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IN VIVO CHANGES IN THE INTESTINAL LIPID PEROXIDATION AND THE CRYPT RESPONSE TO WHOLE-BODY *GAMMA* IRRADIATION IN DIETHYLNITROSAMINE-TREATED MICE

ZMIANY *IN VIVO* W PEROKSYDACJI LIPIDÓW ORAZ ODPOWIEDZI KRYPT JELITOWYCH MYSZY NAPROMIENIOWANYCH NA CAŁE CIAŁO PROMIENIOWANIEM *GAMMA* PO ZATRUCIU NITROSODIETYLOAMINĄ

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Studies were carried out to elucidate lipid peroxidation and crypt survival in the small intestinal mucosa of mice pretreated per os with diethylnitrosamine (DENA) and whole-body gamma irradiated. Results show that DENA lowered the total value of mean lethal radiation dose for crypt cells, and the agent was enable to sensitize crypts to γ-rays. Present data suggest that gamma radiation- and/or DENA-induced pro-oxidant shift(s) is a risk factor for crypt survival.

INTRODUCTION

Diethylnitrosamine (DENA) has been shown to be a potent carcinogenic agent and promoter of lipid peroxidation and/or pro-oxidant shift(s) at many tissue and organ sites in rodents [3, 23, 32]. Because of the possible induction of lipid peroxidation in the gastrointestinal tract of DENA-treated animals [2, 16], the interaction between DENA and other lipid peroxidation producers, such as *gamma* radiation is of interest. Experiments examining the acute post-irradiation effect(s) have recently shown that DENA is enable to increase the risk of *gamma* rays-induced injuries in a short-term radiation carcinogensis model [11, 17]. Interestingly, a short-term acute *gamma* irradiation exposure was also noted to mitigate lipid peroxidation in the liver of mice pretreated with DENA, and it also increased the activity of superoxide dismutase and glutathione-linked enzymes [13].

It is widely believed that DENA induces reactive oxygen and nitrogen species (RONS) such as superoxide ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\bullet}$), and peroxynitrite ($ONOO^-$), the end product of reaction between nitric oxide (NO) and superoxide radical [3, 4, 23, 32]. Since RONS have been shown to be involved in gastrointestinal cancers [26], the crypt of small intestinal mucosa was recognized as a major target for both ionizing radiation- and/or chemical-induced injuries [28]. Although murine crypts, which contain a small number of stem cells, are enable to regenerate the intestinal mucosa after *gamma* irradiation exposure [14], few attempts have been made to elucidate the

harmful effect(s) of lipid peroxidiation and/or pro-oxidant shift(s) in the small intestinal crypt(s) of DENA-treated and *gamma* irradiated animals.

The major goal of this study was therefore to compute the probability of the survival of intestinal crypts for a specific variable of lipid peroxidation in the small intestinal mucosa of B6C3F1 mice. The multiple nonlinear regression models were frequently employed in the computation processes to fit the mean lethal radiation dose for crypt cells into the experimentally obtained results from *gamma* irradiated and/or DENA-insulted animals.

MATERIALS AND METHODS

Animals and experimental protocols

Male *B6C3F1* mice (10–12 wks) were divided into 4 groups of 30 mice each, and they were housed under conventional conditions with food and water *ad libitum*. The animals were treated with either a water solution of diethylnitrosamine (0.01, 0.1, or 1.0 mg DENA/kg b.w) or normal saline (control) daily for 21 days, and 24 h later they were given a single dose of whole-body *gamma* rays (2.5, 5.0, 7.5 or 10.0 Gy) using a ^{60}Co-source (2.0 Gy/min). The animals were sacrificed by cervical dislocation on day 3.5 post-radiation, and the small intestine was removed from the mice, and it was prepared for histological and/or biochemical analysis. Briefly, the small pieces of intestine samples were fixed in *Carnoy's* fixative, and cut into 5 μm thickness after paraffin embedding and stained with hematoxylin and eosin. The number of regenerating crypts that survived was counted under light microscope using the method of *Withers* and *Elkind* [33]. Thiobarbituric acid-reactive substances (TBARS) were determined as markers of lipid peroxidation and/or pro-oxidant shift(s) in the small intestine mucosa of mice as described elsewhere [13, 25]. Protein content was also measured by the method of *Lowry* and co-workers [21].

