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Modifications in Repair and Expression of Potentially Lethal Damage (α-PLD) as Measured by Delayed Plating or Treatment with β-araA in Plateau-Phase Ehrlich Ascites Tumor Cells after Exposure to Charged Particles of Various Specific Energies

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The ability of Ehrlich ascites tumor cells (EAT cells) to repair potentially lethal damage (α-PLD) as demonstrated by either an increase in survival after delayed plating or a decrease in survival after treatment with β-arabinofuranosyladenine (β-araA) was investigated after exposure to protons, deuterons, ^{3}He, ^{4}He, and heavy ions of various specific energies. A significant amount of repair or fixation was observed after delayed plating or treatment with β-araA, respectively, in cells that were exposed to protons of 6–21 MeV energy, reflecting mainly variations in the survival curve shoulder width. Four-hour treatment with 80 μM/liter β-araA resulted in an exponential survival curve for all proton energies tested. A decrease in particle energy increased killing and caused a reduction in D_q without a significant change in D_0. The survival curve obtained after exposure of cells to 3.4 MeV protons had only a small shoulder and was only slightly modified by either delayed plating or treatment with β-araA, suggesting a decrease in the induction rate of α-PLD. Similar results were also obtained after exposure to deuterons and ^{4}He ions. The results are interpreted as indicating the importance of the specific particle energy and the δ-electron spectrum in the induction of α-PLD. When the results of delayed plating of cells exposed to protons, deuterons, or helium ions were pooled, an exponential relationship between D_q and penumbra radius was indicated. After exposure to ^{40}Ar ions of 18 MeV specific energy, a shouldered survival curve was obtained, and β-araA significantly enhanced killing by modifying D_q as well as D_0, a result that also suggests induction of repairable damage by the δ particles produced and interaction of lesions induced within the core of the ion path with penumbra lesions. Based on these results a model is proposed assuming that α-PLD results from interaction, during the course of repair, of pairs of DNA lesions induced within a distance d_i. The model assumes (a) the existence of a critical separation distance d_{ic}, with the property that pairs of lesions induced with separation distance shorter than d_{ic} (expressed as number of base pairs) will always be expressed as lethal, and (b) the existence of a *maximum* separation distance d_{im},

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with the property that pairs of lesions induced with separation distance larger than d_{im} will not interact. Further, it is assumed that the interaction of lesions induced at a distance d_i such that $d_{ic} \leqslant d_i \leqslant d_{im}$ depends on the postirradiation conditions employed. It is thought that d_i decreases if cells are kept under conditions preventing proliferation (DP) and that it increases after treatment with β-araA. In both cases variation in d_i is attributed to treatment-associated alterations in chromatin structure. The observed reduction in the induction of PLD with decreasing particle energy is interpreted as an increase in the fraction of pairs of lesions induced at distances shorter than d_{ic}, and a concomitant decrease in the fraction of pairs of lesions induced at distances between d_{ic} and d_{im}. These hypothetical lesions are thought either to be located within the particle's penumbra or to arise from combinations of ionizations induced in the particle's core and its penumbra. Finally, the possibility of distinction between LET-dependent effects in D_0 and particle specific energy-dependent effects in D_q is explored.

INTRODUCTION

Radiation-induced cell killing is found to increase with increasing ionization density or linear energy transfer (LET), reaching a maximum at values of about 100–200 KeV/μM (*1–3*). It is generally assumed that this increase in effect is due to a modification in the spatial distribution of ionization events and is accompanied by a reduction in the oxygen enhancement ratio (OER), a fact that renders high-LET radiations very attractive for the treatment of human tumors. A further advantage for radiation therapy expected from the use of densely ionizing radiations is a reduction of the importance of repair reactions as usually observed at the cellular level, such as repair of potentially lethal damage (PLD) (*5–14*) or split-dose recovery (*6, 15, 16*). Repair of PLD has been demonstrated in tumors *in vivo* (*10, 17, 18*) and is considered a significant parameter in the outcome of radiation treatment. It has been suggested that the poor response to radiation of certain tumors might be due to an increased capability to repair PLD (*19–21*). It is conceivable, therefore, that information on the induction and repair of PLD after exposure to radiation of various LET might help in designing improved methods and treatment protocols using charged particles or heavy ions.

The ability of cells to repair PLD can be experimentally tested, among other methods, by delaying plating of plateau-phase cells (*22–24*) or by exposing cells after irradiation to conditions or treatments that enhance killing such as, for example, treatment in anisotonic salt solution (*25–27*) or with β-arabinofuranosyladenine (β-araA) (*28–30*). The latter treatment protocols also reveal types of potentially lethal lesions that are normally repaired in cells plated immediately after irradiation. Although all types of lesions that can be fixed by a certain postirradiation treatment and expressed as lethal are per definition potentially lethal (*31*), evidence has been provided that different treatments may affect different types of lesions (*32, 33*). In plateau-phase Chinese hamster V-79 cells, postirradiation treatment in hypertonic medium or β-araA, a DNA polymerase inhibitor (*35–37*), was found to affect different types of potentially lethal damage. This distinction was based on differences in the sensitivity of lesions to each treatment and to differences in their repair rates as well as in the way their fixation affected the survival curve parameters (*33, 34*). The term α-PLD has been proposed for the β-araA-sensitive sector of damage whose repair takes place with a $T_{1/2}$ of 1–2 h and leads to the formation of the survival curve *shoulder*. It is distinguished from β-PLD by the specific sensitivity of the latter to treatment in

anisotonic medium as well as by its effect on the survival curve *slope* and the fast repair kinetics ($T_{1/2}$ = 15 min). Furthermore, α-PLD shows repair kinetics similar to those observed in split-dose recovery experiments, suggesting the possible involvement of similar molecular lesions for both cellular repair processes (*33*). Furthermore, it has been proposed that fixation of α-PLD at the G_1/S border and mitosis may cause the survival variations observed through the cell cycle (*32*). It is likely, therefore, that α-PLD comprises molecular lesions underlying fundamental cellular repair reactions and possibly involved in the biochemical action of certain drugs on cell survival as well as in the variation of sensitivity through the cycle (*32–34*).

It is not known to what extent induction and repair of α-PLD are affected by radiation quality, although information about their modification as a function of LET or particle specific energy could be of interest. It is striking that PLD repair-deficient human AT-cells show a response similar to that of normal human fibroblasts at high LET, thus suggesting involvement of PLD in the RBE–LET relationship (*38*).

Results published recently indicate that repair of PLD, as demonstrated by either delayed plating or treatment with β-araA (α-PLD), cannot be observed after exposure of plateau-phase EAT cells to heavy ions in the range between 1 and 10 MeV/amu (*11*). The same results indicated an influence of the specific ion energy (energy per nucleon) in the inactivation cross section. It was therefore asked whether the observed lack of PLD repair is related to the particle specific energy rather than LET.

