Multistage Neoplastic Transformation of Syrian Hamster Embryo Cells cultured at pH 6.70

R. A. LeBoeuf, G. A. Kerckaert, M. J. Aardema, and D. P. Gibson

The Procter and Gamble Company, Miami Valley Laboratories, Cincinnati, Ohio 45239-8707

ABSTRACT

We have reported that the frequency of induction of the morphologically transformed phenotype in early passage Syrian hamster embryo cells is increased in carcinogen-treated cultures when the cells are cultured in media of pH 6.70, instead of the traditionally used pH 7.35. The purpose of the studies reported here was to determine the neoplastic potential of cells derived from colonies of morphologically normal, altered, and transformed phenotypes from untreated and carcinogen-treated cultures generated under low pH conditions. Results from these studies indicate that cells derived from morphologically transformed colonies generated at pH 6.70 following benzo[a]pyrene or 3-methylcholanthrene treatment became established as immortal cell lines at a frequency of 30 and 14%, respectively. In contrast, cells derived from morphologically transformed colonies in untreated cultures and morphologically normal colonies from carcinogen-treated cultures establish as immortal cell lines at a frequency of 3% or less. Acquisition of aneuploidy was associated with immortalization in most cell lines. Ninety-two % of the immortal lines eventually progressed to the neoplastic phenotype upon subsequent cell culture, as assessed by cell injection into newborn hamsters. Approximately 75% of the neoplastic cell lines exhibited anchorage-independent growth in agarose. A comparison of these results to those generated at pH 7.35 by other investigators indicates that pH 6.70 culture of SHE cells is primarily enhancing early stages of the transformation process, i.e., induction of morphological transformation, while the frequency of progression from the morphologically transformed phenotype to neoplasia appears similar between the two pH values. These results support earlier indications that pH 6.70 culture of SHE cell is a useful model system for the study of multistage carcinogenesis.

INTRODUCTION

SHE2 cells have been used extensively to study the process of multistage neoplastic transformation (reviewed in Refs. 1–3). Following carcinogen treatment (both chemical and physical) and occasionally without carcinogen treatment, SHE cells progress towards the neoplastic phenotype through several discrete biological phenotypes (4–6). These include MT, escape from cellular senescence to form an immortal cell line, increased fibroinolyc activity, and the ability to grow in semisolid agar. MT in either clonal or mass culture is associated with an increased probability of neoplastic progression but is not essential for progression to the neoplastic phenotype (4, 6–11). In contrast, the acquisition of immortality appears to be necessary for the progression of nonneoplastic cells to the neoplastic phenotype (12–14). Additionally, a strong positive correlation exists in this system between the capacity for anchorage-independent growth and tumorigenicity (5).

Studies from our laboratory have demonstrated that the frequency of clonal MT induced by carcinogens from several different chemical classes is significantly increased when SHE cells are cultured in media of pH 6.70 compared to 7.35, the pH used historically for SHE cell culture (15, 16). The increase in transformation frequency at pH 6.70 also occurs with a decreased dependency on specific serum lots and cell isolates, relative to pH 7.35 culture of SHE cells. It was also demonstrated that the frequency of spontaneous MT is increased at pH 6.70 relative to pH 7.35 and that these “spontaneous” MT clones revert to normal morphology upon subculturing at pH 7.35 (15–17). The molecular basis of increased MT induced by carcinogens at pH 6.70 compared to pH 7.35 is unknown.

An increasing database indicates that chemical induction of a statistically significant increase in MT colony frequency at pH 6.70 is predictive of the chemical’s carcinogenic potential (15, 18). However, if this endpoint is to be used as an early indicator of a chemical’s carcinogenic potential, we felt it was essential to determine the biological fate of MT colonies induced by carcinogens at pH 6.70. This is particularly important in light of our previous observations of the spontaneous induction and reversibility of this phenotype at pH 6.70 (17) and the reversibility of the MT phenotype induced by certain chemicals (19, 20). The neoplastic potential of cells derived from MT colonies generated at pH 7.35 has been characterized previously (4, 6–9, 20) and thus those studies were not repeated here.

