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# Emergence of Neoplastic Transformants Spontaneously or After Exposure to N-Methyl-N'-Nitro-N-Nitrosoguanidine in Populations of Rat Liver Epithelial Cells Cultured Under Selective and Nonselective Conditions

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***Many studies have shown that cultured rat liver epithelial cells can be neoplastically transformed by repeated or long-continued exposure to chemical carcinogens. These cells also may transform spontaneously in the absence of carcinogen treatment after long-term, continuous passage in culture or after chronic maintenance in a confluent state*** in vitro. ***In this study, we have compared the times of emergence and rates of accumulation of transformed cells in populations of rat hepatic epithelial cells exposed either to a single dose of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, 3 μg/ml culture medium for 30 minutes) or to acetone vehicle alone (3 μl/ml culture medium for 30 minutes). Transformation was compared in cell populations that were passaged continuously once a week as they attained a confluent density (nonselective growth conditions), or that were maintained at a confluent density for 3 weeks between passages once a month (selective growth conditions). Emergence of both spontaneous transformants and transformants induced by MNNG was facilitated by selective growth conditions, as compared with nonselective growth conditions. Transformants were detected both in cultures exposed to MNNG and in cultures exposed only to acetone (solvent controls), but they always emerged earlier in cultures exposed to MNNG (nine population doublings earlier when grown under selective growth conditions and 22 population doublings earlier when grown under nonselective growth conditions). Once transformants were detected, they replaced the nontransformed population more quickly under selective than under nonselective conditions of culture. Cells possessing the ability to grow in soft agar and to produce tumors in syngeneic rats were detected (at about 12 population doublings after treatment) under selective conditions much earlier than under nonselective growth conditions (at about 90 population doublings after treatment). Among MNNG-treated cultures, the fraction of aneuploid cells in the population was correlated significantly with tumorigenicity. In contrast, among acetone-treated control populations, aneuploidy and tumorigenicity were not correlated; populations of aneuploid acetone-treated cells often were not tumorigenic. These observations suggest that MNNG treatment produced a specific type of aneuploidy that was associated with tumorigenicity. (Am J Pathol 1989, 135:63–71)***

Many studies have shown that cultured diploid rat liver epithelial cells can be neoplastically transformed (produce tumors after transplantation into appropriate test animals) by exposure to various chemical carcinogens.[1–6] Transformation of hepatic epithelial cells by exposure to chemical carcinogens frequently has required long-term continuous or episodic exposure to a carcinogen, often combined with extended postexposure culture in the absence of the carcinogen, before tumorigenicity was expressed. Detailed studies by Schaeffer's group indicated that diploid rat liver epithelial cells that were continuously passaged when cell populations reached confluence

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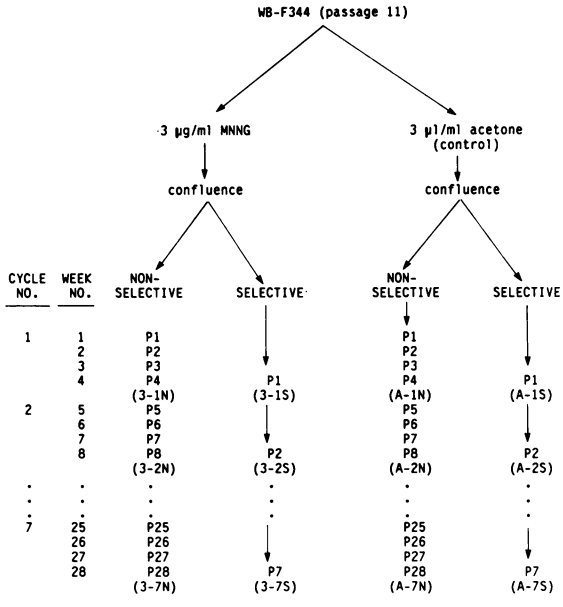


**Figure 1.** *Flow chart for treatment and passaging of WB-F344 rat liver epithelial cells. P, passages/subcultures at the end of a given week; 3-1, 2, . . . 7N, MNNG-treated, nonselective conditions at the end of cycle 1, 2, 3, and so on; 3-1, 2, . . . 7S, MNNG-treated, selective conditions at the end of cycle 1, 2, 3, and so on; A-1, 2 . . . 7N, acetone-treated control, nonselective conditions at the end of cycle 1, 2, 3, and so on; A-1, 2, . . . 7S, acetone-treated control, selective conditions at the end of cycle 1, 2, 3, and so on.*

spontaneously expressed transformation with high probability after they had undergone approximately 100 population doublings in culture, although spontaneous transformation was rare before this time.[7] Borek reported that hepatic epithelial cells also transform spontaneously with high frequency after chronic maintenance of cultures at confluence without feeding or transfer.[8,9] Although expression of spontaneous neoplastic transformation appears to occur much more rapidly in populations of liver epithelial cells maintained at confluence without feeding, studies apparently have not been reported on the emergence of transformants in carcinogen-treated populations of hepatic epithelial cells that are held in a confluent state.