To answer this question, results derived from the same biological system after exposure to charged particles of low z-values (z = 1 to 2) and low specific energy (E = 1–20 MeV/amu) are needed, thus covering the range of LET below the maximum of the RBE–LET effect curve (*39*). Irradiation of cells with particles of low z-values may enable, similar to heavy ion exposure, separation between the biological effects resulting from the primary track (core) and those resulting from δ rays (secondary electrons), particularly in cases where the LET of the dose fraction delivered in the core differs considerably from that delivered by the secondary electrons. For example, in the case of proton beams above 3 MeV energy the ionization density in the core is between 1 and 5 KeV/μM, the δ electrons reaching ionization densities up to 30 keV/μM at the end of their range (*40*). High-energy δ rays are also of interest because they deposit energy into a volume (defined by the tracks of knock-on electrons protruding from the core (*41*)) of considerable dimension (penumbra volume). This property allows experiments designed to study the existence of target molecules within this volume; it is possible that potentially lethal lesions are predominantly induced within or in combination with the penumbra, whereas lethal lesions are more likely induced within the track core in an LET-dependent way (*8*).

In the present report we describe experiments designed to study induction and repair of α-PLD after exposure of plateau-phase Ehrlich ascites tumor cells (EAT cells) to protons, deuterons, ^{3}He, and ^{4}He of various energies. Induction and repair of α-PLD is demonstrated either by an increase in survival after delayed plating (6 h) or by a decrease in survival after treatment with β-araA. The results show a significant decrease in the fraction of cells sustaining only potentially lethal damage as the specific energy of the particle decreases and suggest that this physical parameter may seriously affect the induction rate of α-PLD.

MATERIALS AND METHODS

For all experiments, a line of Ehrlich ascites tumor cells growing in suspension was used (*42*). Cells were subcultured daily at an initial concentration of 2×10^5 cells/ml (50 ml per flask) in A2 medium supplemented with 20% horse serum (GIBCO). Details on the culture conditions and the composition of the medium have been published (*23*). To obtain cells in the plateau phase, a suspension was prepared (10^5 cells/ml) from cells obtained from the subculture and was distributed, in 5-ml portions, in 60-mm petri dishes (Greiner, W. Germany). Cells were growing at 37°C in an atmosphere of 6% CO_2 in air with a generation time of 11 ± 1 h; they reached a plateau after 3 days at a concentration of $2.0 \pm 0.2 \times 10^6$ cells/ml. At this stage approximately 70% of the cells were in a phase with a DNA content equivalent to that of G_1 cells, 10% with that of G_2 cells, and 20% with that of S-phase cells as revealed by flow cytometry measurements (*24*). Cultures reached plateau phase due to exhaustion from the medium of one or more of the essential nutrients (unfed plateau-phase cultures).

For irradiations, plateau-phase cells were concentrated to 2×10^7 cells/ml and were layered on a 0.22 μm pore size membrane filter (Millipore, 50 μl per filter (*11*)). They were exposed to various ions after the medium had been completely absorbed by the agar background on which the filters were kept. For the preparation of the agar used to keep the filters moist, as well as for all other manipulations, conditioned medium (C-med) obtained from replicate plateau-phase cells after filtration was used. This precaution was taken to prevent possible modification in the physiological state of the cells that could interfere with the cellular repair reactions measured. This series of manipulations caused a slight decrease in plating efficiency to 50–60% from the normal 70–80%. In control experiments it was established that this decrease in plating efficiency did not alter the radiation response or the repair ability of the cells.

After irradiation cells were either resuspended in fresh medium, diluted, and plated as previously described (*24*) (immediate plating, IP), or resuspended in C-med and kept for 4–6 h at 37°C before plating to allow repair of potentially lethal damage. Alternatively, to measure fixation of PLD, cells were incubated after irradiation for 4 h in C-med in the presence (10–80 μM) of β-araA (Sigma, Munich), obtained from a 10 mM stock solution prepared in distilled water. β-araA caused a decrease by 10–20% in plating efficiency which was taken into account in the calculation of the surviving fraction. β-araA was very toxic (decrease in PE to 20% or less) if applied to cells that had not reached plateau phase at the time of the experiment. This occurred in the few experiments where the usual growth pattern was not obtained. In this case the results were not analyzed.

Irradiations were carried out at the cyclotron facility of the German Cancer Research Center in Heidelberg (DKFZ). Protons, deuterons, and helium ions at energies between 5 and 20 MeV/amu were used. Details on radiation setup and dosimetry have been published (*43*). Control experiments using groups obtained from a cobalt unit (also located at DKFZ) were periodically performed to monitor the repair ability of the cells during the period in which this work was carried out (over 2 years). No significant modification in the radiation sensitivity or repair ability of the cells was observed.

The type of particles obtained in each radiation facility, the effective nuclear charge (Z_{eff}) calculated according to the formula given by Bichsel (*40*), the parameter Z_{eff}/β^2, the mean restricted LET ($L_{T,100}$), the mean dose averaged lineal energy ($\bar{y}_D$), and the radius of the penumbra (R_{pen}) (*40*) are given in the Table I. The data for restricted LET and lineal energy have been obtained from various sources (*44–47*). Values for $\bar{y}_D$ have not been published for heavy ions in the energy range used. Protons were available with an initial energy of 21 MeV and deuterons with an initial energy of 10 MeV. To reduce proton energy, aluminum foils of various thickness were inserted between the ionization chambers and the cultures under exposure. From the ratio of the charges Q_1 and Q_2 measured in the chambers, the position in the Bragg curve as well as the resultant stopping power could be calculated as reported (*43*). ^{3}He and ^{4}He were available at 28 and 22 MeV, respectively. This energy was that measured before exit of the particles from the vacuum tube and was reduced by the ionization chambers and the air to 26 and 17.6 MeV, respectively. The particle energy was reduced in this case with mylar foils of various thickness, and the position in the Bragg curve and the resultant stopping power were calculated as described for protons (*43*). In one experiment cells were exposed to argon ions delivered from the heavy ion accelerator UNILAC at GSI Darmstadt with specific energy $E = 18$ MeV/amu. The table also shows the mean restricted LET and the mean dose average linear energy for ^{60}Co γ rays.