An initial report of studies designed to address the biological fate of cells derived from MT colonies at pH 6.70 indicated that, with BP treatment, cells derived from 5 of 16 MT colonies became immortal cell lines and 3 of 3 lines tested were neoplastic in newborn hamsters (16). In the studies reported here, colonies of SHE cells with different morphological phenotypes (normal, altered, and transformed) from carcinogen-treated or control cultures were examined for their ability to (a) escape senescence and establish as immortal cell lines, (b) grow in semisolid agarose, and (c) form progressively growing tumors in newborn hamsters. We also describe initial results of the analysis of chromosome changes associated with transformation of these cells to the neoplastic phenotype. A preliminary summary of the results described here has been reported (21).

MATERIALS AND METHODS

Source of SHE Cells. Primary embryo cells were obtained from 13-day gestation Golden Syrian hamsters (Charles River, Wilmington, MA), cultured in pH 6.70 basal medium (see below) containing 10% FBS (GIBCO, Grand Island, NY, or Hyclone Laboratories, Logan, UT) at 37°C in 10% CO2 in air and 90% relative humidity for 24 h and cryopreserved as described previously (15).

Culture Media. The basal medium used for cell culture (Quality Biological Inc., Gaithersburg, MD) is a modified formulation of low (1000 mg/liter) glucose DMEM containing 0.75 g/liter NaHCO3 (resulting in a pH of 6.70 when incubated at 37°C and 10% CO2), containing an equimolar concentration of MgCl2 replacing MgSO4, and a reduced concentration of phenol red (5 versus 15 mg/liter), as described previously (15). For clonal cultures, 20% FBS-containing medium was used and 10% FBS-containing medium was used for serial passage of SHE cell lines. Antibiotics were not present in the culture medium.

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1To whom requests for reprints should be addressed, at The Procter and Gamble Company, Miami Valley Laboratories, P.O. Box 398707, Cincinnati, OH 45239-8707.

2The abbreviations used are: SHE, Syrian hamster embryo; BP, benzo[a]pyrene; FBS, fetal bovine serum; HBSS, Hank’s balanced salt solution; MT, morphologically transformation (transformed); 3-MC, 3-methylcholanthrene; AIG, anchorage-independent growth; DMEM, Dulbecco’s modified Eagle’s medium.
Colonies were injected (s.c.) in the upper back region. The specific lines tested for tumorigenicity were identified by phase contrast microscopy. Additional studies were conducted to exhibit colony formation in agarose, and for those cell lines that were tumorigenic but did not exhibit cytoplasmic spreading. Cells from each line were cryopreserved for further studies.

