereas a culture with a mitotic index of 7.0 per cent had 4 per cent multipolar spindles. Little is known about the mechanisms involved in multipolar spindle formation and the effects of glycine and glucose deficiencies on cell metabolism. It is therefore not possible to relate the observed increase in multipolar spindles to any specific aspect of the experimental procedure at this time.

Some of the morphologically aberrant mitotic figures can be seen in Figs. 2 to 13. Two normal metaphase figures are shown in Fig. 1: A, a polar view and B, a side view of a metaphase plate. Multipolar spindles especially tripolar (Fig. 2) and tetrapolar (Figs. 3 to 5) were commonly seen. An unusual tetrapolar spindle is shown in Fig. 4. Two metaphase plates have formed at right angles to each other. One metaphase plate (seen in polar view) is hypohaploid containing only 12 chromosomes. The diploid chromosome number of rhesus is 42 (12). The other metaphase plate is hyperdiploid. The unequal distribution of chromosomes to the daughter cells is the most common end result of such aberrations (Fig. 5). Unequal distribution of chromosomes also occurs in bipolar divisions (Fig. 6). Apparently cells which have very few chromosomes (Fig. 7) and cells that appear to be polyploid (Fig. 8) may still divide. Precocious movement of chromosomes or chromosomal fragments which fail to become oriented during metaphase were commonly seen (Figs. 8 and 9). Sticky chromosomes and chromosomal bridges at anaphase were rarely found (Figs. 10 and 11). The most common anomalies seen were lagging chromosomes (Fig. 12) and cells with anomalous spindles (Fig. 13).
**TABLE II**

*Frequency Distribution of Nuclear Aberrations in Monkey Kidney (Rhesus) Cultures*

<table>
<thead>
<tr>
<th></th>
<th>Cell suspension I</th>
<th>Cell suspension II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>A</strong></td>
<td><strong>B</strong></td>
</tr>
<tr>
<td><strong>Mitotic abnormalities:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multipolar spindle (per cent)</td>
<td>0-3.0</td>
<td>1-4</td>
</tr>
<tr>
<td>Chromosome bridges (per cent)</td>
<td>0-0.22</td>
<td>0-0.15</td>
</tr>
<tr>
<td>Lagg ing, precocious movement, fragmented chromosomes (per cent)</td>
<td>7-13</td>
<td>10-14</td>
</tr>
<tr>
<td>Scattered chromosomes, anomalous spindle (per cent)</td>
<td>6-9</td>
<td>6-8</td>
</tr>
<tr>
<td>No. of mitotic figures counted</td>
<td>1400</td>
<td>1400</td>
</tr>
<tr>
<td>Total per cent mitotic abnormalities</td>
<td>16-23</td>
<td>18-24</td>
</tr>
<tr>
<td><strong>Other abnormalities:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Giant nuclei (per cent) average</td>
<td>0.56</td>
<td>0.59</td>
</tr>
<tr>
<td>Binucleate cells (per cent) average</td>
<td>1.34</td>
<td>1.61</td>
</tr>
<tr>
<td>Multinucleate cells (per cent) average</td>
<td>0.12</td>
<td>0.09</td>
</tr>
<tr>
<td>Nuclear fragmentation, blebbing, and micronuclei (per cent) average</td>
<td>0.28</td>
<td>0.36</td>
</tr>
<tr>
<td>Nuclear inclusions (per cent) range</td>
<td>0-0.16</td>
<td>0-0.14</td>
</tr>
<tr>
<td>Phagocytosis (per cent) range</td>
<td>0.1-0.18</td>
<td>0.1-2.3</td>
</tr>
<tr>
<td>No. of cells counted</td>
<td>10,000</td>
<td>10,000</td>
</tr>
<tr>
<td>Total per cent non-mitotic abnormalities</td>
<td>3.2</td>
<td>3.8</td>
</tr>
</tbody>
</table>

* Group A, same as in Table I.
† Group B, same as in Table I.
§ Group C, same as in Table I.
¶ Mitotic abnormalities: 7 cultures were examined in each group scoring 200 anaphase and metaphase mitotic figures per culture.
¶¶ Other abnormalities: 5 cultures were examined in each group scoring 2,000 cells per sample.

**Non-Mitotic Abnormalities:**

It is difficult to quantitate accurately the frequency distribution of atypical cells for the variation from suspension to suspension was high and the incidence of certain aberrations low (Table II). The average percentage of non-mitotic atypical cells was approximately 3 to 4 per cent in suspension I and 6 to 7 per cent in suspension II.
Multinucleate cells (Fig. 14) and giant cells with highly polyploid nuclei (Fig. 15) were common in all cultures. Many nuclei assumed irregular shapes with excessive lobing and fragmentation (Figs. 14 and 15). Various types of nuclear inclusions were seen; however, the frequency with which they occurred was very low (Figs. 16 to 19). A strange type of inclusion (Figs. 18 and 19) was present in two of fifteen suspensions studied. The inclusion appeared to form from an invaginated surface of the nuclear membrane with an intricate system of “radiations” emanating from this surface. The “radiations” were rich in ribonucleoprotein while the nucleoli of such inclusion-bearing nuclei were pale and vacuolated.

