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Genetics

Frequency of UV-induced neoplastic transformation of diploid human fibroblasts is higher in xeroderma pigmentosum cells than in normal cells

(DNA excision repair/cell cycle dependence/somatic cell mutagenesis/anchorage independence/fibrosarcomas)

VERONICA M. MAHER, LAURIE A. ROWAN, K. CHARLES SILINSKAS, SUZANNE A. KATELEY, AND J. JUSTIN MCCORMICK

Carcinogenesis Laboratory, Fee Hall, Department of Microbiology and Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824

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ABSTRACT **If neoplastic transformation of diploid human cells results from carcinogen-induced mutations, cells deficient in excision repair of UV-induced DNA damage should be significantly more sensitive to transformation by UV light than normal cells. We tested this hypothesis by irradiating fibroblasts from a xeroderma pigmentosum patient (XP7BE, complementation group D) with low doses of UV light (254 nm) and cells from a normal person with much higher doses and comparing the frequency of transformation to anchorage independence. Both sets of cells exhibited a dose-dependent increase in transformation which corresponded to a dose-dependent decrease in survival. At doses that caused equal cell killing, the frequency of anchorage-independent cells was approximately equal. Colonies of XP7BE and normal cells isolated from agar, propagated, and injected into X-irradiated athymic mice produced fibrosarcomas in 100% of the animals. Normal cells irradiated shortly before the onset of DNA synthesis exhibited a high frequency of anchorage-independent cells; cells irradiated in early G_1 showed no increase over background. These results agree with those we observed for UV induction of 6-thioguanine-resistant mutants in these cells and support the hypothesis that anchorage independence results from mutations induced by DNA replication on a damaged template.**

A fundamental question in carcinogenesis is whether the initial event leading ultimately to the neoplastic transformation of normal cells into tumor-forming cells results from damage to DNA. In other words, is DNA the basic target for carcinogen action? A wealth of data demonstrating a high correlation between the mutagenic and the carcinogenic properties of various chemicals supports the hypothesis that somatic cell mutations are involved in the process leading to neoplasia (see ref. 1). The fact that cells derived from xeroderma pigmentosum (XP) patients who have a genetic predisposition to sunlight-induced skin cancer and whose cells are deficient in rate of excision repair of UV-induced DNA damage (2) are significantly more sensitive than cells from normal persons to UV-induced mutations also supports the hypothesis (3–7). However, the genetic markers used for these mutagenesis studies—i.e., resistance to 8-azaguanine and 6-thioguanine (3–6) or diphtheria toxin (7)—act only as models for the kinds of mutational events by which carcinogens are considered to affect cellular processes or structures involved in neoplastic transformation.

A more direct approach to determining whether DNA is the principal target is to compare the frequency of UV-induced neoplastic transformation of normal diploid human fibroblasts (NF) with that of XP cells to see if the latter are significantly more sensitive. If DNA is the target, the cells that cannot repair UV-induced DNA damage or can excise it only very slowly will be at a much higher risk of being transformed. By analogy with the results of our UV-induced mutagenesis studies (3–6), if the two populations initially receive the same level of DNA damage, the cells with the more rapid rate of excision should exhibit the lower frequency of transformation.

We have taken this approach, using the assay we developed to quantitate the linear dose-dependent carcinogen-induced transformation of diploid human fibroblasts to anchorage independence (8–10). We have found that, when these anchorage-independent colonies are isolated from agar and the cells are pooled, propagated as monolayers, and injected subcutaneously into sublethally X-irradiated athymic mice, they yield fibrosarcomas. In extensive studies (to be reported elsewhere) to characterize these fibrosarcomas and determine the conditions required to obtain tumors in 100% of the animals within 10 days, we have found that the tumors are composed of human cells and are transplantable and invasive. Although the majority persisted for many weeks until they were finally excised, a few were found to regress. This regression appears to be related to the number of anchorage-independent cells injected rather than being an intrinsic property of the tumor (11). Using this assay we have found that XP cells are significantly more susceptible than normal cells to UV-induced transformation.

