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Proliferation of Lethally Damaged Mammalian Cells Irradiated by Fast Electrons, Fast Neutrons, α Particles, and Pions

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Proliferation of lethally damaged tumor cells was determined after irradiation with fast electrons, fast neutrons, α particles, and pions in the peak and plateau of the depth dose curve. The method used was measurement of the proliferation of the total population, N_{tot}, and the subpopulation of viable cells which are able to grow to macrocolonies after irradiation, N_m. The difference between these populations ($N_{tot} - N_m$) then represents the lethally damaged cells. The number of cell divisions after irradiation decreases with increasing absorbed dose in the same way for all the different types of radiation if related to the survival of cells, S. Consequences for the type of lethal radiation damage in mammalian cells and for radiotherapy are discussed.

INTRODUCTION

Quantitative analyses of biological reactions following different types of radiation have been made mainly by determining survival curves. These show the survival S as a function of absorbed dose, where S is the relative fraction of single cells which are able to proliferate up to a visible macrocolony. More information about the biological reactions leading to lethality of damaged cells can be obtained from a quantitative measurement of the proliferation of the subpopulation which is not able to do so after irradiation, i.e., the relative fraction 1-S.

The method of analyzing cell growth curves by subdividing into two fractions of surviving and nonsurviving cells was described in general by Elkind and Whitmore (*1*). A mathematical description of the growth of these two subpopulations was given by Okumura and Uchiyama (*2*), assuming that the proliferation of nonsurviving cells decreases with time after irradiation and with increasing absorbed doses. A cell loss from the subpopulation of nonsurviving cells has also been assumed. An attempt has been made here to determine the extent of proliferation of lethally damaged mammalian cells after irradiation with fast electrons, fast neutrons, α particles, and pions. We intend to compare these quantitative data on cell proliferation capacity after irradiation with those on molecular reactions in the same type of cells, such as single- and double-strand breaks in the DNA and their repair under certain metabolic conditions. Such investigations are in progress in our institute.

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With ascites tumor cells similar to those we have used in the *in vitro* experiments described here, it is possible to produce tumors in mice either as ascites tumors in the abdomen or as solid tumors in the leg muscle. Experiments of this kind are also in progress in our institute. By comparing such data *in vivo* and *in vitro*, additional information may be obtained, e.g., on cell loss *in vivo*, which seems to be absent in the *in vitro* cultures used here.

Experiments with fractionated doses are also in progress in both systems *in vitro* and *in vivo* to gain more quantitative information on tumor growth kinetics and the capacity for repair and cell proliferation in these more complicated situations.

In practical treatment of human tumors with ionizing radiation the capacity for cell proliferation after irradiation may influence the outcome of the treatment, since it could be related to recurrence of the tumor or to metastasis. A measurement of this capacity following different types of radiation may therefore help in the comparison of such treatments.

MATERIALS AND METHODS

Preparation of the Cells

Ehrlich ascites tumor cells were grown in suspension in a well-defined nutrient medium to the stationary phase as described elsewhere (*3*), where a final cell concentration of 2×10^6 cell/ml was reached. Most of the cells were in the G_1 phase of the cell cycle and were able to survive in this medium more than 48 hr at a temperature of 37°C with negligible cell loss. One feature of this nutrient medium should be mentioned because of its importance to these investigations. The usual buffer system of sodium bicarbonate aerated with 6% CO_2 was used to maintain the pH value of the cell suspension within tolerable limits, around 7.4. In addition HEPES buffer was added to a final concentration of 20 mmole/liter to ensure that the pH value was maintained at a constant value during manipulations of the cell suspension in air or during long-term irradiation in closed vessels as described later.

For irradiation the cell suspension was cooled to 6°C to exclude dose-rate effects due to repair of potentially lethal damage and to recovery from sublethal damage during irradiation. No influence on the radiation sensitivity of the cells was observed at temperatures between 37 and 6°C when high absorbed dose rates were employed over irradiation times of a few minutes' duration. No influence on cell survival was detected if cells were kept at 6°C for up to 48 hr before, during, or after irradiation.

Irradiation with α Particles

For irradiation with α particles, cells of the suspension were allowed to settle by gravitation on a filter paper disc with such a low cell density that there was negligible layering of cells.

