Loss of Controlled Nuclear Division in BHK21 Cells
Passed in Vivo

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SUMMARY

Low-passage BHK21/C13 cells respond to cytochalasin B (CB) by undergoing limited or controlled nuclear division. These cells respond to CB as normal cells do since nuclear division usually occurs only once. Premature chromosome condensation, a result of mitoses in highly multinucleate cells, occurs in less than 0.5% of the cells. When they are inoculated into weanling hamsters, s.c. tumors appear within 3 to 4 weeks with as few as 10³ cells. When these cells are returned to cell culture they respond to CB with uncontrolled nuclear division and premature chromosome condensation. All cultured tumors respond in this manner regardless of the number of cells originally inoculated into animals. This suggests at least two possibilities: (a) that loss of controlled nuclear division in BHK cells is closely associated with or required for tumorigenicity, with in vivo passage selecting for a rare tumorigenic variant or (b) that loss of controlled nuclear division is secondary to tumorigenicity and results when cells are passed in vivo, i.e., in vivo passage has the direct effect of causing cells to lose controlled nuclear division.

If the first possibility is operating, then it would be expected that a very small fraction of BHK21/C13 cells show uncontrolled nuclear division (approximately 1 of 1000 CB-treated cells). Also, clones of C13 should be nontumorigenic if only 1 of 1000 cells is tumorigenic. Extensive examination of CB-treated C13 cells shows 1 of 1250 cells to be highly multinucleated although not as highly multinucleated as tumor cells. This provides some evidence in support of the first possibility. However, three separate clones of C13 cells were found to be tumorigenic providing evidence supporting the second possibility.

BHK21/C13 and various BHK21 tumors all appeared to grow to concentration densities markedly higher than hamster embryo fibroblasts. However, the tumor cells usually grew to the same density as did BHK21/C13 or only slightly higher. This suggests that loss of contact inhibition is not sufficient for loss of controlled nuclear division. It also suggests that the theoretical 1 of 1000 tumorigenic cells and 1 of 1250 cells with uncontrolled nuclear division do not overgrow the “normal” cells because all cells grow to similar densities.

INTRODUCTION

Previous reports have shown a differential response to CB² between normal and neoplastic cells (9-12) or between normal and DNA virus-transformed cells (6, 16, 18). In the presence of CB, a mold metabolite that prevents cytokinesis, nuclear division in normal cells is limited to 1 or perhaps 2 rounds (6, 9-11, 18). Thus, only binucleate and occasionally tetraneucleate cells are formed. In neoplastic and DNA virus-transformed cells, nuclear division is relatively uncontrolled and highly multinucleate cells are observed following CB treatment (6, 9, 11, 16, 18). It has also been shown that chromosome pulverization or PCC, an abnormality associated with multinucleation (1, 4, 5, 8, 14, 15), is observed frequently in CB-treated neoplastic (9-11) and virus-transformed cells but is absent in CB-treated normal cells. The PCC observed seems to be a direct function of both the high degrees of multinucleation and continued presence of mitoses in multinucleated cells which lead to asynchrony among the nuclei (11). In normal cells, nuclear division becomes greatly reduced after the 1st division and thus PCC cannot be observed (11).

In earlier reports uncontrolled nuclear division and PCC were reported in various continuous cell lines (10-12), but in a line of BHK21 cells obtained from the American Type Culture Collection (BHK21/C13) nuclear division appeared controlled and PCC was infrequent (9). The present report shows a loss of control of nuclear division in BHK21/C13 when passed in vivo and returned to culture. Studies on contact inhibition show no difference in ultimate concentration densities achieved by various lines of BHK21 cells before and after in vivo passage. Also, BHK21/C13 cells that formed colonies in agar maintained normal control of nuclear division.
F. J. O’Neill

MATERIALS AND METHODS

Cell Cultures. Low passage BHK21/C13 cells obtained from the American Type Culture Collection have been described in previous reports (2, 3). The cells were obtained at passage 58 from original cloning and are diploid. They were used for in vitro and in vivo studies prior to the 10th passage in this laboratory and showed no Mycoplasma (9). BHK21/ IV₃, BHK21/IV₄, BHK21/IV₅, and BHK21/IV₆ are in vitro established lines resulting from s.c. tumors produced by inoculation of BHK21/C13 cells at concentrations of 10⁴, 10⁵, 10⁶, and 10⁷, respectively. HaEF were obtained by trypsinizing whole decapitated embryos from outbred hamsters. All cultures were propagated in Eagle’s medium with 10% heat-inactivated calf serum.