MATERIALS AND METHODS

Cells. The normal cells (designated NF812) were initiated from foreskin material of a newborn infant as described (12). The XP7BE cells [from complementation group D (2)], were provided by J. E. Trosko. Both strains had undergone 14 passages by the time they were used. The cloning efficiency of the cells on plastic without a feeder layer was 30–50% for NF812 and ≈30% for XP7BE.

Media. Cells were routinely cultured in Ham's F-10 or Eagle's minimal essential medium with 15% fetal bovine serum and stored in liquid N_2 as described (12). However, for these experiments, the cells were cultured in Eagle's minimal essential medium supplemented with 25 mM Hepes (pH 7.4), 1 mM sodium pyruvate, nonessential amino acids (Flow, McLean, VA), 50 μg of gentamycin sulfate per ml (Sigma), and 15% fetal bovine serum (Sterile Systems, Logan, UT) because this medium was used in the soft agar assay (9). The medium used for selecting thioguanine-resistant cells has been described (5, 12).

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Abbreviations: NF, normal diploid human fibroblasts; XP, xeroderma pigmentosum; S phase, semiconservative DNA synthesis phase of the cell cycle.

UV Irradiation. The medium was removed from the cells attached to the surface of 100-mm-diameter dishes, and the cells were rinsed and irradiated as described (5). The incident dose was 0.1 J/m^2 per sec for XP cells and 0.2 J/m^2 per sec for NF as determined with a light radiometer (International, model IL570; Newburyport, MA). After irradiation, the cells were refed with fresh medium and allowed to undergo cell replication in the original dishes.

Cytotoxicity Assay. Cells from exponentially growing cultures were trypsinized briefly (0.25% trypsin; GIBCO) and resuspended in culture medium; 100–1,000 cells were plated in 100-mm-diameter dishes (five dishes per dose). They were incubated for 16 hr before being irradiated. The survivors formed colonies in the original dishes and the survival was determined as described (5, 12).

Mutagenesis Protocol. Cells from exponentially growing cultures were trypsinized, resuspended in culture medium, and plated into 100-mm-diameter dishes at a density of 1–3.7 $\times 10^5$ cells per dish (15–20 dishes per determination). The number of cells per dish was determined from the expected survival so as to have at least 1.5 $\times 10^6$ surviving target cells in each set of dishes. The cells were incubated for 16 hr before being irradiated as described. The surviving cells were allowed to undergo three population doublings in the original dishes. The increase in cell numbers was monitored and the extent of replication was calculated as described (5, 12). At the end of three doublings, the cells from each set of 15–20 dishes were pooled and subcultured 1:8 to maintain exponential growth. After six doublings, the progeny were pooled and a portion of the cells (10^6) were again subcultured 1:8 and allowed to continue exponential growth. If the frequency of 6-thioguanine-resistant cells was to be determined, 1–2 $\times 10^6$ cells were assayed as described (5). The rest were frozen for future use (12). After a total of nine doublings, the progeny were again pooled and 2 $\times 10^6$ cells were assayed for the frequency of anchorage-independent cells.

Synchronized Populations. For studies on the effect of allowing time for excision repair prior to S phase, NF812 cells were grown to confluence, re-fed once 24 hr after reaching confluence, and then maintained for 72 hr without refeeding. Cells were released from confluence, plated at 100 or 300 cells per 100-mm dish (five dishes per determination) and at 1 and 3.6 $\times 10^5$ cells per 100-mm dish (18 dishes per point), and allowed to proceed through the cell cycle. A parallel set of cells was released 17 hr later and similarly plated. [Autoradiographic studies of incorporation of [^{3}H]thymidine by NF812 cells released from confluence and plated at approximately this latter density indicated that semiconservative DNA synthesis begins $\approx$24 hr after plating (6).] When the second set of cells had attached and elongated (5 hr later), both sets were irradiated with 6 J/m^2, one in late G_1 (3 hr prior to onset of S) and the other in early G_1 (20 hr prior to S). After irradiation, cells were allowed to replicate in the original dishes and were pooled and propagated as described above.

