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Alteration of the Survival Response of Two Human Colon Carcinoma Subpopulations to X-Irradiation by *N,N*-Dimethylformamide[1]

John T. Leith,[2] Lynn A. Gaskins, Daniel L. Dexter, Paul Calabresi, and Arvin S. Glicksman

Radiation Biology Laboratories, Department of Radiation Oncology, Rhode Island Hospital [J. T. L., L. A. G., A. S. A.]; Department of Medicine, Roger Williams General Hospital [D. L. D., P. C.]; and Sections on Radiation Medicine and Medicine, Brown University, Division of Biology and Medicine, Providence, Rhode Island 02912

ABSTRACT

Two subpopulations of tumor cells obtained from a single human colon adenocarcinoma have been examined with regard to their intrinsic sensitivity to X-irradiation and to how this intrinsic radiosensitivity might be altered by growth in the polar solvent, *N,N*-dimethylformamide (DMF). These subpopulations, designated clones A and D, differ significantly in their radiation sensitivity. Using the single-hit multitarget equation to fit the survival responses, clone A exhibited survival parameters: extrapolation number (n) = 6.3; quasi-threshold dose (D_q) = 2.50 Grays; and mean lethal dose (D_o) = 1.36 Grays. These parameters for clone D were: n = 2.3; D_q = 1.27 Grays, and D_o = 1.56 Grays. Alteration of intrinsic radiosensitivity by DMF was then studied by adapting colon tumor cells to continuous growth in Roswell Park Memorial Institute Tissue Culture Medium 1640 containing 0.8% DMF. This treatment promotes differentiation of these colon cancer cells and is associated with an increased cell culture doubling time of 50 hr (20 hr for control cultures). It was found that, for cells grown in DMF medium, irradiated in fresh complete medium, and replated into fresh complete medium, survival parameters were: clone A—n= 5.2, D_q = 1.91 Grays, and D_o = 1.15 Grays; clone D—n = 2.2, D_q = 0.96 Grays, and D_o = 1.21 Grays. For tumor cells grown in DMF medium, irradiated in fresh complete medium, and replated into medium containing 0.8% DMF, survival parameters were: clone A—n = 2.8, D_q = 1.07 Grays, and D_o = 1.03 Grays; clone D—n = 2.0, D_q = 0.82 Grays, and D_o = 1.19 Grays. Survival responses of cells grown in fresh complete medium, irradiated in fresh complete medium, and replated into medium containing 0.8% DMF were not different from control responses. Using the D_q as an index of the sensitization occurring in the clinically relevant dose range, the pretreatment in DMF medium sensitizes both clone A and D cells to subsequent X-irradiation by a factor of approximately 1.3, whereas pre- and posttreatment in DMF medium increases this factor for clone A to 2.3 and for clone D to 1.5. Therefore, growth of colon cancer cells in DMF medium prior to irradiation, which results in cell differentiation, actually increases the sensitivity of cells to X-rays. Furthermore, subsequent reculturing of irradiated cells in DMF-containing medium compared to regular growth medium results in a further augmentation of the response.

INTRODUCTION

A number of laboratories including our own have reported that DMF[3] induced the differentiation of cultured tumor cells including murine erythroleukemia, melanoma, fibrosarcoma, and rhabdomyosarcoma, and human myeloid leukemia and colon carcinoma cells (1, 2, 4, 5, 13, 20). We have suggested that biological modifiers such as DMF, which induce an alteration of the malignant phenotype of cancer cells, can also alter their response to therapeutic agents (10).