In this report, we have compared the timing of expression of neoplastic transformation, both spontaneous and after a single exposure to MNNG, in populations of rat liver epithelial cells that were either passaged continuously as they reached confluence or subjected to temporary periods at confluence between passages. Except for differences in the frequency of passaging, all groups were maintained under as nearly identical conditions as possible. These studies show that subjection of cultured populations of hepatic epithelial cells to periods of confluence greatly accelerates the time of emergence and the rapidity of overgrowth of transformed subpopulations in both MNNG-exposed and control cultures. Emergence of tumorigenic variants is closely associated with acquisition of aneuploidy in MNNG-treated cell populations, but not in acetone-treated control populations. Although the acetone-treated populations developed large fractions of aneuploid cells, tumorigenicity was not closely related to the level of aneuploidy, suggesting that MNNG treatment produces a specific, tumorigenic type of aneuploidy in these cells.

## Materials and Methods

### Cell Lines and Tissue Culture

The diploid epithelial cell line, WB-F344, which was isolated from the liver of an adult, male Fisher-344 rat as previously described,[10] has phenotypic properties similar to those of proliferated bile ductular ("oval") cells. All cells were routinely cultured in Richter's improved minimal essential medium with zinc option, insulin (4.0 mg/L), and glutamine (Irvine Scientific, Santa Ana, CA), and supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 2.6 mM sodium bicarbonate, gentamicin (0.04 mg/ml), and 10% fetal bovine serum (HyClone Laboratories, Logan, UT). Cells were incubated in a humidified incubator at 37 C and 5% $CO_2$ in air. Plating densities were always $4 \times 10^5$ cells in 10 ml of medium per 100 mm tissue culture dish. Cells were passaged using a 0.25% trypsin solution in $Ca^{2+}$-, $Mg^{2+}$-free Hanks' Balanced Salt Solution (GIBCO Laboratories, Grand Island, NY).

### Treatment Protocol

Exponentially growing WB-F344 cells at passage 11 (approximately 28 population doublings since establishment) were treated once with either 3 µl of acetone/ml culture medium (solvent controls), or 3 µg of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) dissolved in 3 µl acetone/ml culture medium. After growth to confluence, each treatment group of cells was subcultured according to two different conditions of cultivation termed nonselective and selective (Figure 1). Under nonselective conditions, cultures were passaged once per week. In contrast, cultures grown under selective conditions were passaged only once every 4 weeks, with weekly feedings of fresh medium (10 ml/100 mm dish) to prevent depletion of nutrients and reduced pH. Thus, the selectively grown cells were held at confluence for 3 weeks between passages. At any point during the experiment, each of the resultant treatment groups consisted of duplicate 100 mm dishes.

Each 4-week period is referred to as one cycle of growth for both selective and nonselective conditions. Plating efficiencies were determined for cells from each treatment group at the end of each cycle. Either 500 or

1000 cells were plated onto each of five replicate 100 mm dishes, the colonies were allowed to grow to macroscopic size, and were then fixed, stained, and counted. During each cycle, nonselective cultures were passaged four times, whereas selective cultures were passaged only once. Population doublings, therefore, differed considerably between selective and nonselective groups. At the end of each cycle, aliquots of cells from each treatment group were assayed for anchorage-independent growth in soft agar, chromosome counts were made, and the population distribution of DNA content per cell was evaluated by flow cytometry. Karyotypes were made on selected populations. Cells were assayed for tumorigenicity at the end of each cycle, beginning with cycle 3.

### *Anchorage-Independent Growth Assay*

This assay was done as previously described.[11] In general, either 12,500 or 50,000 cells were seeded in 2 ml of 0.3% agar medium onto a 0.5% agar base layer in 60 mm tissue culture dishes. Approximately 24 hours after plating, 1 ml of medium with or without 50 ng/ml epidermal growth factor (EGF) was added gently onto the agar gel. Cultures were incubated for 4 weeks with weekly additions of 0.5 ml of fresh medium (with or without 10 ng/ml EGF) per plate. Colonies were then stained with nitro blue tetrazolium and the colonies larger than 0.1 mm in diameter were counted.