Phagocytosis occurred regularly during the first few days of cellular outgrowth. Many injured and dying cells were engulfed into the cytoplasm of the epithelial cells. Fig. 20 shows a pycnotic cell engulfed in the cytoplasm of a dividing cell. Nuclei were often found surrounding the phagocytized cell (Figs. 21 and 22). Fig. 23 shows a nucleus which has completely engulfed a pycnotic cell—"nuclear phagocytosis." One might speculate as to the fate of such a cell. Can the nucleus “digest” and utilize the chemical components of the phagocytized cell? Does the DNA of the phagocytized cell maintain its biological properties and affect the genetic make-up of the epithelial cell? Are the nuclear inclusions seen in uninfected cultures related to a similar type of nuclear phagocytosis?

Cultures of group C exhibited a higher rate of phagocytosis than those of groups A and B. This is probably due to an increase in the number of damaged cells in the culture as the result of glycine and glucose deficiencies. In HeLa colonies infected with Eastern equine encephalomyelitis, a large proportion of the surviving cells were reported to be phagocytizing the virus-killed and injured cells (13). Since phagocytosis has been observed in as high as 2 to 3 per cent of the cell population (Table II), the phenomenon must be common. This “feeding” of cells should be considered when studying the nutrition and growth of tissue culture cells.

**DISCUSSION**

The nutrient medium of a culture provides more than the essential sources of energy and nutrients (8, 9, 14, 15). During the first 2 to 3 days the cells are adjusting, attaching and preparing for division, and little growth takes place. The peak of mitotic activity of monkey kidney cell cultures occurs during the 3rd to 4th day, then rapidly falls if the medium is not changed (12). A high mitotic rate can be maintained for several days with regular replenishment (Table I, groups A and B). Monkey kidney cultures subjected to a deficient medium stop dividing within 48 hours (Table I, group C). When replenished with complete medium such cultures show a burst of mitotic activity after a lag period of 18 to 20 hours. Preliminary studies using colchi-
cine indicated at least 30 per cent of the cells enter mitosis 18 to 27 hours after replenishment. The work of Rappaport (8, 9, 15) in developing a completely synthetic medium and defining certain growth-limiting factors, has enabled this approach to synchronized growth in monkey kidney cells. It would be interesting to study when, during the lag period, deoxyribonucleic (DNA) synthesis occurs. Hornsey and Howard (16) have made autoradiographic studies of Ehrlich ascites cultures using C14-labelled adenine. DNA synthesis, which occurs during interphase, was completed 6 hours before the cell entered into mitosis.

The investigations described here have indicated that pleomorphism is not the result of long term culturing alone, but is prevalent as soon as cells adjust to "in vitro" growth. In young cultures (6 to 8 days after seeding) 16 to 40 per cent of the dividing cells were abnormal. DNA-Feulgen measurements of sectioned kidney tubule cells prior to trypsinization were compared to measurements made of 10-day-old cultures of cells taken from the same monkey (17). Approximately 18 per cent of the nuclei of cultured cells were aneuploid as contrasted with typically diploid nuclei of the kidney sections.

The nuclear aberrations leading to aneuploidy, polyploidy, and general pleomorphism have been extensively studied and described (18-22). Multipolar spindles, anomalous spindles, lagging, fragmented, and sticky chromosomes, and nuclear fragmentation have been found in cultures from normal and cancerous tissues. Clones derived from single cell isolates were also found to exhibit nuclear aberrations and heteroploidy upon continued subculturing (23).

In the short term cultures used in the present study a wide range of variations in the degree of cellular abnormalities was noted from experiment to experiment (different monkey kidney cell suspensions). In one suspension approximately 21 per cent of the dividing cells were aberrant and 3 to 4 per cent of non-mitotic abnormalities existed in the cultures. In contrast, a second suspension contained an average of 33 per cent aberrant dividing cells while 6 to 8 per cent of the non-mitotic cell population had abnormalities. This suggests that factors such as handling during and after trypsinization, the health of the monkey, and perhaps the presence of latent viruses (24, 25) may affect the degree of heterogeneity developing in monolayer cultures. The process of trypsinization leaves the cells extremely fragile to simple manipulations of pipetting and centrifugation (6). The pH, the ionic balance of the medium, and the properties of the glass surface may affect the attachment, outgrowth, and survival of the cells, (8, 9). In addition to these stresses, the cells are deprived of the efficiency of a circulatory system, and are no longer exposed to the spectrum of hormonal factors which may be essential for the maintenance of certain synthetic activities. The adaptation of highly specialized cells to "in vitro" life must involve adjustments leading to a more flexible
type of metabolism. Under the conditions of the present experiments, cultures grew readily for a few weeks, but not indefinitely; hence some nutritional inadequacies existed, which might have been reflected in the cytological alterations.