This hypothesis was supported by results from a study in which 2 subpopulations of tumor cells obtained from a single mouse mammary adenocarcinoma (9) were exposed to the polar solvent DMF and were then X-irradiated. DMF sensitized these cells to the effects of ionizing radiation (17). The objectives of the experiments reported in this communication were 2-fold. First, we felt it was important to determine if this sensitization effect could be achieved with human cancer cells, specifically human colon carcinoma cells. In this regard, we chose to investigate the effects of DMF on the intrinsic X-ray sensitivity of 2 sublines of the DLD-1 human colon carcinoma cell culture line, which we have designated clone A and clone D (6). Clone A, clone D, and the parent DLD-1 cells have been well characterized with regard to their responses to antineoplastic agents and heat (3, 10, 18). Furthermore, we have reported previously several studies in which the effects of DMF on these cells have been described. The polar solvent alters the morphology and growth characteristics of the human colon cancer cells, reduces their tumorigenicity, induces a better differentiated phenotype as shown by increased expression of carcinoembryonic antigen and colonic mucoprotein antigen, and modulates the levels of purine metabolizing enzymes in these cells (6-8, 13). Second, as the extent to which enzymes responsible for DNA replication and repair are retained or lost in the process of differentiation is not well defined (15), and since the effects of DMF on these cells are reversible (6, 8, 13), we felt it important to determine whether reculturing irradiated cells in medium containing the polar solvent, as compared to regular growth medium, further augmented the response of these cells to ionizing radiation.

MATERIALS AND METHODS

Cell Cultures. The DLD-1 human colon cancer cell line was established from a heterogeneous carcinoma of the colon, and clones A and D were isolated from the parent DLD-1 cell line as described previously (6). The cells are routinely cultured using RPMI 1640 supplemented

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[2] To whom requests for reprints should be addressed, at Brown University, Box G, Providence, R. I. 02912.

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[3] The abbreviations used are: DMF, *N,N*-dimethylformamide; RPMI 1640, Roswell Park Memorial Institute Tissue Culture Medium 1640; n, extrapolation number; D_q, quasi-threshold dose; D_o, mean lethal dose.

with 10% fetal calf serum and antibiotics (Grand Island Biological Co., Grand Island, N. Y.) as has been described previously in reports from our laboratory (6).

Treatment of Colon Cancer Cells with DMF Prior to X-Irradiation. Cells were exposed to 0.8% DMF (v/v; Sigma Chemical Co., St. Louis, Mo.) in RPMI 1640. Cells were cultured in the presence of DMF for 3 to 5 passages before the start of the X-ray experiments. We have reported in a number of studies that this concentration of DMF is not toxic to clone A or D cells and that the treated cells continue to grow exponentially until the cultures become confluent. Doubling times for our untreated cells are about 20 hr, and doubling times for our DMF-treated cells are about 50 hr (6). We have also reported that no significant selection of DMF-resistant subpopulations occurs during this exposure period (7). In routine passages, greater than 90% of cells attach and grow in DMF medium when cells are plated at a density of 3×10^4 cells in 25-sq mm tissue culture flasks.

X-Irradiations. Immediately prior to irradiation, the DMF medium was removed from all of the cell culture flasks, and 5 ml of complete RPMI 1640 (without DMF) was added. Pairs of flasks (25 sq mm; Falcon Plastics, Oxnard, Calif.), *i.e.*, a flask with untreated control cells and one with DMF-treated cells, were irradiated simultaneously with a Picker X-ray machine. The conditions of irradiation were: 100 kVp; 10 ma; focal spot to target distance, 33 cm; added filtration, 1 mm aluminum; and dose rate, 1 Gray/min (1 Gray = 100 rads = 1 J/kg). Flasks were irradiated at ambient temperature (about 20°), and exposure doses were measured using a Victoreen R-meter (Victoreen Instrument Co., Cleveland, Ohio). Absorbed doses were obtained using appropriate temperature and pressure corrections and a Roentgen-to-rad conversion factor of 0.96. All irradiations were complete within approximately 0.5 hr.

