Quantitative Studies of Radiation Transformation with the A31-11 Mouse BALB/3T3 Cell Line

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ABSTRACT

A cloned mouse embryo-derived fibroblast cell line was used to study morphological transformation induced by X-rays and 254-nm ultraviolet light (UV). The transformation frequency increased exponentially with increasing dose from 10 to 400 rads for X-rays and 1.0 to 7.5 J/sq m for UV exposure. Split-dose X-ray exposures led to an enhancement in transformation at total doses below 100 rads and a reduction at doses of 300 to 400 rads. The induced transformation frequency varied among serum lots and was very dependent upon the initial cell density. Spontaneous transfectants were observed in 10 of 22 consecutive experiments; the spontaneous transformation frequency was generally about 1 to 2 x 10^-5 as compared to induced frequencies which ranged up to 3 x 10^-3 for X-rays and 7.5 x 10^-4 for UV exposure. Further results indicate that this cell line has several potential advantages over the mouse 10T½ line for studies with relatively weak in vitro carcinogens such as radiation. These include (a) a reduced overall expression time for the appearance of transformed foci (4 weeks); (b) a high cloning efficiency (50 to 60%); and (c) the fact that about 20 times as many viable cells may be plated per dish for optimal results, allowing transformation frequencies as low as 10^-10 to be measured easily. On the other hand, there was more variability in the results among experiments with the 3T3 cell line.

INTRODUCTION

Ionizing and UV radiation have long been known to be mutagens as well as potent carcinogens both in experimental animals and in humans. The mutagenic effects of radiation are thought to result from the production of DNA damage and the subsequent action of molecular repair processes. Recently, there has been considerable interest in the study of the induction of oncogenic transformation in vitro by radiation. This interest has evolved in part from the observations that most chemical carcinogens are mutagens (19), that they bind to DNA and induce DNA damage, and that they induce DNA repair processes which are similar to those induced by radiation. Indeed, a number of chemical carcinogens have been classified as "X-ray-like" or "UV-like," depending on the characteristics of the DNA excision repair they induce (8, 22). Radiation, therefore, may act as a model for the action of many chemical carcinogens at the level of DNA. Radiation has the advantage that the dose to the critical sites in the cell can be precisely controlled and measured, and, for UV exposure in particular, the specific characteristics of the DNA damage and repair processes induced are well known.

The initial studies of X-ray transformation in vitro were carried out with primary hamster embryo cell cultures (4, 6). Recently, however, interest has centered on the use of established mouse embryo fibroblast cell lines which are highly sensitive to the postconfluence inhibition of cell division (7, 17, 20, 21, 25). Such cell lines offer several advantages over primary cultures. Being established lines, they can be grown in large quantities. The cells can be cloned, and cells from the same cloned population may be used by many laboratories. This factor has facilitated the reproducibility of results with mouse fibroblast lines among different laboratories (11, 20, 25, 26). To date, studies of radiation transformation have been largely confined to the 10T½ mouse fibroblast system developed by Reznikoff et al. (23, 24).

This report describes the use of a cloned 3T3 mouse fibroblast cell line for studies of transformation induced by X-rays and UV. This cell line has several potential advantages over the 10T½ line. These include a reduced expression time for the appearance of transformed foci, owing largely to a lesser number of cell divisions required for expression. This factor, coupled with their high cloning efficiency, allows the seeding of many more cells per dish prior to exposure to the carcinogen. This is particularly important for carcinogens such as radiation, which induce relatively low transformation frequencies in vitro but have a significant lethal effect on the cells, as it allows the measurement of transformation frequencies an order of magnitude lower than is readily feasible with 10T½ cells.