Irradiation was carried out with a ^{241}Am source mounted at a fixed position 2 mm above the cell layer. The absorbed dose delivered by the 4.7-MeV α particles to the single cell layer was determined using a thin-walled extrapolation ionization chamber under identical conditions (*3*). Comparison with the absorbed dose cal-

culated from the particle fluence and energy spectra measured with a solid-state detector resulted in differences of about 10%. Reproducibility of the absorbed dose in single irradiations should be on the order of ±1%. The absorbed dose rate was 45 Gy/min so that repair of potentially lethal damage was negligible during irradiation at room temperature.

Irradiation with Fast Neutrons

Neutrons with an energy of 14 MeV were produced by bombarding a tritium target with 600-kV deuterons. The cell suspension was placed in plastic tubes which were rotated in air on a wheel 50 cm from the neutron target in a thin plastic box kept at 6°C during irradiation. The absorbed dose of fast neutrons has been measured with a tissue equivalent ionization chamber (*4*) under equivalent irradiation conditions. The γ contamination in the neutron beam was less than 5% and was determined using a carbon ionization chamber and a proportional counter device. From international intercomparison of these instruments (*5, 6*), an uncertainty in the accuracy of this absorbed dose determination of about ±5% can be expected. The precision (reproducibility) of the single irradiations, however, was of the order of ±1%. The absorbed dose rate was 0.1 Gy/min and repair during irradiation was negligible at 6°C.

Irradiation with Fast Electrons

The cells were irradiated in plastic dishes with a broad (10 × 10 cm) beam of 30-MeV electrons from a betatron. A 2-cm layer of Lucite was placed in front of the cell suspension to position it at the maximum of the depth dose curve so that all cells received the same absorbed dose.

Dosimetry of the fast electrons at this point was made by an extrapolation ionization chamber (*7*), resulting in an uncertainty of about ±2% in the determination of absorbed dose. Comparison with a special ferrous sulfate solution (*8*) irradiated simultaneously confirmed this value within these limits using the G value for fast electrons of 15.3×10^{-2} eV^{-1} as recommended by ICRU (*7*).

The absorbed dose rate was about 10 Gy/min and repair during irradiation was negligible at 6°C.

Irradiation with Fast Pions

A vertical beam of fast pions (πE 3) at the Schweizer Institut für Nuklearforschung (SIN, Villigen) was used. The initial momentum was 180 MeV/c with a momentum spread of about $\Delta p/p = 0.07$ (*9*). The absorbed dose rate was about 0.05 Gy/min in the peak of the depth absorbed dose curve. As tested in separate experiments repair of potentially lethal damage was negligible at 6°C during irradiation.

The depth absorbed dose curve measured in a large water tank on the central axis of the beam is shown in Fig. 1. From the measurement of the ionization charge in a small tissue-equivalent ionization chamber the absorbed dose was determined using the same W values as for 14-MeV neutrons (*10*), neglecting the change of W

with particle type and energy. This may introduce an uncertainty of about ±4% of the absolute absorbed dose values in the plateau and peak region of the depth dose curve. Reproducibility of single doses monitored by a transmission ionization chamber in front of the water phantom seemed to be within less than 1%.

In this large water tank a smaller cylindrical tank 16 cm in diameter and 26 cm long was aligned to the beam's central axis, which was kept at 6°C and contained a plastic tube with walls 2 mm thick to support the irradiation vessels with the cell suspension. The irradiation vessels were flat cylindrical dishes which could be closed tightly. The walls were 3 mm thick (see Fig. 1C), and the bottom and top of the vessels were made of 1-mm-thick Lucite. The top and bottom of the vessels had only a small effect on the depth dose curve because their density differed from that of water; this resulted in a maximum shift of about 2 mm at position XII of cell layers. In contrast, the 3-mm-thick walls and the 2-mm-thick support had a large influence. Therefore, the depth dose curve was also measured in the presence of a 5-mm-thick cylindrical plastic tube which simulated conditions of the actual irradiation of the cell suspension. Due to the larger electron density of the plastic, pions traversing this material are stopped earlier than in water. A shift of the maximum of the depth dose curve and a broadening of the peak can be seen in Fig. 1B.

The cell suspension was transferred to the irradiation vessels (volume, 4 ml). The cells settled by gravity to the bottom of the vessel within a few minutes; in this way they represented a cell layer less than 1 mm thick perpendicular to the beam axis. The positions of these layers (I to XV) are indicated in Fig. 1.