The survival curve shoulder width D_q is used throughout this paper as a parameter describing the amount of potentially lethal damage repair taking place after exposure to the individual particles and particle energies as described in Table I. This was found to be a satisfactory first approximation for the interpretation

TABLE I

Physical and Radiobiological Parameters of the Radiations Used in EAT Cell Experiments

Radiation type	*Facility*	Z_{eff}	Z^2_{eff}/β^2	$\bar{L}_{T,100}$ *(keV/μm)*	$\bar{y}_D$	*RBE (initial slope)*	*Shoulder length delayed plating (Gy)*	R_{pen} *(μm)*
^{60}Co, γ	DKFZ	—	—	0.22	1.5	1.0	3.0 ± 1.0	—
Protons, 21 MeV	DKFZ	1.0	24	1.4	4.2	1.3	2.6 ± 0.2–0.6	5.8
Protons, 11 MeV	DKFZ	1.0	42	2.4	5.7	1.9	2.0 ± 0.5	2.4
Protons, 6.4 MeV	DKFZ	1.0	74	3.6	8.6	2.6	1.7 ± 0.3–0.7	1.2
Protons, 3.4 MeV	DKFZ	1.0	143	5.0	11.8	3.9	1.1 ± 0.35	0.5
Deuterons, 5 MeV/n	DKFZ	1.0	94	4.4	9.9	3.1	1.8 ± 0.5	0.9
^{3}He, 8.6 MeV/n	DKFZ	2.0	222	11.2	~24	6.0	1.7 ± 0.45	1.7
^{3}He, 5.0 MeV/n	DKFZ	2.0	378	17.3	~37	8.3		0.9
^{3}He, Bragg P.	DKFZ	1.9	2,900	70	~125	7.8		0.03
^{4}He, 4.4 MeV/n	DKFZ	2.0	425	19	~41	8.9	1.0 ± 0.4	0.7
^{4}He, 2.9 MeV/n	DKFZ	2.0	650	27	~51	10.3		0.4
^{4}He, 1.4 MeV/n	DKFZ	1.98	1,340	45	~70	13.6	0.5 ± 0.3	0.15
^{40}Ar, 18 MeV/n	GSI	17.4	8,120	450			$(1.0 \pm 0.5) \times 10^6$	4.6
^{84}Kr, 2.9 MeV/n	GSI	22	79,000	2910			$(0.0 + 0.3) \times 10^6$	0.4
^{238}U, 6.5 MeV/n	GSI	48	170,000	7610			$(0.8 \pm 0.5) \times 10^6$	1.2

Note. Listed are the type of radiation used in EAT cell experiments; the radiation facility (DKFZ = Deutsches Krebsforschungszentrum Heidelberg, FRG; GSI = Gesselschaft fur Schwerionenforschung Darmstadt, FRG); the effective particle charge $Z_{eff} = Z \cdot (1 - \exp(-0.95\, v_r))$, where Z is the atomic number of the particle, v_r is the relative velocity given by $v_r = v/v_0\, \bar{Z}^{2/3}$, v is the particle velocity, and $v_0 = e^2/h$ the average velocity of the H-electron (see Ref. (*12*)); the parameter Z^2_{eff}/β^2 with $\beta = v/c$; the track average restricted linear energy transfer $L_{100,T}$; and the mean linear energy $\bar{y}_D$, as obtained from measurements in spheres of 1 μM diameter (see Ref. (*61*)); the relative biological effectiveness (RBE) of initial slope values of delayed plating survival curves (with reference to ^{60}Co γ rays, details of RBE measurement in Ref. (*8*)); the shoulder length of delayed plating survival curves; and the penumbra radius R_{pen} of the particle's track according to calculations by Chatterjee and Magee (*41*). $\bar{y}_D$ values also were obtained partly by the relationship $\bar{y}_D = 9.8\bar{L}_D$, with L_D the dose averaged LET and therefore regarded as approximate only.

of the results since neither delayed plating nor treatment with β-araA significantly affected D_0 (except for argon irradiation), provided cultures had reached plateau phase and β-araA toxicity was moderate (less than 30% decrease in plating efficiency).

RESULTS

Proton and Deuteron Irradiations

Figure 1 shows survival curves of cells exposed to 21 MeV protons and plated either immediately (IP) or 6 h later (DP). Also shown in the figure are survival curves obtained after a 4-h postirradiation incubation with 10 and 60 μM β-araA. As evident from the results, plateau-phase EAT cells are able to repair PLD if plating is delayed. This repair activity is manifested mainly by an increase in the shoulder width without a significant effect on the slope, a result different from that reported for other cell lines (*22*). Incubation with 10 μM β-araA prevented this repair ability, but in addition it also fixed damage normally repaired in cells plated immediately after irradiation. This fixation of PLD (α-PLD) was also manifested by a decrease in D_q, with D_0 chang-

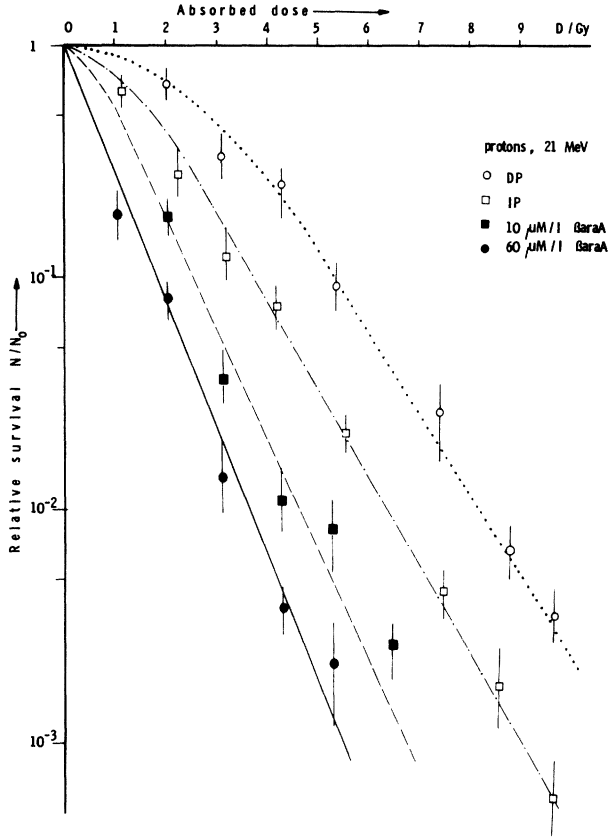

FIG. 1. Survival curves of EAT cells exposed to various doses of 21 MeV protons and plated either immediately (IP, open squares), after 6 h incubation in the plateau phase (DP, open circles), after 4 h treatment with 10 µm/l β-araA (solid squares), or after 4 h treatment with 60 µm/l β-araA (solid circles).

ing only slightly; it was even higher after incubation with 60 μM β-araA, and the resulting survival curve was essentially exponential. Due to the low cell concentration in these experiments ($1-2 \times 10^5$ cells/ml), significantly lower amounts of β-araA were needed to remove the shoulder compared to those required for cells treated in the plateau phase at their final density without further manipulations (*28–30*). The dependence of the β-araA effect upon cell concentration is a known property of the drug and has been discussed (*28*). The results indicate that a significant proportion of the damage inflicted by 21 MeV protons in EAT cells is only potentially lethal, it can be fixed by β-araA, and its repair causes the formation of the survival curve shoulder.