MATERIALS AND METHODS

Maintenance and Passaging of Cells. The 3T3 mouse embryo fibroblast cell line designated A31-11 was kindly provided by Dr. T. Kakunaga at the National Cancer Institute, Bethesda, Md. This line is a subclone derived from the A31 clone of the BALB/3T3 cell line (1). The cells were grown and maintained in a humidified 5% CO2 atmosphere in Eagle's minimal essential medium (Grand Island Biological Co., Grand Island, N. Y.; Catalog No. F-15) supplemented with 10% fetal calf serum heat inactivated for 30 min at 56°C, penicillin (50 units/ml) and streptomycin (50 µg/ml). Stock cultures were grown in 60-mm plastic Falcon Petri dishes. The initial cell inoculum received from Dr. Kakunaga was allowed to reach confluence and was then subcultured and distributed into 10 dishes. The initial cell inoculum received from Dr. Kakunaga was allowed to reach confluence and was then subcultured by a 1:20 dilution. We have designated the dishes resulting from this subculture as containing Passage 1 cells. Subsequently, the cells were passaged at weekly intervals by detaching them with an 0.25% trypsin solution, resuspending them in complete medium at a 1:20 dilution, and reseeding into new dishes (roughly 10^5 cells/dish). The culture medium was routinely renewed on the fifth day after seeding.
Transformation Assay. The cells were seeded into replicate 100-mm plastic Falcon Petri dishes. The cell numbers were adjusted such that approximately 4000 to 7000 viable (colony-forming) cells were seeded per dish after correction for the cloning efficiency and the expected toxicity of the particular treatment. Routinely, 10 replicate dishes were seeded for each treatment group. The cells were irradiated 20 hr later and were returned to the incubator to allow the transformed foci to develop. The nutrient medium was renewed on the third and seventh days after irradiation, and thenceforth at twice-weekly intervals. Four weeks after irradiation the cultures were fixed in Bouin’s solution and stained with trypan blue. Nontreated control cultures handled in the same manner were included in each experiment.

In parallel with the above cultures, three 100-mm dishes were seeded from a 1:50 dilution of the same cell suspension (100 to 140 viable cells) in each treatment group in order to determine the actual cloning efficiency and toxicity of the particular treatment. These dishes were irradiated and returned to the incubator for 8 days prior to fixation and staining. The number of viable colonies was counted, the surviving fraction was determined, and this value was used to calculate the actual number of viable cells seeded in the transformation dishes. The transformation frequency was determined by dividing the total number of transformed foci scored in a given treatment group by the total number of viable cells seeded, and it is therefore expressed as transformants per viable cell. Transformed foci were recognized as dense piled-up colonies of cells appearing overlying the normal monolayer (Fig. 1). The criteria used to score a colony as transformed were those described by Kakunaga (12) for the A31-714 strain; their characteristics are similar to those of type III foci in the 10T½ cell system (23, 25).

Cells from these foci grow in soft agar and form tumors (fibrosarcomas) with high frequency upon reinjection into syngeneic hosts. The edge of a transformed focus is shown in Fig. 2. Classically, the densely piled-up cells form a swirling irregular pattern and appear stellate as compared with the normal cells.

Irradiation. X-irradiation was carried out at room temperature with a Philips MG-100 industrial unit operated at 100 kV and 9.6 ma with 0.795-mm aluminum filtration. The dose rate to the cells was 78 rads/min. The cells were irradiated overlaid with medium 20 hr after initial seeding. UV irradiation was carried out at room temperature in a specially constructed irradiator containing a bank of 5 GE G8T5 germicidal lamps, a shutter, and a collimator designed to minimize inequalities of dose at the edges of the dishes. Over 90% of the UV irradiation was emitted from these lamps at a wavelength of 254 nm. The dose rate to the cells was 0.38 J/sq m sec. The overlying nutrient medium was removed prior to UV irradiation and replaced with fresh medium immediately thereafter.

RESULTS

Characteristics of Nontreated Cells. A growth curve for nontreated strain A31-11 cells is shown in Chart 1. After an initial lag period of about 24 hr after seeding, the cells multiplied exponentially with a doubling time of 12 to 13 hr. The cells reached a stable confluency at a density of 2 to 3 x 10⁶ cells/60-mm Petri dish (8 to 12 x 10⁶ cells/sq cm surface area).

The spontaneous transformation frequencies observed in nontreated cells in 22 consecutive experiments are tabulated in Table 1. In 18 of these experiments, the spontaneous transformation frequencies were <0.1 to 0.2 x 10⁻⁴. In the 2 experiments (Experiments 5 and 12) in which spontaneous transformation frequencies exceeded 0.4 x 10⁻⁴, the cells used were of relatively high passage number. The cloning efficiencies in these 22 experiments ranged from 42 to 95%, generally falling in the range of 50 to 60%.

