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TEST OF RADIATION DAMAGE ENHANCEMENT DUE TO INCORPORATION OF BrUdR INTO DNA USING CHROMATID ABERRATIONS

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ABSTRACT

Monte Carlo track structure calculations, leading to an estimation of the magnitude of enhancement of radiation damage due to the incorporation of the halogenated pyrimidine, bromodeoxyuridine (BrUdR) a thymine analog, into DNA have been made. The increase in the yield of double strand breaks for various degrees of substitution in one (monofilarly) or both strands (bifilarly) have been calculated. To test these calculations, quantitative selected radiation-induced aberrations have been obtained in Chinese hamster (V79) fibroblast chromosomes having various patterns of BrUdR substitution following irradiation with 250 kV X rays. Free "breaks" and achromatic lesions "gaps" show no appreciable sensitizations, but breaks involved in chromatid interchanges show significant enhancement though of lower magnitude than theoretical predictions.

INTRODUCTION

This paper attempts to correlate the early biophysical effects of radiation induced-damage obtained using Monte Carlo track structure calculations with experimental data, chromosome aberrations being the biological end point.

When ionizing radiation interacts with living cells a long chain of events ensues which may lead to cell death, mutation or transformation. The quest toward an understanding of the mechanisms of damage caused by ionizing radiation has been pursued in the multidisciplinary field of radiobiology which combines knowledge of physics, chemistry and biology. Chromosomal structural change was one of the earliest systems for the quantitative study of the effects of radiation (1,2). These provide a visible and quantitative measure of the effect on the cell. Recent developments have made it possible to explain the chromosomal structural changes in terms of the molecular theory of radiation effect (3). A central theme of the molecular theory of radiation-induced chromosomal structural change is that the DNA double strand breaks correspond to the primary breaks that lead to aberrations (4,5).

The conceptual framework here is that the interaction of the ionizing particle results in deposition of energy at the target site and this subsequently can lead to the induction of many forms of damage (6-8). In spite of great advances made in the past two decades to further our basic understanding of the processes which lead to biological damage, there remain many questions as to the mechanisms linking the initial physico-chemical damage to the final cellular effect. The paper by Charlton and Nikjoo (this volume) presents a method and examines the complexity of attempting to model the very basic initial interactions at very short times ($< 10^{-12}$ s). We have used the same modelling technique to obtain the data for the theoretical calculations of this study. The physico-chemical stages of radiation can be envisaged as inducing a variety of types of damage, such as single strand breaks, double strand breaks, base damage and cross-links. Most single strand breaks and base damage are repaired very rapidly (9-14). Double strand breaks are thought to be the most important lesion participating in radiation-induced damage. As these are not repaired very efficiently, they lead to the induction of chromosomal aberrations (15-20).

Halogenated pyrimidines, such as BrUdR and IUdR, which are analogs of thymidine, incorporate into DNA and enhance the radiation effect (21-25).

This property has been explored both for clinical interest in certain tumor treatments and for identifying the mechanism(s) involved in radiation-induced damage (26-32). To understand the radiosensitization effect, a number of investigations have been reported on the loading effect of the DNA by bromine or iodine analogs (28,29,33-36). These studies which use either a cellular system, measuring radiosensitivity in terms of survival fraction, or chromosome aberrations, sometimes, but not always, show quantitative differences in sensitization between monofilarly and bifilarly substituted DNA. These differences have been attributed to the level of substitution and chromatin packing.

Because of the semi-conservative nature of DNA replication, chromosomes with contrasting forms of substitution can readily be obtained. Natural DNA contains thymine (T) in both strands and is therefore TT (Fig. 1a). If bromodeoxyuridine is present for one complete synthesis/replication period (S phase), both the chromatids will have DNA with thymine in one strand (T) and bromouracil replacing some thymines in the other (B). So the chromosome constitution is TB/TB (Fig. 1b).

If the bromodeoxyuridine is removed and a further round of replication permitted in the presence of thymidine, the chromosome constitution will be TT/TB. If, however, the bromodeoxyuridine remains for the second round, the chromosome constitution will be TB/BB (Fig. 1c). In either case, the chromatids can be differentially stained and aberrations produced in TT, TB or BB chromatids recorded.

Using these two protocols, it should be possible to produce experimental sensitization factors for TT/TB and TB/BB and, by inference (since TB is common to both protocols) TT/BB. This we have attempted, so that the factors can be compared with the theoretical predictions.