Figure 2 shows survival curves of cells exposed to various energy protons, obtained as described under Materials and Methods, and plated *immediately* after irradiation. Reduction in particle energy from 21 MeV originally to 11.3 MeV resulted in a potentiation of cell killing mainly reflected by a change in the survival curve shoulder width. This effect was even more pronounced after exposure to 6.2 MeV protons,

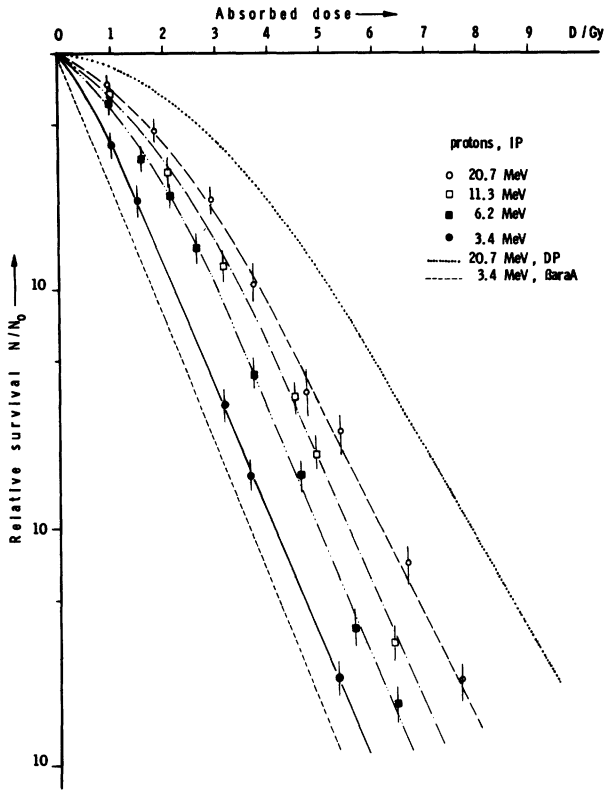

FIG. 2. Survival curves of cells exposed to various doses of protons of various energies as indicated. Survival curves obtained after delayed plating of cells exposed to 20.7 MeV protons or β-araA treatment of cells exposed to 3.4 MeV protons are shown for comparison (dotted lines).

and exposure to 3.4 MeV protons resulted in a survival curve with only a small shoulder (D_q = 0.2 ± 0.2 Gy). Also drawn in the figure are the survival curves obtained after delayed plating and after treatment with 60 μM β-araA for comparison. Thus a significant increase in the radiation effect was observed after a moderate increase in LET which was approximately 1.4 KeV/μM for 20.7 MeV protons and increased to 10 KeV/μM for 3.4 MeV protons (see Table I), a range usually covered by X rays generated at various tube voltages (*48*). The survival curve obtained after exposure to 3.4 MeV protons was only slightly different from that obtained after exposure to 20.7 MeV protons and treated with β-araA (60 μM, 4 h), suggesting a reduction in the induction of α-PLD, which, due to the small change in LET, could be tentatively attributed to a different parameter of the particle, determined by its energy.

To test the hypothesis that the observed reduction in D_q with decreasing particle energy was due to a reduction in the induction rate of α-PLD, cells were exposed to various doses of 21 and 3.4 MeV protons and plated either 6 h after irradiation (DP) or after 4 h incubation with 60 μM β-araA. The results obtained are shown in Fig. 3. Similar to the results shown in Fig. 1, delayed plating of the cells caused an increase and treatment with β-araA caused a decrease in D_q. It is of interest, however, that

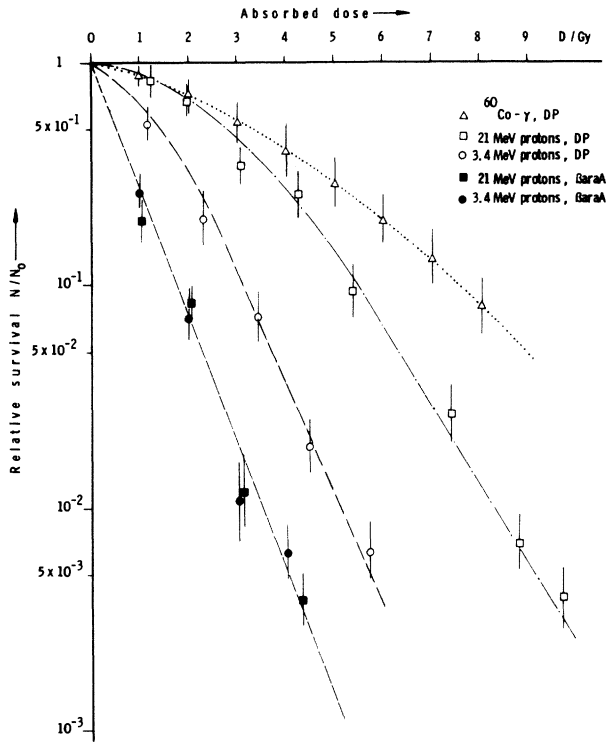

FIG. 3. Survival curves obtained after delayed plating or β-araA treatment of cells exposed to various doses of 21 and 3.4 MeV protons as indicated. The open triangles show a survival curve obtained with cells delayed-plated after exposure to ^{60}Co γ rays.

fixation of α-PLD caused by β-araA resulted in the same survival curve independent of the particle energy. This result supports the assumption that with decreasing particle energy smaller amounts of α-PLD are induced. The survival curve of cells exposed to ^{60}Co γ rays and plated 6 h later is also plotted in the figure.

In Fig. 4 a set of results, similar to those of Fig. 1 but obtained after exposure of cells to 10.2 MeV deuterons (specific particle energy E = 5 MeV/amu) is shown. Here again, repair and fixation of PLD taking place after delayed plating and treatment with β-araA, mainly affected D_q with only a small effect on D_0. Treatment with 60 μM β-araA resulted in an exponential survival curve. Thus α-PLD again appears to be involved in the formation of the survival curve shoulder after exposure to deuterons and to be affected mainly after delayed plating.

Helium Irradiations

Results obtained when plateau-phase EAT cells exposed to 5.3 MeV helium ions were subsequently plated either immediately (IP) or after a 6-h incubation in the plateau phase (DP) are shown in Fig. 5. Also shown are the results obtained after incubation for 4 h with 60 μM β-araA.