Cloning. BHK21/C13 cells were cloned in Falcon cloning wells (Microtest II dishes). Cells were diluted in culture medium so that there were approximately 10 cells/0.4 ml. Each of 30 wells was then inoculated with 0.2 ml and the cells were allowed to attach. Wells that contained only 1 cell, via microscopic examination, were then marked and allowed to grow until there were enough cells to explant to culture flasks. The cloning cycle was then repeated. Three clones were selected and examined for tumorigenicity or controlled nuclear division.

Growth in Agar. Cells were propagated in agar by a variation of the method of MacPherson and Montagnier (7). Five ml of a solution of growth medium in 0.6% agar (Baltimore Biological Laboratories, unwashed agar) were allowed to gel in multiple 60-mm Falcon Petri plates. Then 1 x 10⁵ cells/ml were suspended in growth medium containing 0.3% agar and 2 ml of cell suspension were added to each plate. An additional 2 ml of such medium (without cells) were added to the plates every 5 days. On Day 14, the number of colonies per plate was scored. On Day 12, colonies were picked from the agar with a Pasteur pipet and the cells were propagated in 1-oz prescription bottles. When these were confluent the cells were transferred to 8-oz bottles or assayed for control of nuclear division.

Multinucleation and Chromosome Studies. These studies were performed by methods previously described (9, 10, 17).

Contact Inhibition. From 0.1 to 0.5 x 10⁴ cells (BHK or HaEF) were plated onto small replicate culture flasks or 60-mm Falcon Petri plates and incubated in normal medium. The cultures were propagated for 8 to 10 days and the media changed every 2nd day. Duplicate replicate cultures were harvested periodically by detachment with Versene, trypsin (10, 11, 13). The suspended cells were washed in medium, suspended in phosphate-buffered saline, treated with trypsin blue, and counted in a hemacytometer. The cell counts were normalized to indicate the number of cells per sq cm in situ. The fact that 60-mm Falcon Petri dishes measure only 50 mm in diameter has a slight effect on the computed cell density. All counts were made using the 50-mm figure.

In Vivo Passage. Outbred weanling hamsters (4 weeks old) were inoculated s.c. with BHK21/C13 cells at concentrations of 10⁴, 10⁵, 10⁶, and 10⁷ cells. Four animals were used for each dilution of cells, and resulting tumors were returned to culture (1 from each inoculation dilution) by the following procedure. The tumor was aseptically excised and cut into small pieces with sterile scissors. This material containing fragments and suspended cells was placed in a trypsinizing flask containing 0.15% trypsin, and the material was agitated for 25 min with a magnetic stirring bar. The suspended tumor cells were centrifuged, explanted into 8-oz prescription bottles with normal medium, and incubated. Within 24 to 48 hr complete monolayers were formed. The cells were propagated for up to 5 passages and continued to grow rapidly. Their responsiveness to CB was very similar at all passages tested.

RESULTS

Multinucleation and PCC. When treated with CB at a concentration of 1.0 to 1.5 µg/ml, BHK21/C13 cells and cells derived from colonies formed in agar showed a large number of cells with 2 nuclei and very few with 3 or 4 nuclei (Table 1; Fig. 1). Very few, if any, cells could be detected with more than 4 nuclei (Fig. 1). PCC was also not significantly increased after administration of CB (Table 2).

When tumors produced by BHK21/C13 were returned to tissue culture, all responded to CB with a loss of control of nuclear division (Fig. 2). Table 1 shows the frequency of multinucleation of cultured tumors derived from 4 different concentration of cells. All show high degrees of multinucleation. At least 37% of all tumor cells contained at least 5 nuclei and 23% showed 7 or more nuclei. In Table 2, PCC is shown in relatively high frequencies. PCC occurred in at least 16% of all tumor cells as compared to 0.8% for BHK21/ C13 cells.

Contact Inhibition. HaEF, BHK21/C13, and all the tumors produced by BHK21/C13 were propagated beyond the stage of confluence to determine their ultimate concentration densities. As shown in Chart 1, HaEF exhibited significantly more contact inhibition than any of the BHK lines. Interestingly, BHK21/C13 and the cell lines of tumors produced from it were relatively non-contact inhibited. Thus, our BHK21/C13 cells grow to similar concentration densities whether they show control of nuclear division or have been passed in vivo.