Dependence of Radiation Transformation on Initial Cell Density. The results of an experiment (Table 1, Experiment 4)
are presented in Chart 2, in which the cells were seeded at densities varying from 5,000 to 1.2 × 10^6 total cells per 100-mm dish prior to irradiation with 400 rads of X-rays. When one corrects for a cloning efficiency of 62% and for the fact that 400 rads yielded a surviving fraction of 20.1% in this experiment, the actual numbers of viable cells seeded ranged from 625 to 125,000/dish. As can be seen in Chart 2, the transformation frequency declined continuously with increasing cell numbers. As the change was minimal between 2,000 and 10,000 viable cells, cell numbers were adjusted in subsequent experiments such that an estimated 4,000 to 7,000 viable cells were seeded per dish for all control and treatment groups.

**Dose-Response Relationships.** Survival curves for A31-11 cells exposed to various doses of X-rays or UV are shown in Charts 3 and 4. The cells were irradiated 20 hr after seeding at low density, while they were in early exponential growth.

The results of 22 consecutive transformation experiments are presented in Table 1; these represent all experiments performed with this cell line during a 14-month time interval. The transformation frequencies induced by 400 rads of X-rays were determined in 15 of these experiments and by 7.5 J/sq m of UV in 5 experiments. As can be seen in Table 1, there was considerable variation among experiments in the transformation frequencies induced by the same dose of radiation. The data in Table 1 indicate that this variation can be ascribed in part to 2 factors: (a) the particular serum lot in which the cells were grown; and (b) the level of spontaneous transformation in control cultures. The mean transformation frequency induced by 400 rads is significantly lower with Serum Lot C than with Serum Lot B (p < 0.05), whether or not the experiments with a high spontaneous frequency (Table 1, Experiments 5, 12, and 18) are excluded. Similarly, the mean induced frequency in Experiments 5, 12, and 18 is significantly higher than the mean of the remaining 12 experiments (p < 0.01).

The data from 4 X-ray transformation experiments in which the cells were irradiated with varying doses ranging from 10 to 600 rads are plotted in Chart 5. The dashed line was drawn by eye through the data for 3 experiments in which the induced transformation frequency was relatively high (Table 1, Experiments 8, 11, and 12). The transformation frequency in these experiments appears to increase approximately exponentially with doses up to 400 rads. The shape of the dose-response curve in the fourth experiment (Table 1, Experiment 15) is very similar to that for the other experiments, even though the induced transformation frequency was systematically lower by a factor of about 5.

A dose-response curve for UV-induced transformation is shown in Chart 6. Again, the transformation frequency rose approximately exponentially with doses up to 7.5 J/sq m.

The distribution of transformed foci in these experiments generally followed Poisson statistics for transformation frequencies below 10^-3; scoring of foci was more difficult with higher frequencies in groups where lethality was great (over 90%). Seeding of transformed foci appeared to occur not infrequently in these groups, and was difficult to recognize when many foci were present. For this reason, this cell system appears most valuable for studying transformation at low doses or by weak in vitro carcinogens.
**Transformation frequencies observed following single or split-dose X-irradiation**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>5/5 rads</th>
<th>10 rads</th>
<th>12.5/12.5 rads</th>
<th>25 rads</th>
<th>25/25 rads</th>
<th>50 rads</th>
<th>50/50 rads</th>
<th>100 rads</th>
<th>150/150 rads</th>
<th>300 rads</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>2.4</td>
<td>1.4</td>
<td>2.6</td>
<td>1.7</td>
<td>1.4</td>
<td>5.1</td>
<td>5.1</td>
<td>8.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>1.8</td>
<td>0.9</td>
<td>2.3</td>
<td>1.9</td>
<td>3.9</td>
<td>2.7</td>
<td>8.2</td>
<td>4.1</td>
<td>11.0</td>
<td>14.0</td>
</tr>
<tr>
<td>12</td>
<td>0.9</td>
<td>0.8</td>
<td>2.4</td>
<td>2.0</td>
<td>3.5</td>
<td>1.8</td>
<td>2.5</td>
<td>3.0</td>
<td>6.3</td>
<td>11.0</td>
</tr>
<tr>
<td>15</td>
<td>0.6</td>
<td>0.3</td>
<td>0.4</td>
<td>&lt;0.2</td>
<td>0.8</td>
<td>0.2</td>
<td>1.9</td>
<td>0.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Doses given as single or split (x/x) exposures with 5 hr between them. The significance of the differences between the single and split-dose exposure frequencies were determined by a paired t test on the data from the first 3 experiments in which the single-dose frequencies were relatively similar. The p values are: 10 rads, < 0.05; 25 rads, < 0.05; 50 rads, < 0.01; 100 rads, < 0.5; 300 rads, < 0.02.