METHODS

Monte Carlo Track Structure Calculations

The approach is to first simulate the stochastic tracks of electrons and then calculate the energy deposited in the target volume. We have compiled a comprehensive set of data on energy deposition by ionizing radiations in cylindrical targets for use in biophysical models of the action of radiation on

biological targets (37-40). These data have been used to calculate the yield of single and double strand breaks in a model of DNA (41-44). Model number three of Charlton and Nikjoo (this volume) was used to calculate effects in bifilarly bromine-substituted DNA. A fifth model was developed, by modifying model number three, to calculate the enhancement when DNA was monofilarly substituted. Unlike the experimental protocol, the present simulation work does not distinguish on which template the substitution was made. In this calculation the sensitization effect was due to incorporated

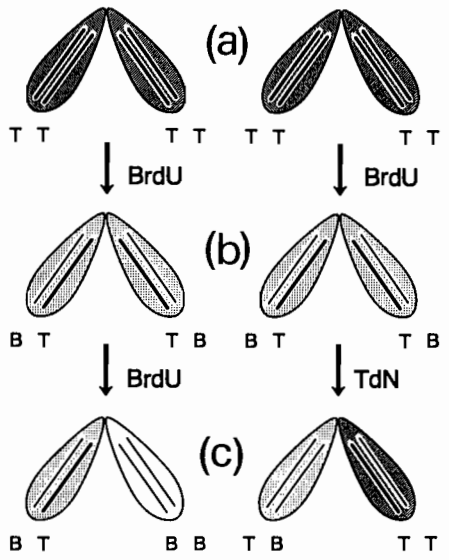

FIG. 1. Protocols for obtaining substituted chromatids. The group on the left of the Figure 1 is the protocol for producing the TB/BB chromatids where one chromatid (BB) is bifilarly substituted and the other (TB) monofilarly substituted. The BB chromatid is light (l) and the TB chromatid is dark (d). The group on the right of the figure shows the protocol for obtaining the TT/TB chromatids. In this case BrUdR (BrdU) was removed and normal thymidine medium was added for the second cycle replication. At the end of 2nd replication one chromatid is monofilarly substituted (TB) and the other unsubstituted (TT). The TB chromatid is light (l) and TT is dark (d). The heavy lines show the unsubstituted template. Fig. 1a shows the 1st generation, Fig. 1b the 2nd generation, and Fig. 1c the 3rd generation.

bromine inducing SSB and DSB calculated by replacing T's by the bromine analog with a 50% probability of filling on one strand of the DNA only, and assuming the threshold energy of 15 eV for bromine activation with a probability of 0.5 to produce an SSB. A suitable number of 20 keV monoenergetic electrons were simulated and subsequently were used to calculate the enhancement ratio when bromine was added monofilarly and bifilarly. Enhancement is the quotient of the effect after to that of the effect before the addition of the bromine.

A Biological Test

Cells of Chinese hamster, V79-379A, were grown in Falcon T75 culture flasks using Eagles MEM supplemented with 10% FCS, L-glutamine and antibiotics. Under these conditions, they have a modal cycle time of ≈ 8.5 h. The flasks were divided into two batches:

Batch A; (TB/BB protocol). BrUdR (final concentration, 10 μg/ml, 3.25×10^{-5} M) was given and 17 h later they received 0.9 or 1.5 Gy X ray irradiation (250 kV, 14 mA, HVL 1.2 mm Cu, 0.76 Gy/min). Cells were sampled at 3 h post-irradiation, following a 2 h colcemid accumulation (0.05 μg/ml).

Batch B; (TT/TB protocol). BrUdR (concentration as above) was given for 9 h. The medium was removed, the cells rinsed with Earle's BSS and complete medium containing added thymidine (10 μg/ml, 4.13×10^{-5} M) restored. At 17 h, they were irradiated along with the flasks of Batch A, receiving the same absorbed doses, and then sampled at 3 h following 2 h colcemid.

Air dried chromosome preparations were stained with fluorescence-plus-Giemsa methods to produce "harlequin" sister-chromatid differentiation. Care was taken to ensure that the pale chromatids (BB for batch A, TB for batch B) were not under-stained, for that could lead to under-scoring of achromatic lesions. Aberrations were scored in fully differentiated second-division chromosomes and chromosome groups. Only three of several types of chromatid-type aberrations were recorded and the following procedure was adopted: Slides were traversed until at least 400 unambiguous chromatid breaks ("discontinuities") were collected for each dose, and allocated to the light (l) or dark (d) chromatid. In about 12% of cases, the break

was accompanied by an exchange of sister-chromatids, seen as a color-jump at the point of breakage (c*).