Four irradiations (A to D) were done with cell layers at all the indicated positions with increasing absorbed dose at a standard depth in the plateau region: A, 0.8 Gy; B, 1.6 Gy; C, 2.4 Gy; D, 3.2 Gy. In the plateau region some vessels were exchanged after the first irradiation (0.8 Gy) and some stayed during all irradiations, resulting in a total absorbed dose of 8 Gy. In this way a dose–effect curve ranging from 0.8

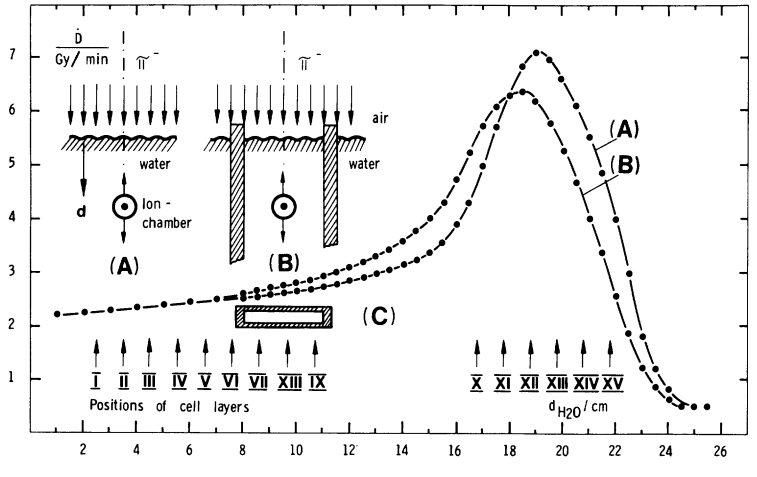

FIG. 1. Depth dose curve for a vertical beam of fast negative pions in water (A) and influence (B) of a plastic cylinder simulating the support and the wall of cuvettes (C) for the irradiation of cell suspension. Indicated are the positions of cell layers (I–XV) during irradiation. (Dose rate × 100).

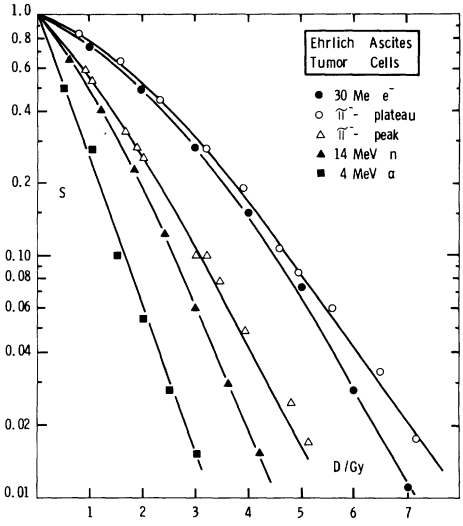

FIG. 2. Survival S of stationary Ehrlich ascites tumor cells as a function of absorbed dose in water, D, treated with different types of radiation.

to 8 Gy was obtained with many experimental points (see Fig. 2). The cell suspensions positioned in the peak of the depth dose curve (ps. X–XV) were each irradiated only once. In this way many dose–effect curves consisting of four different doses each were obtained. The samples from pos. X, XII, XIV, and XV resulted in the same dose–effect curves within the experimental uncertainties if the absorbed doses were determined from the depth dose curve measured with the plastic phantom (Fig. 1B). Therefore all points are fitted by a single dose–effect curve as shown in Fig. 2. The cell suspensions irradiated at pos. XI in the peak were used for measurements of cell-cycle progression by pulse cytofluorometry. Cells irradiated at position XIII were used for measurement of cell proliferation as described later in detail.

The different dose rates and different RBEs at these positions in the pion beam were compensated for by using these different procedures in the plateau and peak of the depth dose curve.

Determination of Dose–Effect Curves

After irradiation the cells were warmed to 37°C and were diluted into fresh medium. They were mixed with nutrient agar (0.4% final agar concentration) at 37°C and 1.5 ml was plated on nutrient agar (0.5% agar) containing the same concentration of nutrients. The agar dishes were then kept at room temperature for 5 min to allow the surface agar to solidify. Dilution in nutrient medium was carried out so that between 50 and 200 macrocolonies appeared per agar dish after 14 days of incubation at 37°C. The number of macrocolonies N_m relative to this number of the unirradiated control N_{m0} is given as the survival $S = N_m/N_{m0}$ without any further correction in Fig. 2.

Measurement of Total Cell Proliferation

From the unirradiated control and the irradiated samples a cell suspension in fresh nutrient medium with an initial concentration of $N_{total} = 2 \times 10^4$ cell/ml was prepared (two petri dishes with 10 ml for each absorbed dose). These dishes were incubated at 37°C in an atmosphere of air containing 8% CO_2. About every 12 hr one of these two dishes was stirred by vigorous sucking with a pipet to dissolve any clusters of cells. This was checked by microscopic observation. By using this method, changes in pH due to stirring and manipulation in air as well as other metabolic factors were kept to a minimum.