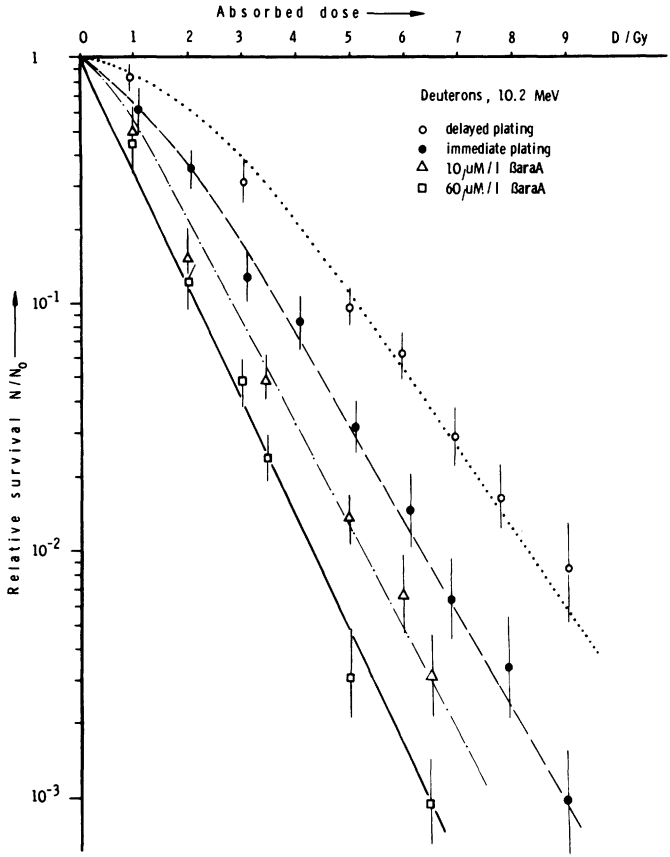

FIG. 4. Survival curves of cells exposed to various doses of 10.2 MeV deuterons and plated either immediately (IP, solid circles), after 6 h incubation in the plateau phase (DP, open circles), after 4 h treatment with 10 μM β-araA (open triangles), or after 4 h treatment with 60 μM β-araA (open squares).

The survival curve of cells plated immediately after irradiation was almost exponential ($D_q = 0.15 \pm 0.2$ Gy). Similar to the results obtained with low energy protons, delayed plating caused only a small increase in cell survival mainly reflected by an increase in D_q. Treatment with β-araA eliminated the shoulder from the survival curve but caused a small increase in D_0 as well ($D_0 = 0.42 \pm 0.03$ Gy).

The survival curves of cells exposed to ^{4}He ions of various energies and plated immediately after irradiation are shown in Fig. 6. Also shown in the figure are the delayed plating curve after exposure to 17.6 MeV ^{4}He and the β-araA treatment curve after exposure to 5.3 MeV and 60 μM β-araA. Decrease in particle energy mainly caused a decrease in D_q. In this experiment, exposure of cells to 5.3 MeV ^{4}He resulted in a survival curve very similar to that obtained after treatment with β-araA. The variations in the β-araA effect observed in Fig. 5 vs Fig. 6 may be due to variations in the distribution of the cells in the cycle at the time of irradiation, as has been briefly outlined under Materials and Methods. Similar to the results in Fig. 5, delayed plating caused only a small increase in survival and affected mainly the shoulder width.

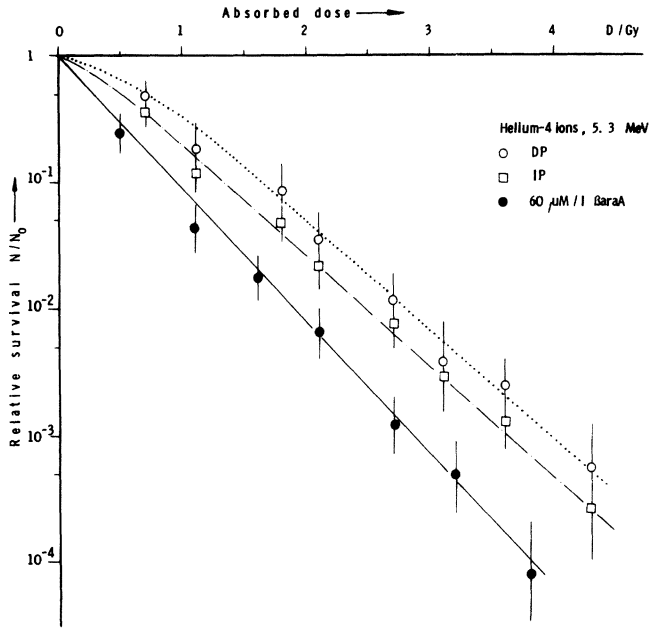

FIG. 5. Survival curves of cells exposed to various doses of 5.3 MeV ^{4}He ions and plated either immediately (IP, open squares), after 6 h incubation in the plateau phase (DP, open circles), or after 4 h treatment with 60 μM β-araA (solid circles).

In Fig. 7 survival curves obtained after exposure of cells to ^{4}He ions at 17.6 or 5.3 MeV and plated either 6 h after irradiation or after a 4-h treatment with β-araA are shown. β-araA eliminated in both cases the shoulder of the survival curve; it did not significantly affect the D_0 after exposure to 17.6 MeV ions ($D_0 = 0.55 \pm 0.05$ Gy) but it slightly decreased it after exposure to 5.3 MeV ions ($D_0 = 0.45 \pm 0.05$ Gy) (open circles vs closed circles).

A compilation of results obtained after exposure of cells to ^{3}He ions at various energies and postirradiation conditions is shown in Fig. 8. The results obtained with cells plated immediately after irradiation indicate an increase in killing with decreasing ion energy from 26 to 15 MeV, followed by a decrease again for cells irradiated near the Bragg peak position. It is assumed that this decrease in effectivity at low particle energy is due to molecular overkill, i.e., to a limited number of lesions available (a term proposed by J. Kiefer, GSI Scientific Report 1985, GSI 86-7, p. 246). Delayed plating after exposure to 26 MeV ^{3}He ions resulted in an increase in survival and caused mainly an increase in shoulder width. There is a significant increase in LET with decreasing ^{3}He ion energy ranging from 11.2 KeV/μM for 26.0 MeV ions to 70 KeV/μM for ions near the Bragg peak. Thus, for exposures to ^{3}He and ^{4}He ions, D_q is primarily affected by the specific energy while D_0 is more closely related to LET (*8*).

^{40}Ar Irradiations

In Fig. 9 the results obtained after exposure of cells to 18 MeV/amu ^{40}Ar ions and plated either immediately, after a 6-h incubation in the plateau phase, or after a 4-h

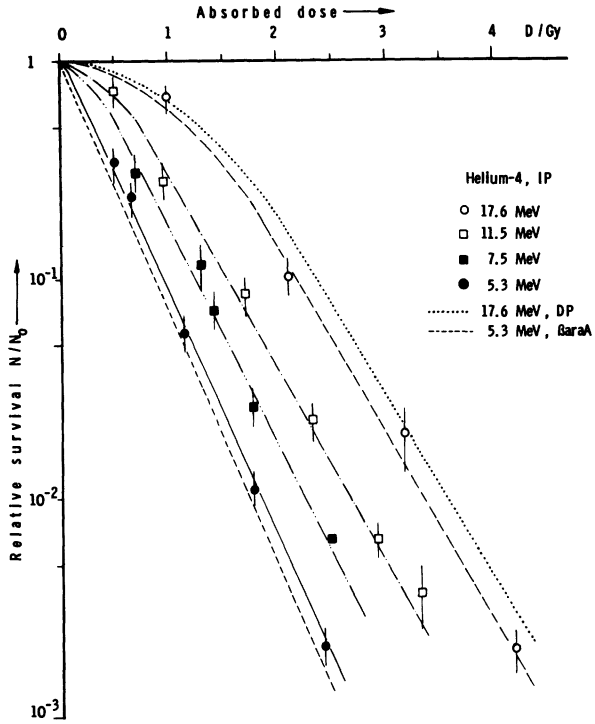

FIG. 6. Survival curves of cells plated immediately after irradiation with ^{4}He at various doses as indicated. Survival curves obtained after delayed plating of cells exposed to 17.6 MeV ^{4}He, or β-araA treatment of cells exposed to 5.3 MeV helium are shown for comparison.