Postirradiation Cell Manipulations. Immediately after irradiation, the medium overlaying the monolayers was poured into 15-ml centrifuge tubes. The monolayers were washed with 2 ml of 0.03% trypsin-EDTA (Grand Island Biological Co.) which was also collected. Then cultures were trypsinized at 37° with 3 ml of 0.03% trypsin-EDTA for 10 min. The trypsinate was added to the centrifuge tube, an equal amount of complete RPMI 1640 was added, and the samples were centrifuged at 100 rpm for 5 min. The supernatant was discarded, and the pellet was resuspended in 2 ml of complete RPMI 1640 and counted by hemocytometer. Depending on the survival level, sufficient tumor cells were seeded into 60-mm culture dishes (Corning Glass Works, Corning, N. Y.) to insure a level of about 50 surviving colonies per dish. After seeding, dishes were incubated at 37° in a humidified incubator with a 95% air and 5% CO_2 environment. To assess the surviving number of colonies, dishes were gently washed once with physiological saline, and then fixed and stained with 0.5% crystal violet in absolute methanol for 12 min. After staining, the number of colonies per dish was assessed using the criterion that a colony must possess at least 50 cells to be scored. The use of this criterion meant that dishes undergoing the various treatments had to be fixed at different times. The incubation times allowed for the various conditions were from 10 to 14 days and insured that the slowest growing colonies had reached the 50-cell-per-colony stage.

Statistical Analysis. In each experiment, 5 replicate dishes were obtained per dose point for all of the radiation conditions (*e.g.*, control cells, control cells replated into fresh medium containing 0.8% DMF, DMF pretreated cells replated into fresh medium, and DMF pretreated cells replated into fresh medium containing DMF). Two independent experiments were done for the clone D cells, and 3 for the clone A cells. For analysis, the means of the calculated survivals at each dose point were used. The parameters in the single-hit multitarget equation ($S/S_o = 1 - (1 - e^{-D/D_o})^n$) were obtained by fitting the data in the exponential region of the response curves to a linear least-squares regression equation. This equation was then used to determine n, D_q, and D_o. The 95% confidence limits on these values were determined using standard statistical procedures (12).

RESULTS

In Charts 1 and 2, we present the actual survival curves for the various experimental conditions for clone A (Chart 1) and clone D (Chart 2). In Table 1, we have summarized the results for the calculated radiation survival parameters, together with the error limits, for all the experimental conditions studied. It may seen that, while there is a trend towards a decrease in n, particularly for the clone A treatment conditions, these values are not statistically different. This trend is also seen in the D_os, but again these values are not statistically different. However, there are significant differences among the conditions in terms of the D_qs. For clone A, the D_qs for the 4 treatment conditions decrease as one goes from untreated cells to untreated cells recultured in DMF-containing medium, to cells treated with DMF and recultured in fresh complete medium without DMF, and then to DMF-treated cells recultured in medium with DMF. The slight decrease in D_q seen when control cells are X-irradiated and replated into DMF-containing medium can be thought of as a "drug shock" control experiment. While a small effect is seen, it is not statistically different from the control value for untreated cells. Conversely, the D_qs seen for the cells pretreated with DMF and recultured either into fresh complete medium or medium containing 0.8% DMF are statistically different from the control values, indicating that the pretreatment growth of cells in DMF-containing medium is critical for the full expression of the radiosensitizing effect of DMF. A similar effect is seen in clone D cells, but there is some overlap of 95% confidence limits. These "shoulder" changes induced by DMF are further considered in the discussion. Also in Table 1, we have listed the plating efficiencies (and their standard errors) obtained for these experimental conditions. These plating efficiencies refer to the survival (colony formation) of unirradiated cells in each treatment condition, and all survival data