When this occurred, the latest passage cryopreserved period cultures were thawed and passaged for over 3 weeks and the cells became very large with marked senescence. The results are shown in Table 1. These data indicate that cells derived from BP- or 3-MC-induced MT colonies escape senescence and form immortal cell lines at a 10 and 4 times higher frequency, respectively, compared to cells derived from normal or altered colonies from untreated cultures (24). Statistical significance for these data sets. Cells derived from carcinogen-treated cultures (3%) or MT colonies from untreated cultures escaped senescence following serial passage. The results are shown in Table 1. These data indicate that cells derived from BP- or 3-MC-induced MT colonies escape senescence and form immortal cell lines at a 10 and 4 times higher frequency, respectively, compared to cells derived from normal or altered colonies from untreated cultures. However, a 4-fold increase observed with 3-MC compared to controls probably represents a biologically significant increase. The increased frequency of MT colonies escaping senescence observed with BP treatment is statistically significant (P = 0.0071), as indicated by a one-sided Fisher’s exact test (24), whereas that for 3-MC (P = 0.19) is not, compared to the frequency of MT colonies isolated from untreated cultures. Assuming a 4-fold increase was maintained for 3-MC-induced MT colonies escaping senescence and form immortal cell lines at a 10 and 4 times higher frequency, respectively, compared to cells derived from normal or altered colonies from carcinogen-treated cultures (3%) or MT colonies from untreated cultures (3%) (i.e., “spontaneous” transmortal colonies). The increased frequency of MT colonies escaping senescence observed with BP treatment is statistically significant (P = 0.0071), as indicated by a one-sided Fisher’s exact test (24), whereas that for 3-MC (P = 0.19) is not, compared to the frequency of MT colonies isolated from untreated cultures. However, a 4-fold increase observed with 3-MC compared to controls probably represents a biologically significant increase. The increased frequency of MT colonies escaping senescence observed with BP treatment is statistically significant (P = 0.0071), as indicated by a one-sided Fisher’s exact test (24), whereas that for 3-MC (P = 0.19) is not, compared to the frequency of MT colonies isolated from untreated cultures. However, a 4-fold increase observed with 3-MC compared to controls probably represents a biologically significant increase. Assuming a 4-fold increase was maintained for 3-MC-induced MT colonies escaping senescence, compared to untreated MT colonies, 100 colonies/treatment type would have to be cloned to establish significance at the P < 0.05 level with a power of 80%. This fact, in combination with the labor intensiveness of cloning studies, highlights some of the difficulties in establishing statistical significance for these data sets. Cells derived from morphologically normal colonies in untreated cultures did not escape senescence. The moderate increase in frequency (3%) of established cell lines generated from normal or altered colonies from BP-treated cultures or MT colonies from untreated cultures, compared to morphologically normal colonies from untreated cultures (0%), is not statistically significant and is of uncertain biological significance at this time.

The colony isolates that senesced, 80% went through 4 to

RESULTS

Twenty to 30 clones of morphologically normal (Fig. 1, A–C), altered (Fig. 1, D–F), and MT phenotypes (Fig. 1, G–I) from untreated, BP-treated, or 3-MC-treated cultures were ring isolated, detached, and examined for their ability to escape senescence following serial passage. The results are shown in Table 1. These data indicate that cells derived from BP- or 3-MC-induced MT colonies escape senescence and form immortal cell lines at a 10 and 4 times higher frequency, respectively, compared to cells derived from normal or altered colonies from carcinogen-treated cultures (3%) or MT colonies from untreated cultures (3%) (i.e., “spontaneous” transmortal colonies). The increased frequency of MT colonies escaping senescence observed with BP treatment is statistically significant (P = 0.0071), as indicated by a one-sided Fisher’s exact test (24), whereas that for 3-MC (P = 0.19) is not, compared to the frequency of MT colonies isolated from untreated cultures. However, a 4-fold increase observed with 3-MC compared to controls probably represents a biologically significant increase. Assuming a 4-fold increase was maintained for 3-MC-induced MT colonies escaping senescence, compared to untreated MT colonies, 100 colonies/treatment type would have to be cloned to establish significance at the P < 0.05 level with a power of 80%. This fact, in combination with the labor intensiveness of cloning studies, highlights some of the difficulties in establishing statistical significance for these data sets. Cells derived from morphologically normal colonies in untreated cultures did not escape senescence. The moderate increase in frequency (3%) of established cell lines generated from normal or altered colonies from BP-treated cultures or MT colonies from untreated cultures, compared to morphologically normal colonies from untreated cultures (0%), is not statistically significant and is of uncertain biological significance at this time.

Of the colony isolates that senesced, 80% went through 4 to
TRANSFORMATION OF SHE CELLS AT pH 6.70

Fig. 1. Representative colony and cell morphology of cloned normal, altered, and transformed colonies. Shown are ×10 (A, D, and G) and ×45 (B, E, and H) photomicrographs of fixed and stained colonies and ×75 (C, F, and I) photomicrographs of colonies in situ of morphologically normal (A, B, and C), altered (D, E, and F), and transformed (G, H, and I) colonies. Scale, 1.0, 0.22, and 0.13 mm for the ×10, ×45, and ×75 magnification photomicrographs, respectively.

