Characterization of a Quantitative Assay for the in Vitro Transformation of Normal Human Diploid Fibroblasts to Anchorage Independence by Chemical Carcinogens

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ABSTRACT

We have characterized an assay for the quantitative measurement of the frequency of conversion to anchorage-independent growth of N-acetoxy-2-acetylaminofluorene-treated normal human diploid fibroblasts. We investigated the effects of the following parameters on the absolute number and on the frequency of anchorage-independent colonies scored: (a) the number of cells seeded per dish; (b) the type of posttreatment medium; (c) the number of population doublings allowed posttreatment prior to seeding in suspension; and (d) the carcinogen dose.

The assay was linear over the range of $1.9 \times 10^3$ to $3.8 \times 10^4$ cells seeded per 60-mm dish for both total colonies scored and the induced frequency of anchorage-independent growth. The medium used posttreatment affected both the frequency and the kinetics of appearance of the anchorage-independent phenotype. The number of population doublings and the number of days allowed posttreatment prior to assaying for anchorage-independent growth potential also influenced the frequency of recovery of this phenotype. Under standardized conditions, the assay yielded a dose-response relationship for transformation to anchorage independence over the concentration range of 0 to 10 $\mu$M N-acetoxy-2-acetylaminofluorene.

INTRODUCTION

NHDF44 have been transformed to Al in vitro by both physical and chemical carcinogens (3, 9, 12, 14, 17, 18). The varied and sometimes complex nature of the protocols used has made it difficult to assess which aspects of the procedures are particularly important. A quantitative study of the induction of the transformation event would allow one to more accurately evaluate the influence of the various culture conditions or protocols, as well as to gain a better understanding of aspects of the development of the Al phenotype. Although a number of markers are traditionally associated with oncogenic transformation in murine cell systems, the conversion to Al has been considered the one most closely related to tumorigenicity (1, 7, 10). Indeed, we have shown elsewhere that N-Ac-AAF-treated cells recovered as colonies from Al growth conditions and grown in monolayer to sufficient numbers for tumorigenicity testing in athymic mice formed tumors at the site of injection in 4 of 10 such cell strains tested (21).

Our transformation protocols were derived from that originally reported by Mili and DiPaolo (12). Our intention was to evaluate the efficacy of the various pre- and posttreatment conditions in this apparently complicated procedure and to better characterize an assay system based on it. We have reported on the role of synchronization, the pretreatment conditions, elsewhere (20). Here, we have characterized the Al colonies scored and investigated the influence of the number of cells seeded per dish on the number of foci scored and the resulting frequency of Al obtained following a 3 $\mu$M dose of N-Ac-AAF. We also evaluated quantitatively the contribution of 3 posttreatment media to the acquisition of Al growth in terms of the absolute frequency and the kinetics of its development in carcinogen-treated and untreated cultures. Under standardized conditions, a dose-response relationship for N-Ac-AAF was then established.

MATERIALS AND METHODS

Media and Serum. Eagle's minimum essential medium (Grand Island Biological Co., Grand Island, N. Y.) was routinely used for all media compositions except as noted. Stock minimum essential medium supplemented with 1 mM sodium pyruvate, nonessential amino acids (0.1 mM each), and 2 mM glutamine (all obtained from M. A. Bioproducts, Walkersville, Md.) was designated GM. Roswell Park Memorial Institute Medium 1629, 2x concentrated, was obtained from Grand Island Biological Co. Low calcium and Dulbecco's modified Eagle's medium lacking arginine and glutamine were obtained from Biologs, Northbrook, Ill. Medium designated 8x 20% is GM supplemented with 0.8 mM each nonessential amino acids (2 ml/100 ml medium of M. A. Bioproducts solution, Catalogue No. 13-114), essential amino acids (2 ml/100 ml medium of M. A. Bioproducts solution, Catalogue No. 13-606), essential vitamins (2 ml/100 ml M. A. Bioproducts solution), and 20% (v/v) FBS (Reheis lot No. S4606; Armour Pharmaceutical, Phoenix, Ariz.). Medium designated 8x 1% is 8x 20% reduced to 1% FBS concentration, and 10% CM is GM containing the essential amino acids and vitamins at the concentrations indicated, supplemented with 10% FBS final v/v concentration. Adjustments to maintain a neutral pH of the various media were made by the addition of either NaHCO₃ (Fisher Scientific Co., Pittsburgh, Pa.) or 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid buffer (Sigma Chemical Co., St. Louis, Mo.). Antibiotics were not used (6). Dialyzed FBS was freshly prepared at not longer than 4-week intervals as described (20).