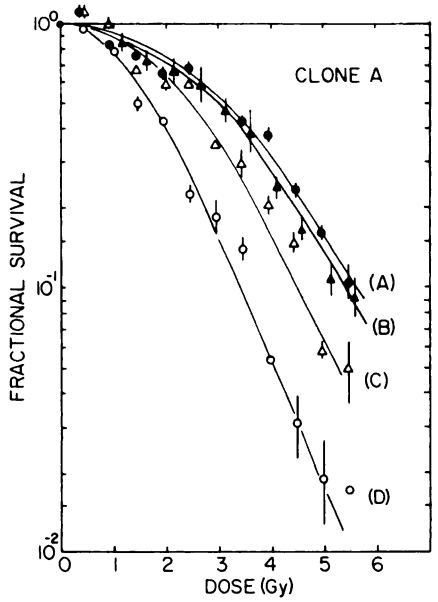

Chart 1. Survival response of clone A human colon carcinoma cells after X-irradiation. *Curve A*, response of irradiated control cells; *Curve B*, response of control cells irradiated and recultured in complete fresh medium containing DMF (0.8%); *Curve C*, response of cells grown prior to irradiation in DMF (0.8%), irradiated, and recultured in complete fresh medium; *Curve D*, response of cells grown prior to irradiation in DMF (0.8%), irradiated, and replated in complete fresh medium containing DMF (0.8%). *Points*, means; *bars*, S.E.

are calculated using the appropriate unirradiated plating efficiency.

DISCUSSION

A straightforward way to assess the effect of DMF on the intrinsic radiation sensitivity of clone A and clone D cells is to compare the doses needed to achieve the same level of survival for the various conditions. We have examined 2 levels of survival (50% and 10%) for these isoeffect comparisons. As these comparisons are the ratio of radiation doses, each having its own error, we have determined the propagated 95% confidence limits on the isoeffect comparison values obtained. For clone A cells, at 50% and 10% survival, respectively, the isoeffect values are 1.05 ± 0.05 (S.E.) and 1.06 ± 0.05 for control cells replated into DMF-containing medium, 1.27 ± 0.09 and 1.24 ± 0.04 for DMF pretreated cells replated into fresh complete medium (without DMF), and 1.92 ± 0.40 and 1.63 ± 0.11 for DMF pretreated cells replated into DMF-containing medium. For clone D cells, again at the 50% and 10% survival levels, values were 1.07 ± 0.04 and 1.07 ± 0.05 for control cells replated in DMF-containing medium, 1.30 ± 0.16 and 1.30 ± 0.04 for DMF pretreated cells replated into fresh complete medium (without DMF), and 1.43 ± 0.18 and 1.33 ± 0.04 for DMF pretreated cells replated in DMF-containing medium. Therefore, cells adapted for growth in DMF medium show a sensitization when exposed to X-rays. This sensitization is increased when clone A cells are replated in DMF-containing medium after irradiation (*e.g.*, the enhancement factor is 1.92). For clone D cells, there is a smaller increase for DMF pretreated cells X-irradiated and replated into DMF-containing medium (to 1.43). This augmentation of the DMF-induced increase in radiosensitivity by growth of irradiated cells in DMF-containing medium after X-ray treatment suggests that both pre- and posttreatment is the preferred schedule for the most advantageous effect. As these findings are very similar when either the 50% or 10% survival levels are compared, it appears that the action of DMF in these cells is essentially dose modifying (*i.e.*, approximately the same isoeffect ratios will be obtained at any level of survival examined).

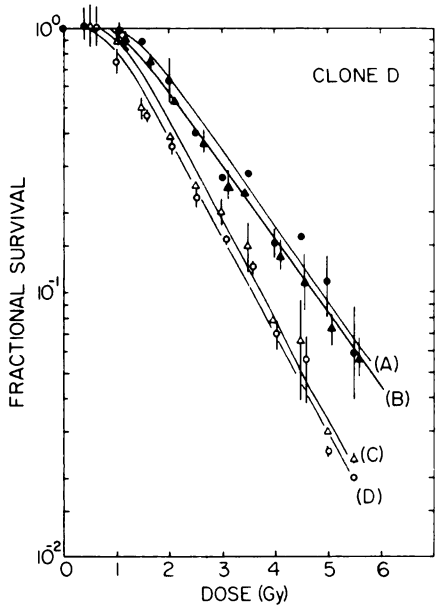

Chart 2. Survival response of clone D human colon carcinoma cells after X-irradiation. *Curve A*, response of irradiated control cells; *Curve B*, response of control cells irradiated and recultured in complete fresh medium containing DMF (0.8%); *Curve C*, response of cells grown prior to irradiation in DMF (0.8%), irradiated, and recultured in complete fresh medium. *Curve D*, response of cells grown prior to irradiation in DMF (0.8%). *Points*, means; *bars*, S.E.