From this suspension 0.1–0.4 ml was withdrawn for determination of the total cell concentration N_{total} with an electronic cell counter (Coulter, Ltd.). The results of such measurements are shown as an example in Fig. 3 for cells being irradiated in the peak of the pion depth dose curve (pos. XIII in Fig. 1). After an initial lag time of about 12 hr, which was in part produced by cooling cells to 6°C, the unirradiated control suspension increased exponentially with a cell doubling time of 12 hr. The final cell concentration of $N_{total} = 2 \times 10^6$ cell/ml may be due to either the consumption of one component in the nutrient medium or excretion of a chalone-type substance which limits cell proliferation at this concentration. The increase in total cell number for irradiated cells proceeds in a dose-dependent way. A quantitative analysis will be given in the final section.

Measurement of Proliferation of Surviving Cells

From the same petri dishes used for total cell proliferation as described previously, small amounts (0.1 ml) were withdrawn and after suitable dilution were plated on nutrient agar as described. Dishes were incubated for 14 days at 37°C in air with 6% of CO_2; then macrocolonies N_m were counted (N_0 = number of macrocolonies at $t = 0$). The ratios N_m/N_0 shown in Fig. 4 at $t = 0$ are identical to the survival S shown in Fig. 2. As can be seen from Fig. 4 the lag time for the control cells and cell doubling time are identical with those obtained for total cell proliferation in Fig. 3. This indicates that the plating efficiency of the cells on nutrient agar (about 0.8) remains constant throughout the experiment.

ANALYSIS AND DISCUSSION OF THE RESULTS

From a comparison of the data in Figs. 3 and 4 some general conclusions can be drawn. The doubling time of surviving cells (see Fig. 4) after a few cell divisions is identical to that of an unirradiated control up to absorbed doses of about 6 Gy corresponding to a survival of about 0.006 (see data for pion irradiation in Fig. 6). This has been observed with all types of radiations (Fig. 6). Only in the case of electron irradiation where survival was less than 10^{-3} a small reduction in the cell doubling time of about 10% was observed. This indicates that after a few cell divisions irradiated cells which are able to grow to a macrocolony are indistinguishable from unirradiated cells in this respect within the experimental uncertainties of the measurements shown in Fig. 4.

From a quantitative comparison of the data for the total population given in Fig. 3 and for the surviving cells in Fig. 4, information about the behavior of the cells

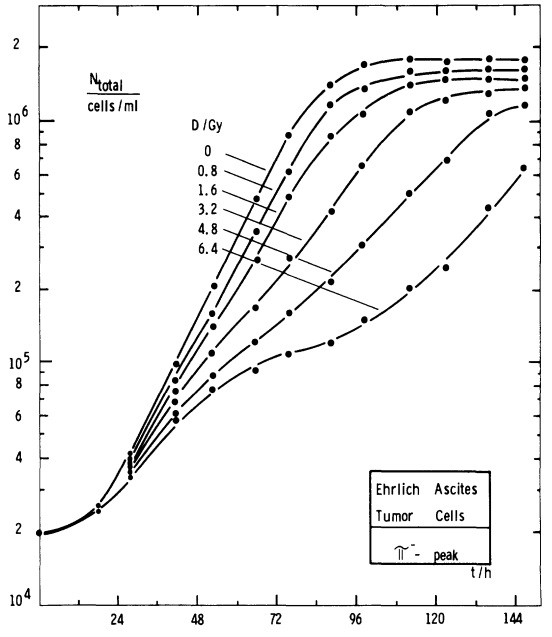

FIG. 3. Cell proliferation at 37°C in liquid nutrient medium of the whole cell population N_{total} measured with an electronic cell counter (Coulter, Ltd). Controls ($D = 0$) and cells irradiated with various absorbed doses in the peak of the pion depth dose curve.

which are lethally hit can be obtained. This is explained in Fig. 5. As an example, the cell proliferation of the total population irradiated with 4.8 Gy with pions (from Fig. 3) and the corresponding proliferation curve for surviving cells from Fig. 4 are drawn together in Fig. 5.