10 passages (12 to 30 population doublings) after colony isolation, which represents approximately 26 to 44 population doublings from the single-cell stage. The remaining 20% went through only 1 or 2 passages prior to senescence. Approximately one third of the BP-induced lines escaping senescence exhibited rapid cell proliferation, increased cell culture densities (2–4 × 10⁶ cells/T-25 flask at passage every 3 to 4 days), and MT of the mass culture immediately following colony isolation.
TRANSFORMATION OF SHE CELLS AT pH 6.70

Table 1  Frequency of immortal SHE cell lines generated from colonies of different morphological phenotypes

<table>
<thead>
<tr>
<th>Treatment-Phenotype</th>
<th>Frequencya (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None-normal</td>
<td>0/33 (0)</td>
</tr>
<tr>
<td>None-transformed</td>
<td>1/29 (3.4)</td>
</tr>
<tr>
<td>BP-normal</td>
<td>1/30 (3.3)</td>
</tr>
<tr>
<td>BP-altered</td>
<td>1/28 (3.8)</td>
</tr>
<tr>
<td>BP-transformed</td>
<td>9/30 (30)</td>
</tr>
<tr>
<td>3-MC-transformed</td>
<td>3/21 (14)</td>
</tr>
</tbody>
</table>

* Lines generated/colonies isolated.

with little indication of a population crisis. A representative phenotype of these cell lines is shown in Fig. 2B. Early passage normal SHE cells are shown in Fig. 2A. With a plating density of $1 \times 10^3$ cells/T-25 flask and assuming 100% plating efficiency and 3 days between passages, this represents approximately 5 population doublings and a doubling time of 14.4 h. Approximate doubling times were calculated for these lines based on cell counts during passaging. The majority of BP-induced lines which escaped senescence (approximately two thirds of the cultures) doubled approximately 3 to 4 times in 3 days, with a resultant doubling time of 24 h ($0.6-1.0 \times 10^4$ cells/T-25 flask at passage every 3 to 4 days), and then entered into a visible crisis at passage 4–10. The crisis was characterized by an increased population doubling time and a marked cytoplasmic spreading of approximately 75% of the cells. The crisis period lasted for approximately 3 to 6 passages prior to establishment of the cell lines, which was indicated by increased cell proliferation and increased saturation density at confluence. These cell lines exhibited either a MT phenotype (Fig. 2B) or a more ordered pattern of growth, which formed an organized monolayer upon reaching confluency (Fig. 2C). Occasionally, within the crisis population only one or two viable MT clones were visible on a background of senescent cells. Once these clones appeared, cells of identical morphology overtook the culture within 2 passages. The cell lines derived from these cultures were MT (Fig. 2B) and reached high saturation densities. With additional passages, the cell lines with the transformed morphologies (approximately 80% of the lines) routinely reached saturation densities of $5-8 \times 10^6$ cells/T-25 flask.

Ten of the clones with the MT phenotype from BP-treated cultures were analyzed at passage 1 or 2 after colony isolation for chromosome number. In all of the cultures, greater than 75% of the cells examined had 44 chromosomes, compared to normal early passage SHE cells, which had 70% of the cells with 44 chromosomes. In addition, no increases in structural aberrations were observed in any of these cultures, compared to control. Seven of the immortal cell lines from BP-treated cells and cells from the spontaneous transformant UT-T1 were examined for chromosome number at the same passage(s) tested for tumorigenicity. As shown in Table 2, all of the immortal cell lines examined except BP-T6 and BP-T8 were highly aneuploid, with less than 30% of the cells having 44 chromosomes. This change in chromosome number preceded the acquisition of tumorigenicity in most of the cell lines examined.

