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THE INTERACTION OF RADIATION AND INTERCALATING AGENTS IN NORMAL BONE MARROW CELLS AS EVALUATED BY SPLEEN COLONY ASSAY TECHNIQUE: THE EFFECTS OF BLEOMYCIN SULFATE AND ACTINOMYCIN D.

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ABSTRACT

The relationship between the way in which normal hemopoietic stem cells respond to irradiation alone or in the presence of bleomycin sulfate (BLM-S) and actinomycin D (ACT-D) was investigated. Single doses of BLM-S at 0.3 mg/kg and ACT-D at 0.10 mg/kg body weight were injected intravenously 1-6 hours prior to whole body irradiation and treatment was repeated twice more with time intervals. When assessed by survival of spleen colony forming units (CFU-S) of bone marrow cells (BMC), BLM-S alone caused only 10% reduction in survival compared to controls. There was not a significant difference in survival fraction (SF) when treatment with BLM-S was repeated twice more. On the other hand , ACT-D alone caused a 45% reduction in SF after the first injection and only a 10% reduction after the third injection. Increase in survival might be due to resistance induced in BMC after treatments with the drugs. The difference between the SF of BMC of mice exposed to doses of 1-3 Gy whole body irradiation was statistically significant with a p-value <0.05. When used in combination with radiation, neither BLM-S nor ACT-D caused a synergistic or additive effect. Although survival was seen to be lower for ACT-D treated animals, the effect was not as pronounced as expected. A significant change in the results was also not observed for fractionated doses of gamma rays in the presence of BLM-S and ACT-D injected at various time intervals. Results obtained from the administration of drugs at various time intervals before irradiation does not suggest a specific time for drug treatment prior to irradiation. These results also suggest that no potentiating effect is likely to be produced by a combination of BLM-S or ACT-D and radiation therapy in bone marrow cells. We therefore believe that these drugs induce a modest resistive response to the effects of radiation on bone marrow cells by a mechanism which is not yet understood. Therefore, using this agent repeatedly for cancer treatment might not cause severe adverse biological effects in bone marrow stem cells.

Key words: Biological effects, Radiation, Bleomycin sulfate, Actinomycin D, Bone marrow cells, Clonogenicity.

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INTRODUCTION

Radiobiology of normal tissues has always been a great concern to radiobiologists and radiotherapists following application of radiation and chemotherapeutic agents for cancer treatments. Some chemicals are used alone or in combination with radiation as sensitizers or additives in order to increase the mortality of malignant cells. Although most chemotherapeutic agents are designed to act selectively and accumulate in malignant tissues, they might also cause adverse biological effects.

Bleomycin is a cytotoxic glycopeptide isolated from *Streptomyces verticillies*. [1] It is an antibiotic that has antitumour activity against squamous cell carcinoma and malignant lymphomas. [2] The mode of action of BLM-S is shown to be due to the effects on DNA synthesis [3,4] and its cytotoxicity is related to DNA damage, chromosome breakage, and inhibition of protein synthesis.[5-7] Bone marrow depression is a very rare complication during clinical use of BLM-S [8] and studies in rats have shown that daily administration of BLM-S at high dose levels for a prolonged period of time does not produce significant changes in the peripheral blood picture.[9] One reason for the low hemopoietic cytotoxicity of this agent seems to be that following intravenous injection of BLM-S to rats, the level of drug in the marrow does not reach high values, possibly due to the presence of some BLM-S inactivating enzymes.[10] As well as being cytotoxic, BLM-S interferes with cell progression when applied to G_2 cells.[11,12] It has been shown that BLM-S induces molecular and cytological effects similar to ionizing radiation, [13,14] such as induction of DNA strand breaks, G_2 delay and chromosome aberrations.[13,15-18]

It is suggested that the combination of bleomycin treatment with radiotherapy would give an improved and synergistic therapeutic effect, as bleomycin causes a scission of DNA and inhibits DNA synthesis as well as DNA repair. [15] It was also shown that the presence of BLM-S before or during irradiation of bacteria and mammalian cells in culture lead to a cumulative effect[19,20] as well as a reduction in survival of epithelial cells.[21] After experiments with murine solid tumors, Begg et al.[22] found no difference in survival between combined treatment with BLM-S and radiation and radiation alone. It was also shown that doses near the LD_{50} (200 mg/kg) had a slight effect on CFU-S after 24 h in normal mice. [23] Maier and Schmid [24] and Kurten and Obe [25] found bone marrow of mice to be unaffected by BLM-S treatment at therapeutic dose range, probably because of selective accumulation in epithelial cells.