### *Tumorigenicity*

Cells were harvested by trypsinization from plates that had reached confluence. Before cells were injected into animals, they were incubated overnight in serum-free medium. For tumorigenicity assays, $1 \times 10^6$ cells in 0.1 ml of $Ca^{2+}$-, $Mg^{2+}$-free Hanks' Balanced Salt Solution were injected into either the peritoneal cavity or the dorsal subcutaneous tissue of day-old Fisher-344 rats. Animals were sacrificed when tumors became evident or at the end of 1 year. All tumors were confirmed by histologic examination. Cell lines were established from tumors as previously described,[12] except that tissue digestion with collagenase and hyaluronidase was omitted and medium with 10% fetal bovine serum was used.

### *Flow Cytometry*

The DNA content of cells in the populations of each treatment group was quantified by flow microfluorimetry using an Ortho ICP 22A flow cytometer (Ortho Instruments, Westwood, MA). At the end of each cycle, cells from each treatment group were trypsinized to yield a single cell suspension, washed in an anticlumping buffer (0.1 M NaCl, 11.0 mM glucose, 5.6 mM $Na_2HPO_4$, 5.4 mM KCl, 0.4 mM CA ($NO_3$), 0.4 mM $MgSO_4$),[13] passed through a 16-gauge syringe, and fixed by dropwise addition of cold 95% ethanol to a final concentration of 70%. Immediately before analysis, fixed cells were resuspended in the anticlumping buffer at a concentration of $1 \times 10^6$ cells/ml, incubated with RNAase for 30 minutes, and stained with propidium iodide (20 $\mu$g/ml cell suspension).

### *Chromosome Counts and Karyotypes*

Chromosome preparations were made from exponentially growing cells. Ethidium bromide was added 2 hours before harvest to a final concentration of 7 $\mu$g/ml to decrease chromosome condensation. Colcemid was added 0.5 hours before harvest to a final concentration of 50 ng/ml to disrupt spindle formation and increase chromosome spreading. Cells were harvested by trypsinization, hypotonically shocked in 75 mM KCl for 10 minutes at 37 C, and fixed in 3 methanol:1 acetic acid. Cell suspensions were dropped onto cold, wet glass microscope slides, and dried in an oven at 70 C for 20 minutes.

For chromosome counts, at least 50 mitotic figures were counted for each treatment group at the end of each cycle. For Q-banding, slides were stained for 10 minutes in 0.5% (w/v) quinacrine dihydrochloride, washed for 5 minutes in deionized water, and mounted wet for examination and photography.

## *Results*

Table 1 summarizes the data on colony forming efficiency in soft agar (with and without added EGF), tumorigenicity after transplantation of cells into newborn syngeneic rats, and mean latent period for tumor detection during each cycle of treatment. Neoplastic transformation occurred in all groups, both in MNNG-treated and in acetone-treated controls, and under both selective and nonselective conditions of growth. Transformation was detected earlier in cell populations cultured under selective conditions than in cultures that were cultured under nonselective conditions. MNNG-treated cells transformed earlier than did the acetone-treated control cells under both selective and nonselective conditions. Figure 2 shows the results of regression analysis for the cumulative fraction of animals with tumors as a function of cumulative population doublings for each of the four treatment groups. The slopes of the regression lines for the two populations grown under selective conditions (MNNG-treated and controls) did not differ significantly from each other, but their elevations (ie, y-intercepts) were significantly different ($P < 0.001$). These regressions suggest that, for populations grown

Table 1. *Colony-Forming Efficiency in Soft Agar, Tumorigenicity, and Tumor Latency of the Treatment Groups at the End of Each Cycle*

| | MMNG treated | | | | Controls | | | |
|---|---|---|---|---|---|---|---|---|
| | Mean CFE (%) | | | TL | Mean CFE (%) | | | TL |
| Cycle no | +EGF | −EGF | TUM | (wks) | +EGF | −EGF | TUM | (wks) |
| Nonselective conditions | | | | | | | | |
| 1 | 0 | 0 | ND | | 0 | 0 | ND | |
| 2 | 0 | 0 | ND | | 0 | 0 | ND | |
| 3 | 0.003 | 0 | 0/8* (0%) | | 0 | 0 | 0/10 (0%) | |
| 4 | 0.001 | 0 | 0/8 (0%) | | 0 | 0 | 0/5 (0%) | |
| 5 | 0.002 | 0 | 1/7 (14%) | 52.0 | 0 | 0.003 | 0/5 (0%) | |
| 6 | 0.480 | 0.039 | 9/13 (69%) | 27.4 | 0.079 | 0.001 | 6/16 (38%) | 34.3 |
| 7 | 1.282 | 0.632 | 7/7 (100%) | 10.9 | 2.962 | 0.264 | 3/9 (33%) | 15.7 |
| Selective conditions | | | | | | | | |
| 1 | 0 | 0 | ND | | 0 | 0 | ND | |
| 2 | 0 | 0 | ND | | 0 | 0 | ND | |
| 3 | 0.001 | 0 | 1/6 (17%) | 51.0 | 0 | 0 | 0/8 (0%) | |
| 4 | 0.003 | 0 | 2/10 (20%) | 51.0 | 0 | 0 | 1/6 (17%) | 51.0 |
| 5 | 0.083 | 0.020 | 5/10 (50%) | 34.2 | 0.001 | 0.001 | 1/5 (20%) | 27.0 |
| 6 | 0.506 | 0.082 | 5/6 (83%) | 33.6 | 0.005 | 0.002 | 6/8 (75%) | 7.5 |
| 7 | 0.546 | 0.160 | 9/9 (100%) | 10.8 | 0.512 | 0.122 | 7/11 (64%) | 15.7 |