The total number of cells N_{total} is the sum of the surviving cells N_m (which can grow to a macrocolony) and the number of cells which are not able to do so, N_n:

$$N_{total} = N_m + N_n.$$

Therefore, by subtracting the values of N_m from the curve N_{total}, the proliferation of the population N_n can be obtained; this is drawn in Fig. 5 as a broken line.

As can be seen from such curves for N_n the lag time is not different from that of the population N_m and at low absorbed doses the cell doubling time is about the same for N_m and N_n (Fig. 3). At high absorbed doses cell proliferation in the population N_n is retarded.

The difference between N_{total} and N_m also shows that cell proliferation in the population of N_n is inhibited after a certain number of cell divisions, reaching a final concentration of $N_{n,\infty}$. This value stays constant for up to 200 hr; cell lysis is negligible under these conditions. In the example shown in Fig. 5 the lethally damaged cells are able to increase in number by a factor of $N_{n,\infty}/N_{n0} = 9$. This factor represents the mean value of a Poisson distribution of such numbers if measured by single cell observations and their growth after irradiation under identical conditions.[1]

[1] K. Krespin and M. Nüsse, private communication.

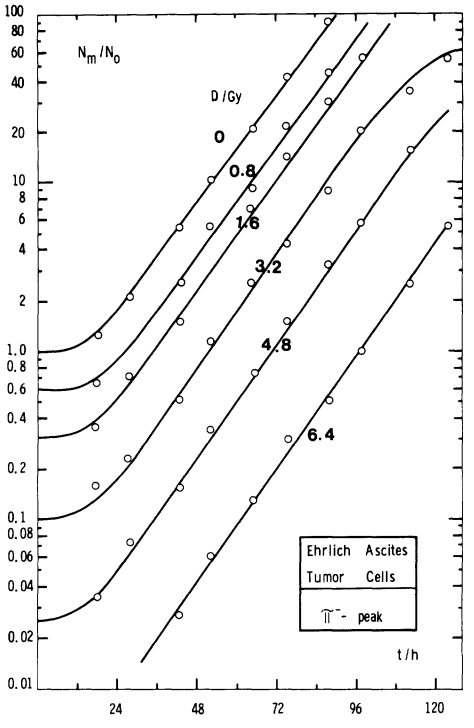

FIG. 4. Proliferation of viable cells N_m which are able to grow to a macrocolony as a function of length of time in liquid nutrient medium (see Fig. 3).

In Fig. 6 the results of such analyses for different absorbed doses and different types of radiations are collected. The cell proliferation factor of lethally damaged cells $N_{n,\infty}/N_{n0}$ is given as a function of survival S of the cells to exclude the dose-dependent RBE of the different types of radiation. As can be seen all data obtained with a wide variety of radiation fall on one curve within the experimental uncertainties.

The survival S is a measure for the probability of cell population survival and 1-S is the probability of death due to lethal damage. As can be seen from Fig. 6 this probability 1-S correlates with the number of cell divisions which lethally damaged cells can undergo after irradiation regardless of the type of radiation used. At high survival values a large number of what appear to be cell divisions can occur at the normal rate, indicating that the lethal damage is not located in any of the molecular structures necessary for cell proliferation or cell division or in the DNA carrying the necessary information for these processes. Damage in the DNA which is responsible for more differentiated functions of the cell and is less specific for cell division must therefore be considered as that which finally leads to cell death. The biochemical mechanisms of such reactions are as yet unknown but are now under investigation in our institute.

At high absorbed doses and corresponding low survival of $S = 10^{-4}$ the curve approaches $N_{n,\infty}/N_{n0} = 2$. This means that one cell division can be performed in these highly damaged cells with the lethal radiation damage expressed during the next