It is also important to assess the effects of DMF on modification of the shoulder (D_q) region of the X-ray survival curve. The width of this shoulder has been taken as an index of the ability of the cell to accumulate sublethal radiation damage (11) and is the region of interest in terms of the clinical application of dose-modifying agents in cancer therapy. The data in Table 1 allows us to examine the effects of DMF on the shoulder width. Using the ratio of D_qs for the various conditions, it may be calculated that the relative sensitization of clone A cells in the various conditions is 1.14 ± 0.19, 1.31 ± 0.22, and 2.34 ± 0.86 for control cells replated into DMF-containing medium, DMF pretreated cells replated into complete fresh mecium (without DMF), and DMF pretreated cells replated into DMF-containing medium, respectively. Values are the propagated 95% confidence limits. For clone D tumor cells, values are 1.11 ± 0.61, 1.32 ± 0.51, and 1.55 ± 0.59 for control cells replated into DMF-containing medium, DMF pretreated cells replated into complete fresh medium (without DMF), and DMF pretreated cells replated into DMF-containing medium. It is therefore clear that significant sensitization in the low-dose region of the survival curve has been caused by DMF.

Table 1

Calculated survival curve parameters after X-irradiation of human colon cancer cells in vitro

Survival values from replicate experiments were analyzed using a linear least-squares regression of the unweighted data points in the exponential region of survival.

	Clone A				Clone D			
Treatment condition	*n*	D_o (Grays)	D_q[a] (Grays)	PE[b] (%)	*n*	D_o (Grays)	D_q (Grays)	PE (%)
Non-DMF treated cells								
Medium without DMF	6.3 (2.3–16.9)[c]	1.36 (0.40)	2.50 (0.20)	80.8 ± 12.5[d]	2.3 (0.7–7.4)	1.56 (0.66)	1.27 (0.44)	80.7 ± 1.88
Medium with DMF	4.9 (0.8–13.2)	1.37 (0.28)	2.19 (0.21)	41.5 ± 16.8	2.2 (0.8–7.2)	1.48 (0.22)	1.14 (0.34)	38.8 ± 5.2
DMF pretreated cells								
Medium without DMF	5.2 (1.6–16.8)	1.15 (0.36)	1.91 (0.29)	66.0 ± 23.1	2.2 (1.4–3.6)	1.21 (0.18)	0.96 (0.16)	75.6 ± 27.1
Medium with DMF	2.8 (0.6–12.2)	1.03 (0.42)	1.07 (0.47)	41.7 ± 15.2	2.0 (1.4–2.9)	1.19 (0.14)	0.82 (0.13)	48.2 ± 8.19

[a] $D_q = \ln(n \times D_o)$.
[b] PE, plating efficiency for zero dose.
[c] Numbers in parentheses, 95% confidence limits.
[d] Mean ± S.E.

We feel that it is significant that DMF apparently has its greatest effect on the colon tumor subline with the biggest shoulder (D_q; clone A) in terms of sensitization. Such an effect has also been demonstrated in studies on mouse mammary adenocarcinoma sublines, in that the line with the largest D_q (line 66) also showed a greater response than the line with the smaller D_q (line 67; Ref. 17). If DMF proves to be an agent that is preferentially effective on cell lines whose irradiation survival curves have large shoulders, this could be of therapeutic significance, particularly from the viewpoint of sequential combined modality therapy.

