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Correlation of Radiation-Induced Micronucleus Frequency with Clonogenic Survival in Cells of One Diploid and Two Tetraploid Murine Tumor Cell Lines of the Same Origin

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Whether the micronucleus assay can be used as a predictive tool to estimate the intrinsic cellular radiosensitivity depends on whether the results correlate with clonogenic survival. In the present study we investigated the influence of the ploidy on the correlation of the two assays by comparing cells of one diploid and two tetraploid murine tumor cell lines of different radiosensitivities. A definite correlation between survival and micronucleus frequency is evident for all three cell lines investigated. The rank order of the frequency of micronucleus expression correlates with that of clonogenic survival in the cell lines studied. No influence of DNA content on the radiosensitivity and the relationship between the micronucleus frequency and clonogenic survival could be observed. Thus our results are promising for the clinical application of the micronucleus assay as a predictive measure of radiosensitivity. However, it is necessary to consider such factors such as cell proliferation kinetics which may influence the correlation between micronucleus frequency and clonogenic survival. © 1997 by Radiation Research Society

INTRODUCTION

The micronucleus assay has been used as a reliable and rapid test for detecting genetic damage induced by radiation or cytotoxic agents (*1–4*). Because of its simplicity, this assay is expected to be of clinical relevance for predicting the cure of cancer by radiation therapy in individual cancer patients (*1, 5–9*).

A good correlation between micronucleus frequency and clonogenic survival in cells of tumor cell lines has been reported by several investigators (*2, 4–7, 9, 10*). However, some conflicting results also exist. No correlation between micronucleus frequency and clonogenic survival has been observed in some human tumor cells (*11*) and human lymphoblastoid cell lines (*12*). Slavotinek *et al.* (*12*) reported that the rank order of the frequency of micronuclei in the cells of their cell lines did not coincide with that for clonogenic survival. In another report, in which a linear correlation was observed between micronucleus frequency and clonogenic survival in a series of human renal cell carcinomas, the relationships varied between different cell lines (*9*).

In many of these experiments, cells with different biological characteristics have been used, and this could be one of the factors complicating the interpretation of the results. In the present study we have examined the relationship between the micronucleus frequency and clonogenic survival in cells of three murine tumor cell lines of the same origin but of different radiosensitivities. All the cell lines showed similar population doubling times and similar cell cycle distributions. These conditions enabled us to investigate the correlation without any conflicting effects from differing cell characteristics. In addition, since one cell line was diploid and two were tetraploid, it was interesting to examine the influence of the change in ploidy on the radiosensitivity.

MATERIALS AND METHODS

Cells and Cell Culture

Three murine tumor cell lines of the same origin were used. The cell line SHA_3K_4-I_c (I_c) is derived from a squamous cell carcinoma originally established from a rectal carcinoma of a mouse and was kindly provided by G. E. Adams (Medical Research Council Radiation and Genome Stability Unit, Didcot, Oxfordshire, UK). The cell lines SHA_3K_4-II_{14} (II_{14}) and SHA_3K_4-III_{19} (III_{19}) are started as primary cultures from allotransplants of I_c tumor cells on nude (nu/nu) mice (*13*). These two cell lines stemmed from tumor passages 14 (II_{14}) and 19 (III_{19}).

Cells of all three cell lines were grown as monolayers in Eagle's minimum essential medium (Gibco) supplemented with 20% fetal calf serum (Gibco) and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Flow Cytometry and Estimation of Chromosome Numbers

The DNA content was analyzed by flow cytometry as described previously (*14*) using an ICP22 flow cytometer (Phywe, Germany). Murine lymphocytes were used as a standard for determining the DNA content,

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TABLE I
Cell Proliferation after Irradiation and Treatment with Cytochalasin B