**DISCUSSION**

These results indicate that the A31-11 mouse embryo-derived fibroblast line may be useful for quantitative studies of radiation transformation. Although there was considerable variation among different experiments in the transformation frequencies induced by the same radiation dose, the results within
a given experiment appeared internally consistent and reproducible. The shape of the dose-response curves for X-ray and UV transformation (Charts 5 and 6) are similar to those obtained with the 10T½ mouse fibroblast line (7, 25) and with the primary hamster embryo system (4). However, the A31-11 cells appear significantly more sensitive to low doses of radiation than do the 10T½ cells. Transformation by doses in the range of 10 to 50 rads or 1.0 to 5.0 J/sq m of UV can be measured readily (Charts 4 and 6), whereas significant results have been obtained with 10T½ cells at these dose levels only by irradiating very large numbers of culture dishes (100 to 200/point), as have Miller and Hall for X-rays (20) and Chan and Little (7) for UV, or by application of a promoting agent such as 12-O-tetradecanoylphorbol-13-acetate (16, 21).

The A31-11 line has several other characteristics which offer potential advantages over the 10T½ line: (a) the routine cloning efficiency is about 3-fold higher (50 to 60% versus 20%); (b) about 20 times as many viable cells may be plated per dish for optimal results (4000 to 7000 versus about 300), allowing the detection and quantification of lower transformation frequencies; and (c) the overall expression time for the development of transformed foci is shorter (4 versus 6 weeks). This shorter expression time results not only from the fact that fewer rounds of cell division are needed to reach confluency and that the doubling time of A31-11 cells is shorter (Chart 1) but also because the transformed foci appear to develop more rapidly after confluency is reached. The first 2 characteristics offer a particular advantage with weak in vitro carcinogens, especially those such as UV and X-irradiation, for which the potential to induce transformation in vitro is low in relation to their lethal effects on cells.

The rapid fall-off in transformation which occurred with higher initial cell densities (Chart 2) is very similar to that observed with 10T½ cells (25). The difference is that it occurred only at cell densities above 10,000 cells/dish with the A31-11 cells, whereas it was observed with densities above 400 cells/dish with 10T½ cells (25). On the other hand, there is no true plateau on the curve in Chart 2. Therefore, the induced transformation frequency in A31-11 cells may be very sensitive to relatively small differences in initial cell density, although an analysis of the data in Table 1 in relation to initial cell numbers indicates that most of the variation among the present experiments cannot be explained on this basis. The 10T½ cells thus have the advantages of a lesser dependence on initial cell density, greater reproducibility among experiments, and a lower spontaneous transformation frequency.

The observation that the induced transformation frequency declines rapidly with increasing initial cell densities has been interpreted as resulting from the fact that carcinogen-treated cells must undergo a defined number of rounds of cell divisions in order to express transformational damage (25). Experiments with 10T½ cells indicate that in this cell line one round of cell division is necessary for the fixation of X-ray induced damage, whereas an additional 12 rounds of cell division are required for expression of this damage (17, 25). The results in Chart 2 would suggest that only 8 or 9 additional rounds of cell division were necessary for expression of X-ray damage in A31-11 cells (increase from 10 to about 7 x 10⁶ cells/100-mm dish). The number of cell divisions required for expression has also varied in several other systems. Kakunaga found that more than 4 cell generations were necessary for the expression of transformation in the A31-714 strain of 3T3 cells after exposure to 4-nitroquinoline 1-oxide (13) or 3-methylcholanthrene (14), whereas at least 13 generations were required for the development of transformation in human diploid cells treated with 4-nitroquinoline 1-oxide (15).