Cells. Neonatal foreskin samples were obtained from routine circumcisions under sterile conditions and placed in culture by either of 2 methods. In the first method, the tissue was cut into increasingly smaller pieces with scalpels while being transferred through four 100-mm dishes (Falcon Plastics, Oxnard, Calif.), each containing 10 ml GM at 20% FBS. In the fourth dish, the tissue pieces were pressed into grooves cut in the plastic disc with the scalpels. Over a 2- to 3-week period, during which the media was changed every 4 to 5 days, a sufficient number of cells

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4 The abbreviations used are: NHDF, normal human diploid fibroblasts; Al, anchorage independence (anchorage independent); N-Ac-AAF, N-acetoxy-2-acetylaminofluorene; GM, growth medium; low calcium medium, low calcium-containing Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; CM, complete medium; AAS, amino acid starvation; DIR, density-inhibition release; DMSO, dimethyl sulfoxide; PDPT, population doublings posttreatment.

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populated the dish to allow transfer by trypsinization to a 75-sq cm flask (Falcon Plastics). At this time, the medium was removed, the culture was rinsed with Earle's balanced salt solution, and 1 ml of 0.2% trypsin (1:2500; Difco Laboratories, Detroit, Mich.) was added. After 3 to 5 min at 37°, 10 ml of GM at 10% FBS were used to make a single-cell suspension, and a 75-sq cm flask was seeded. Cells were considered to be at 3 to 5 population doublings as judged by the cell number and the amount of tissue initially present.

The second method was that described by Reigner et al. (16). Briefly summarized, minced tissue pieces were prepared as above and then incubated at 37° for 1 hr in GM containing 0.8% collagenase (135 units/mg; Worthington Biochemical Corp., Freehold, N. J.). Postincubation, the preparation was centrifuged and resuspended 2 times in GM at 20% FBS, and a 75-sq cm flask was seeded.

By either method, cells exhibiting both fibroblastic and epithelial morphologies populated the primary cultures. After the first trypsinization, however, essentially only cells with a fibroblastic morphology were observed. The second method was used in later experiments, as larger numbers of cells were obtained in the primary cultures, which thus produced larger numbers of cells at 6 to 10 population doublings postestablishment in culture.

**Transformation Protocols.** Fibroblasts between 6 and 10 population doublings were used for all experiments. An abbreviated description of the AAS and DIR synchrony methods used prior to treatment is presented below. The detailed protocols have been published elsewhere (20).

A preconfluent or freshly confluent (24 hr) 75-sq cm flask of NHDF was subcultured 1:4 into Dulbecco's modified Eagle's medium minus arginine and glutamine at 10% dialyzed FBS for the AAS synchronization. The cultures were refed 24 hr later with GM at 10% FBS. Ten hr postrefeeding, the cultures were treated for 15 min at 37° with the appropriate dose of N-Ac-AAF in DMSO (0.003 to 0.03% final DMSO concentration). The carcinogen was then removed, and the cells were washed with Earle's balanced salt solution and refed with conditioned medium. Fourteen hr later, the treated and untreated (DMSO only) cultures were subcultured 1:2 into the appropriate posttreatment medium (either 8x 20%, 8x 1%, or 10% CM). At each subsequent confluence, cultures were passaged 1:10. Cells were seeded in suspension and scored for AI growth potential at the appropriate number of PDPT as described below.