treatment with β-araA are shown. Although the mean LET is well above 100 KeV/μM (see Table I) and thus beyond the maximum in the LET–RBE effect curve (*8*), a small but significant shoulder was observed as well as a significant potentiation of killing by β-araA application. The inset in the figures shows the survival of immediately plated cells at a fluence $F = 10^7$ particles/cm^2 and various thicknesses of absorber foil (Mylar) inserted between the beam exit window and the irradiated cells. Survival increased from 10% at $d = 0$ μM to almost 70% when the foil was thick enough to absorb the beam completely. The residual killing (about 30%) observed, if not due to biological variability, may result either from energetic δ rays and particles from nuclear reactions originating in the absorber or from straggling in the primary particle beam. Further experiments are required to establish the contribution of each of the above-mentioned effects.

The observed reduction in survival suggests that (a) the probability of an effect per track decreases with increasing LET, if straggling can be considered to be negligible, and (b) the response should be in the saturation of the RBE–LET relationship. Thus PLD appears to be induced, repaired, or fixed, depending on the postirradiation conditions which cannot be attributed to the ionization density within the primary ion track but rather to the relatively large specific energy, which is well above the values used in helium-ion irradiations. ^{238}U ions at an effective energy of 6.5 MeV/amu

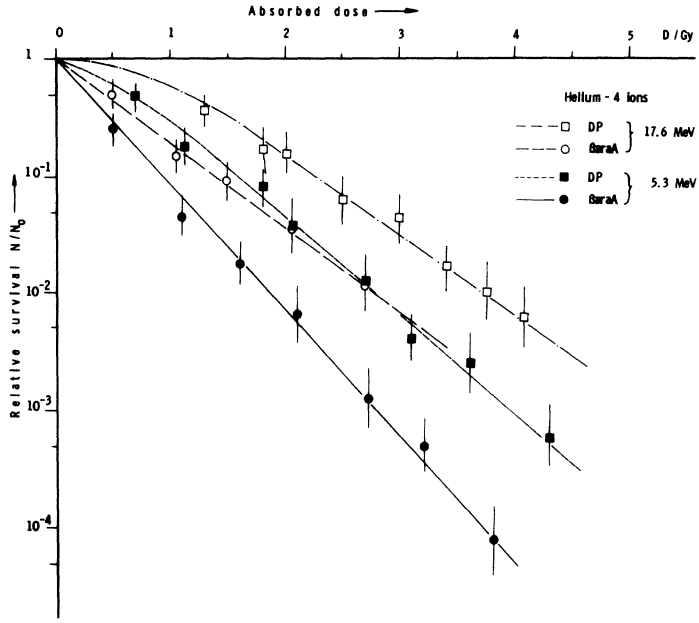

FIG. 7. Survival curves obtained after delayed plating or β-araA treatment of cells exposed to various doses of 17.6 or 5.3 MeV ^{4}He ions as indicated.

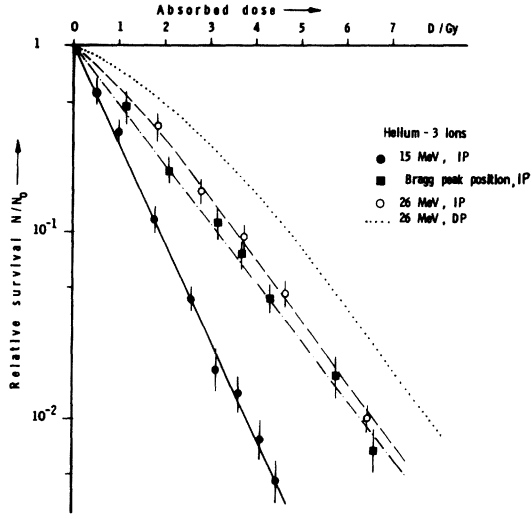

FIG. 8. Survival curves obtained after immediate plating of cells exposed to various doses of ^{3}He ions at 26 MeV, 15 MeV, or near the Bragg peak position. The dotted line shows delayed-plating survival of cells exposed to 26 MeV ^{3}He ions for comparison.

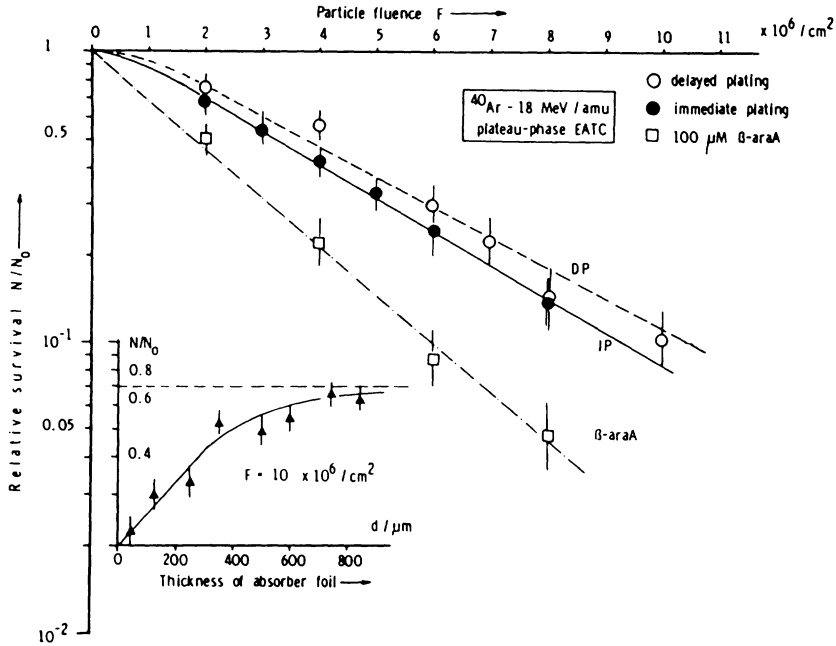

FIG. 9. Survival curves of cells exposed to various particle fluences of ^{40}Ar ions at 78 MeV/amu specific energy and plated either immediately (IP, solid circles), after 6 h incubation in the plateau phase (DP, open circles), or after 4 h treatment with 100 μM β-araA (open squares). Inset shows cell survival (IP) for various thicknesses of absorber foil.

showed only low induction and repair of PLD, whereas no evidence for repair of PLD was obtained with heavy ions of specific activity below 5 MeV (*11*) (see also Table I).