Immortal cell lines progressed to the neoplastic phenotype with subsequent culture with a frequency of 92% (Table 2). Fifteen immortal cell lines were established from these studies.
Table 2  Neoplastic potential of SHE cell lines

<table>
<thead>
<tr>
<th>Cell line*</th>
<th>Passage tested</th>
<th>Neoplastic potential</th>
<th>Animals with tumors/survivors*</th>
<th>Time to tumor (weeks)*</th>
<th>Cells with 44 chromosomes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Third passage SHE cells</td>
<td>3</td>
<td>-</td>
<td>0/20 (observed to 2 years)</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>UT-T1*</td>
<td>48, 115</td>
<td>+, +</td>
<td>1/5, 3/3</td>
<td>16, 5</td>
<td>7, NA</td>
</tr>
<tr>
<td>BP-normal</td>
<td>37</td>
<td>+</td>
<td>3/3</td>
<td>6</td>
<td>NA</td>
</tr>
<tr>
<td>BP-altered</td>
<td>40</td>
<td>+</td>
<td>3/3</td>
<td>5</td>
<td>NA</td>
</tr>
<tr>
<td>BP-T1</td>
<td>16, 28, 48, 101</td>
<td>+, +, +</td>
<td>0/5, 0/5, 1/2, 7/9</td>
<td>13, 22, 4, NA</td>
<td></td>
</tr>
<tr>
<td>BP-T2</td>
<td>16, 28, 45</td>
<td>+, +, +</td>
<td>0/5, 0/4, 6/6</td>
<td>6, 24, 23</td>
<td></td>
</tr>
<tr>
<td>BP-T3</td>
<td>16, 28, 45, 115</td>
<td>+, +</td>
<td>0/1, 0/6, 1/2, 9/11</td>
<td>13, 22, 4, NA</td>
<td></td>
</tr>
<tr>
<td>BP-T4</td>
<td>28, 41</td>
<td>+, +</td>
<td>1/1, 4/4</td>
<td>6, 5</td>
<td>NA</td>
</tr>
<tr>
<td>BP-T5</td>
<td>44, 69</td>
<td>-</td>
<td>0/1, 7/9</td>
<td>0, NA</td>
<td></td>
</tr>
<tr>
<td>BP-T6</td>
<td>76, 85, 120</td>
<td>-</td>
<td>0/4, 0/5, 4/5</td>
<td>80, 66, 82</td>
<td></td>
</tr>
<tr>
<td>BP-T7</td>
<td>33, 69, 78</td>
<td>+, +</td>
<td>0/3, 2/3, 7/7</td>
<td>6, 6</td>
<td>NA, 0, NA</td>
</tr>
<tr>
<td>BP-T8</td>
<td>38, 76, 117</td>
<td>+, +</td>
<td>0/3, 0/2, 0/8</td>
<td>90, 72, NA</td>
<td></td>
</tr>
<tr>
<td>3-MC-T1</td>
<td>26, 43</td>
<td>+</td>
<td>0/1, 2/3</td>
<td>6</td>
<td>NA</td>
</tr>
<tr>
<td>3-MC-T2</td>
<td>54</td>
<td>+</td>
<td>2/3</td>
<td>3</td>
<td>NA</td>
</tr>
<tr>
<td>BP-T1-TD1</td>
<td>2</td>
<td>+</td>
<td>1/1</td>
<td>2</td>
<td>NA</td>
</tr>
<tr>
<td>BP-T2-TD1</td>
<td>2</td>
<td>+</td>
<td>6/6</td>
<td>2</td>
<td>NA</td>
</tr>
<tr>
<td>BP-T3-TD1</td>
<td>4</td>
<td>+</td>
<td>2/2</td>
<td>2</td>
<td>NA</td>
</tr>
</tbody>
</table>