Cells synchronized by DIR were grown to confluency, refed, and left undisturbed for 3 days. The cells were then subcultured 1:2 and treated as described above at 24 hr postsubculturing. Fourteen hr later, the cells were subcultured 1:2 into one of the 3 posttreatment media and subsequently passaged at 1:10 upon reaching confluence.

**AI Seeding and Scoring.** Confluent cultures at the appropriate PDPT were trypsinized, and a single-cell suspension containing the appropriate number of cells was prepared in 5 ml of low calcium medium at 10% FBS. For routine experiments, 3.6 x 10⁴ cells were seeded per dish above a 2% agar base (Bacto-Agar; Difco), which was prepared from a 1:1 (v/v) mixture of 4% washed agar stock and 2x concentrated Roswell Park Memorial Institute Medium 1629 at 40% FBS, of which 3 ml were poured into each 60-mm dish (Falcon Plastics). The washed agar stock was prepared by vigorously stirring a known weight of agar in triple-distilled water every 1 to 2 hr for 8 hr (4 to 5 times). Each time the agar settled, the liquid and floating material was aspirated, more water was added, and the process was repeated. Following the last aspiration, the 4% stock was prepared on a wt/wt basis and autoclaved. Usually, 10 to 20 dishes were seeded per experimental point; during the 28-day period allowed for colony formation in suspension, 1 to 2 ml of fresh low calcium medium at 10% FBS were usually added on the 14th day to each dish for refedding purposes.

Scoring of the colonies growing in suspension was done 28 days postseeding by visually scanning each dish completely under x4 magnification. Colonies exhibiting the carcinogen-induced morphology (as defined in "Results") were considered positive if they contained not less than 10 to 15 distinct cells. This lower limit was pragmatically determined, as an unequivocal determination of the number of cells in larger colonies proved difficult, while a real limit was required for the assay. It was our experience that approximately 60 to 70% of colonies scored as positive in the treated cultures contained too many cells to accurately count, whereas in the untreated cultures only about 20% of the positively scored colonies were of such proportions. The background frequency was therefore probably elevated by the setting of such a visual cell number limit; on the other hand, such an established limit accurately defined the criteria for scoring positive foci.

**Survival Determinations.** The survival of treated cells was determined by a standard clonogenic potential assay. Cultures synchronized and treated as described were seeded at 1000 cells/100-mm dish (Falcon Plastics) at 14 hr posttreatment in GM at 10% FBS. Two weeks later, the plates were fixed and stained, and the number of colonies containing more than 50 cells was determined. Survival was calculated by correcting for the cloning efficiency in the untreated control dishes.

**RESULTS**

**Identification of Carcinogen-induced AI Colonies.** In both treated and untreated cultures, 2 morphologically distinct types of AI colonies were observed. These were characterized either as colonies exhibiting less dense cell packing with a rough-edged periphery (Fig. 1) or as smooth, dense colonies (Fig. 2). Therefore, it was first necessary to differentiate those colonies which were carcinogen induced and potentially tumorigenic from the others and, if possible, to identify them morphologically.

In a series of preliminary experiments, it was observed that the smooth-edged, densely packed colonies as depicted in Fig. 2 arose very rapidly postseeding and reached sizes within 5 to 6 days that the other type of colonies required several weeks to attain. Under our conditions, these smooth-edged colonies appeared to initiate from occasional small aggregates of cells present upon initial seeding, although they clearly could continue to grow beyond the initial size of the aggregation. Other data from our laboratory have further demonstrated that these apparently normal cell-derived colonies or "spheroids" incorporate [³H]thymidine only in their outer 1 to 2 cell layers, while the interior progressively becomes necrotic (19). Furthermore, we observed that these colonies began to disintegrate and fragment when held in suspension cultures for increasing periods of time over 28 days, presumably as a result of their necrotic interiors.

