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Synergistic Cell Cycle Kinetic Effect of Low Doses of Hyperthermia and Radiation on Tumour Cells*

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Abstract—*In the present work asynchronous PNJ cells, an* in vitro *line of a mouse mammary tumour, were treated with small doses of heat* (41.5°C *for* 60 min) *and radiation* (200 rad), *either alone or in combination with variable sequence and when no and a* 24-hr *interval was interposed between the treatments.*

The effects were evaluated by growth curves, the relative survival and cell cycle kinetic changes by means of flow cytometry.

When hyperthermia preceded radiation, a more lethal effect was observed than when the opposite sequence was used. An interval of up to 24 hr *did not change the effect of the combined treatment.*

When the cells were treated with either hyperthermia or radiation, the alteration of the cell cycle was found to be only small and short-lived, while the combined treatment induced a considerable accumulation of cells with G_2 DNA content which could be traced in the DNA histograms for several days. The kinetic effect was at least as pronounced and extended in time when a 24-hr *interval was introduced between the treatments as when one treatment was immediately followed by the other.*

The results suggest that different heat- and radiation-sensitive mechanisms are involved in the progression of cells through the cell cycle and in the lethal damage of the cells. Another explanation is that changes in the cell cycle distribution occur at a lower treatment level than changes in the survival.

INTRODUCTION

Most *in vitro* studies on hyperthermia have analysed the cell-killing by studying the colony-forming ability of the cells after treatment. As both heat and radiation induce cytokinetic changes in the treated cells, some laboratories have investigated these heat and /or radiation induced alterations by means of flow cytometry (FCM) [1–6].

Such cell kinetic investigations have shown some differences in the distribution of cells in the cell cycle after hyperthermia as compared to X-irradiation. In both cases, the cells appear to accumulate in the G_2 phase, but at different times after the treatments. However, the amount of cells in the S phase was more pronounced after hyperthermia than after X-irradiation [2, 7, 8]. It has also been found that with a combination of the two modalities the radioresistant S phase cells were made more radiosensitive than the G_1 cells by hyperthermia.

Studies on the effect of sequence and interval on the interaction of hyperthermia and radiation [7, 9–14] have shown that the importance of the sequence is related to the temperature used. At lower temperatures (40–43°C), hyperthermic treatment following X-irradiation seemed to be the most effective sequence, and an interval of 10–30 min between the two treatments resulted in a decrease in the potentiating effect [7, 10]. When higher temperatures were used, hyperthermia preceding X-irradiation was usually slightly more effective. Still there seemed to be a rapid fall in the potentiating effect of hyperthermia, when the interval between the two treatments was increased [7].

The aim of the present investigation was to study the survival of asynchronous PNJ cells *in vitro* and the cell cycle kinetic effect as evaluated by means of FCM after the combination of low doses of hyperthermia and

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radiation with and without a 24-hr interval interposed between the treatments.

MATERIALS AND METHODS

Tumour

The PNJ cells used in the present study are an *in vitro* established cell line of the hyperdiploid transplantable ascites tumour developed in our laboratory from a mammary carcinoma HB growing isologously in the C3H strain of mice.

The cells are grown in Falcon tissue-culture flasks in Basal Medium Eagle (BME) (GIBCO) supplemented with foetal calf serum (FLOW) and antibiotics (100 i.u./ml of penicillin and 100 μg/ml of streptomycin). The cultures are aerated with a mixture of 95% humidified air and 5% CO_2 and maintained at 37°C.

The PNJ cells used for explantation were obtained from stock cultures by simple shaking, and counting of the cells was performed in a haemacytometer. In the growth curve and the FCM study 2×10^5, and in the survival study 500 PNJ cells were explanted into 25 cm^2 Falcon flasks containing 10 ml BME supplemented with 10% calf serum and antibiotics. These cultures were used in the experiments.

Most of the PNJ cells obtained from stationary stock cultures are accumulated in the G_1 phase of the cell cycle. Therefore, in order to achieve asynchrony the explanted cells were allowed to grow for four days before they were used in the experiments.

In the FCM study, the tumour cells were harvested and counted at various times after treatment by trypsinization (1% trypsin MERCK, 20,000 i.u./g, 30 min at 37°C).

Treatment

Heat treatment of the cell cultures was performed by submersion in a water bath. Temperature equilibrium was obtained within 4 min. During treatment the variation in temperature was less than ± 0.05°C measured with a certified thermometer.

Radiation

Two hundred and fifty kV, 15 mA, 2 mm Al filter, field-source distance 36 cm. Dose rate 500 rad/min. The cells were irradiated in the flasks under the cover of 4 mm fluid placed in a special lucite jig in order to obtain full back scatter. The absorbed dose was measured by lithium fluoride thermoluminiscence, and calibration was performed against a Baldwin–Farmer-ionizing chamber.