* Number of rats with tumors/number of rats inoculated with cells.

CFE, colony forming efficiency; TUM, tumorigenicity (fraction represents number of animals with tumors over number of animals tested); TL, mean tumor latency; EGF: epidermal growth factor; ND, not done.

under selective conditions, tumorigenicity arises in the MNNG-treated populations nine population doublings earlier than in the acetone-treated control populations. Based on the limited number of data points, neither the slopes nor the y-intercepts of the regression lines for the two populations grown under nonselective conditions (MNNG-treated and controls) differed significantly ($P < 0.50$), indicating that no significant differences existed in either the rate of increase of tumorigenicity or its first occurrence for populations grown under nonselective conditions. However, the slopes of the regression lines for each of the populations grown under selective conditions were significantly ($P < 0.05$) steeper than are the slopes for the regression lines for either of the populations grown under nonselective conditions, indicating that tumorigenicity increases significantly faster in the populations grown under selective conditions. As indicated by the cumulative fraction of animals that developed tumors after inoculation with cells, selective conditions potentiated the accumulation of tumorigenic cells. Only the animals inoculated with the MNNG-treated cells reached 100% levels of tumorigenicity by the end of the experiment.

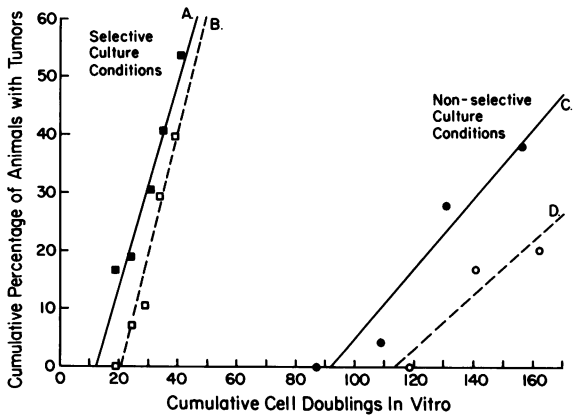


Figure 2. *Cumulative tumorigenicity of the cell populations of each treatment group as a function of cumulative number of population doublings at the end of cycles 3 to 7. Solid lines and filled symbols are for MNNG-treated groups, and dashed lines and open symbols are for control groups. The regression (Model I) equations, coefficient of determination ($r^2$), and significance of slopes are A: $y = -22.3 + 1.83x$, $r^2 = 96\%$, $P < 0.005$; B: $y = -44.4 + 2.12x$, $r^2 = 94\%$, $P < 0.01$; C: $y = -55.5 + 0.605x$, $r^2 = 94\%$, $P < 0.05$; D: $y = -52.4 + 0.460x$, $r^2 = 87\%$, $P > 0.20$. Tests for equality of slopes and elevations of regression lines were performed using Student's t-test, with two fewer degrees of freedom than usual because of the cumulative nature of both axes.*

Table 2 shows the results of correlation analyses of tumorigenicity *vs.* colony-forming efficiencies in soft agar, with or without EGF. For all treatment groups combined, anchorage-independent growth is significantly correlated with tumorigenicity, with a higher correlation coefficient for soft agar growth without EGF than for growth in the presence of EGF. Some of the relevant components of the overall correlation are shown by examining the correlation coefficients of the data when segregated into different groupings. Subdividing the data on colony-forming efficiencies into populations grown under either nonselective or selective conditions or subdividing into MNNG-treated groups increases the correlation coefficients between tumorigenicity and ability to grow in soft agar. In contrast, in control groups there is no correlation between tumorigenicity and soft agar growth with or without EGF. The same lack of significant correlation in acetone-treated control populations is also evident when each of the four