These densely packed, smooth-edged colonies were morphologically distinguishable from those colonies which exhibited a less dense cell packing and a rough-edged morphology, as seen in Fig. 1. Individual cells could be discerned at the periphery of these foci and often throughout the smaller colonies. These morphologically distinctive colonies progressively increased in size throughout the 28-day period of scoring. They were initially identified as the presumptive carcinogen-induced (positive) foci for 2 reasons: (a) the rough-edged colonies appeared more frequently and grew to larger sizes in the carcinogen-treated cultures than in the untreated controls (although they were present in control cultures), and (b) this morphological phenotype was practically identical to that of 2 human tumor-derived cell lines PC-3 (8) and C-143 (5) in the suspension assay as can be seen in Fig. 3. After incubation with [³H]thymidine, labeling occurred throughout the rough-edged colonies rather than in the outermost cell layers alone.

Subsequent experiments established the tumorigenic potential of some of these colonies upon isolation and inoculation into...
nude mice (21); cells derived from 4 out of 10 rough-edged colonies tested produced tumors in athymic mice. We thus conclude that this carcinogen-induced phenotype could represent an early stage in the development of oncogenic potential of these NHDF. Smooth-edged colonies were consistently found to be negative in their tumorigenic potential. These colonies were therefore not scored or included in the data calculations; only colonies exhibiting the rough-edged morphology were scored as positive. The rough-edged foci observed in the untreated control cultures were scored and calculated as the background of the frequencies indicated.

Influence of Cell Number Seeded. Chart 1 illustrates the relationship in the suspension assay between the cell number seeded per dish, and the number of foci scored (Chart 1, A and C) or the frequency of carcinogen-induced Al growth (Chart 1, B and D). The graphed data represent the induced yield of rough-edged colonies. In each case, the background frequency found in parallel nontreated control cultures has been subtracted; these control values are given in the legend to Chart 1. Generally, the background frequencies were 10- to 20-fold lower than the carcinogen-induced frequencies at a 3 μM dose of N-Ac-AAF. Five replicate plates per point of both treated and untreated cultures were seeded at the indicated cell numbers from 1.9 x 10³ to 3.8 x 10⁴ cells/dish, and 3 replicate plates per point were seeded at 1.9 x 10⁵ and 3.8 x 10⁶ cells/dish.

As can be seen in Chart 1, A and C, the number of foci which developed in dishes seeded with treated cells at densities of 1.9 x 10³ to 3.8 x 10⁴ cells/dish increased in an exponential manner, as did the background yield in parallel nontreated cultures. Reflecting this relationship, the frequency of Al growth was essentially linear over this range, as seen in Chart 1, B and D. Extremes at either end of the dilution spectrum (below 1.9 x 10³ or above 3.8 x 10⁴) deviated from linearity.

At 1.9 or 3.8 x 10⁵ cells/dish, it was impractical to effectively count the number of colonies or delineate those which arose independently from aggregates. On the other hand, at 1.9 or 3.8 x 10⁶ cells/dish, the difference between scoring one and 3 colonies greatly affected the resultant frequency and obviously distorted the general trend. Although it is advantageous to score as many colonies as possible in order to reduce the contribution of differences in colony numbers scored from dish to dish, very high cell density reduced the recovered frequency for technical and perhaps biological reasons. On the basis of these findings, 3.8 x 10⁵ cells were seeded per dish in the following experiments.

Role of Posttreatment Environment. In their experiments, Milo and DiPaolo (12) used 8x 20% medium posttreatment, presumably to maximize the expression of the transformed phenotype. Preliminary experiments (data not shown) indicated that GM at 1% FBS was not selective for the growth of treated cultures or clones, in agreement with Borek (3), Sutherland et al. (18), and McKeehan et al. (11). We hypothesize that if the amino acids in the 8x 20% medium were responsible in some manner for the apparently enhanced expression of the transformed phenotype and not the serum concentrations, these effects could be identified by reducing the serum concentration from 20% (8x 20%) to 1% (8x 1%). Therefore, we compared the effects of 8x 20%, 8x 1%, and 10% CM (as a control) media posttreatment on the recovery of Al colonies. Concomitantly, we examined the kinetics of the cells’ ability to grow under Al conditions as a function of the number of PDPT following a 3 μM dose of N-Ac-AAF, as well as in untreated control cultures.