FCM analysis

DNA histograms of the tumour cells were obtained by flow cytometry (FCM) after staining of the cells according to the method described by Vindeløv [15].

The distributions of cells in the G_1, S and $G_2 + M$ compartments from the DNA histograms were estimated as previously described [16].

Cell survival

Treatment of the cultures (500 cells) was performed 3 hr (for cellular attachment) after explantation. Eight to ten days after treatment, the number of colonies (more than 50 cells in close contact) was determined stereomicroscopically after staining of the cultures with toluidine.

Statistical analysis

The tests used were the F-test and Student's t-test.

RESULTS

The treatment consisted in a 1-hr exposure at 41.5°C and/or a radiation dose of 200 rad applied either with no interval, or at a 24-hr interval between the treatments. In the first series of experiments, heat was given before radiation, and in the second series, the cultures were subjected to radiation before heat treatment.

Growth curves

Figure 1 shows the growth curves of the cells after the various treatments. Obviously, the treatments induced a retardation of the growth, most pronounced after the combined treatments. On day 6 after treatment there was a significant difference ($P < 0.001$) between the treated and untreated cells. A significant difference ($P < 0.01$) was also observed between cells treated with the single and the combined schedules.

Cell survival

Table 1 shows the survival of the PNJ cells after the various treatments. The plating efficiency of the untreated cultures was 40%. The effect of the combined treatments was significantly ($P < 0.005$) different from the effect of the treatments given alone and the controls. The cell-damaging effect of heat

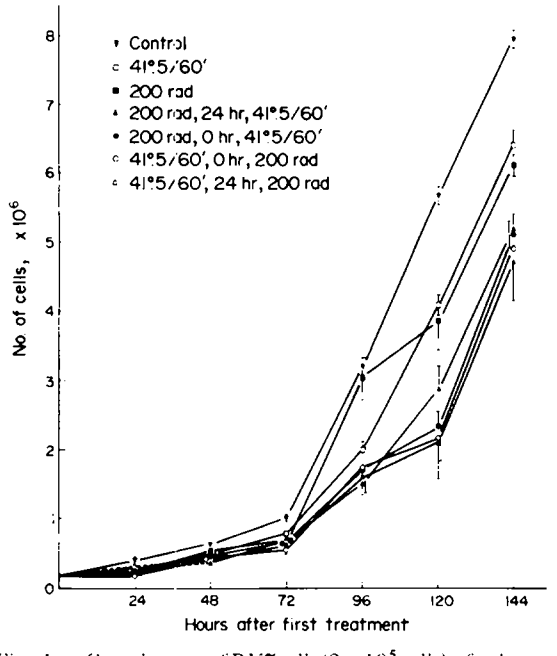

Fig. 1. Growth curves of PNJ cells (2×10^5 cells) after heat—h (41.5°C/60 min) and/or radiation—r (200 rad) with various sequence. ▼controls, □heat, ■radiation, ○heat followed by radiation with no interval, ●radiation followed by heat with no interval, △heat followed by radiation at a 24-hr interval, ▲radiation followed by heat at a 24-hr interval.

given before radiation seemed to be slightly more pronounced than vice versa, and when the results from the combined treatments were compared without considering the interval between the treatments, there was a significant difference ($P<0.005$), the effect of hyperthermia given before radiation being most extensive.

Cell cycle distribution

In untreated PNJ cells the FCM analysis showed that with increasing time after explantation the S level decreased slowly as a consequence of the increasing number of cells and exhaustion of the media, but during the experiment the cultures never reached the plateau phase.

Figure 2 shows the DNA distributions of the PNJ cells after the various treatment schedules. The data extracted from the histograms are shown in Fig. 3.

Figure 3, first column, shows the variation with time in the DNA distribution in non-treated cultures, compared with single heat or radiation treatment. It appears that the number of S cells in the controls is slowly decreasing with time after treatment, whereas heat induces a small accumulation of cells in the S phase. This figure also shows a minor accumulation of cells with G_2 DNA content after radiation.

Figure 3, second column, shows the calculated variations in G_1, S and G_2+M after heat, heat and radiation with no interval, and at a 24–hr interval. Compared with heat treatment alone, the combined treatment induced a marked accumulation in G_2+M, most pronounced after 48 hr, and consequently a decrease in the percentage of cells in G_1. All treatments induced a small accumulation of cells with S DNA content.