How can we explain successful adaptation, survival, and growth of cells with entire chromosomes missing, with duplications of some chromosomes and deletions of others? The possibility that many genes have their effect on the cell early in the life history of the cell, inducing self-regulating systems (plasmagenes), has been suggested (26). These genes would then be less important for the survival of the later generations. Levan (21) and Berman et al. (5) further postulate that in heteroploid cell populations, combinations of certain genetic duplications and/or deletions may lead to greater viability and adaptability for an in vitro existence. They note that cell lines with high chromosomal aberrations and radically different genotypes are generally more resistant to unfavorable conditions.

SUMMARY

A cytological study was made of short term primary cultures of monkey kidney epithelial cells grown in serum-containing (M-H) and synthetic (SM-2) media. The mitotic activity of the cultures reached a peak 3 to 5 days after seeding, and rapidly declined if the medium was not changed. With replenishment at 48 hour intervals a high mitotic rate was maintained through the 8th day. Cultures subjected to a glycine- and glucose-deficient medium showed a sharp decline in mitotic activity within 24 hours. When such cultures were replenished a burst of mitotic activity occurred 20 to 30 hours later.

Mitotic aberrations such as multipolar spindles, anomalous spindles with scattered chromosomes, lagging or precocious movement of chromosomes, chromosome fragments, and chromosome bridges were found in all cultures ranging from 16 to 40 per cent of the dividing cells. Non-mitotic aberrations such as giant nuclei, multinucleate cells, nuclear fragmentation, micronuclei, nuclear inclusions, and phagocytosis were found in approximately 3 to 7 per cent of the non-dividing cell population. The frequency and types of mitotic abnormalities were generally independent of the medium used and the mitotic index of the cultures.

BIBLIOGRAPHY


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ABERRATIONS IN CULTURES OF KIDNEY EPITHELIAL CELLS


EXPLANATION OF PLATES

"Normal" cultures of monkey kidney epithelial cells. All cells were stained with hematoxylin-eosin. × 700.

PLATE 47

Fig. 1. Two normal metaphase figures, A, polar view, B, side view.

Fig. 2. A tripolar anaphase figure.

Fig. 3. A tetrapolar metaphase figure.

Fig. 4. A tetrapolar metaphase with one group of chromosomes oriented in a plane perpendicular to another group. The metaphase plate seen from polar view contains only 12 chromosomes.

Fig. 5. Late anaphase of a tetrapolar division. Note the unequal distribution of chromosomes to the four daughter cells.

Fig. 6. Unequal distribution of chromosomes and cytoplasm in a bipolar division.

Fig. 7. A hypohaploid cell in mitosis. The chromosomes appear thinner than typical metaphase chromosomes.

Fig. 8. A polyploid cell in metaphase. Note the chromosome fragments which have failed to become oriented on the metaphase plate.

Fig. 9. A cell in mitosis showing precocious movement of chromosomes.

Figs. 10 and 11. Cells in anaphase with chromosome bridges.
PLATE 48

FIG. 12. A tripolar anaphase division figure with lagging chromosomes

FIG. 13. A cell with an anomalous spindle. The chromosomes are scattered about in a disorganized fashion.

FIG. 14. A multinucleate cell with four polyploid (or aneuploid) nuclei. The two nuclei on the left have a common bridge between them and may be in the process of fusing. Note the knob-like protrusion coming from the uppermost nucleus. This is probably the manner in which most micronuclei, frequently seen in these cultures, are formed.

FIG. 15. A giant binucleate cell with extensive lobation of the nuclei. There appears to be some interaction or connection between the two nuclei suggestive of fusion.

FIG. 16. A large nucleus containing a commonly seen inclusion. The inclusion appears to have been formed by an invagination of the nuclear membrane encircling cytoplasmic material.

FIG. 17. A cell showing a prominent nuclear inclusion bounded by a dense basophilic membrane.

FIGS. 18 and 19. Nuclear inclusions which appear to form in association with an invaginated surface of the nuclear membrane. Note the intricate system of radiations. The nucleoli of cells having such inclusions are pale and highly vacuolated.

FIG. 20. Phagocytosis in a dividing cell. The phagocytized cell is a pyknotic, dying cell and has been completely engulfed by the dividing cell.

FIGS. 21, 22, and 23. Phagocytosis with nuclear involvement. Figs. 21 and 22. The nucleus is encircling the phagocytized pyknotic cell. Fig. 23. The nucleus has completely engulfed the phagocytized cell ("nuclear phagocytosis"). The nucleoli (N) can readily be distinguished from the phagocytized cell (I).
(Kleinfeld and Melnick: Aberrations in cultures of kidney epithelial cells)