As seen in Chart 2, the 8x 20% and 8x 1% media posttreatment conferred a higher overall frequency of Al colonies over the investigated range of population doublings and days posttreatment, as well as augmenting the frequency at earlier times, compared to the 10% CM. The early effects of the method of

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Chart 1. Effect of the number of cells seeded on: the average number of rough-edged foci/dish (A and C) and the resultant frequency of transformation to Al growth (B and D). The latter is defined as the number of rough-edged foci divided by the number of cells seeded per dish. A and B used AAS synchrony; C and D used DIR synchrony. The 3 posttreatment media are 8x 20% (A), 8x 1% (C), and 10% CM (D). The average frequencies of rough-edged colonies in untreated control dishes were: 8x 20% = 8.2 x 10⁻⁶; 8x 1% = 6.0 x 10⁻⁶; 10% cm = 6.0 x 10⁻⁶; D: 8x 20% = 1 x 10⁻⁶; 8x 1% = 6 x 10⁻⁶; 10% cm = 4 x 10⁻⁶. The results plotted represent the average values obtained for each cell dilution from 4 separate experiments utilizing cells from 4 different PDPT.
The control data in Chart 2, A and B, indicate that the 8× 20% and 8× 1% media generally resulted in an earlier expression of the background frequency of Al growth as compared to the 10% CM medium through 5 PDPT. Thereafter, however, the controls in all cases reached a plateau around $1 \times 10^{-4}$, which was at least 5- to 10-fold lower than the frequencies in the corresponding treated cultures.

**Dose-Response Characteristics.** Chart 3 shows the dose-response relationships obtained for transformation to Al by N-Ac-AAF. There was a generally linear increase in the frequency with increasing doses. The percentage of survival relative to controls for these doses is tabulated in Table 1. The cells in the 8× 20% and 10% CM experiments were scored at 14 PDPT, and those in the 8× 1% were scored at 11–13 PDPT.

**DISCUSSION**

We have presented evidence that Al can serve as a quantitative marker for an early phenotypic change in carcinogen-treated human fibroblasts. When cells isolated from Al colonies were synchrony on expression have been reported elsewhere (20).

The greatest enhancement of expression was produced by the 8× 20% medium which yielded a 4- to 16-fold increase in the frequency of Al colonies above background through 14 PDPT. A kinetically similar 2- to 9-fold enhancement was produced by the 8× 1% medium. In both cases, the enhancement above background was somewhat higher for the AAS synchronized cultures, particularly at earlier times posttreatment. At 24 PDPT, the frequencies for the 8× 20% conditions were still about twice that of the 10% CM cells, when corrected for background.

It was evident from these data that the number of days in culture as well as the number of PDPT might be influencing the development of the Al phenotype. Chart 2, C and D, represents the data from Chart 2, A and B, corrected for background levels and redrawn to express the influence of the number of days posttreatment. In general, the kinetics of expression as measured by time in culture or by PDPT were similar. However, one interesting difference emerged. A comparison of the last points for the 8× 1% media in the AAS (13 PDPT; 85 days) (Chart 2, A and C) and DIR (11 PDPT; 115 days) (Chart 2, B and D) groups reveals that, even though the DIR cells had undergone fewer PDPT, they had been in culture for about a month longer and displayed a nearly 2-fold higher frequency of Al growth. This finding suggests that the number of days posttreatment, as well as the number of cell divisions, may influence the acquisition of Al.