Tumorigenicity. Table 3 shows the tumor-producing dose for the 1st and 2nd in vivo passages. Approximately one-half of the animals inoculated with about 1000 BHK21/C13 cells developed tumors. Two cell lines resulting from separate tumors induced by BHK cells were also inoculated into weanling hamsters. These inoculations represented the 2nd in vivo passage. From Table 3, it can be seen that the dose producing tumors in 50% of the animals was almost 10⁴ for BHK21/IV⁵ while BHK21/IV⁶ was slightly more tumorigenic than C13 cells.

The tumorigenicity of the 3 clones studied appeared to be maintained with the possible exception of clone 3. Clones 1 and 2 were not significantly more or less tumorigenic than parental C13 cells while clone 3 was marginally less tumorigenic than parental C13 cells.

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Table 1
Multinucleation in various BHK21 cell lines treated with CB

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CB concentration</th>
<th>% of cells with nuclei numbers of:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethyl sulfoxide</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>BHK21/C13</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>BHK21/C13</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>BHK21/IVa</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>BHK21/IVa</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>BHK21/IVb</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>BHK21/IVb</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>BHK21/IVb</td>
<td>+</td>
<td>-</td>
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<tr>
<td>BHK21/IVb</td>
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<tr>
<td>BHK21/IVb</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>BHK21/IVb</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>BHK21/agar colony 1</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>BHK21/agar colony 1</td>
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<td>+</td>
</tr>
<tr>
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</tr>
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<td>BHK21/agar colony 2</td>
<td>-</td>
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</tr>
<tr>
<td>BHK21/agar colony 3</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>BHK21/agar colony 3</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

* Dimethyl sulfoxide was used as a control since it is used as the CB solvent (1-3).

Table 2
PCC in various BHK21 lines treated with CB

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>No. of cells with PCC</th>
<th>Total cells scored</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHK21/IVa</td>
<td>DMSOab2</td>
<td>0</td>
<td>237</td>
</tr>
<tr>
<td>BHK21/IVa</td>
<td>CB</td>
<td>51</td>
<td>250</td>
</tr>
<tr>
<td>BHK21/IVa</td>
<td>DMSO</td>
<td>42</td>
<td>250</td>
</tr>
<tr>
<td>BHK21/IVa</td>
<td>CB</td>
<td>2</td>
<td>250</td>
</tr>
<tr>
<td>BHK21/IVa</td>
<td>CB</td>
<td>39</td>
<td>250</td>
</tr>
<tr>
<td>BHK21/C13</td>
<td>DMSO</td>
<td>2</td>
<td>250</td>
</tr>
<tr>
<td>BHK21/C13</td>
<td>CB</td>
<td>2</td>
<td>250</td>
</tr>
</tbody>
</table>

* DMSO was the control since it is used as the CB solvent (1-3). A total of 500 consecutive metaphases were scored for each experiment.

** DMSO, dimethyl sulfoxide.

Growth in Agar. BHK21/C13 cells appeared to form colonies at a rate of 1 in 2500 cells since the average number of colonies per dish was approximately 80. Most of these colonies were small, with about 20 large colonies per dish. The 3 colonies studied were large and grew readily when explanted. As shown in Table 1, all colonies showed normal controlled nuclear division. Thus we were unable to select out cells with uncontrolled nuclear division in this manner. These cells were also tumorigenic at about the same frequency as C13 cells (data not shown).

DISCUSSION

Previous reports from this laboratory have suggested that tumor cells from a variety of sources respond to CB by the formation of highly multinucleated cells and PCC. Evidence has been presented which suggests that PCC is a result of continued mitoses in highly multinucleated cells (9-11). There the presence of PCC is another indicator or assay for loss of control of nuclear division. Normal cells usually form only binucleate cells, nuclear division is greatly reduced, and PCC is therefore absent (9-11). The present report provides data using a more concise experimental system which is consistent with these previous observations. Although BHK21/C13 cells respond to CB similarly to normal cells, tumors produced by these cells respond by becoming highly multinucleated and exhibiting PCC. The results suggest that in this system (a) loss of controlled nuclear division is secondary to tumorigenicity, being a result of the in vivo environment, or (b) inoculation of BHK21/C13 cells in vivo selects a small number of these cells with uncontrolled growth.
F. J. O'Neill

nuclear division that are tumorigenic (perhaps 1 in 1000; see Table 3).