Cell line:	I_c			II_{14}			III_{19}		
Incubation with Cytochalasin B:	48 h			48 h			32 h		
Degree of nucleation (%)	Mononucleated cells	Binucleated cells	Polynucleated cells	Mononucleated cells	Binucleated cells	Polynucleated cells	Mononucleated cells	Binucleated cells	Polynucleated cells
Control	8 ± 2	44 ± 3	48 ± 2	8 ± 3	68 ± 9	24 ± 6	8 ± 1	49 ± 9	43 ± 10
1 Gy	9 ± 3	45 ± 3	46 ± 4	11 ± 3	67 ± 6	22 ± 7	6 ± 1	53 ± 7	41 ± 7
2 Gy	12 ± 4	56 ± 4	32 ± 4	16 ± 5	67 ± 2	17 ± 6	8 ± 4	56 ± 6	36 ± 7
3 Gy	14 ± 5	64 ± 5	22 ± 4	20 ± 8	67 ± 5	13 ± 6	8 ± 3	59 ± 7	33 ± 5
4 Gy	21 ± 14	62 ± 10	17 ± 6	27 ± 10	63 ± 7	10 ± 4	10 ± 5	58 ± 6	32 ± 7

Note. Values are means with standard deviations of two independent quadruplicate experiments.

and the peak G_1-phase channel was considered to represent the diploid cells (DNA content = 2C). Parameters of the kinetics of exponential cells were analyzed by one-parameter flow cytometry using unfixed cells stained with a propidium iodide solution (2.5×10^{-5} *M* in 0.1 *M* Tris, 0.1 *M* NaCl, pH 7.5) including 0.2% Triton X-100 (*15*). The DNA histograms were produced by transfer of the data to a FACScan flow cytometer (Becton Dickinson) equipped with Cellfit software, and the distribution of cells throughout the cell cycle was calculated using the SOBR model (*14*).

The chromosome numbers of the cell lines I_c and III_{19} were estimated microscopically from metaphase spreads stained with 5% Giemsa.

Clonogenic Survival Assay

Cells in exponential growth were used. Twenty-four hours before irradiation, 5×10^5 cells were incubated in 25 cm^2 flasks in the above-mentioned medium. Irradiations were carried out using an X-ray machine (Stabilipan, Siemens, Germany; 240 kV, 15 mA, 0.5 mm Cu filter, dose rate 1 Gy/min) with doses of 1, 2, 3, 4, 6 and 8 Gy, applied at room temperature. Control cells received sham irradiation. The cells were then trypsinized, counted and plated in appropriate numbers for colony formation into 19.6 cm^2 tissue culture dishes (Becton Dickinson). Eight to 9 days later, colonies were fixed with 96% methanol, stained with 1% crystal violet and counted to assess the clonogenic survival. Colonies containing ≥50 cells were counted. Two (for the line II_{14}) or three (for the lines I_c and III_{19}) independent experiments, each performed in triplicate, were carried out for each cell line.

Micronucleus Assay

Cells (5×10^4) were plated into 19.6 cm^2 tissue culture dishes and irradiated with doses of 1, 2, 3 and 4 Gy. Immediately after irradiation Cytochalasin B (Sigma, Germany) was added. Cytochalasin B was dissolved (1 mg/ml) in dimethyl sulfoxide (DMSO; Serva, Germany) and frozen in aliquots. The stock solution was diluted with culture medium to a final concentration of 1.5 μg/ml (*6, 16*). A Cytochalasin B incubation time of 48 h was chosen for the I_c and II_{14} cell lines and 32 h for the line III_{19} to ensure an equivalent binucleation. A shorter Cytochalasin B incubation time in the latter cell line was necessary, since the cells showed little radiation-induced mitotic delay (Table I). The frequency of mononucleated, binucleated and polynucleated (>2 nuclei) cells was estimated by scoring at least 200 cells/dish. After the cells were rinsed with 0.9% NaCl, they were fixed for 20 min with 96% methanol at room temperature, air-dried overnight and stained with 4,6-diamidino-2-phenylindole (DAPI; 100 ng/ml in Tris buffer, pH 7.0; Serva, Germany). Fixation and staining were performed with well-spread attached cells. Scoring was carried out at a magnification of 400× using a microscope equipped with fluorescence (excitation maximum 344 nm, emission maximum 449 nm) and phase contrast. Micronuclei were scored from coded dishes in a blind manner. Two independent experiments carried out in quadruplicate were performed for each cell line. At least 200 binucleated cells per dish (resulting in 1600 binucleated cells/dose) were assessed and the micronucleus frequency per single binucleated cell was estimated. Only micronuclei within well-spread cells were included, with the following criteria for scoring: (1) micronuclei were separated from the main nuclei and nuclear buds were excluded; (2) micronuclei were round to oval-shaped with distinct borders; (3) micronuclei exceeding one-third the diameter of the nuclei were excluded (*1, 11, 16, 17*).