Table 2. *Correlation Analyses of Tumorigenicity with Growth in Soft Agar**

| Group analyzed | N | +EGF | | −EGF | |
|---|---|---|---|---|---|
| | | *r* | *P* value | *r* | *P* value |
| All | 20 | 0.630 | <0.005 | 0.739 | <0.001 |
| Nonselective only | 10 | 0.690 | <0.05 | 0.819 | <0.005 |
| Selective only | 10 | 0.818 | <0.005 | 0.828 | <0.005 |
| MNNG treated only | 10 | 0.942 | <0.001 | 0.864 | <0.005 |
| Controls only | 10 | 0.379 | NS | 0.435 | NS |
| Nonselective, MNNG treated | 5 | 0.986 | <0.002 | 0.907 | <0.05 |
| Nonselective, controls | 5 | 0.678 | NS | 0.560 | NS |
| Selective, MNNG treated | 5 | 0.983 | <0.005 | 0.999 | <0.001 |
| Selective, controls | 5 | 0.552 | NS | 0.556 | NS |

* Percentage data were transformed using Bartlett's Correction to the inverse sine transformation.[14] A two-tailed test was used at the 5% level of significance.
NS, not significant.

treatment groups is analyzed separately. The lack of a correlation between anchorage-independent growth (with or without EGF) and tumorigenicity in all acetone-treated control populations and the presence of this correlation in all of the MNNG-treated populations suggest that spontaneously occurring and MNNG-induced transformants differ with respect to a functional relationship between these two phenotypes.

Figure 3 shows the results of a regression analysis of tumor latency as a function of population tumorigenicity. Tumorigenicity data were expressed as the proportion of animals with tumors from the number of animals tested. These data, as well as all other percentage and proportional data, were transformed using Barlett's Correction to the inverse sine transformation[14] to satisfy assumptions of homogeneity of variances for the statistical tests used. A Model II regression method was used because both the independent and dependent variables were random variables that were not under experimental control.[15] Figure 3A shows that, as the tumorigenicity of the cells in the MNNG-treated populations increased, there was a highly significant decrease in the latency time of the resultant tumors. Figure 3B shows that there was not a significant regression of tumor latency on tumorigenicity for the acetone-treated control populations (ie, 95% confidence limits included a slope of zero).

Figure 4 shows a representative series of flow cytographs that depict the changes in distribution of DNA content/cell in the whole populations of each of the four treatment groups at the ends of cycles 1 through 7. In the MNNG-treated groups (see Figures 4A and 4C), the emergence of aneuploid (subdiploid) cells occurred by the end of cycle 1 or 2, followed by the polyploidization of subdiploid and diploid cells to form subtetraploid and tetraploid subpopulations. With increasing passage of each treatment group, these polyploidized cells became progressively more common, resulting in populations that were predominately subtetraploid by the end of the seventh cycle. However, both acetone-treated control groups showed much more random patterns of ploidy changes in the population with respect to both cumulative population doublings and tumorigenicity. Moreover, there were many occurences of nontumorigenic populations with large fractions of aneuploid cells, especially with large fractions of subtetraploids.

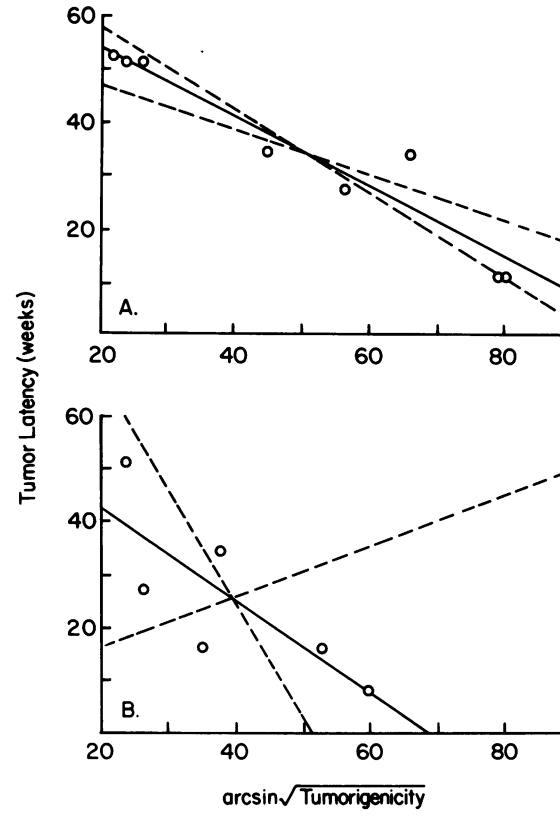


Figure 3. *Model II regression analyses for tumor latency as a function of population tumorigenicity. Dashed lines indicate 95% confidence limits for the slope of the regression line (solid line). Bartlett's Correction[14] of the inverse sine transformation of the tumorigenicity data and Bartlett's three-group method[15] for Model II regression analysis were used.* **A:** *Regression line for MNNG-treated populations: $y = 66.1 - 0.647x$, $r^2 = 93\%$; 95% confidence limits on slope: −0.781 to −0.423.* **B:** *Regression line for acetone-treated control populations: $y = 59.9 - 0.880x$; $r^2 = 61\%$; 95% confidence limits on slope: −2.17 to 0.472.*