Assay for 6-Thioguanine Resistance. The procedures for assaying resistance to 6-thioguanine have been described (5, 6, 12). Reconstruction studies accompanying each assay indicated no loss of mutants by metabolic cooperation. The mutant frequency was determined from the number of resistant colonies corrected for the number of clonable cells plated, as well as by determining the chance of a mutant per dish from the fraction of empty dishes as described (5).

Assay for Loss of Anchorage Dependence. A total of 2 $\times 10^6$ cells (10^5/60-mm dish) were assayed for ability to form colonies in 0.33% agar essentially as described (9) except that Noble agar (Difco) was used. One or 2 ml of medium was added to the dishes the following day and every 10 days or so thereafter as needed to make up for evaporation until the colonies were fully developed. Precautions were taken to maintain a humidity as close to 99% as possible. After 4–5 weeks, the area of the dish was divided into 10 parts and the macroscopic colonies ($\geq$0.1 mm in diameter) were counted with a dissecting microscope. When cells isolated from agar colonies were retested for anchorage independence, 5,000 cells were plated per 60-mm dish (20 dishes).

Isolation of Anchorage-Independent Cells. With the aid of an inverted microscope, colonies were picked from agar by using a Pasteur pipette. Colonies to be pooled and propagated were pipetted into a 250-ml flask.

Assay for Tumor Formation. The ability of cells derived from soft agar colonies to form tumors in athymic mice was examined essentially as described (9). Briefly, 10^7 cells were injected subcutaneously into the subscapular region of sublethally x-irradiated mice BALB/c male (*nu/nu*) athymic mice (five mice per assay). A dose of 325 rads (1 rad = 0.01 gray) from a GE Maxitron 300 kVp x-ray unit was delivered to the animals 24 hr prior to injection. As a positive control, 10^6 cells derived from a human carcinoma cell line (Hs835T) (9) were similarly injected; the negative control was 20 $\times 10^6$ nonirradiated human fibroblasts that had not been selected in agar. The mice were examined for tumors twice weekly for 10 months.

RESULTS

Frequency of Thioguanine Resistance and Anchorage Independence Induced by UV Radiation. As expected (7), the survival data (Fig. 1 *Top*) showed that the XP7BE cells were significantly more sensitive than NF812 cells to the lethal effect of UV; e.g., a dose of 1.1 J/m^2 decreased their survival to 20%, whereas 7.5 J/m^2 was required to cause the same decrease in NF812 cells. Because NF cells excise UV-induced DNA lesions at a significantly faster rate than do XP7BE cells (2), the fact that the two populations exhibited equal survival after different levels of UV irradiation suggests that reproductive death (inability to form a clone) results directly or indirectly from DNA damage remaining unexcised at some critical time after irradiation and that the average number of such DNA lesions remaining in each population at that time was equal (5, 6).

The mutagenicity data [frequency of 6-thioguanine-resistant cells in the population (Fig. 1 *Middle*)] indicated, as expected (3–7), that the XP7BE cells were significantly more sensitive than NF cells to the mutagenic action of UV radiation. We assayed the progeny of XP7BE cells that had been irradiated with 0.3, 0.5, and 0.7 J/m^2 for resistance to 6-thioguanine after these had undergone an expression period of six doublings. We did not assay the progeny of the NF812 populations exposed to 6 or 7.5 J/m^2 for resistance to 6-thioguanine because we had previously determined the dose-response of this particular cell strain in a series of experiments (5, 6). However, for purposes of comparison, representative data from these previous experiments (5) are included (solid circles). The mutant frequencies have been corrected for the cloning efficiency of the cells because we have shown that, under the selection conditions, their cloning efficiency is similar to that of cells plated at cloning density in the absence of selection (13).