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**DISCUSSION**

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### Table 1

<table>
<thead>
<tr>
<th>Method of synchrony</th>
<th>% of survival at following N-Ac-AAF dose</th>
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<tr>
<td></td>
<td>1 μM</td>
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<tr>
<td>AAS</td>
<td>94.0 ± 5.0</td>
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<td>DIR</td>
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* Mean ± S.D. of 3 separate experiments for each condition.
grown in sufficient numbers, 4 out of 10 isolates were tumorigenic in nude mice; these latter results have been reported in detail elsewhere (21). This marker may thus represent an early stage in the development of malignant transformation in human fibroblasts. In agreement with Silinskas et al. (17) and Sutherland et al. (18), we have found Al to appear at early times posttreatment in NHDF, in contrast to its traditionally late appearance in rodent cell transformation systems (1, 2, 7, 10). We have also shown that the Al phenotype appeared in untreated controls at frequencies as high as 2 x 10^{-8}. Further characterization of NHDF transformation in vitro may provide insights regarding these differences between human cells and the rodent cell systems currently in widespread use for the study of in vitro transformation. In particular, it remains to be precisely determined what proportion of the carcinogen-induced Al colonies eventually develop tumorigenic potential and the various steps that may be involved in the conversion of an Al cell to the fully transformed phenotype.

We chose to suspend cells in liquid medium over an agar base rather than plating in semisolid medium for several reasons. In our experience with soft agar, several refeddings were necessary due to dehydration over the 28-day period until growth was scored. If only medium was reintroduced, the semisolid layer sometimes broke up into islands of agar floating in medium. Thus, some cells and colonies were in soft agar, and others were free in the medium. The alternative choice, to refeed with the agar and medium mix, increased the final agar concentration since only water was presumably lost, thereby potentiating osmolarity problems. Carlsson (4) studied the effects of osmolarity on the growth rate and colony size of human tumor cells in agarose cultures. He concluded that semisolid conditions posed serious biological and nutritional problems for the cells that resulted in the "osmolar" and "nonosmolar inhibition" of growth at 2 weeks. In contrast, various colonies forming in liquid suspension above an agar base plate grew with similar kinetics and reached similar maximum sizes.

Based on the number of cells treated and the subculturing procedures, we concluded that the transformation frequency scored in our soft agar conditions (about 1 x 10^{-3}) was not reflecting the number of initiated cells in the treated population (data not shown). For example, about 5 x 10^5 cells were treated in an average experiment. Assuming equivalent exponential growth rates for potentially transformed and normal cells, the presumed number of cells with AI growth potential at the first confluency posttreatment for a 3 µM dose resulting in a 1 x 10^{-3} transformation frequency would be 24. The number of these cells that will be present in the subsequent 1:10 split, of which only 2 flasks were routinely carried, would thus be about 2.4 cells. Clearly, at transformation frequencies on the order of 10^{-3}, one is very fortunate not to lose the transformation event during subculturing. Therefore, we felt our semisolid growth conditions were less than optimal, and we began our investigation of alternatives.

In agreement with Pehl and Stanbridge (15), we have found that untreated NHDF are able to form AI colonies. Our experience and data (Figs. 1 to 3) indicate, however, that there are 2 colony morphologies which develop under Al conditions that must be differentiated in order to identify the carcinogen-induced frequency. Under our conditions, the smooth, densely packed colonies (Fig. 2) appeared to arise from small groups or aggregates of cells present at the time of seeding. Unlike the rough-edged colonies, their frequency was similar in both treated and untreated cultures. Other data from this laboratory have also shown the ability of trypsinsized NHDF to form such densely packed, smooth-edged colonies by agitation of the cultures (19). Sutherland et al. (18) have also found it necessary to identify such aggregates following seeding, and have corrected for these foci in their data.

It remains to be clearly established, however, whether the rough-edged AI colonies found in the controls represent clonal growth from single variant cells present in low passage NHDF, in contrast to the smooth-edged colonies which appear to arise from aggregates of normal cells. Our data, and that of others (15, 17, 18), indicate that this variant population exists, but varying Al conditions apparently affect its frequency of expression (Chart 1). Furthermore, in contradistinction to smooth-edged colonies, the frequency of rough-edged colonies increased with increasing dose in carcinogen-treated cultures (Chart 3). It is clear, however, that colonies or aggregates of apparently normal cells must be differentiated from the less dense, rough-periphery morphology we have identified here as being the carcinogen-induced phenotype in order to properly evaluate its induced frequency. It should also be noted that the rough-edged phenotype illustrated in Fig. 1 in suspension is the same as that observed under semisolid conditions in our hands. The pertinent difference is the frequency of its appearance in suspension, which gives the quantitative relationships reported here.