TABLE II
Parameters of Cell Kinetics

Cell line	Population doubling time[a] (h)	Cell cycle distribution[b] (%) G_1 phase	S phase	G_2/M phase
I_c	15.0	51 (46–56)	43 (40–46)	6 (3–8)
II_{14}	16.4	44 (41–46)	43 (42–43)	13 (12–15)
III_{19}	14.2	46 (46–47)	45 (43–47)	9 (7–10)

[a]Population doubling time was obtained from cell growth curve.

[b]Cell cycle distributions were analyzed by flow cytometry in exponential cells. Values given are means of two independent measurements with the range of values in parentheses.

RESULTS

DNA Content, Chromosome Numbers and Cell Cycle Kinetics

The cell lines I_c and II_{14} are tetraploid (DNA content = 4C), and the cell line III_{19} is diploid (DNA content = 2C). The mean chromosome number of the tetraploid line I_c is 71.7 ± 9.4 and the mean of the diploid line III_{19} is 38.4 ± 11.3 (normal chromosome number of the mouse: 40). Both cell lines showed some Robertsonian translocations.

The parameters of cell cycle kinetics are shown in Table II. It is evident that there are no significant differences between the three cell lines.

Clonogenic Survival

The data for clonogenic survival after irradiation are presented in Table III and Fig. 1. The curves were fitted by eye. Plating efficiency is $I_c < II_{14} < III_{19}$. The tetraploid cell line I_c is the most radiosensitive, and the survival curve shows no evidence of a shoulder. The tetraploid cell line II_{14} and the diploid line III_{19} are comparatively radioresistant and the survival curves have shoulders.

TABLE III
Clonogenic Survival of the Three Cell Lines

Cell line	Plating efficiency (%)	Surviving fraction 1 Gy	2 Gy	3 Gy	4 Gy	6 Gy	8 Gy	Extrapolation number (n)	D_0 (Gy)
I_c	15.9 ± 7.2	0.41 ± 0.05	0.16 ± 0.05	0.095 ± 0.036	0.023 ± 0.011	0.0064 ± 0.0022	0.0015 ± 0.0008	1.0	1.2
II_{14}	29.3 ± 5.7	0.91 ± 0.03	0.62 ± 0.13	0.34 ± 0.05	0.20 ± 0.05	0.052 ± 0.005	0.011 ± 0.002	3.5	1.4
III_{19}	87.0 ± 9.8	0.75 ± 0.11	0.63 ± 0.05	0.44 ± 0.04	0.29 ± 0.04	0.10 ± 0.03	0.036 ± 0.010	2.5	1.9

Note. Values are the means of two (II_{14}) to three (I_c, III_{19}) independent experiments (each done in triplicate) and the standard deviations.