These 1 in 1000 cells which are tumorigenic should also show uncontrolled nuclear division. Thus if the 2nd hypothesis is operating, there should be an occasional CB-treated C13 cell with many nuclei. These rare cells may have gone unnoticed since the analyses made for Table 1 utilized only 250 to 300 cells. We therefore have reexamined numerous slides and after scoring more than 20,000 consecutive cells have detected 16 with at least 7 nuclei (1 of 1,250). None of the cells showed more than 9 nuclei (this contrasts with CB-treated tumor cells which show 5 to 10% with 9 or more nuclei) (data not shown). This observation is at least partially consistent with in vivo passage selecting for preexisting tumor cells with uncontrolled nuclear divisions. If there are occasional tumorigenic cells with uncontrolled nuclear division in the BHK21/C13 population, one might expect these to become the dominant cell type within a short period. However, as shown in Fig. 1, both C13 and tumor cells grow to similar concentration densities, although these concentration densities are much greater than those of HaEF cultures. Consistent with this is the observation of Jarrett and MacPerson (3) showing that highly tumorigenic variants of BHK21 cells did not exhibit in vitro growth advantages over poorly tumorigenic variants.

The question of selection versus the possibility that BHK21/C13 cells undergo an alternation in vivo was examined in 2 other ways. If 1 of 1000 C13 cells is tumorigenic and shows uncontrolled nuclear division, then nearly all subclones should be nontumorigenic since the chances that 1 of these would be selected by cloning would be remote. Also if in vivo passage selects for rare tumorigenic variants, then the tumorigenicity of a 2nd in vivo passage should show a significant increase. However, neither method supports the selection hypothesis. Three separate clones of C13 cells are tumorigenic and, with 1 possible exception, show the same level of tumorigenicity as do their progenitors (Table 3). Also the tumorigenicity of C13 cells undergoing a 2nd in vivo passage was not significantly greater than that of the 1st passage for 1 tumor line and only marginally greater for another (Table 3). These results appear to contrast those of another study in which all subclones of BHK21 cells were nontumorigenic and cells undergoing a 2nd in vivo passage were markedly more tumorigenic than those of the 1st passage (3). Although the cells used in the previous study (3) were also BHK21/C13, they produced tumors with only 1 in 10^6 cells (3). This earlier study (3) utilized young adult hamsters rather than weanlings. Also the tumorigenicity studies were performed intracranially rather than s.c. These differences in experimental procedure may explain the apparently contrasting results.

It is also questionable whether our results rule out the selection hypothesis. Although subclones of C13 cells are tumorigenic, this may be due to the continuous production of tumorigenic variants from nontumorigenic progenitors, perhaps by mutation. The observation that the 2nd in vivo passed C13 cells are not significantly more tumorigenic may result from inefficiency of tumor production with small numbers of cells. Therefore, further studies are necessary to determine whether BHK21/C13 cells lose controlled nuclear division as a result of in vivo passage.

Tumors produced by BHK21/ATCC cells do not grow to higher concentration densities than their progenitors. These cells do grow to significantly higher concentration densities than do normal HaEF. This suggests that loss of contact inhibition per se is not sufficient for loss of control of nuclear division. However, it is possible that loss of contact inhibition is a requirement for loss of controlled nuclear division since there seems to be some association between these phenomena (11).

Finally, the observation that occasional variants of C13 cells, which are able to grow in agar, maintain normal control of nuclear division suggests the following. The ability to grow in agar does not result in the expression of uncontrolled nuclear division; therefore these phenomena may be separate. They also indicate a fundamental difference between tumor cells and cells that have been grown in agar, i.e., tumor cells show a loss of controlled nuclear division.

ACKNOWLEDGMENTS

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REFERENCES


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Nuclear Division Loss in BHK21 Cells


Fig. 1. Binucleation in BHK21/C13 cells treated with CB (1 µg/ml) for 7 days. Cells were permitted to grow on coverglasses and then fixed in situ.

Fig. 2. Multinucleation in BHK21/IV cells treated as in Fig. 1. This field is somewhat unusual since it does not contain mitoses. When mitoses are present in highly multinucleated cells, they are often associated with PCC.
Loss of Controlled Nuclear Division in BHK21 Cells Passed \textit{in Vivo}

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