The transformation frequency data (Fig. 1 *Bottom*) indicated that the XP7BE cells were also significantly more sensitive than the NF cells to UV-induced loss of anchorage dependence. These data were obtained by assaying the progeny of the irradiated cells after an expression period of about nine population doublings. However, in the majority of cases, the frequency of anchorage-independent cells was assayed twice—i.e., after 9

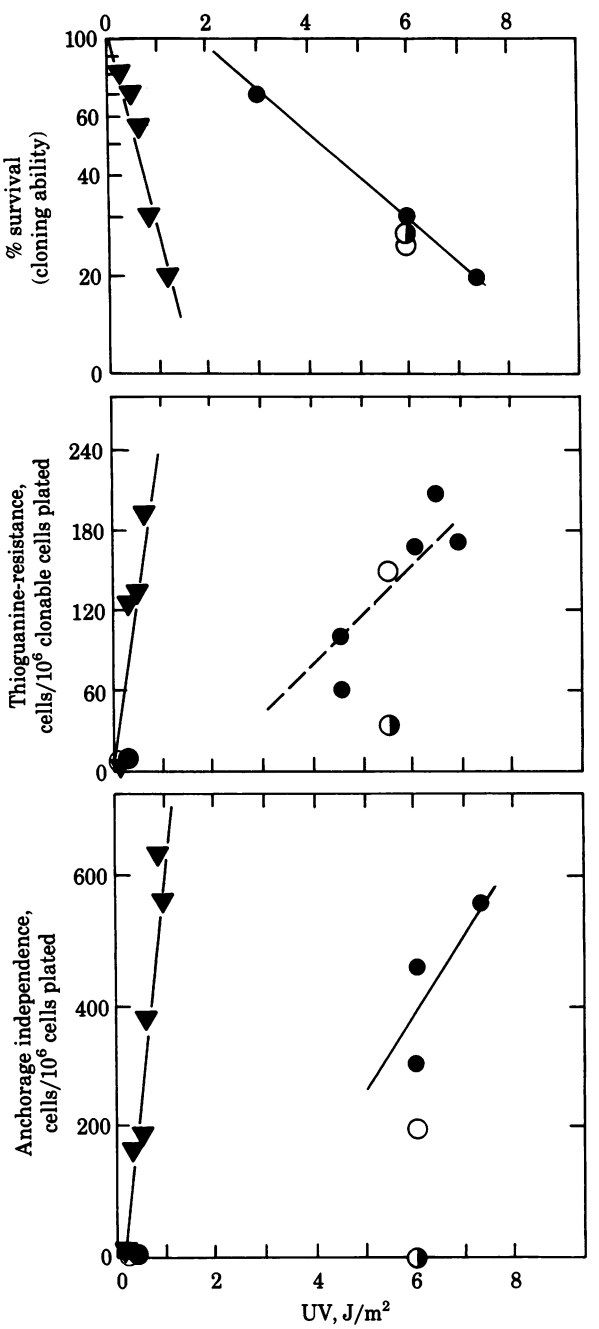

FIG. 1. Cytotoxicity, mutagenicity, and transforming activity of UV radiation in normal (circles) and XP cells (XP7BE) (triangles). The frequency of thioguanine resistant cells was assayed after six doublings; that of anchorage-independent cells, after nine doublings. The former were corrected for cloning efficiency on plastic. Solid symbols, populations irradiated in exponential growth; open circles, cells synchronized by release from confluence and irradiated shortly before onset of S phase; half-solid circles, cells irradiated 18–20 hr prior to S. See text for details. The background frequencies of thioguanine-resistant cells were $<10^{-6}$ in the XP7BE populations and $5–10 \times 10^{-6}$ in the NF812 populations. The background frequencies of anchorage-independent cells were $<10^{-5}$. Lines were fitted by eye.

and 11 doublings—and yielded approximately the same frequencies both times (data not shown). (Note that these data are the observed frequencies of agar colonies counted directly without a correction factor.) No soft agar colonies were observed in the dishes plated with progeny of unirradiated NF812 cells or unirradiated XP7BE cells in the experiment in which doses of 0.9 and 1.1 J/m^2 were used. In the XP7BE experiment using 0.3–0.7 J/m^2, the background frequency was $<10^{-5}$.