Whilst searching for breaks, all achromatic lesions ("gaps") encountered were also recorded and l,d allocated. A "buffer" category of gap/break was provided for those aberrations where an unequivocal decision could not be made. For analysis, this buffer was merged with gaps. All chromatid interchanges observed during break search were classified for symmetry and completeness, and the presumptive break-points were assigned l/l, l/d, d/d. About 2-3% showed mixed breaks, having a color-jump break at one end.

Since we included isolated chromosomes and incomplete cells in our scoring, and only scored selected aberrations, absolute frequencies, expressed as aberrations per cell can not be given (not that such values mean very much in a situation where observed yield fluctuates with sample time). Instead, we scored for each dose treatment the total aberration involvement of a random sample of at least 200 large metacentric chromosomes (of which there are 4 per cell) and provide "aberrations per metacentric" for comparative purposes.

RESULTS

Table I is a summary of computational data showing the bifilar and monofilar enhancement ratios for various bromine loadings. The data show the ratios TT/BB for bifilarly and TT/TB for monofilarly substituted DNA strands. The bifilarly substituted ratio TT/BB was obtained from model number three of Charlton & Nikjoo (this volume) and the monofilarly substituted ratio TT/TB from model number five. The data in brackets show the effect plus the contribution due to Auger electrons.

Table II summarizes the experimental results. Only non-color jump lesions have been included in the calculations. It is seen that for the TB/BB situation (BB light), neither free breaks nor gaps show any evidence of excess BB involvement. Conversely, the breaks/lesions involved in interchanges show a highly significant excess, a factor exceeding 2. For the TT/TB situation (TB light), free breaks at 0.9 Gy show a significant excess in TB. This is absent at 1.5 Gy and absent for gaps at both doses. Interchanges show significant excess at both doses, though the factor is lower (about 1.5) than for TB/BB.

TABLE I
Enhancement Ratios Calculated for Various Bromine Loading in the DNA

Br %	Bifilar TT/BB	Monofilar TT/TB
10	1.40 (1.40)	1.19 (1.19)
20	1.64 (1.66)	1.34 (1.40)
30	1.85 (1.90)	1.51 (1.53)
40	2.12 (2.18)	1.65 (1.69)
50	2.25 (2.36)	1.73 (1.77)
60	2.46 (2.57)	1.84 (1.89)
70	2.69 (2.81)	2.00 (2.60)

Table III gives the absolute aberration yields. The chromosomes show hardly any sensitization for the TT/TB situation but a very obvious one for TB/BB.

DISCUSSION

Starting from a physical basis for radiation-induced DNA damage we have attempted to present a simple model for calculating the effect of monofilar substitution of the DNA strand and compare it with experimental chromosome aberration data. In calculating these data we have made the following assumptions for the sensitization effect:

1. Energy is deposited in the target volume discretely by individual tracks.
2. SSB are produced as a result of a threshold energy deposition of 17.5 eV by a single ionization or excitation.
3. SSB's interact to produce a DSB.
4. DSB is the primary damage.
5. Repair and other primary damage is not considered.
6. Energy for bromine activation is 15 eV.
7. All the biological damage is mediated via chromosome aberrations.

TABLE II
Dominance of Lesions in Light (l) Chromatids

Treatment	Dose (Gy)	l	d	l/d	Prob.
BREAKS TT/TB	0.9	202	153	1.32	0.009**
	1.5	143	120	1.19	0.16
TB/BB	0.9	236	214	1.10	0.30
	1.5	211	219	0.96	0.70
GAPS TT/TB	0.9	587	625	0.94	0.28
	1.5	288	329	0.88	0.10
TB/BB	0.9	350	324	1.08	0.32
	1.5	177	204	0.87	0.17
INTERCHANGES	0.9	214	150	1.43	0.0008***
TT/TB	1.5	147	91	1.62	0.0003***
TB/BB	0.9	216	101	2.14	1.1×10^{-10}***
	1.5	175	79	2.22	1.7×10^{-9}***

Breaks showing color-jumps have not been included in the table or the calculations.

The data from theoretical calculations show the increase in yield of DSB's for various degrees of substitution in one (monofilar) or both (bifilar) strands. Calculated data show a greater sensitization effect than the experimental data. For each experimental substitution situation, there are two quite different sensitization factors, dependent upon the particular aberration category chosen. We cannot be certain that either represents a relevant value.

If we accept the results for breaks and for achromatic lesions (gaps) then, with the sole exception of TT/TB breaks at 0.9 Gy, there is no evidence for any sensitization by either mono- or bifilar bromouracil incorporation. On the other hand, if we accept the results from interchange breaks/lesions then TB>TT $\approx$ 1.5 and BB>TB $\approx$ 2.2.