Dactinomycin is also an antibiotic of the actinomycin group which is produced by various species of streptomyces. [26,27] This drug is usually used in chemotherapy and intercalates into double-stranded DNA, preferentially into (dG.dC) regions. [28] As a consequence of this complex formation, DNA-dependent nucleic acid polymerases are inhibited. [27] It is used for cancer treatment of various malignancies such as rhabdomyosarcoma, Ewing's sarcoma and osteogenic sarcoma. [27] Elkind [29] showed that a dose of ACT-D after irradiation leads to a higher level of survival than using each of these agents individually, because radiation selectively kills G_2 cells, while these cells are insensitive to ACT-D. It is shown that ACT-D reduces repair of radiation-induced potentially lethal damage (PLD) and sublethal damage (SLD), whereas adriamycin from the same group of ACT-D only prevents repair of potentially lethal damage temporarily without any significant effect on repair of sublethal damage. By doing experiments with solid tumors, Twentyman et al. [30] could not find a specific time for administration of ACT-D before irradiation. However, they observed enhanced reaction of some normal tissues to radiation. It was also shown that mitomycin C, an analogue of ACT-D, enhances radiosensitivity of normal tissue and produces accumulative cytotoxicity in oxygenated cells. [31,32] A recent report from Hassen and Recs [33,34] indicates that antibiotics such as actinomycin D can have two different modes of action on progenitors of bone marrow cells. They can either produce cellular damage directly, or stimulate differentiation of normal human marrow myeloid progenitor cells. In a study with patients suffering from malignant melanoma, it was shown that actinomycin D is not metabolized to a great extent and accumulates in nucleated cells. [26] Human studies show that approximately 30% of ACT-D is excreted from the body during a week. [26] Therefore one of the most important side effects of this drug may be bone marrow depression.

In the work reported herein, we have concentrated on determining the possible side effects induced by BLM-S and ACT-D treatment in normal bone marrow cells when used alone or in combination with single or fractionated radiation doses and the effect on survival of spleen colony forming units (CFU-S) in mice. [35]

MATERIALS AND METHODS

Animals

Syrian white female mice were purchased from Pasteur Institute, Tehran. Mice were housed in metal cages in good condition and given standard mouse pellet and water *ad libitum* 3 days before beginning the experiments. All experiments were performed using eight week old mice. More than 1000 mice were used in

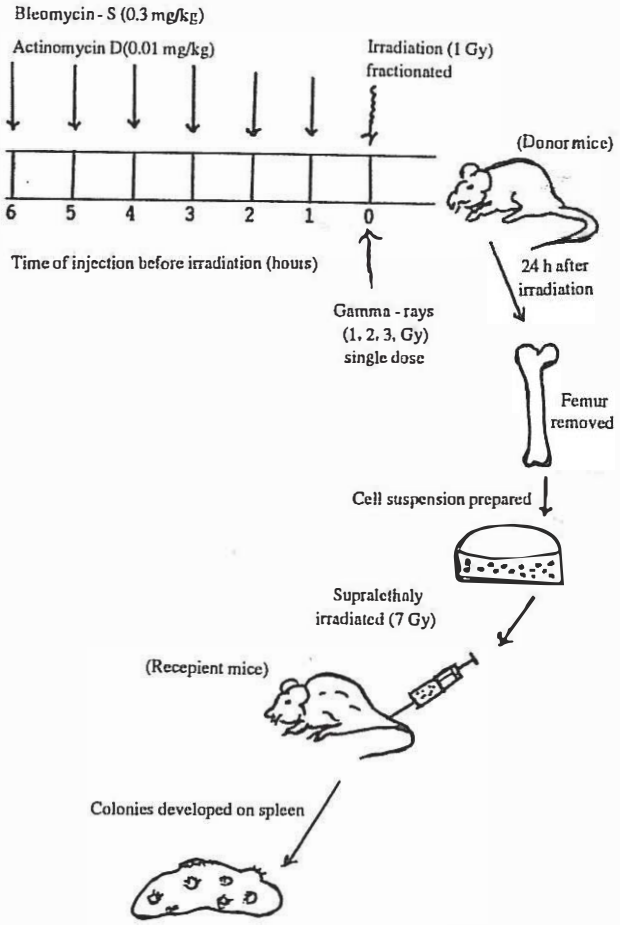

Fig. 1. Diagrammatic representation of the experimental design.