The data shown as solid symbols in Fig. 1 indicate that, to achieve a particular degree of cell killing, mutagenesis, and transformation, the NF cells had to be exposed to ≈8-fold higher doses of UV radiation compared to the XP7BE cells. As discussed above with regard to equal cell survival, this is the result expected if induction of anchorage independence as well as thioguanine resistance results ultimately from DNA damage remaining unexcised in the cell at some critical time after irradiation and if, because of the difference in their respective rates of excision repair, the average number of lesions remaining at this critical time is approximately equal in the two populations.

Effect of Time for Excision Repair Before S on the Frequency of Anchorage Independence in Repair-Proficient Normal Cells. A cellular event that could be responsible for "fixing" transformation is semiconservative DNA replication on a template containing unexcised lesions. In a separate study (6) we showed that, when synchronous populations of NF812 cells are irradiated shortly before the onset of S phase, the frequency of induced 6-thioguanine resistant cells is ≈8-fold higher than in the same cells treated ≈18 hr prior to S. No such difference was observed when the target cells were virtually incapable of excision repair. One set of data points from that mutagenesis study with synchronized cells is included in Fig. 1 *Middle* for comparison (open circle, cells irradiated 1 hr prior to S; half-solid circle, cells irradiated 18 hr prior to S). These data suggest that mutations to 6-thioguanine resistance result from replication on a DNA template containing unexcised lesions.

To determine if a similar cell cycle effect occurred for induction of transformation, we irradiated populations of NF cells in early and late G_1 and assayed them for survival and, after the necessary expression period (9), for frequency of anchorage-independent cells. Unirradiated control populations were similarly carried through the protocol and assayed for survival and for anchorage independence. As shown in Fig. 1 *Top* and *Bottom* as open and half-solid symbols, the survival of the two populations did not differ significantly and was similar to that of cells irradiated in exponential growth. In contrast, the transformation frequencies showed a strong cell cycle dependence. The cells irradiated with 6 J/m^2 ≈3 hr prior to onset of S phase yielded ≈200 anchorage-independent cells per 10^6 cells plated; cells irradiated ≈20 hr prior to S gave no cells capable of soft agar growth out of 2×10^6 cells plated. The unirradiated cells also gave no colonies out of 2×10^6. In the mutagenesis experiments with synchronized cells from which the data in the middle panel were taken (6), the frequency of mutant cells did not decrease completely to the background level. However, in that experiment, the cells irradiated in G_1 had somewhat less time for excision repair before onset of S than was available in these transformation experiments.

Cloning Efficiency in Soft Agar of Anchorage-Independent Cells Induced in the Two Cell Strains. We compared the ability of NF812 and XP7BE cells derived from soft agar colonies to form colonies when reassayed in agar a second time. Colonies formed by the progeny of XP7BE cells irradiated with 0.9 and 1.1 J/m^2 and NF812 cells irradiated with 6 and 7.5 J/m^2 were isolated and allowed to attach to plastic. When such cells grow onto plastic, they have an epithelium-like appearance but, as they multiply and fill the dish or are subcultured, they exhibit a typical fibroblastic appearance and growth pattern. After about seven population doublings on plastic, we found no significant difference between NF812 and XP7BE in ability to form colonies in soft agar when reassayed in agar the second time. The average number of colonies observed per dish was

250/5,000 cells (5%). However, the number of colonies that develop in agar when the progeny of anchorage-independent cells are reassayed depends, in part, on the number of nontransformed but viable cells transferred from the original agar along with the colonies (9).