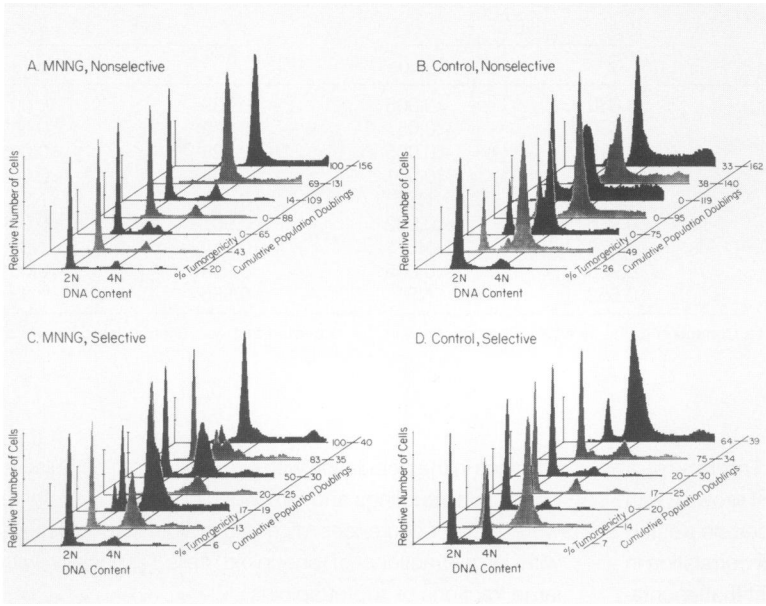


**Figure 4.** *Histograms of DNA content/cell determined by flow cytometry for the cell populations of the four different treatment groups examined at the end of each of the seven cycles of the experiment. Tumorigenicities and cumulative population doublings are also indicated for each population. Cells were harvested from confluent cultures only.*

## *Chromosome Counts and Karyotypes*

Chromosome counts of at least 50 mitotic figures from each of the four treatment groups at the end of each of the seven cycles yielded population distributions of cell ploidy that corroborated the findings of flow cytometry of DNA. The percentage of aneuploid cells in each population was estimated by determining the percentage of mitotic figures with a chromosome count outside the normal rat euploid numbers ± 5%, ie, 2N equals 42 ± 2 and 4N equals 84 ± 4. Figure 5 shows the results of a Model II regression analysis of population tumorigenicity as a function of the percentage of aneuploid cells in each population. For MNNG-treated populations, Figure 5A shows that, as the percentage of aneuploid cells in the population increased, there was a significant ($P < 0.05$) increase in population tumorigenicity. In contrast, for acetone-treated control populations, Figure 5B shows that there was no significant dependency of tumorigenicity on the percentage of aneuploid cells in the population.

Detailed karyotypic analyses are incomplete, but preliminary studies indicate that both spontaneous and MNNG-induced transformants contain nonrandom translocations and numerical abnormalities. These studies will be published elsewhere.

## *Discussion*

In this study, we found that temporary maintenance of hepatic epithelial cell populations at confluence before passaging (selective growth conditions) reduced the number of population doublings required for cells to express the transformed state, irrespective of whether the cells were exposed to MNNG or to acetone vehicle only. Transformants emerged in both MNNG-treated and acetone-treated cell populations subjected to selective growth conditions 80 to 90 population doublings earlier than in comparably treated cell populations that were passaged continuously just as they reached confluence (nonselective growth conditions). These results show that the selective pressures imposed by periods of confluent growth have a stronger influence on the expression of transformation in hepatic epithelial cell populations than do the number of accumulated population doublings (or cell births) in the cultured populations (Figure 2). We have repeated this protocol on several separately derived clonal and nonclonal diploid rat liver epithelial cell lines and have consistently achieved MNNG-induced transformation after 4 to 8 selective cycles (Tsao MS, unpublished observations, 1987–1988). In previous studies, we demonstrated that 11 repeated treatments with 5 μg/ml MNNG during 11 successive passages under nonselective growth conditions over a period of approximately 20 weeks (approximately 35 population doublings) were required to make the same diploid parental cell line (WB-F344) tumorigenic.[16] Only a single treatment with 3 μg/ml MNNG and three passages during 12 weeks (12 population doublings) were required to make WB-F344 cells tumorigenic when the cells were grown under selective conditions. In contrast, after a single treatment with 3 μg/ml MNNG, 20 passages (approximately 109 population doublings) were required for expression of transformation under nonselective growth conditions. Acetone-treated

