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**Ionizing Radiation-Induced 6-Thioguanine
Resistant Clones in Synchronous CHO Cells**

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ABSTRACT

When cultured Chinese hamster ovary (CHO) cells are exposed to acute doses of ionizing radiation at different times during the cell division cycle, there is a characteristic cell-cycle response for radiation-induced cell killing and induced resistance to 6-thioguanine. For cell killing the sensitive periods of the cell cycle are the G1, G2, M and early S periods, as others have reported. For mutation induction the sensitive stage is the G1 period with the maximum sensitivity near the boundary between G1 and the S period. Cells appear to be very refractile to induction of 6TG resistance in other periods of the cell cycle. These results suggest that chromosomal rearrangements of the X chromosome are most likely to occur in the G1 period before the hypoxanthine-guanine-phosphoribosyl-transferase (HGPRT; EC 2.4.2.8) gene replicates, most likely due to genetic recombination. Clones resistant to 6TG-induced by X-rays are most likely induced by a different mutagenic pathway than ones stimulated by ultraviolet (UV) or ethylnitrosourea treatments since the response in the cell cycle is quite different.

KEY WORDS: ionizing radiation, synchronized CHO cells, mutations

INTRODUCTION

The HGPRT-CHO system has been used successfully in recent years in asynchronous systems as a well-defined system to test for environmental mutagenesis from a variety of agents (1). The system offers some advantages over the earlier methods of measuring the induction of resistance to 8-azaguanine (2,3). The variants obtained after mutagenesis treatment and an expression time of 7 days or more are mutants in the vast majority of cases (1,4).

We have been interested in the induction of clones resistant to 6-thioguanine in synchronous cells from a variety of chemical and physical agents that damage the cells "randomly" (5,6), and also BUdR and other similar agents that damage the cell nucleus "non randomly" in certain regions (7). In these experiments it is important to note that the synchrony is achieved without the use of drugs which, we expect, would alter the normal responses of cells to environmental mutagens in the cell cycle. The discovery of sensitive periods in the cell cycle would be important for the practical reason that many cells in the body are in a non-dividing G_0 state and might or might not be more sensitive than in logarithmic growth. In addition, the elucidation of mechanisms of mutagenesis might be easier if the biochemistry of all the cells in the population at one time in the cell cycle was similar.

Previous work on mutagenic effects of X-rays in the cycle reported: no cyclic difference (8,9), enhancement in the G2 (10), sensitivity at the G1/S interphase (11), and M, G1 and G1/S border (12). These previous works suffered either from an inadequate number of measurements in the cell cycle or use of 8-azaguanine (8AZ) in an unreliable way, or use of chemical treatments to induce synchrony. We report here that there is a pronounced sensitivity to mutation induction in the G1 stage of the cell cycle with the maximum sensitivity near the end of the G1 period and at the beginning of the S

period, before replication of the HGPRT gene or genes.

MATERIALS AND METHODS

Cell Culture Conditions

The CHO cell line was received from L. Kapp and R. Klevecz, City of Hope Medical Center, Duarte, California, and was selected for use on the cell-cycle analyzer (Talandic Research Corporation).

This cell line has been shown to be free of pleuropneumonia-like organisms (PPL0), and has a modal chromosome number of 21. The population doubling time of the cells in these experiments was 11.5 hr under optimum conditions with a 4-hour G1 period, 6-hr S period, and a G2 plus M period of 1.5 hours.

The cells are grown either in a CO₂ incubator in closed or open tissue culture flasks at 37°C, or in large closed roller bottles, in McCoy's 5A media supplemented with 15% fetal calf serum and 100 units/ml of penicillin and 100 µg/ml of streptomycin, and 1mM HEPES buffer to slow pH changes during experiments.

Irradiation Conditions

Either asynchronous populations of CHO cells in 90-cm dishes in the middle of logarithmic growth or synchronous populations of cells in 75-cm² tissue culture flasks were washed two times with Puck's Saline A (PSA) and irradiated with 50 kVp X-rays from a Picker X-ray machine (after the covers of the dishes or the tops of the flasks were removed with a glass cutter). The dose rate was 300 rads per minute as determined with ferrous sulfate dosimetry. The beam was filtered with 2.5-mg/cm² aluminum and had a half-value thickness of 8 mm of H₂O. The beam was isotropic over the irradiation area within 5% as determined with LiF thermoluminescent dosimeters. The cells were irradiated with all but a thin layer of the PSA removed at room temperature. After irradiation, the cells were immediately trypsinized and

plating-efficiency determined. At least 2×10^6 cells were then grown for the "expression period" described below. Aliquots of cells were used for Coulter cell volume spectroscopy, flow cytometry, or incorporation of tritiated thymidine to analyze cell cycle position at the time of irradiation (7).