Cell Proliferation

The rate of nucleation in cells with treated Cytochalasin B after irradiation is summarized in Table I. The most radioresistant cell line, III_{19}, shows comparatively little mitotic delay, as judged by the small dose-dependent decrease in polynucleated cells and a small increase in mononucleated cells. No distinct difference in the mitotic delay is observed between the cell lines I_c and II_{14} despite a difference in radiation sensitivity between these cell lines. In both cell lines a clear dose-dependent decrease in polynucleated cells is observed.

Micronucleus Frequency

Figure 2 shows dose–response curves for micronucleus expression in the three cell lines. All cell lines show a linear increase in the number of micronuclei per binucleated cell with increasing X-ray doses. The micronucleus frequency of the I_c line (4C) is nearly twofold that of the II_{14} (4C) and III_{19} lines (2C). On the other hand, no significant difference in frequencies is seen between the tetraploid cell line II_{14} and the diploid cell line III_{19}, although the diploid line III_{19} appears to have a slightly lower frequency. These data are consistent with the results of the assay of clonogenic survival. There is no apparent influence of ploidy on the micronucleus frequency.

The data points were fitted best by a straight line according to the relationship $y = a + bD$. The slopes and standard errors are: I_c, $y = 0.26\ (\pm\ 0.04) + 0.61D\ (\pm\ 0.04)$; II_{14}, $y = 0.13\ (\pm\ 0.02) + 0.28D\ (\pm\ 0.02)$; III_{19}, $y = 0.05\ (\pm\ 0.01) + 0.23D\ (\pm\ 0.01)$, where y is the frequency of micronuclei, a and b are constants, and D is the radiation dose.

Relationship between Clonogenic Survival and Micronucleus Frequency

In Fig. 3, the micronucleus frequencies (after subtraction of the control values) of all three cell lines are plotted as a function of the corresponding surviving fractions. The data were fitted with the program SigmaPlot (Jandel). Almost all data are lying within the 95% confidence interval of the linear regression curve (correlation coefficient, $r = -0.9892$: Spearman rank correlation).

Figure 4 shows the correlation between survival and the number of cells with at least one micronucleus (damaged cells). The correlation coefficient is $r = -0.9624$ (Spearman rank correlation). Again, the 95% confidence intervals are presented in the figure.

DISCUSSION

Whether the micronucleus assay can be used as a predictive measure to estimate the intrinsic radiosensitivity of tumor cells depends on whether the results correlate in rank order with the clonogenic survivals (*5, 7, 11*). Under optimal conditions, both tests should correlate in a quantitative way.

Several authors have reported a good quantitative relationship between micronucleus frequency and clonogenic survival (*5–7*). Shibamoto *et al.* (*6*) observed a good correlation between micronucleus frequency and clonogenic survival in cells of several human and murine tumor cell lines *in vitro* and *in situ*, while Bakker *et al.* (*5*) and Stap and Aten (*7*) demonstrated the same quantitative relationships in different cell lines *in vitro*. In the three cell lines we investigated, the rank order of radiation-induced micronu-

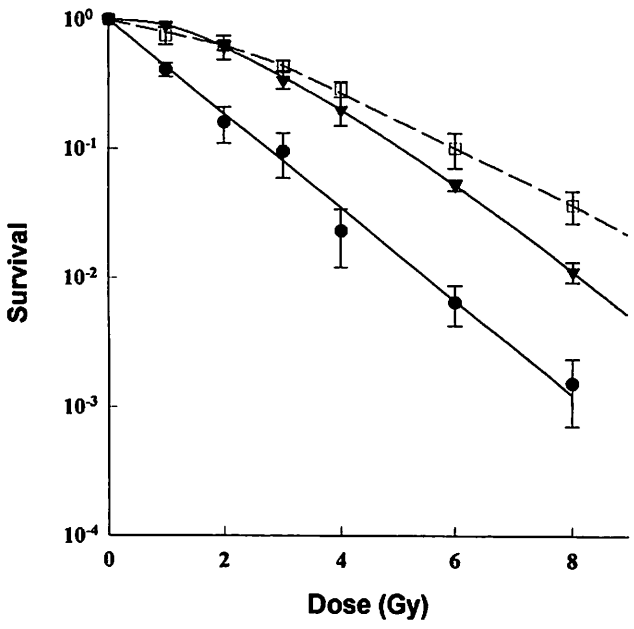

FIG. 1. Clonogenic survival curves for the three cell lines: (●) SHA_3K_4-I_c, (▼) SHA_3K_4-II_{14} and (□) SHA_3K_4-III_{19}. Each symbol and bar represent the mean value and standard deviation of two to three independent triplicate experiments.