Assaying the Anchorage-Independent NF and XP Cells for Tumorigenicity. The four populations of progeny cells derived from the sets of pooled colonies of XP7BE or NF812 cells (exposed to 0.9 and 1.1 or 6 and 7.5 J/m^2, respectively) were propagated to $\approx 6 \times 10^7$ cells. One-sixth were frozen in liquid N_2 for future use; the rest were assayed for tumorigenicity in athymic mice (10^7 cells per mouse; five mice per assay). With three of the sets, five of five animals developed tumors $\approx$1 cm in diameter within 10 days; the fourth set gave tumors in four of five animals. Frozen cells from the latter population were returned to culture, propagated to $\approx 6 \times 10^7$, and assayed in a second set of five animals. This time five of five animals developed tumors within 10 days. All of the mice injected with Hs835T cells (10^6 cells per mouse) developed tumors within 10 days. None of the animals injected with 2×10^7 unirradiated cells of either cell strain developed tumors. (They have been examined twice a week for the past 10 months.)

Four of these tumors were excised, one from each set of animals injected with NF812 cells or XP7BE cells. A tumor formed in mice injected with Hs835T cells was also excised. Half of each tissue was prepared for histological examination; the other half was used to return cells to culture for future use. The fibroblast-derived tumors were diagnosed as fibrosarcomas; the tumor resulting from Hs835T cells was diagnosed as a carcinoma.

Cells derived from one of the fibrosarcomas induced by injecting the progeny of UV-irradiated XP7BE cells were tested to see if they retained their characteristic sensitivity to the killing action of UV radiation. The survival curve of these tumor-derived cells was superimposable on that of parental XP7BE cells, indicating that the cells forming the tumor were still repair deficient.

DISCUSSION

A reasonable explanation for the fact that, to obtain equal cell killing and equal frequencies of transformation (and mutagenesis) in the two cell strains, the normal cells had to be irradiated with 8-fold higher doses of UV is the following. Low doses induced low numbers of DNA lesions in the XP7BE cells, but these cells were unable to excise many of the potentially cytotoxic, mutagenic, and transforming lesions before these were permanently "fixed" in the cells. High doses induced a high number of lesions in the normal cells but, before these could be permanently fixed, the repair-proficient cells were able to reduce the number to a level approximating that remaining in the XP7BE cells. The fact that allowing NF cells substantial time for excision before DNA synthesis eliminated the potentially mutagenic and transforming effect of UV radiation suggests that DNA synthesis on a template still containing unexcised lesions is the cellular event responsible for fixing the mutations and transformation.

Support for this idea was reported by Kakunaga (14). He showed that, when confluent cultures of a mouse cell line were exposed to 4-nitroquinoline-1-oxide and then allowed to carry out excision repair but not to replicate, the potential for foci formation was gradually eliminated. However, when they were allowed to undergo a single population doubling before attaining confluence, additional time in confluence did not decrease the transformation frequency.

Our results with synchronized cells indicated that the repair-proficient cells were able to eliminate the majority of the potentially mutagenic and transforming lesions induced by UV radiation, if allowed sufficient time before DNA synthesis. Yet, the cytotoxicity data indicated that cell survival was not significantly affected by the amount of time available for excision repair before the onset of DNA synthesis. These results cannot be explained by assuming that the NF cells do not eliminate potentially lethal lesions induced by UV radiation because we (5, 15) and others (16, 17) have demonstrated with density-inhibited cultures that they do. Furthermore, their survival after a given dose of UV radiation is so much higher than that of XP cells. Instead, we interpret these results, and the similar results of our more extensive studies using UV irradiation (15) or the 7,8-diol 9,10-epoxide of benzo[*a*]pyrene (18) as the DNA damaging agents, as indicating that cell killing is not determined principally by the number of lesions remaining unexcised at the time of DNA replication. Rather, we suggest that reproductive death results from inability to carry out required protein synthesis because of faulty or blocked transcription from DNA containing unexcised lesions.