* Cell line designations refer to culture treatment-morphology type of the original isolated colony.
* The cell lines were passaged twice weekly with a plating density of 1 x 10^6 cells/T-25 flask. Each passage represents 4 to 6 population doublings.
* Latency period is the duration after cell injection before a palpable (>3 mm) tumor formed.
* Time to tumor (weeks) = the approximate passage shown to be neoplastic (untreated T, and 3MC-T2). Three cell lines also failed to form colonies in agarose when plated at 1 x 10^6 cells/plate in 10 plates. Interestingly, the 3 cell lines which are AIG negative but tumorigenic at the cell number tested in our studies exhibit a monolayer morphology, as shown in Fig. 2C. Where examined, the cloning efficiency in semisolid agarose increased with additional passages in culture for the neoplastic cell lines. Overall, 75% of neoplastic cell lines exhibit the AIG phenotype and all AIG-positive cell lines were neoplastic. As expected, tumor-derived cell lines exhibited a substantial cloning efficiency (40%) in semisolid agarose, whereas early passage SHE cells did not form viable colonies. Early passage SHE cells formed colonies in agarose, as shown in Fig. 3A, at an average frequency of 0.2% but, upon isolation with a Pasteur pipette of 13 colonies from several experiments, viable subcultures could not be established. Based on this characteristic, this colony type from both early passage and established cell lines was not included in AIG cloning efficiency frequencies. In contrast, cell lines capable of inducing tumors upon injection gave rise to viable colonies, as shown in Fig. 3B and occasionally as shown in Fig. 3C. These colonies (16 isolated) formed viable cultures upon isolation and plating. Colonies of the Fig. 3C type were predominant with tumor-derived cell lines. Plating efficiencies of the various cell lines on plastic ranged from 10 to 40% when 100 cells/60-mm plate were seeded.

Twelve lines progressed to the neoplastic phenotype, one has not (BP-T8, passage 117 at the time of this report), and two lines (a BP- and a 3-MC-induced MT-derived cell line) died during an incubator failure. We were unable to obtain viable cell lines for these two cultures from frozen stocks. Typically, cell lines can be established routinely from our frozen stocks. The reason for these exceptions is unknown. All tumors induced by the cell lines were diagnosed as differentiated or undifferentiated fibrosarcomas and all, if let grow long enough, were lethal to the animal. In several cases, fibrosarcomas were found at sites distant from the site of injection. The passage number at which neoplasia was demonstrated, the tumor latency period, and the percentage of animals with tumors varied among the cell lines, as summarized in Table 2. The colony morphology type from which the lines were established did not correlate with progression rate or neoplastic potency. Tumor-derived cell lines were established in culture from the fibrosarcomas induced by several of the cell lines. A representative morphology of the tumor-derived lines is shown in Fig. 2D. All of these were found to be highly neoplastic when injected into newborn hamsters, with a latency period of less than 2 weeks and a frequency of 100% tumor-bearing animals. These results, in total, are consistent with increased neoplastic progression with increased cell proliferation and enrichment/selection of the neoplastic population of cells upon proliferation in vivo.

Based on results reported by Barrett et al. (5) of a 95% correlation between the property of AIG in semisolid agar and the neoplastic phenotype, we screened the various cell lines for their ability to grow in semisolid agar at various passages, as an indication of neoplastic potential. The results from these studies are shown in Table 3.