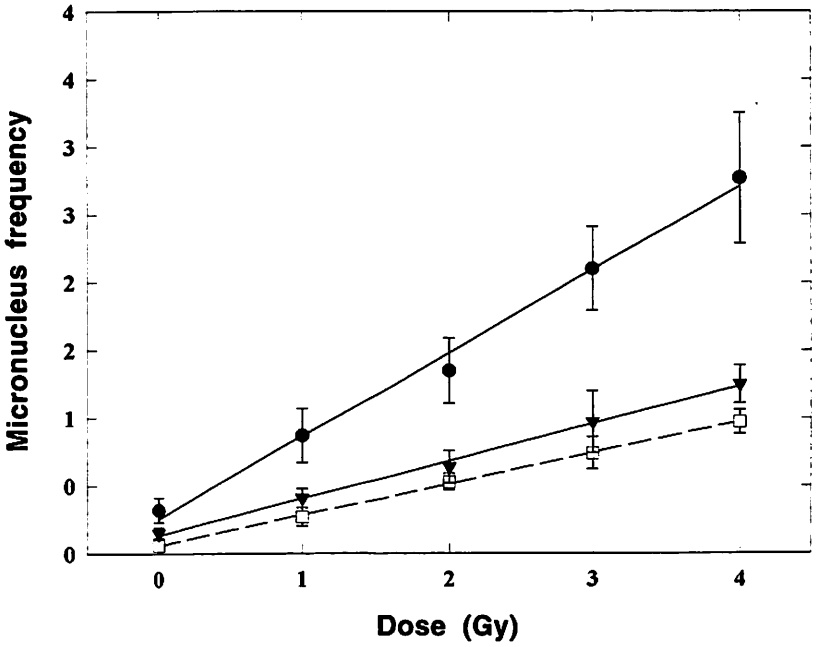

FIG. 2. Dose–response curves for micronucleus frequency in the three cell lines: (●) SHA_3K_4-I_c, (▼) SHA_3K_4-II_{14} and (□) SHA_3K_4-III_{19}. Each symbol and bar represent the mean value and standard deviation of two independent experiments (each quadruplicate).

cleus frequency correlates with the clonogenic survival, both when the number of micronuclei per binucleated cell is plotted as a function of survival (Fig. 3) and after plotting the number of cells with micronuclei as a function of survival (Fig. 4). These results are encouraging for the clinical application of the micronucleus assay as a predictive measure for estimating tumor radiosensitivity.