Figure 3, third column, similarly shows the variations when radiation preceded heat treatment. Again it is seen that the combined treatment induced a considerable accumulation of cells in G_2+M, a small accumulation of cells in S, and a pronounced decrease of cells in G_1. It will be observed that the maximum accumulation in G_2+M is reached at different times after the first treatment in the combined series depending on the interval.

DISCUSSION

In the present work we have investigated the effect on tumour cells *in vitro* of combined

Table 1. Survival of the PNJ cells after treatment with hyperthermia and radiation with variable sequence and at varying intervals

Treatment	Relative survival* ± S.D.	
Controls	1.00 ± 0.06†	
Hyperthermia (41.5°C/60 min)	0.58 ± 0.07§	
Radiation (200 rad)	0.59 ± 0.05§	
41.5°C/60 min—0 hr—200 rad	0.20 ± 0.01‡	0.20 ± 0.02‡‖
41.5°C/60 min—24 hr—200 rad	0.22 ± 0.02‡	
200 rad—0 hr—41.5°C/60 min	0.26 ± 0.02‡	0.27 ± 0.04‡‖
200 rad—24 hr—41.5°C/60 min	0.27 ± 0.05‡	

*Mean of at least 3 cultures.
†Plating efficiency 40%.
‡Significantly different from controls and single treatments ($P<0.005$).
§Significantly different from controls ($P<0.005$).
‖Significantly different ($P<0.005$).

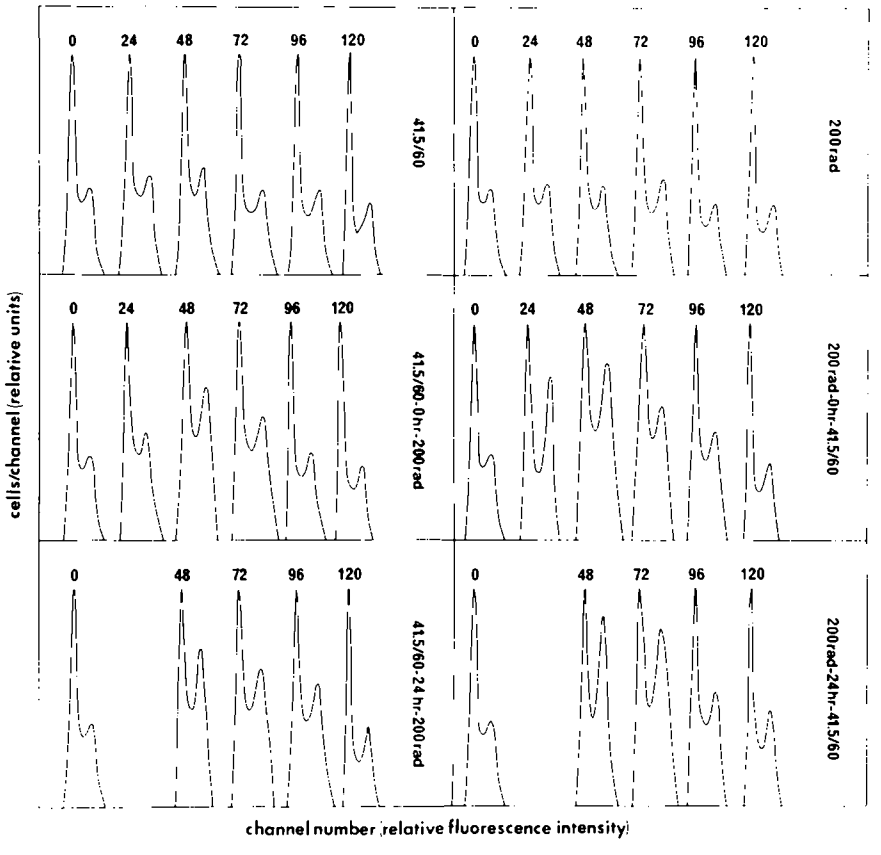

Fig. 2. DNA histogram of the treated PNJ cells at various hours after first treatment.