Five (42%) of the 12 neoplastic cell lines exhibited AIG at the approximate passage shown to be neoplastic (untreated T, BP-altered, BP-T4, BP-T5, and BP-T7), 2 of 12 (17%) were not tested for AIG at the tumorigenic passage but were AIG positive when tested at a later passage (BP-T1 and BP-T2), 2 of 12 (17%) were AIG negative at a tumorigenic passage but subsequently expressed the AIG phenotype (BP-normal and BP-T3), and 3 of 12 (25%) are AIG negative (BP-T6, 3MC-T1, and 3MC-T2). These three cell lines also failed to form colonies in agarose when plated at 1 x 10^6 cells/plate in 10 plates. Interestingly, the 3 cell lines which are AIG negative but tumorigenic at the cell number tested in our studies exhibit a monolayer morphology, as shown in Fig. 2C. Where examined, the cloning efficiency in semisolid agarose increased with additional passages in culture for the neoplastic cell lines. Overall, 75% of neoplastic cell lines exhibit the AIG phenotype and all AIG-positive cell lines were neoplastic. As expected, tumor-derived cell lines exhibited a substantial cloning efficiency (40%) in semisolid agarose, whereas early passage SHE cells did not form viable colonies. Early passage SHE cells formed colonies in agarose, as shown in Fig. 3A, at an average frequency of 0.2% but, upon isolation with a Pasteur pipette of 13 colonies from several experiments, viable subcultures could not be established. Based on this characteristic, this colony type from both early passage and established cell lines was not included in AIG cloning efficiency frequencies. In contrast, cell lines capable of inducing tumors upon injection gave rise to viable colonies, as shown in Fig. 3B and occasionally as shown in Fig. 3C. These colonies (16 isolated) formed viable cultures upon isolation and plating. Colonies of the Fig. 3C type were predominant with tumor-derived cell lines. Plating efficiencies of the various cell lines on plastic ranged from 10 to 40% when 100 cells/60-mm plate were seeded.

DISCUSSION

Due to the reversibility of the MT phenotype under certain chemical exposure and culture conditions (17, 19, 20) and the need to establish whether the MT phenotype induced under pH 6.70 culture of SHE cells is associated with an increased neoplastic potential, compared to cells from morphologically normal colonies, we characterized the neoplastic potential of cells derived from colonies of different morphological phenotypes generated at pH 6.70. The results reported here demonstrate that cells derived from carcinogen-induced MT colonies have a greater probability of progressing to the neoplastic phenotype, compared to carcinogen-exposed morphologically altered and normal colonies and MT colonies from untreated cultures. Morphologically normal colonies from untreated cultures did not establish as immortal cell lines, which is consistent with previous reports (25). The fact that 70 to 80% of MT colonies
from carcinogen-treated cultures and 97% of MT colonies from untreated cultures senesce indicates that the MT phenotype is, however, heterogeneous with regards to its association with neoplastic potential. Consequently, cell line formation is a more accurate predictor of eventual neoplastic potential than is the morphologically transformed phenotype observed under these experimental conditions. However, the MT phenotype in this model should be considered to be indicative of a higher probability of cell line formation, in that the frequency of cell lines which establish from MT colonies (13 of 80) pooled across treatment groups is significantly greater (P = 0.0024), compared to cell lines which establish from morphologically normal colonies (1 of 63).

Morphologically transformed colonies generated by a combination of BP and phorbol ester treatment of SHE cells cultured at pH 7.35 have been reported to establish as cell lines with a frequency of 20–30% (20), frequencies similar to those reported here for carcinogen-treated cultures. Once cell lines are established at pH 6.70, regardless of the colony phenotype from which they were derived, they progress with varying rates from a nonneoplastic to the neoplastic phenotype with high frequency (92%). Similar frequencies have been reported by others for lines generated at pH 7.35 (6, 10). Thus, a comparison of results obtained with MT colonies generated at pH 6.70 versus pH 7.35 indicates that the frequency of progression to immortality and subsequently to neoplasia from the MT phenotype appears similar between the two pH culture conditions. This observation, in conjunction with our previous studies which demonstrate that the frequency of carcinogen-induced MT phenotype can be at least 10- to 20-fold greater at pH 6.70 compared to pH 7.35, depending on the chemical and concentration of carcinogen employed (15), would indicate that acidic culture is influencing an early stage of the neoplastic process, i.e., preimmortalization. Recent studies from our laboratory have indicated that pH 6.70 decreases gap junctional intercellular communication in SHE cells, compared to pH 7.35 (26). Changes in gap junctional intercellular communication have been shown to affect expression of the morphologically transformed phenotype in other transformation models (27–29) and, thus, may be involved in the increased MT frequency observed at pH 6.70, compared to media of higher pH.