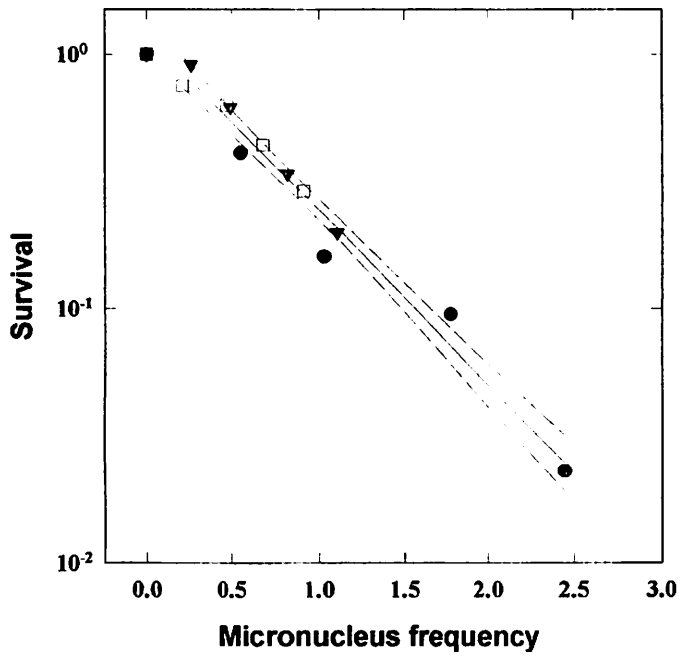

FIG. 3. Correlation between clonogenic survival and radiation-induced micronucleus frequency (after subtraction of the control value): (●) SHA_3K_4-I_c, (▼) SHA_3K_4-II_{14} and (□) SHA_3K_4-III_{19}. The solid and dashed lines represent a fitted regression line and 95% confidence intervals, respectively.

Some authors, however, have reported a missing quantitative relationship between the micronucleus frequency and clonogenic survival (*9*, *11*, *12*). Bush and McMillan (*11*) investigated cells of four human tumor cell lines of widely differing radiosensitivities and showed that the micronucleus test could not rank the cells in the same order of radiosensitivity as the clonogenic survival. Slavotinek *et al.* (*12*) reported a similar result using cells of six human lymphoblastoid cell lines.

Several factors exist which may influence this correlation. The percentage of scorable binucleated cells after irradiation and treatment with Cytochalasin B can vary between cell lines (*12*, *16*). Slavotinek *et al.* (*12*) speculated that this variation in the size of the scorable population, perhaps due in part to interphase cell death after radiation exposure, might be one reason for the lack of correlation. Fuhrmann *et al.* (*16*) also pointed out the importance of cell proliferation and its extent for the interpretation of the results. Although they did not use cytokinesis block with Cytochalasin B, Kaffenberger *et al.* (*18*) stated that a close correlation between cell death and the occurrence of micronuclei after irradiation could be established only when the progression of cells through the cell cycle and the biological characteristics of micronuclei were taken into account. We have chosen individual incubation times with Cytochalasin B for each cell line to obtain comparable numbers of binucleated cells and to avoid interference from the formation of polynucleated cells. The distribution of cells in the phases of the cell cycle at the time of irradiation may also have an effect on the quantitative relationship

between the micronucleus frequency and clonogenic survival. It is well known that cells irradiated in different phases of the cell cycle show different radioresponses (*19*). Bush and McMillan (*11*) referred to the possibility that, in cells of cell lines which contain a high percentage of S-phase and G_2/M-phase cells, micronucleus expression may not correlate with colony formation because the damaged G_2/M-phase cells and some of the damaged S-phase cells which form chromatid-type aberrations would still form colonies if only one daughter cell was affected. However, these uncertainties should not play a role in our investigation, because the three cell lines we used had very similar proliferation kinetics.

With respect to DNA ploidy, Revell (*20*) showed that any deviation from euploidy can upset the relationship between micronucleus frequency and clonogenic survival. He observed a better survival in cells of hyperdiploid than diploid cell lines after irradiation and speculated that the former had an enhanced capacity to tolerate the loss of their genetic material in the formation of micronuclei. Shibamoto *et al.* (*6*) observed no clear correlation between the DNA index and micronucleus frequency, and Schmid (*21*) pointed out that chromosome number does not seem to play a crucial role with respect to micronucleus formation after an exposure to the mutagen Trenimon. Similarly, Schwartz (*22*) did not observe a correlation between DNA content and radiosensitivity in cells of seven human squamous cell carcinoma lines. On the other hand, Sontag *et al.* (*23*) reported that the DNA content did correlate with the mean inactivation dose in cells of nine mammalian cell lines. These results demonstrate that the relationship between DNA ploidy and micronucleus frequency or chemo- and radiosensitivity is still a matter of controversy. Our results show that there is no significant difference in micronucleus frequency between the diploid cell line III_{19} and the tetraploid one II_{14}, although the line III_{19} had a slightly better survival and lower micronucleus frequency. This suggests that in our cell lines variation in ploidy does not disturb the correlation between micronucleus frequency and clonogenic survival.