Computation processes and statistics

All raw data for crypt count were extracted from our previously published paper [11]. The crypt surviving fraction S(D) was calculated by the equation originally described by *Grudziński* [11]:

$$S(D) = 1 - \{1 - [\exp(\delta - \alpha_1 Dr + \alpha_2 Dr^2 - \beta_1 Dch + \beta_2 Dch^2)]\},$$

where Dr is the total dose of *gamma* radiation (Gy), Dch is the total dose of NDEA (mg/kg b.w), a_1, a_2 are the *gamma* radiation curve coefficients (Gy^{-1}, Gy^{-2}), β_1, β_2 are the NDEA curve coefficients $(mg/kg\ b.w)^{-1}$, $(mg/kg\ b.w)^{-2}$, and δ is the associated radiation/NDEA hybrid coefficient, the constant value for each curves S(D). All the estimated coefficients were previously discussed in details [11]. In the present studies, a double minus log transformation of the crypt mortality probability was linear with radiation dose, so that the survival curves were employed to compute the mean lethal dose (Do) for crypt cells as described by *Henry* and *Potten* [14]. The multiple nonlinear regression model for a best fit to the total amount of thiobarbituric acid reactive substances (TBARS) and/or crypt-surviving fractions as a function of the Do value(s) were used as test for trend. The *Mann-Whitney* U test was made to compare the estimated derived from all fitted curves. A significance level of the 0.05 was used throughout. The results were presented as the 3D-topographical projection of lipid peroxidation, which it provided a simple graphic-based method to estimate a dose-dependent response of intestinal crypts to lipid peroxidation and/or pro-oxidant shift(s) in mice pretreated with DENA and whole-body *gamma* irradiated. Two-dimensional plots (2D) were also employed to elucidate nonlinear regression models and confidence levels.

RESULTS AND DISCUSSION

Diethylnitrosamine (DENA) being one the most extensively studied mutagenic carcinogen, and considered to have no threshold for its hepatocarcinogenic effects was presently found to produce a substantial increase of lipid peroxidation in the small intestinal mucosa of mice (Figs. 1, 2A). The agent also elevated lipid peroxidation in other tissues of mice and rats including liver, kidney and spleen, and DENA-induced pro-oxidant shift(s) was mitigated by spermidine and/or N^G-Nitro-L-arginine methyl ester, a non-selective inhibitor of inducible nitric oxide synthase (iNOS) [12]. It should be noted that DENA also increased the level of lipid peroxide products, and it decreased antioxidant enzyme activities (superoxide dismutase and catalase) in the liver of cancer-bearing animals [19]. It is known that CYP2A6 or CYP2E1 is mainly involved to metabolize this carcinogen towards its ethylating electrophiles [6, 31], however DENA was also shown to trigger a number of other reactive oxygen and/or nitrogen species [3, 23, 26, 32].