Repair Capacity and Specific Particle Energy

The results presented in the previous paragraphs show a predominant involvement of the shoulder width as the parameter modified when either repair of PLD, as observed after delayed plating, or fixation of PLD, as caused by incubation with β-araA, took place. Small variations in slope observed after exposure to helium ions can be at least partly attributed to biological variations and were not observed to the same extent in every experiment; the significant changes in slope after exposure to argon ions and treatment with β-araA, on the other hand, may be due to peculiarities in the inactivation mechanism of this type of radiation.

It has been shown previously (*28, 29, 33, 34*) and is also suggested by the results reported here, that after exposure of cells to sparsely ionizing radiation β-araA-mediated fixation reaches its maximum at survival levels corresponding to an exponential line. It is reasonable, therefore, to consider the shoulder width of the survival curve, as obtained after delayed plating as a measure of the total amount of PLD repaired. Since this type of PLD is sensitive to β-araA it can be classified as α-PLD based on the criteria discussed elsewhere (*33, 34*). Because D_q and therefore also the amount

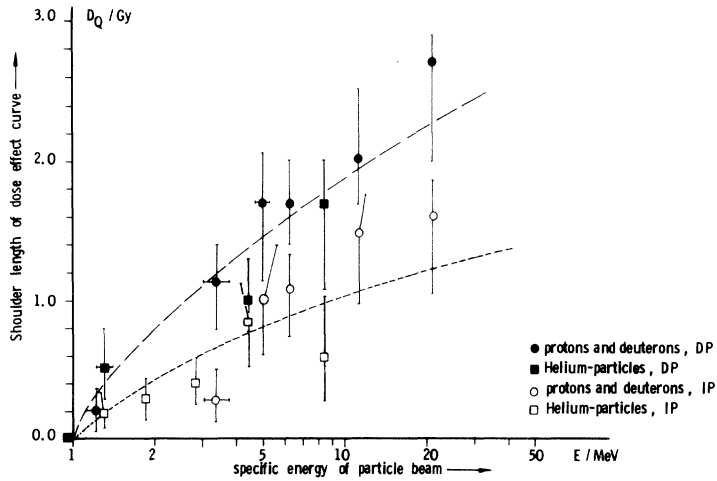

FIG. 10. Survival curve shoulder width D_q for various specific particle energies and treatment conditions as indicated. Lines drawn do not imply functional dependence and are shown only to indicate tendencies.

of α-PLD induced and repaired were found to strongly depend on particle energy, we tried to establish a possible relationship with the specific particle energy or the penumbra radius. The data, extracted from the results shown in previous figures, are summarized in Figs. 10 and 11. A tendency for D_q to increase with increasing specific energy both in cells plated immediately (IP) and in cells plated several hours after irradiation (DP) is observed (Fig. 10). It is not possible from these results, however, to draw any conclusions as to the functional dependence of D_q on specific energy. A clearer picture arises when D_q is plotted as a function of the penumbra radius R_{pen} as shown in Fig. 11 for the results obtained after delayed plating. The data points in the log–linear

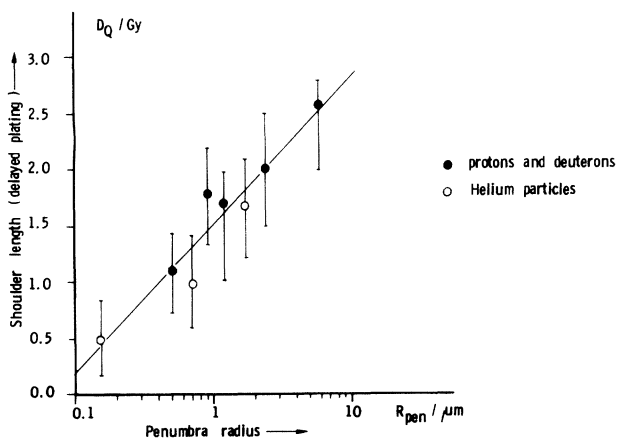

FIG. 11. Shoulder width of survival curves obtained with delayed-plated cells after exposure to protons, deuterons, or ^{4}He particles. The line was obtained by linear regression of D_q and ln R_{pen} values; it indicates zero shoulder width at $R_{pen} = 0.073\ \mu M$ corresponding to 0.85 MeV specific energy and agrees with previously published results (*58*).

plot can be reasonably fitted by a straight line, suggesting a logarithmic dependence of D_q and R_{pen} and thus of the amount of α-PLD induced with increasing penumbra radius.

Calculations indicated that velocity spreading and the resulting LET straggling were negligible for deuterons and helium ions of all energies used in this work. Similar results were also obtained with high energy protons. Some variation was found for 3.4 MeV protons estimated to result in straggling in LET from 7.9 to 13.1 KeV/μM ($L = 10.54 \pm 2.6$ KeV/μM). This resulted in an uncertainty in the calculation of the penumbra radius which was found to vary from 0.3 to 0.9 μM with a mean value of 0.5 μM. This variation, however, does not significantly affect the interpretation of results offered in the next section.

DISCUSSION

The results presented in the previous section indicate a reduction in the induction of α-PLD with decreasing particle energy. This reduction occurred within a narrow range of restricted LET values after exposure to protons (between 1 and 5 KeV/μM; full LET values L_∞ range from 2 to 10 KeV/μM) and suggests that the specific energy of the particle with its associated penumbra may considerably affect the rate of induction of PLD (see also Fig. 10). Similar conclusions were also drawn after exposure of cells to ^{4}He (LET between 19 and 45 KeV/μM) and deuterons. It is necessary, therefore, that theoretical models of radiation action used to fit similar results consider the character and the density of ionizations within the track, as for example that proposed by Chatterjee and Magee (*41*).

The experimental protocols used in this work focused on changes in response to variation of particle energy of a form of PLD associated with the survival curve shoulder width and the increase in cell survival observed after delayed plating (α-PLD). Other forms of PLD uncovered by different postirradiation treatments may show different responses, as indicated by the results obtained after exposure of Chinese hamster V-79 cells to neutrons. In this case similarities were established between α-PLD and damage involved in the interaction effect observed after exposure to mixed neutrons and γ irradiation but not for β-PLD (*49*).

A decrease in D_q with increasing ionization density has been observed with a number of mammalian cell lines (*13, 38, 52*) and was attributed to modifications in the induction of sublethal damage. Theoretical and experimental evidence support the hypothesis, however (*53, 62*), that the formation of the survival curve shoulder can also be attributed to repair of potentially lethal damage (α-PLD).