populations were spontaneously tumorigenic after 21 population doublings under selective conditions of growth and after 114 population doublings under nonselective growth conditions. Thus, neoplastic transformation was expressed earlier when selective growth conditions were used. MNNG treatment significantly shortened the time of emergence of transformants under both selective and nonselective growth conditions. These findings indicate that both MNNG-treatment and selective-growth conditions influence the emergence of transformed liver epithelial cells, and suggest that the effects of MNNG-treatment and selective-growth conditions are independent and additive. Multiple repeated exposures to MNNG[16] mimicked the action of periodic intervals of maintenance at confluence on the timing of expression of the transformed state in these cell lines, although the mechanisms by which transformation is potentiated may differ under the two conditions.

Although the mechanisms by which either MNNG-treatment or selective-growth conditions potentiate the emergence of transformants in populations of hepatic epithelial cells are not clear from this study, our findings suggest that the development of aneuploidy is a key event in the MNNG-treated populations. However, in the acetone-treated control populations, development of aneuploidy appears to be a necessary, but insufficient indicator of tumorigenicity. In MNNG-treated populations, aneuploidy appears to be initiated by the development of paradiploid cells that results from the loss or gain of one or more chromosomes due to aberrant distribution of chromosomes at mitosis. Subsequently, hypodiploid cells appear to undergo polyploidization to form the subtetraploid, tumorigenic subpopulation. Polyploidization may also be followed by further losses and/or additions of individual chromosomes. The pattern of change to ultimately subtetraploid tumorigenic populations, which is shown by flow cytometry and chromosome enumeration, is consistent with previous observations by us[16] and others[7] of a subdiploid-to-subtetraploid transition of aneuploid variants during transformation of cultured hepatic epithelial cells, and of the predominant subtetraploid DNA content of cells from the tumors produced in syngeneic animals inoculated with uncloned tumorigenic populations.[12,16] These observations, especially the findings reported here on the rapidity with which subtetraploid subpopulations replace the original diploid culture population, and that such cells populate the resultant tumors strongly suggest that the development of aneuploidy is essential for the neoplastic transformation of rat liver epithelial cells treated with MNNG. However, the absence of this relationship among the acetone-treated control populations (Figure 5) suggests that MNNG treatment produces a specific type of aneuploidy associated with tumorigenicity.

Previous studies by Schaeffer and Polifka,[7] in which cultured rat liver epithelial cells were maintained *in vitro*

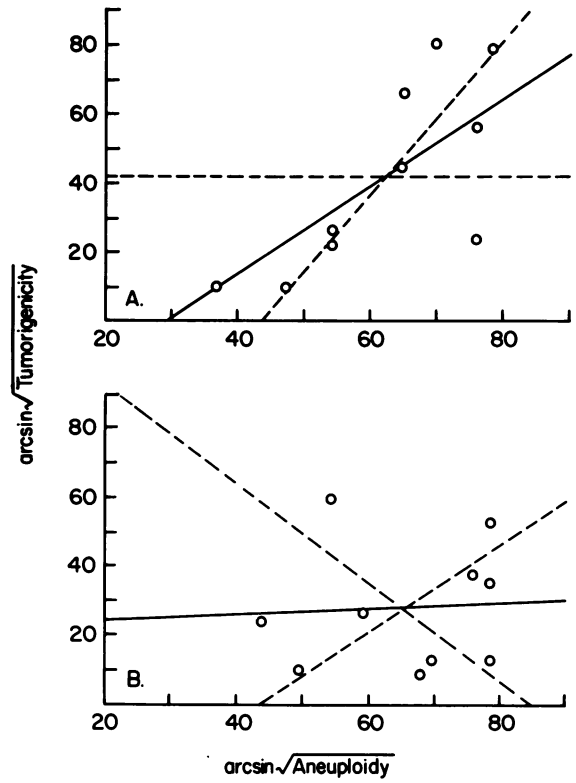


**Figure 5.** *Model II regression analyses for tumorigenicity as a function of the percentage of aneuploid cells in the population. Bartlett's Correction[14] of the inverse sine transformation was used for both the tumorigenicity data and the proportion of aneuploid cells in each population. Dashed lines indicate 95% confidence limits for the slope of the regression line (solid line).* **A:** *Regression line for MNNG-treated populations: $y = -37.4 + 1.28x$, $r^2 = 55\%$; 95% confidence limits on slope: 0.0079 to 2.24.* **B:** *Regression line for acetone-treated control populations: $y = 23.1 + 0.0779x$, $r^2 = 1.2\%$; 95% confidence limits on slope: −1.43 to 1.26.*