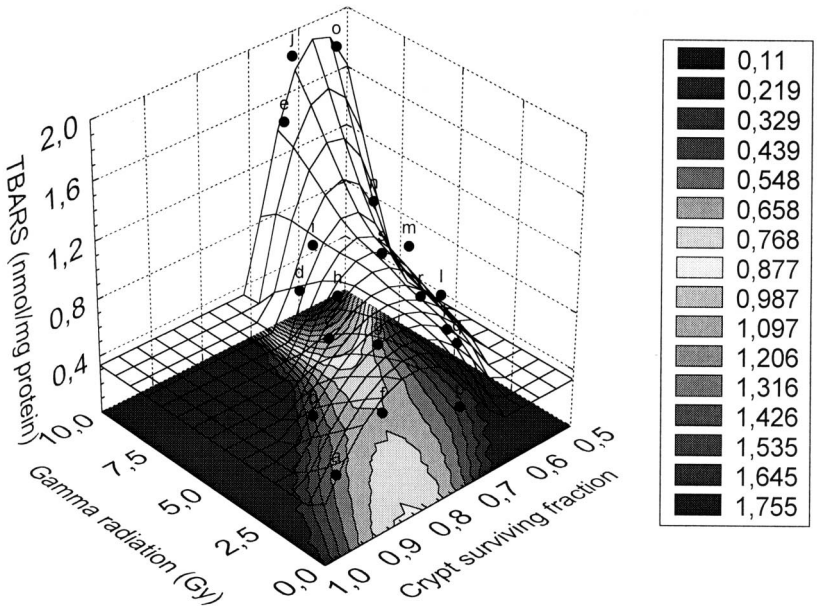

Fig. 1. Three-dimensional (3D) plot of lipid peroxidation and crypt surviving fraction after *gamma* irradiation and DENA exposures.
All data have been smoothed and topographically projected to obtain a graphical-based matrix. The values of TBARS (nmol/mg protein) were expressed as legend. Solid points are experimental data produced by normal saline (control) or DENA (0.01, 0.1, or 1.0 mg/kg b.w) in sham (control) or *gamma* irradiated (2.5, 5.0, 7.5, or 10.0 Gy) mice. The points represent mean ± SEM (n = 6 per point of irradiation), and they were assigned as follow (radiation Gy/NDEA mg/kg b.w): a – 0/0, b – 2.5/0, c – 5.0/0, d – 7.5/0, e – 10.0/0, f – 0/0.01, g – 2.5/0.01, h – 5.0/0.01, i – 7.5/0.01, j – 10.0/0.01, k – 0/0.1, l – 2.5/0.1, m – 5.0/0.1, n – 7.5/0.1, o – 10.0/0.1, p – 0/1.0, q – 2.5/1.0, r – 5.0/1.0, s – 7.5/1.0, t – 10.0/1.0.