Synchronization Methods

Cells were grown for several days in logarithmic growth phase in roller bottles turned at 0.5 rev/min. They were subcultured into one or more roller bottles at 2 to 3×10^7 cells per bottle. After 24 hr, the cells were synchronized in a 37°C room using a "Cell Cycle Analyzer" (Talandic Research Corporation, Pasadena, California), an apparatus patterned after the instrument developed by Klevecz (13). The mitotic cells were usually shaken off and collected at 1-hour intervals for 18 hours or more. Other details of the synchrony technique and cell life cycle progress analysis after mitotic detachment are described elsewhere (6,7).

Determination of Percent Survival and Selection for 6TG Resistance

In all of the experiments the plating efficiency of cells after the various treatments and times was determined by plating about 100 viable cells for each 90-mm tissue culture dish. After growth in normal medium for 7-8 days, the dishes were stained with 1% methylene blue and the number of visible colonies counted.

Clones resistant to 6TG were determined after continuous logarithmic growth in glass roller bottles for 8 days. At that time, 10^5 cells were plated in 90-mm tissue culture dishes containing $5 \mu\text{g/ml}$ of 6TG in the media. After 8 days of growth the number of visible colonies was determined as above. In unirradiated controls this corresponded to two subcultures of the roller bottles to 2×10^6 cells per bottle after 2 and 5 days. However, in the irradiated samples at higher doses, the cells were only subcultured after

5 days, or not subcultured at all.

RESULTS

Asynchronous Cells

When logarithmically growing populations of CHO cells are exposed to 50 kVp X-rays, one determines the dose response curve for killing and mutagenesis as shown in Figure 1. The survival curve is shouldered and the induction of resistance to 6TG is curvilinear with dose. These types of data are similar to those reported by others for exposure of both CHO and V79 cells to more energetic X-rays (14-17). It is interesting to note that the average RBE for 50 kVp X-rays, compared to 250 kVp X-rays (1), is about 2 which is as expected from the RBE vs LET data of Cox et al. (18).

Synchronous Cells

When cells are synchronized without the use of drugs into approximately 1-hour intervals in the cell cycle, the ionizing radiation-induced reproductive death during the cycle follows a now characteristic pattern. Figure 2 gives the percent survival for different times in the cycle at 600 and 750 rads of ionizing radiation. This type of "age response" for radiation-induced reproductive death is similar to that reported by others for Chinese hamster cells with a 3-4 hour G1 period (19-22).

The results for radiation-induced resistance to 6TG for five different doses of radiation at different times in the cell life cycle is given in Figure 3. There are many more mutations induced in the G1 period than at other defined stages of the cell cycle. The sensitive period appears to be at maximum late in the G1 period and appears to extend into the early S period at high doses. Notice that the G2-M period does not appear to be sensitive as is the case for radiation-induced reproductive death.

The dose response curve for cells irradiated in early S, G1, and late S

is given in Figure 4. It appears that the majority of the mutations produced in asynchronous cells are from cells in the G1 stage of the cell cycle at the time of radiation exposure.

DISCUSSION

There is a pronounced cell-cycle dependence for the induction of clones resistant to 6TG by acute exposure to X-rays. The most sensitive period for the induction of resistant clones is the later G1 and early S stage of the cell cycle. Most of the mutations produced in asynchronous populations are due to events occurring in G1 populations since the induction of mutations for logarithmic cells is quite similar to the G1 results. These results may apply to most mammalian cell systems because the results of Watanabe and Horikawa with HeLa cells (11) show a sensitive period near the end of G1 in chemically synchronized cells, and the recent results of Jostes (12) also suggest an increased G1 sensitivity.