The molecular basis of the MT phenotype is not known but is believed to be induced by a broad spectrum of genetic changes, including point and chromosomal mutation, chromosome aberrations, and changes in gene expression (1, 30, 31), and has been reported to follow single hit kinetics (3, 32). Also, as determined by fluctuation analysis, spontaneous escape from senescence in serial cultures is a rare, single, heritable event (25). However, the events responsible for MT and immortality are not necessarily the same, in that not all MT colonies give rise to immortal cell lines and, conversely, not all immortal cell lines have the MT phenotype. Instead, there probably exist several molecular pathways to immortality (33).

Possible evidence for an immortalization event occurring subsequent to MT exists in those cultures (approximately two thirds of the BP cultures studied here) which go through a crisis period and in which a subpopulation of cells, or in some cases clones, within the “crisis” population can be identified which exhibit proliferation. These cells or clones will overtake the population and establish as a cell line. Additional evidence of immortalization events occurring subsequently to MT comes from the cytogenetic examination of cultures at the first or second passage following MT colony isolation. In 10 of 10
cultures examined at the first or second passage following colony isolation, greater than 75% of the cells examined had 44 chromosomes, a distribution similar to that observed in normal early passage SHE cells. In contrast, most of the immortal cell lines which ultimately progress to the neoplastic phenotype are aneuploid, as has been reported previously (34). This change in karyotype can be observed prior to the acquisition of the neoplastic phenotype. This suggests that the "immortalization" event occurred subsequent to the isolation of the MT colony, assuming that immortality and aneuploidy are associated phenomena in this system, an association which generally appears to be the case (4, 8). Alternatively, other cell lines never go through a visible crisis but rather have enhanced proliferation characteristics similar to established cell lines immediately following colony isolation. The latter situation suggests that either (a) the genetic change which led to MT also induced immortality or (b) either two independent events occurred simultaneously or the second event occurred prior to the phenomenon of population crisis. One phenotype which has been reported to correlate well with neoplastic potential in SHE cells is the property of AIG (5, 14). In our experiments, all AIG-positive cell lines were neoplastic and 75% of the neoplastic cell lines were AIG positive. The slightly lower frequency of neoplastic lines capable of AIG observed in our studies, compared to the 95% reported previously by Barrett et al. (5), may be due to differences in the experimental methods employed, i.e., the use of supplemented agars (5) compared to our use of agarose. It is unlikely that the lower correlation in our studies is due to a lack of sensitivity of the AIG assay in that, for those cell lines which were tumorigenic but AIG negative, the same number of cells were examined for both the AIG and tumorigenicity endpoints. The fact that all of the tumorigenic but AIG-negative cell lines had a monolayer, less fibroblast-like morphology suggests that pH may influence the specific cell types which form cell lines. This may in turn result in a spectrum of phenotypes at the various stages of progression, some of which may not have been observed previously in this system at pH 7.35, i.e., tumorigenic but AIG negative.

In summary, we report that cells derived from colonies of the MT phenotype induced by BP or 3-MC at pH 6.70 are associated with an increased probability of progressing to the neoplastic phenotype, compared to cells derived from other colony phenotypes. The acquisition of immortality appears to be essential for the progression to the neoplastic phenotype, as has been reported previously by others (12-14). These results, in combination with our previous results demonstrating an increased frequency of induction of the MT phenotype by carcinogens at pH 6.70 compared to pH 7.35, indicate that pH 6.70 culture of SHE cells is a useful and reproducible model system for the study of neoplastic transformation of cells in culture.

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REFERENCES


Multistage Neoplastic Transformation of Syrian Hamster Embryo Cells Cultured at pH 6.70