For the selection of Al cells, the results in Chart 1 indicate that the useful range of the assay is between 1.9 x 10^3 and 3.8 x 10^3 cells seeded in suspension per 60-mm dish. Within this range, an increase in cells per dish was reflected in more foci scored per dish, which resulted in a stable Al frequency. This relationship must be established if the Al frequency is to have quantitative relevance. It should be noted that the Al frequency was defined as the number of foci scored divided by the number of cells seeded in suspension. In a system such as this, which requires multiple subculturings and necessitates discarding cells, it is possible that the frequency scored following a number of subculturings posttreatment is not that which would be found if all cells and progeny were saved and scored.

Furthermore, one is assuming equivalent growth kinetics of potentially transformed and nontransformed cells. The apparent stability of the carcinogen-induced phenotype in the 8x 20% cultures between 14 and 24 PDPT (54 to 118 days) suggests that an early acquisition of this phenotype is not a competitive disadvantage, at least over these time periods. It has been suggested that if the treated cells are not called upon to express their potential for Al growth, this carcinogen-induced event will be diluted out (17). Whether or not this phenomenon will occur above 24 PDPT in these conditions remains to be determined.

It has been our experience, and that of others, that the absolute Al frequency can vary from one series of experiments to another (17, 18). We attribute this to varying culture conditions, in particular the FBS. Therefore, the comparative value of multiple experiments increases with the similarity of the conditions. In this series, we used the same fibroblast strain within the same lot of serum (Reheis Lot S4606; Armour).

The posttreatment media altered the appearance kinetics of the Al phenotype, as well as the overall frequency, as seen in Charts 1 and 2. The influence on these kinetics of the number

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* R. J. Zimmerman and J. B. Little, unpublished observations.
of PDPT versus the number of days posttreatment remains to be more clearly defined. The observed contribution of the high concentration of 0.8 mm nonessential amino acids present in the 8x 20% and 8x 1% media was contrary to our expectations. The mechanisms behind this have been made by McKeehan et al. (11), however, that the multiplication of normal human lung fibroblasts was inhibited by an excessive concentration of amino acids, whereas the SV40-transformed counterparts displayed an optimal rate of multiplication over a wider range of concentrations of isoleucine, leucine, methionine, phenylalanine, threonine, tyrosine, and valine. It has also been suggested that amino acids are the limiting growth factor for confluent, transformed cells in vitro and not serum (13).

The preliminary dose-response data shown in Chart 3 must be further investigated. There is no a priori reason to assume that maximum expression of the AI phenotype occurs at the same time for different doses of N-Ac-AAF. As is indicated in Chart 2 as well, the posttreatment media also influenced the acquisition of the AI phenotype. Furthermore, in an effort to hold some parameters constant, we did not study the effect of various media on the growth of cells in suspension (under AI conditions). Based on the reports of similar assays, however, the media composition would not be predicted to be either prohibitive or exclusive. It is far more likely that the serum factors are the limiting controlling elements.

Questions such as those posed above should now be studied in a quantitative rather than a qualitative manner, since clearly there exists a wide range of observations that need to be more fully investigated and compared among the various protocols used for the transformation of human fibroblasts to AI growth in vitro.

REFERENCES

Fig. 1. Colonies representative of the rough-edged morphology identified as the carcinogen-induced (positive) Al phenotype in NHDF at 28 days after seeding in suspension culture. Cells were treated with 3 μM N-Ac-AAF and seeded at 14 PDFT. Phase-contrast, x 40.

Fig. 2. Morphological phenotype characteristic of the smooth-edged colonies apparently derived from normal cells at 28 days after seeding in suspension culture. Phase-contrast, x 40.

Fig. 3. Morphological phenotype of human tumor-derived cells (PC-3) in suspension culture at 21 days after seeding. Phase-contrast, x 40.
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