Other factors which may have an impact upon the relationship between micronuclei and clonogenic survival are the quantitative differences in the conversion of chromosomal fragments into micronuclei and karyotypic instability (*11*, *12*). Bush and McMillan (*11*) also speculated that functional hemizygosity may influence the correlation. Slavotinek *et al.* (*12*) suggested that one possible reason for the lack of correlation between induced micronuclei and survival in their lymphoblastoid cell lines might be the unusual radiosensitivity and instability of the cells. The lymphoblastoid cell lines could change from diploid to tetraploid during the course of an experiment. Also, a lack of correlation between micronucleus formation and survival may result from the occurrence of apoptosis (*24*).

In our experiments the chromosome numbers were analyzed in one tetraploid and one diploid cell line. Both showed abnormal chromosomal numbers with some

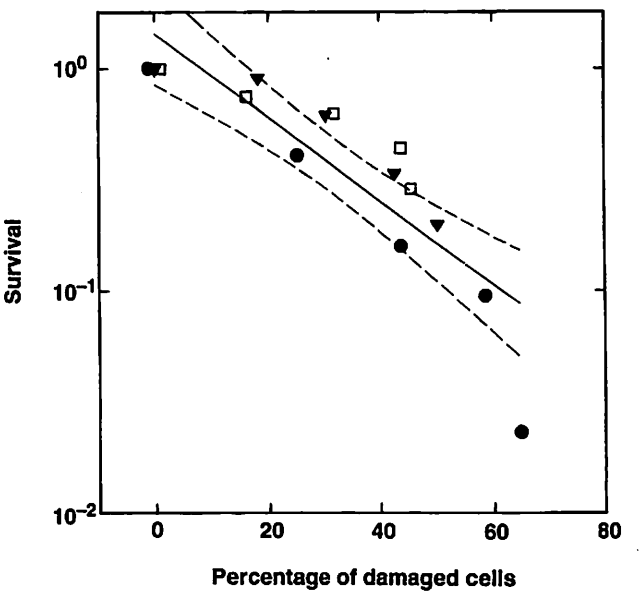

FIG. 4. Correlation between clonogenic survival and percentage of damaged cells (cells with at least one micronucleus after subtraction of the control value): (●) SHA_3K_4-I_c, (▼) SHA_3K_4-II_{14} and (□) SHA_3K_4-III_{19}. The solid and dashed lines represent a fitted regression line and 95% confidence intervals, respectively.

Robertsonian translocations. Tumor cell lines frequently carry genomic instability, and its influence on micronucleus expression cannot be excluded completely even when cell lines of the same origin are used. Therefore, when cells of different types or different properties are investigated, it might be necessary to consider these factors.

The analysis of cell proliferation revealed that the most radioresistant cell line, III_{19}, showed the least mitotic delay. Interestingly, despite a difference in the shapes of the survival curves and the micronucleus frequencies, the cell lines II_{14} and I_c did not differ with respect to mitotic delay (Table I). This result indicates that the differences in mitotic delay seem to have less influence on the relationship between cell killing and micronucleus induction.

In summary, we obtained a good correlation between micronucleus frequency and clonogenic survival in cells of three murine tumor cell lines of the same origin but different ploidy. This is an encouraging result for the clinical application of the micronucleus assay as a predictive measure of radiosensitivity. However, it appears necessary to consider the effects of some factors, such as cell proliferation kinetics and the response of the cells to Cytochalasin B, which may influence the correlation between clonogenic survival and micronucleus frequency.

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