Milo *et al.* (19) recently reported transforming normal human fibroblasts with UV radiation (254 nm). However, in contrast to our results, they reported that: (*a*) the cells must be irradiated just at the beginning of S; (*b*) the cell killing cannot exceed 50% if a dose response is to be obtained; (*c*) growth medium that selects for transformed cells must be used; and (*d*) an expression period of 20–25 doublings must be allowed. The explanation for the difference between their results and ours is not likely to be that the target NF cells we used were atypical because we have obtained a linear dose-dependent response in numerous other NF cells with our protocol and various chemical carcinogens (ref. 9; unpublished data). More recently, we confirmed the present results with UV radiation in another NF cell strain and in another XP strain (unpublished data). Direct comparison between our results and those of Milo *et al.* (19) is difficult because the methods they used to estimate the cytotoxic effect of UV radiation differed significantly from our technique.

Sutherland *et al.*, using a different protocol, also found induction of anchorage independence in NF cells with multiple exposures to UV radiation (254 nm) (20) and, more recently, to longer wavelengths (21). They failed to obtain a dose-response and the cells taken from agar did not yield tumors. Nevertheless, their results are evidence that nucleic acids are the principal target for induction of anchorage independence. Because the frequencies they found at low doses were much lower than predicted from those observed at high doses, they suggested that DNA excision repair might be acting to decrease the frequency of such transformation. The comparative data for NF and XP cells in our present study confirm that prediction. Furthermore, the results of our tumorigenicity assays extend the hypothesis to include that DNA is the critical cellular target for neoplastic transformation.

In addition to the information that can be gained by analyzing the 8-fold difference between XP and NF cells in Fig. 1, it is useful to compare the frequencies of anchorage-independent cells with those of 6-thioguanine-resistant cells. Resistance to thioguanine is a well-characterized mutation marker. As noted (9), the induction of anchorage independence in human fibroblasts has many characteristics in common with introduction of 6-thioguanine resistance, including a similar expression curve. The similarity of the dose-responses for the two phenotypes is obvious and supports the idea that acquisition of anchorage independence (transformation) in human cells occurs as the result of a single mutational event.

The frequencies differ by a factor of $\approx$2.5. In our earliest experiments, using propane sultone (9), the difference was $\approx$22-fold. However, a ratio of 2.5 is much closer to the ratios

we now routinely observe with a number of agents. Part of the reason for the higher value reported in the early experiments can be attributed to the use of a different lot of serum and a different type of agar. Small technical differences in the way the cells were handled may also have contributed to the difference. A third significant difference between our present experiments and those with propane sultone is that, in the case of those early experiments, the agar colonies per dish were so numerous that only a representative fraction of them were counted and the calculated numbers may have been systematically overestimated. In subsequent experiments with propane sultone, in which fewer cells per dish were assayed and every macroscopic colony was counted, the frequency was 1/4th of the earlier value (9).

If we assume that anchorage independence results from a mutation, we might speculate about the size of the DNA target involved. The observed number of 6-thioguanine-resistant colonies (selected on plastic at a density equivalent to 10^4 cells per 60-mm dish) has been corrected for the cells' intrinsic cloning efficiency (i.e., 30–50%); the number of anchorage-independent cells (selected at a density of 10^5 cells per 3 ml of soft agar), on the other hand, was determined directly from the observed number of agar colonies without correction. There is no simple way to determine the "intrinsic cloning efficiency" of anchorage-independent cells under the condition in which 10^5 cells are plated. If the cloning efficiency in agar were less than 100%, the frequencies would have to be increased accordingly. Nevertheless, the relationship between the frequencies observed for 6-thioguanine resistance as well as anchorage independence in the two strains that differ in DNA repair capacity supports the hypothesis that mutations are involved in transformation.

Note Added in Proof. We irradiated XP12BE cells ≈18 hr or ≈1 hr prior to the onset of S phase and compared the frequencies of 6-thioguanine resistant cells and anchorage-independent cells induced. As expected for this strain which is virtually devoid of excision repair capability, we found no significant difference between the frequencies at the two times of irradiation.

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