TABLE III
Absolute Aberration Yields

Substitution	Dose (Gy)	No. of metacentrics scored	Total aberrations	Abs. per metacentric
TT/TB	0.9	200	8	0.04
	1.5	600	62	0.10
TB/BB	0.9	200	29	0.15
	1.5	400	107	0.27
Control	0.9	200	9	0.05
	1.5	200	19	0.10

It is difficult to reconcile these differences between aberration categories with currently accepted ideas of aberration origin. If there is a common pool of breaks from which all structural changes are formed, then any sensitivity bias should affect all aberrations equally. Perhaps some kind of differential repair of BrUdR-enhanced breaks occurs between the formation of interchanges and the intrachange/break/gap categories.

A second worrying feature of this data appears when we consider the absolute aberration frequencies in relation to absorbed dose. We find consistently that, for a given dose, TT/TB chromosomes are, overall, much less sensitive to aberration production than TB/BB chromosomes by a factor 2 - 3. This tells us immediately that the monofilarly substituted chromatid (TB), common to both protocols, does not have a fixed sensitivity. Rather, its response in terms of aberration production/involvement is dependent in some way upon the constitution of its sister-chromatid. TB with a BB sister always has more aberrations than TB with a TT sister. From this it follows that the data do not support a model of sensitization based simply on the amount of BrUdR incorporated. We have to conclude that the TB aberration frequency is conditional upon the substitution condition present in its sister. This has interesting implications for the theory of aberration formation.

However, it is only fair to point out that the two TB chromatids are not identical with regard to their mode of formation. For TB/BB, bromouracil-containing DNA is built onto a template containing endogenous thymine, but for TT/TB, exogenous thymine-containing DNA is built onto a bromouracil-containing template. These differences could affect the packing and condensation of the chromatin, but whether this would produce aberration frequency disparities of the magnitude we observe is questionable. Because of this difference in absolute frequency of the common TB chromatid, we cannot use it to produce a common overlap value which will allow us to predict the sensitivity effect for TT→BB.

We have not measured the actual degree of substitution we obtained with an exposure concentration of 10 μg/ml for one or two cycles. Extrapolations from published measurements where Chinese hamster cells were used, indicate an expectation of about 60% thymine replacement (45).

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DISCUSSION

Schneiderman, M. The labeling protocol, irradiation, and subsequent measurements of strand breaks do not take account of cell cycle effects. Your protocol would allow you to look only at cells that can recover from radiation induced division delay.

Nikjoo, H. I do not think that cell cycle is a major factor influencing the observations for the following reasons: 1) We are not measuring strand breaks but structural chromatid-type aberrations which are not necessarily the same thing. 2) Obviously we can only observe and score these in cells that come to metaphase. 3) Mitotic delay is not, at these doses (up to 2.0 Gy), an "all or nothing" phenomenon. The mitotic index never reaches zero and cells continue to proceed, at varying rates, into the cycle, but obviously arrive at division in different order and different relative frequencies than they would have done if no dose was given. 4) In any case, we sample at intervals through G_2 in a multiple sample time regime up to 5 h post-irradiation. Immediately after irradiation we replace the medium with one containing

thymidine instead of BrUdR. This allows us to recognize S phase cells by virtue of the TT patches and bands which they carry. By 5 h post irradiation, the fraction of S cells is 50-60% at 1.5 Gy - higher (>80%) in controls. Thus post-S (G_2) and S-cells are progressing into metaphase to be scored. 5) Whilst we cannot claim to have sampled all of G_2, we obviously have cells from most, if not all, developmental stages (this, after all, is the principle of a multiple sampling regime). There is no variation in the frequency of true discontinuities or of achromatic lesions in the light or in the dark strand (BB or TB) at any sample time even though there are, as expected, differences in absolute yield of aberrations is, as expected, much lower in S-cells, so we do not have as much data. Mid-early S cells have much lower frequencies of chromatid aberrations and we have insufficient data to perform suitable tests. 6) There is, as far as I know, no evidence for interphase death in V79 fibroblasts at these dose levels for lower LET radiations (high-LET might be different). Therefore, all cells eventually "recover" from mitotic delay - but to sample the whole of G_2 would involve sampling up to probably 20 h and beyond (there are few very slow cells in V79). The period 0-6 h is the region most people sample for G_2 cells and chromatid-type aberrations. We have covered most of this - so I doubt that the few percent of G_2 cells missed are likely to make much difference.