There are at least two major ramifications of the existence of a distinct "age response" for mutation induction in mammalian cells: first, its importance for establishing the risk estimates for environmental mutagens and carcinogens and second, the possible advantage of synchronous cell populations in mechanistic studies. In the former case, if the sensitive stage corresponded to the G1 period, it is quite likely that most of the cells in the mammalian body in G_0 might be more sensitive than supposed from studies of cells in culture in asynchronous growth. Preliminary experiments in our laboratory on stationary phase cultures, which simulate G_0 , suggest that these cultures are more sensitive to mutagenesis, so the increased risk for mutagenesis might be also true for G_0 cells in vivo.

It is important to realize that differential stage sensitivity to mutagenesis will be important in repair studies (for example, dose fractionation

studies) because cell progression and differential stage sensitivity would influence the interpretation of results. In future studies it will continue to be important to use synchronous populations of cells that are synchronized by selective rather than chemical or inductive methods, to avoid possible results deriving from an interaction between the chemical used to synchronize and the agent that is being tested. Another factor that has been in dispute is the choice of 6TG over 8AZ for drug selection. We are in agreement with others who suggest that 6TG is the preferred method because the variables seem easier to control. However, the recent results of Jostes (12) suggest that in careful studies 8AZ results may be similar to 6TG results.

Studies of the mutagenic effects of other environmental mutagens show results somewhat similar to those found above with X radiation. For example, Riddle and Hsie (4) have shown a greater sensitivity to UV in the G1 period of the cycle for 6TG resistance. We have confirmed and extended their results to induced resistance to Oua or diphtheria toxin and find that G1 is quite important, especially in the case of induced resistance to diphtheria toxin (6). However, after exposure to the chemical carcinogen ENU there is no "age response" for induction of 6TG resistance (5) although at high doses the G1 period is slightly more sensitive for the induction of Oua resistance. Thus, we are forced to conclude at this time that the "age response" for mutation induction by environmental mutagens in mammalian cells is mutagen specific and may be test specific. The results above are results obtained with "random" mutagenesis of the cell DNA. Results with the "non-random" mutagenesis agent BUdR give a different time-dependent pattern, characteristic of the type of induced resistance presumably related to the replication of regions within parts of the nucleus (7).

What mechanism facilitates X-ray-induced mutations in the G1 and the

early S phase but does not efficiently lead to mutations in the rest of the cell cycle? The answer appears to be related to three observations. First, the peak for induced mutations drops precipitously after the gene or genes associated with HGPRT have replicated (7). Second, the effects are not really point mutations, but most likely chromosomal rearrangements and deletions (23). Third, induced genetic recombination and spontaneous mitotic recombination occur most readily at the G1 period and before gene replication in the S phase (24,25). These observations suggest that the "late G1," "early S" mutagenesis sensitivity in mammalian cells is associated with genetic recombination induced by X-rays leading to chromosomal rearrangements or deletions, or that a form of efficient post replication repair exists in mammalian cells. Because there is little evidence for a process after X-rays suggested by the latter hypothesis, it appears that the former hypothesis may be most likely, especially since it is strongly supported by yeast data where detailed genetic analyses have already been made. It is interesting to note that this process of genetic recombination can lead to a homozygous recessive diploid only in the G1 and S periods before the gene replicates (25). The data here with the "recessive mutation" resistance to 6TG and other data after UV exposure for induction of dip^r (6) suggest that genetic recombination in G1 and the early 'S' period may be important in the induction of mutations in mammalian cells by environmental mutagens.

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(A)

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(B)

Fig. 1 A. Percent survival of cells exposed to various doses of X-rays.
B. The frequency of induced 6-thioguanine resistant clones per 10⁵ viable cells as a function of the acute dose of filtered 50 kVp X-rays.

Fig. 2 Percent survival of cells after a dose of 600(●) and 750(o) rads at 300 rads/min for different times after mitotic selection. (x) Percent survival of a population of cells exposed to 750 rads after 8 days of growth following radiation exposure.

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Fig. 3 The frequency of induced 6-thioguanine resistant clones per 10^5 viable cells 8 days after irradiation at 300 rads/min at different times after mitotic detachment. ◇ = 150 rads, ▲ = 300 rads, □ = 450 rads, ● = 600 rads, and ○ = 750 rads.

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Fig. 4 The frequency of induced 6-thioguanine resistant clones per 10^5 viable cells 8 days after irradiation for three times in the cell cycle: the G1 period, the early S period, and the late S period.

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