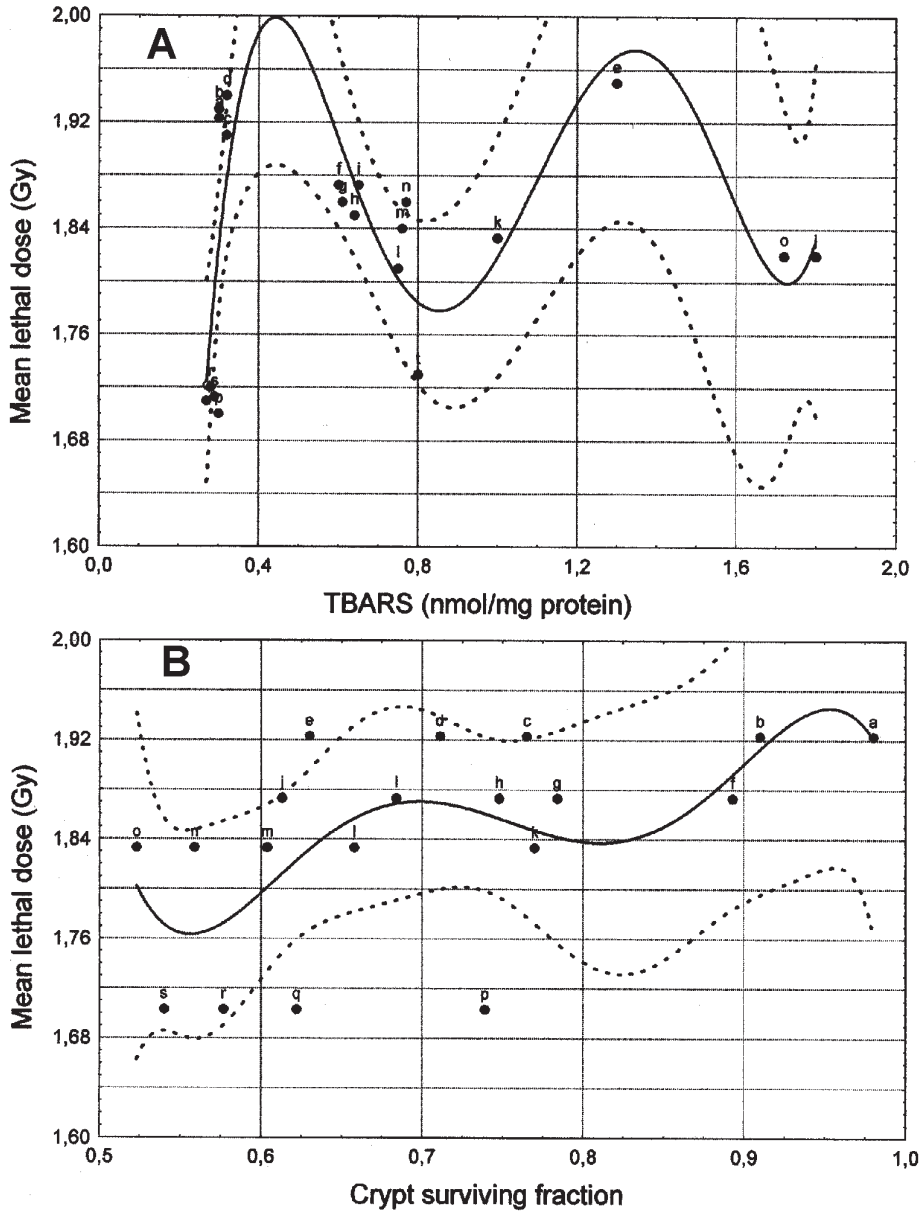

Fig. 2. Estimates of the mean lethal radiation dose (Do) as a function of DENA and *gamma* radiation doses.
The solid line shows the nonlinear regression of the observed TBARS (A) or crypt surviving fraction (B) plotted against Do after exposing mice to DENA for 21 days and irradiated from a cobalt-60 source. The dashed lines are 95 % confidence levels for the nonlinear regression line and described the range which the regression values will fall a calculated percentage after repeated measurements. See figure 1 for points (a-t) description.