In Table 1, the plating efficiencies at zero dose for all 4 of the experimental conditions studied are listed. Compared to untreated cells, there is a reduction in the plating efficiency in control cells, replated into DMF-containing medium, in DMF pretreated cells replated into fresh complete medium without DMF, and in DMF pretreated cells that were replated in medium containing DMF. For clone A, this amounts to 49%, 18%, and 48% decreases, respectively, for the conditions of control cells replated into DMF medium and for the 2 conditions of DMF pretreatment. For clone D, these values are 52%, 7%, and 40%, respectively, for control cells replated into DMF medium and for the 2 conditions of DMF pretreatment. On a statistical basis, there is no difference between the 2 cell lines in their responses to DMF. There is the possibility that the reductions in plating efficiency may be associated with selection of cells with somewhat different growth properties in DMF. However, the fact that the plating efficiency results are similar for the 2 lines argues that the augmented response of clone A cells to posttreatment with DMF cannot be attributed to selection. Also, the fact that the survival curves for clone D for 2 different conditions of DMF exposure are similar, although the plating efficiences are different, argues that we have not inadvertantly selected for a particular subpopulation. The major point is that DMF pretreatment has significantly reduced the number of cells surviving X-irradiation compared to irradiated cells not pretreated with the polar solvent.

Changes in the shoulder regions of the survival curves suggest that the increased sensitivity observed with DMF pretreatment may be due to the decreased ability of sensitized cells to repair sublethal radiation damage. This could result from a decrease in the activity of repair enzymes. We have shown that the activity of adenosine deaminase in clone A cells is reduced 11-fold by DMF treatment, so enzyme levels can be significantly affected by exposure to the polar solvent (7). If repair enzyme activities are decreased by DMF exposure, then replating the DMF-pretreated cells after irradiation into medium without DMF should allow these enzymes to return to their normal levels, because all of the effects of DMF in these cells have been shown to be reversible (6, 8, 13). We are currently investigating the role of repair enzymes in this system.

The mechanism whereby DMF sensitizes these cultured human colon cancer cells to X-irradiation is not known. One important question is whether this sensitization is related in any way to the induction of differentiation that is produced in these cells with the same concentration of DMF. Results from other laboratories suggest that this may be the case. For example, Hill *et al.* (14) have shown that a B16 melanoma that was more differentiated with respect to pigment (melanin) production was also more sensitive to X-irradiation than was a less pigmented variant. Lavin *et al.* (16) have reported that cultured mouse neuroblastoma cells were more sensitive to far UV light after they had been induced to differentiate by growth in serum-free medium. This same group also obtained survival curves for UV treatment of differentiated and undifferentiated mouse neuroblastoma cells from the same clone. At a dose of 50 ergs/sq mm, only 10% of neuroblastoma cells induced to differentiate by serum deprivation survived, whereas 70% of undifferentiated cells survived such an exposure. Both studies suggest that there is a relationship between radiation sensitivity and the degree of differentiation of target cells. DMF induces many alterations of the malignant phenotype of cultured human colon cancer cells as well as maturational changes in these cells as measured by altered expression of markers such as carcinoembryonic antigen (8). One can therefore hypothesize that the sensitization of these carcinoma cells to X-rays may be related in some way to the differentiation process occurring with DMF exposure. Also, both ionizing radiation and dimethyl sulfoxide (chemically similar to DMF) produce single-strand breaks in DNA, and it is possible that the observed effects are mediated through such a damage process (19). A causal relationship remains to be demonstrated.

In summary, these experiments have shown that sensitization to X-irradiation of 2 subpopulations isolated from a human colon carcinoma cell line was produced when cells were grown in DMF prior to exposure. This pretreatment with DMF yields an enhancement factor of about 1.3 for both lines and occurs when irradiated cells are recultured in complete fresh medium without DMF. If, on the other hand, irradiated DMF-pretreated cells are recultured in medium again containing 0.8% DMF, there is a further augmentation of the sensitization effect, particularly for the clone A tumor cells. For clone A, this factor is now 1.8 to 1.9, whereas for clone D it is 1.4 to 1.5. This finding points to a significant biological difference between the 2 colon cancer sublines with regard to their ability to respond to the continued presence of DMF after X-irradiation and again emphasizes the heterogeneity of human tumors. Furthermore, these data imply that, if DMF were to be used as an adjunct in radiation therapy, then both pre- and posttreatment with DMF will give the optimum result.

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