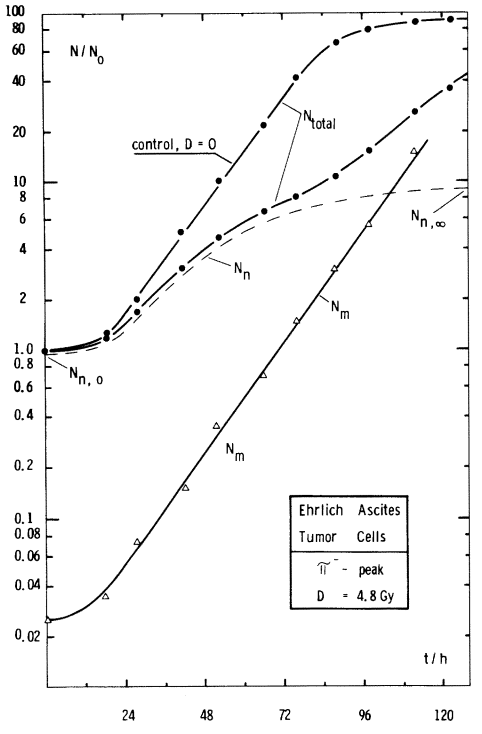

FIG. 5. Cell proliferation of the total cell population N_{total} after an absorbed dose of 4.8 Gy (from Fig. 3) and proliferation of the viable cells N_m relative to the initial number of unirradiated cells N_0. The dotted line indicates the proliferation of lethally damaged cells N_n which cannot grow to a macrocolony. Indicated is the initial (N_{n0}) and the final number of lethally damaged cells ($N_{n,\infty}$).

cell cycle. This is in accordance with some measurements which follow these lethally damaged cells through a few cell cycles after irradiation by cytofluorometry.

Proliferation data for cells irradiated with α particles have not yet been obtained since the irradiation of a single cell layer did not yield a sufficiently large number of cells for such experiments. If cells after α-particle irradiation react in a different way, this should have been seen earlier with fast neutrons since about half the absorbed dose of fast neutrons is imparted to matter in the LET interval of α particles (*11*). (See note added in proof.)

Other cell lines may proliferate after irradiation in a different way even if the dose–effect curves are comparable due to different energy metabolism and corresponding time constants (*2*). However, all these cell lines have shown considerable cell loss in the nonsurviving fraction, and the final number of cell divisions could not be estimated with sufficient accuracy. After completing our investigations we learned of similar new experiments with X irradiation in Chinese hamster ovary cells;[2] they reported negligible cell loss in culture. Those results agree in general with the data presented here but show larger numbers of $N_{n,\infty}/N_{n0}$ at corresponding

[2] H. Jung, private communication.

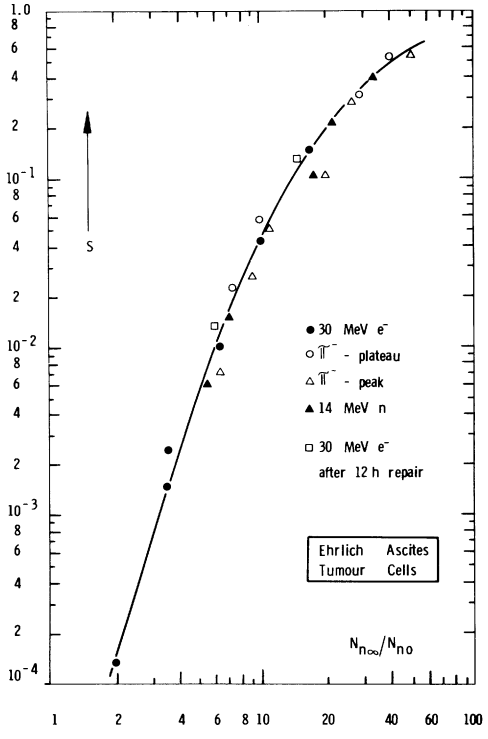

FIG. 6. Cell proliferation factors $N_{n,\infty}/N_{n0}$ for lethally damaged Ehrlich ascites tumor cells for different absorbed doses represented by the corresponding survival S of the cells (see Fig. 2) for different types of radiation. The measured points scatter within the experimental uncertainties around the indicated line of a best fit of all data.

survival S. The difference might be due to different feeding procedures for the two cell lines after irradiation.

When the results obtained with *in vitro* cultures are compared with cell growth curves *in vivo* (*1, 12–19*), the main difference is that cell loss occurs regularly in the nonsurviving fraction of cells *in vivo* and can be influenced by changing the energy metabolism of the tissue (*20*). From measurements of the radiation-induced growth delay in rhabdomyosarcoma (*21*), it seems that the extent of cell loss as a function of time after irradiation is dependent on the LET of the radiation used.

Further investigations with the same cell lines used both *in vitro* and *in vivo* are therefore necessary to achieve quantitative data for application in a complete mathematical model such as that developed by Okumura and Uchiyama (*2*) for experimental tumors. The cell line used here is suitable for such investigations *in vivo* and corresponding experiments have been started.

From well-understood *in vivo* tumor models certain statements and predictions for clinical tumor therapy could be made; this has been shown by several authors (*11, 21–25*). Further quantitative knowledge of the different parameters seems to be needed, however, especially for radiations from different LET.

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Note added in proof. Meanwhile data for α-particles have been obtained which are in accordance with the curve shown in Fig. 6.

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