Although the small intestinal mucosa did not play a major role to metabolize diethylnitrosamine in rodents [30], the results of the present study evidenced that murine crypts are very sensitive targets for DENA-and/or *gamma* radiation-induced pro-oxidant shift(s). As shown in figures 1 and 2A, irradiation of mice increased lipid peroxidation in the small intestinal mucosa of animals pretreated with or without DENA, and the agent lowered crypt-surviving fractions in post-irradiated mice. A present attempt to study the mean lethal radiation dose (Do) for crypt cells showed a decrease of its total value (Gy) in *gamma* irradiated and DENA-treated animals (Fig. 2). Interestingly, the minimum Do occurred at the highest DENA dosage (points p-s), however, crypt cells in the gut have been found to be most radio-resistant in mice insulted with DENA at 0.01 or 0.1 mg/kg body weight (Fig. 2B). It is now believed that all cells of the proliferating compartment in the crypt undergo a step-by-step differentiation and/or maturation from stem cells to the fully functional cells on the intestinal villus [20, 28]. Since reduction in overall crypt cellularity can be attributed to acute post-irradiation cell death (e.g. apoptosis), and *N*-nitroso compounds have been previously found to induce apoptosis, and they also targeted stem (clonogenic) cells in mice crypts [15, 29], it was hypothesized that the crypt survival of *gamma*-irradiated mice depends not only on the number of clonogenic cells in each intestinal crypts, but also on the radio-sensitivity of clonogenic cells in the crypt. It should be emphasized that DENA enhanced *in vivo* ^{60}Co-radiation-induced carcinogenesis, and the carcinogen also elevated glutathione S-transferase positive foci, a marker of pre-neoplastic changes in the liver tissue of irradiated rats [17]. In other recent studies, DENA was shown to promote the over-expression of radiation-induced p53 and *c-myc* oncoproteins in cultured human uroepithelium [22], and the agent increased the expression of γ-glutamyl transpeptidase in post-*gamma* irradiated rat liver tissues [27]. The compound has been previously reported to elevate a number of pro-carcinogenic lesions in whole-body *gamma* irradiated animals, such as DNA single-and/or double-strand breaks [5] and sister-chromatid exchanges [8]. Furthermore, the *mdr1b* gene, a stress-responsive DNA fragment, was recently elevated by DENA and radiation exposure [18], and it was further suggested that pretreatment of mice with DENA might also change p53-mitigated stem cell responses to *gamma* radiation-induced lesions. This suggestion was further supported by data reported earlier wherein crypt cells were found to be depleted in *gamma* irradiated and nitrite-pretreated mice [9, 10]. Since both DENA and *gamma* radiation elevated iNOS enzyme and nitric oxide (NO) levels in liver tissues and macrophages [1,24], and NO was found to prevent *gamma* radiation-induced cell cycle arrest [7], the question of the regulatory role of RONS including NO in murine crypts requires detailed investigation.

In conclusion, the present results show that diethylnitrosamine (DENA) and ^{60}Co-*gamma* irradiation induced lipid peroxidation (TBARS) in the small intestinal mucosa of mice. The agent was found to decrease crypt survival in post-irradiated animals, and it also mitigated the total value of mean lethal radiation dose. Both nonlinear logistic and/or polynomial regression models were computed to predict lipid peroxidation and crypt survival in DENA-treated and *gamma* irradiated mice, and the results of these studies were illustrated in the topographically projected matrix of lipid peroxidation and crypt surviving fractions.

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Summary

Studies were carried out to elucidate lipid peroxidation and crypt survival in the small intestinal mucosa of mice pretreated *per os* with either diethylnitrosamine (DENA) (0.01, 0.1, or 1.0 mg kg/b.w) or normal saline daily for 21 days, and whole-body *gamma* irradiated (2.5, 5.0. 7.5, or 10.0 Gy) following post-DENA and/or post-saline (control) exposures. Results show that DENA lowered the total value of mean lethal radiation dose for crypt cells, and the agent was enable to sensitize intestinal crypts to γ-rays. Present data suggest that *gamma* radiation- and/or DENA-induced lipid peroxidation and/or pro-oxidant shift(s) is a risk factor for murine crypt survival.

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Streszczenie

Przeprowadzono badania w celu analizy peroksydacji lipidów oraz przeżycia krypt w błonie śluzowej jelita cienkiego myszy zatruwanych *per os* N-nitrozodietyloaminą (DENA) (0.01, 0.1, or 1.0 mg kg/m.c) przez okres 21 dni oraz napromieniowanych na całe ciało (2.5, 5.0. 7.5, or 10.0 Gy) promieniowaniem *gamma* po ekspozycji na DENA. Myszy otrzymujące *per os* fizjologiczny roztwór chlorku sodowego (0.9 % NaCl) stanowiły kontrolę w doświadczeniu. Rezultaty wykazały, że DENA obniża letalną dawkę promieniowania *gamma* dla komórek krypty jelitowej oraz zwiększa wrażliwość krypt na promieniowanie gamma. Obecne wyniki sugerują, że pro-oksydacyjne oddziaływanie DENA i/lub promieniowania *gamma* jest czynnikiem ryzyka dla przeżywalności krypt jelita cienkiego.

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