Considerable evidence has been provided indicating a significant contribution of δ rays in the cellular response at high ionization densities (*54–56*). Delta rays, similar to fast electrons, deliver only a minor part of the dose at ionization densities above 10 KeV/μM (*48*) and may therefore induce predominantly repairable lesions (*8*). Results reported by other investigators also suggest, directly or indirectly, the importance of penumbra properties on the ability of the cells to repair. For example, a significant amount of split-dose recovery was observed after exposure of cells to 10 MeV/amu helium and lithium ions, but no repair could be observed after exposure to boron and carbon ions at energies less than 10 MeV/amu (*59*). No difference in

PLD repair was observed between cells exposed to Bragg peak carbon ions (median specific energy approximately 100 MeV/amu, well above the value where saturation of the repair capacity is expected) and X rays (*10*); no difference in the kinetics of PLD repair could be established in EMT6 cells irradiated either with ^{60}Co γ rays or with 160 MeV/amu helium ions (mean LET about 10 KeV/μM) (*14*), and, except for 8.3 MeV/amu argon, PLD was usually observed in 10T1/2 cells exposed to heavy ions at energies above MeV/amu (*60*). Furthermore, detailed analysis of results obtained with human T-1 cells exposed to carbon, neon, and argon beams at different positions in the plateau and the extended Bragg peak region of the beam indicated that at least three variables, i.e., particle fluence, velocity, and charge, are required to describe the observed biological response (*54*).

The type of damage induced by δ rays may be different from that induced in the core of a charged particle. This has also been suggested by the results obtained at the chromosomal level after exposure to V-79 cells to heavy ions (Fe ions, 13 MeV/amu, L = 2100 KeV/um; U ions, 3.4 MeV/amu, L = 140 KeV/um).[1] Since, along with the typical observations such as breaks and exchange aberrations, chromosome disintegration was also observed, the induction of bulky lesions in the particle core was proposed. This finding can be explained by the widening of the track close to the dimensions of chromatin at higher particle charges (*57*) thus inducing damage at extended parts of the DNA.

Unlike the clear separation of track core and penumbra effects after exposure of EAT cells to heavy ions (*11*), a clear separation of effects after exposure to protons and ^4He ions is not as straightforward due to partial overlapping of the energy distribution of energy deposition events in the core and the δ-ray segment. The results presented, however, suggest that a difference in the effect may exist and determine, to a considerable extent, the response of cells to these types of radiations.

For an interpretation of the effects observed in the induction and repair of PLD as a function of the particle energy, a set of hypotheses has been formulated about the nature of α-PLD. They will be discussed here only briefly; a more elaborate description including a mathematical analysis and a comparison with existing models of radiation action will be presented in a later paper.

The basic assumption of this interpretation is that radiation induces a type of damage (other types of damage are not excluded but are not considered in this analysis) involving interaction of pairs of DNA lesions induced within a distance d_i (similar arguments can also be developed for clusters of lesions induced at mean distance d_i apart, but the discussion will be confined to pairs for simplicity). The interaction is understood as spatial overlapping of the DNA segments that undergo structural modifications simultaneously (unwinding, digestion, polymerization, etc.) in the course of repair of *each* individual lesion. As a result of this interaction of lesions destabilization is assumed to be caused in the DNA, inducing permanent alterations in the molecule (e.g., strand separation) and giving rise to chromosomal aberrations (*50, 51*), finally leading to loss of proliferative capacity of the cell.

[1] W. Muller, Chromosomenaberrationen der Chinesischen Hamster-Zellinie V79 nach Rontgen-und Schwerionenbestrahlung. GSI Report 85-3, 1985.

The parameter d_i corresponds to the linear separation (e.g., number of base pairs) between the lesions in the DNA. The fraction of pairs of lesions induced with a linear separation d_i is assumed to be a function of the physical properties of the radiation used. We propose the existence of a critical separation distance d_{ic} between lesions, defined entirely by the *biological* characteristics of the system, with the following property: pairs of lesions with DNA separation distance shorter than d_{ic} will be expressed as lethal, even under the most favorable conditions for PLD repair, whereas pairs of lesions with a separation distance larger than d_{ic} but shorter than d_{im} (d_{im} is the maximum distance between lesions where interaction is still possible under the postirradiation conditions employed) will be potentially lethal (α-PLD in this set of experiments). Potentially lethal damage will be successfully repaired if the cell is kept under postirradiation conditions that prevent interaction between the individual lesions, i.e., by preventing cell cycle progression or altered metabolism-associated structural chromatin alterations. Minimum interaction (i.e., expression only between pairs of lesions separated by d_{ic} or less), and therefore maximum cell survival, will be expected under conditions preventing cell cycle progression-associated structural chromatin alterations or DNA functions (e.g., transcriptions, replication, etc.) as, for example, in cells maintained in the plateau phase of growth after irradiation. When cells are plated in fresh medium after irradiation lesions, except for those closer than d_{ic} or further apart than d_{im} ($d_{ic} \leqslant d_i \leqslant d_{ia}$ where $d_{ia} \leqslant d_{im}$), they may interact and result in the survival levels observed after immediate plating. It is assumed that this additional interaction of lesions between d_{ic} and d_{im} is due to structural alterations occurring in the DNA as a result of the initiation of activities associated with cell progression. Postirradiation incubation of cells with β-araA further increases the effective distance at which interaction between pairs of lesions is possible ($d_{ic} \leqslant d_i \leqslant d_{im}$). This is due to the inhibition by β-araA of DNA polymerization (*36–38*), without affecting other repair-related enzyme activities (e.g., exonuclease, topoisomerase activities, etc.). In this way, amplification of DNA denaturation is likely to increase the segment of DNA structurally affected and, correspondingly, the number of lesions interacting.

Evidence for this possibility has recently been obtained in experiments designed to study the effect of β-araA on radiation-induced chromosome damage, using the premature chromosome condensation technique in plateau-phase CHO cells. Except for inhibition in the rejoining of chromosome breaks, irradiated chromosomes in the presence of β-araA showed some elongation and extensive denaturation, particularly close to the broken ends, compared to unirradiated but β-araA-treated controls, or to irradiated but not β-araA-treated samples (G. Iliakis and Pantelias, unpublished observations). Such pairs of interacting lesions can be induced within the track core or the penumbra of charged particles, but also by combination between ionizations induced in the track core and the penumbra.

We propose that the observed decrease in the induction of PLD with decreasing particle energy observed in our experiments was due to an increase in the fraction of pairs of lesions induced at distances shorter than d_{ic}, and therefore expressed as lethal, and to a concomitant decrease in the fraction of pairs of lesions induced at distances between d_{ic} and d_{im} (i.e., a decrease in the amount of PLD induced). Since the observed reduction of PLD induced with decreasing particle energy correlated well with

changes in the penumbra radius (rather than in LET), it is proposed that the hypothetical pairs of lesions are located mainly within the particle's penumbra, or they arise from combinations of ionizations induced in the particle's core and its penumbra.

In summary: It has been observed that exposure of cells to heavy ions of energies higher than a few MeV induce a δ-ray or penumbra-associated repairable damage component. The results presented here also suggest that similar phenomena may be involved in cell inactivation caused by protons, deuterons, and helium ions, affecting the induction of lethal vs potentially lethal lesions (α-PLD). These observations may be important in the evaluation of the expected benefit by the clinical application of protons or heavy-ion beams.

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