with precise accounting for the number of culture passages and cell population doublings, indicated that these cells spontaneously transformed with high probability after more than 100 continuous population doublings *in vitro*. In agreement, Sato et al[17] passaged seven separate lines of rat liver epithelial cells continuously after establishment *in vitro*. Morphologic transformation (evidenced by nuclear atypia, mitotic abnormalities, and aneuploidy) was first noted after the 33rd passage (the number of population doublings was not given), with most lines transforming between passages 48 and 61. Tumorigenicity was acquired between passages 60 and 115. Montesano's studies of spontaneous transformation in continuously cultured rat liver epithelial cells (presented in Table 1 of reference 18) also showed that the probability of spontaneous transformation increased dramatically after continuous culture for more than 38 weeks (the number of passages or population doublings was not given). When hepatic epithelial cell populations were exposed to carcinogens early in their *in vitro* history, Schaeffer and coworkers found that carcinogen-exposed populations

expressed the transformed phenotype several population doublings earlier than would occur spontaneously.[2,7,19] Our results on continuously passaged cultures not allowed to remain at confluence (nonselective growth conditions) corroborate these earlier findings.

Borek's studies showed that diploid rat liver epithelial cells transform spontaneously after simply holding them at confluent cell densities for a relatively long period with infrequent feeding.[8,9] She used a clone derived from an adult rat liver epithelial cell line that had been continuously subcultured for 18 months since its establishment in culture. When these cells were continuously passaged as they reached confluence, transformation was not observed (the number of continuous passages was not stated). However, when cells were allowed to remain at confluent densities and were refed only at 21-day intervals, anchorage-independent tumorigenic variants emerged 30-to-40 days after the beginning of the experiment. Our results confirm Borek's observation of the potentiating effect of confluence on emergence of spontaneous transformants in hepatic epithelial cell populations. In addition, our studies show that confluent conditions also promote the expression of transformation in carcinogen-treated cultures. Borek hypothesized that the spontaneous transformation she observed was the consequence of nutritional stress (nutrient depletion) and exposure of the cells to their own metabolic products and lowered pH.[8] However, our results indicate that it is the condition of confluence, and not variation in nutrition or pH of medium, that potentiates the expression of transformation because we were careful to maintain fresh medium on confluent cultures to maintain a stable level of nutrients and metabolic products in the cells' environments.

The reason selective culture conditions potentiate the emergence of transformed cells is not entirely clear, but several possibilities may explain the phenomenon. Our nonselective growth conditions (continuous passage without periods of confluence) favor the accumulation of altered cells that proliferate most rapidly during exponential growth (ie, that have the shortest cycle time). Conversely, temporary maintenance of cell populations at confluence between passages (selective growth conditions) favors the accumulation of altered cells that can continue to proliferate in an environment that suppresses the growth of more normal cells. Our previous studies with clones of chemically transformed rat liver epithelial cells showed that most subtetraploid variants grew more slowly than the paradiploid/diploid cells, including wild-type cells.[20] Because most transformed cells in these rat liver epithelial cell populations are subtetraploid, as indicated by flow cytometry, it seems most likely that their selective growth is favored as a result of their being insensitive to inhibition of growth in confluent cultures.

Correlation analysis (Table 2) shows that MNNG-treated transformants express a relationship between colony-forming efficiency in soft agar and tumorigenicity, whereas no such significant correlation exists for the spontaneous transformants of the acetone-treated controls. Similarly, there is a significant relationship between tumor latency and tumorigenicity (Figure 5) for MNNG-treated populations, but not for the acetone-treated control populations. These observations may indicate both the existence of a group of biological parameters that can distinguish between the MNNG-induced and the spontaneous transformants, and that MNNG-induced and spontaneous transformants differ phenotypically. However, we found no apparent differences in the histology or frequency of metastases among tumors formed from MNNG-exposed or spontaneously transformed cells.

Spontaneous transformation occurring in the absence of an inciting agent often complicates studies designed to analyze the mechanism of induced neoplastic transformation. Spontaneous acquisition of tumorigenicity by subpopulations of cells in test animals or cell cultures can pervert the sensitive detection of carcinogenic chemicals and complicate the analysis of their mechanism of action. Thus, spontaneous transformation has been viewed universally as unwanted noise in transformation systems. However, the analysis of the process of spontaneous transformation in molecular and cellular terms may be as important as understanding the mechanism of carcinogen-induced transformation because both mechanisms give rise to a common final phenotype, ie, tumorigenicity. The techniques of cell growth and transformant identification reported here should facilitate the investigation of the potential distinctiveness that may exist between carcinogen-induced and spontaneous transformants.

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