Taken as a whole, these results indicate that, although cell proliferation is required to express transformation in vitro, its exact amount may vary with cell strain and perhaps also with the particular carcinogen used. The reasons for the rise in transformation seen with very low cell densities (Chart 2) are not clear. We have seen a similar phenomenon with 10T½ cells exposed to X-rays, and Haber et al. (10) reported finding no minimum cell density below which transformation induced by benzo(a)pyrene was independent of initial cell density in 10T½ cells.

The spontaneous transformation frequency (Table 1) appears to be finite in A31-11 cells, and to be about 1 to 2 x 10⁻⁵ under usual conditions. This is in the same general range as the spontaneous transformation frequency recently reported by one laboratory for 10T½ cells (18), and it compares with a spontaneous mutation frequency of about 0.5 to 1.0 x 10⁻⁵ reported at the hypoxanthine-guanine phosphoribosyltransferase locus in both Chinese hamster and human diploid cells (2, 3, 9). Several laboratories, including ours, have reported finding no spontaneous transformation with 10T½ cells (20, 24, 25), although the experimental design has generally been such that spontaneous frequencies of less than 1 to 2 x 10⁻⁴ (an order of magnitude higher than those measured in A31-11 cells) would not be detected in any given experiment, it appears to be lower in the 10T½ than in the 3T3 cells. The 2 experiments (Table 1, Experiments 5 and 12) in which the spontaneous transformation frequencies observed in A31-11 cells were substantially higher than 2.0 x 10⁻⁵ both involved relatively high-passage cells (Passages 7 and 10). The slightly higher levels found in Experiments 16 and 18 (3 and 4 x 10⁻⁶) involved Passage 4 and 5 cells. Although other factors might account for these observations, the results suggest that transformation experiments with the A31-11 line should be carried out only with very-early-passage cells (Passages 1 to 4).

There was considerable apparent variability among experiments in the transformation frequencies induced by a single dose of 400 rads (Table 1). The data suggest, however, that much of this variation can be ascribed to 2 factors, serum and the level of spontaneous transformation. As can be seen in Table 1, the 2 experiments in which the spontaneous frequencies were unusually high (Experiments 5 and 12) were also associated with high induced levels of transformation. However, as can be seen in Experiments 8, 10, and 11, the converse was not always the case; high induced frequencies were observed in experiments where the spontaneous level was low. The association of an enhanced sensitivity of these cells to transformation by 4-nitroquinoline 1-oxide with high spontaneous transformation frequencies has also been observed by Kakunaga. In addition, the experiments performed with Serum Lot B (Table 1) yielded higher overall X-ray-induced transformation frequencies than did those performed with the 2 other serum lots, whereas Lot C appeared to give the most consistent results. These 2 factors appear to account for much, but not all of the variability. As is evident in the data plotted in

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2. T. Kakunaga, personal communication.
Table 2 and Chart 5, however, the results relative to each other within a given experiment were consistent and reproducible despite differences in the absolute transformation frequencies induced.

The findings reported here for the A31-11 cells generally confirm previously reported results for radiation transformation in the 10T½ mouse fibroblast line and in primary hamster embryo cells. Of particular interest are the split-dose X-ray results shown in Table 2 and Chart 7. Borek and Hall (5) initially showed that the transformation frequency induced in primary hamster cells by total X-ray doses of 50 and 75 rads was enhanced if the radiation was given as a split exposure with 5 hr between doses. Terzaghi and Little (26), on the other hand, found that transformation was reduced in 10T½ cells when doses of 300 or 800 rads were given as split exposures; there was no difference following a total dose of 150 rads. Recently, Miller and Hall (20) compared the transformation frequencies induced in 10T½ cells by single and split exposures of X-rays over the entire range of 30 to 800 rads. They found that transformation was enhanced in the split-dose groups with total doses of 30 to 100 rads, whereas it was reduced with doses above 200 rads. The present results with the A31-11 cells thus confirm these previous findings and suggest that the enhancement in transformation which occurs with split-dose exposure to low doses of X-rays is a general phenomenon and is not related to a specific cell strain or experimental condition.

ACKNOWLEDGMENTS

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REFERENCES

Fig. 1. Transformed focus overlying monolayer of normal cells. Culture fixed and stained 4 weeks after exposure to 400 rads of X-rays. Trypan blue, × 3.

Fig. 2. Edge of transformed focus shown in Fig. 1. Trypan blue, × 75.
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