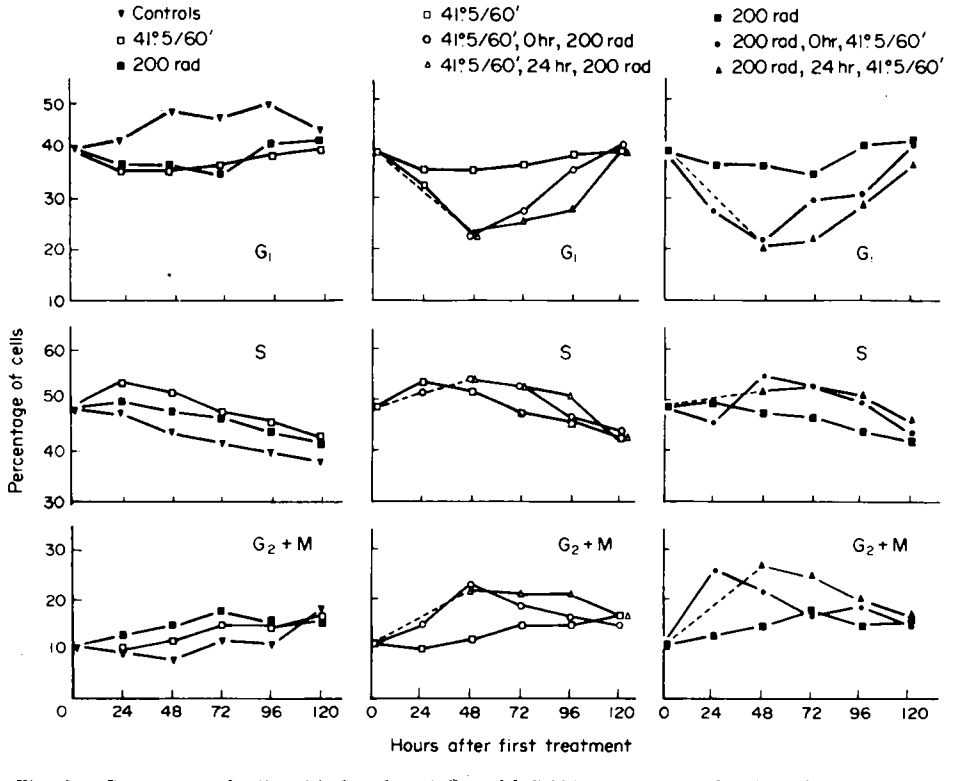

Fig. 3. Percentages of cells with G_1, S and G_2+M DNA content as a function of time after first treatment (heat and/or radiation). Symbols as in Fig. 1.

low doses of hyperthermia and radiation by means of growth curves, colony-forming ability and flow cytometry (FCM).

The growth curve shows that all treatments induced a delay in the growth, most pronounced after the combined treatments. The survival curves and the FCM study show that the delay in the growth is due to both a temporary arrest of the cells in the cell cycle and a cell-killing effect. As seen from the growth curves the growth rate of the treated and untreated cells are very much the same after 96–120 hr whereas the changes in the DNA histograms are most pronounced at 48 hr and then they fade out. However, as the DNA histograms reflect the proliferative activity of the cell population it is reasonable to expect changes in the DNA distribution to occur prior to changes in the growth curves.

The study of the colony-forming ability showed that the combined treatments had a more pronounced effect on cell survival than when the modalities were given alone. The study also showed that an interval of up to 24 hr did not change the effect of the combined treatments. This seems to be in contrast to most previous reports [7, 10], in which the thermal sensitization appeared to decrease with increasing interval between the two modalities with a lost potentiation, when this interval exceeded 10–180 min.

In our study, hyperthermia given before radiation was significantly more effective than the reverse sequence. This finding seems to be controversial in that some laboratories find more severe effects when radiation precedes heat [3]. In general, however, when low temperatures are used (41–43°C), hyperthermic treatment before radiation seems to be the most effective sequence. The reason why we found that hyperthermia before radiation was more effective could be that the PNJ cells in our study are more sensitive to 41.5°C/60 min than the cultures used by others.

The effect of hyperthermia given before radiation was found to be more than just additive (a relative survival of 0.20 against the expected survival of 0.34), whereas radiation before heat did not seem to be more than additive.

The FCM study demonstrated that a combination of a small dose of radiation with a small dose of hyperthermia, both of which when applied alone only induced slight changes in the distribution of cells over the cell cycle, leads to a considerable accumulation of cells with G_2 DNA content. Furthermore, it seemed that the cell kinetic changes after the second treatment were as pronounced when a 24-hr interval was interposed between the treatments as when one treatment was followed immediately by the other. The cell kinetic response seemed to be largely the same no matter whether heat was given before or after radiation.

Although other laboratories [1–6] have shown that both hyperthermia and radiation induce an accumulation of cells in certain phases of the cell cycle, it is impossible from our study to tell whether the distributional changes are real or simply due to a selective phase-dependent cell kill and cell disintegration.

As shown in Figs. 4 and 5, the cell kinetic changes seem to be more than additive in all the combinations, which suggests that the first treatment induces a sensitizing effect of the second treatment on the cell cycle which lasts for more than 24 hr.

The results of the present experiments suggest that the progression of cells through the cell cycle and the mechanisms involved in the lethal damage of the cells are different with respect to sensitivity to heat and radiation. Furthermore, it seems that heat or radiation applied alone induces a latent damage to the cells which is disclosed only in the combined schedules.

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