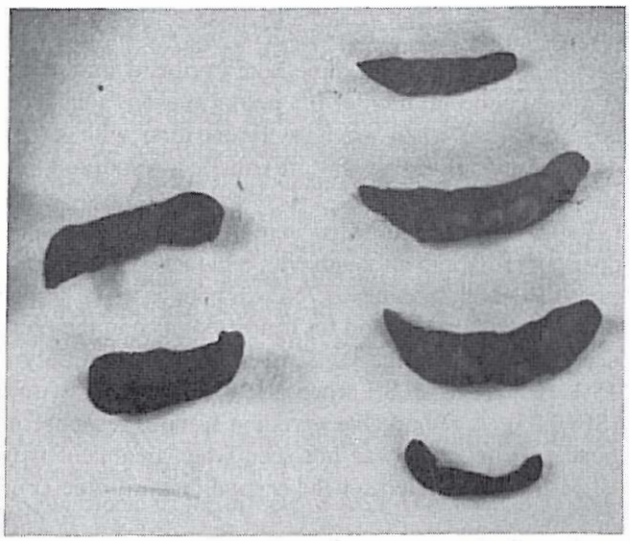

Fig. 2. Photomicrograph showing colonies developed on mouse spleens. Spleens without any colony were removed from mice in which transplantation failed and died 4 or 5 days after bone marrow grafting.

this study. More than 200 of them were irradiated or treated with ACT-D and BLM-S, and the rest were recipients of bone marrow transplantation.

Drugs

Actinomycin D (ACT-D), composed of 0.5 mg dactinomycin and 20 mg mannitol, which is commercially available as Iyovac cosmegen (Merck) and bleomycin sulfate (Nippon Kayaku Co.) was used in this study. After dilution with sterile physiologic buffer, BLM-S at a final concentration of 0.3 mg/kg and ACT-D at 0.10 mg/kg body weight were injected intravenously in the tail. Drugs were administered at various time intervals from 1-6 hours prior to gamma-irradiation.

Irradiation

Mice were irradiated with a cobalt 60 gamma-ray therapy unit (Teratron 780 c, Canada). Treated and control animals were irradiated at a dose of 195 cGy/min at a source sample distance (SSD) of 63 cm at room temperature. The radiation field was 30 × 30 cm and mice were irradiated in a cardboard box in groups of thirty. Irradiation was carried out either as a single whole body dose of 1, 2, and 3 Gy or as a fractionated regimen of 1 Gy at each fraction. The time interval between the first and second fraction was three days and between the second and third fraction was two days due to limitation in using the radiation source.

Assay Procedures

A flow diagram of the experimental design is shown in Fig 1. The assay for CFU-S was carried out by a method first described by Till and McCulloch. [35] For *in vivo* experiments the bone marrow of 6 femora was aspirated from mice in a group of three which had received ACT-D and BLM-S alone or in combination with radiation 1-6 hours prior to irradiation. Marrow suspensions were prepared 20 hours after irradiation under sterile conditions for injection into recipients. Nucleated cells in suspension were counted with a coulter counter (S-coulter counter, England). Groups of 9 recipients were given 700 cGy whole body irradiation prior to bone marrow transplantation. A known number of bone marrow cells in 0.3 ml suspension was injected intravenously in the tail of recipients. Spleens were removed 10 days after transplantation, fixed in methanol for 48 h and counted for determination of CFU-S survivals. A photomicrograph of such colonies on the spleen is shown in Fig. 2. By knowing the number of inoculated cells and counting the colonies on each sample, survival fraction was calculated using the following formula:

SF= Number of colonies counted/ Number of inocu-

Table I. Detailed results showing mean survival of bone marrow cells after exposure to gamma rays alone or in the presence of 0.3 mg/kg bleomycin sulfate and 0.01 mg/kg actinomycin D injected at various time intervals from 1-6 hours prior to irradiation.

Treatments	Dose Fraction D1 (1 Gy)	D2 (1+1 Gy)	D3 (1+1+1 Gy)
Control	0.977 ± 0.030	0.970 ± 0.034	0.936 ± 0.025
Saline	0.989 ± 0.029	0.945 ± 0.031	0.964 ± 0.031
Gamma-rays	0.775 ± 0.035	0.961 ± 0.027	0.864 ± 0.030
Bleomycin Sulfate	0.851 ± 0.035	0.803 ± 0.021	0.876 ± 0.017
Actinomycin D	0.530 ± 0.017	0.684 ± 0.016	0.916 ± 0.026
BLM-S (1)* + Gamma	0.804 ± 0.011	0.752 ± 0.015	0.904 ± 0.020
BLM-S (2) + Gamma	0.858 ± 0.016	0.755 ± 0.027	0.936 ± 0.025
BLM-S (3) + Gamma	0.781 ± 0.023	0.686 ± 0.011	0.930 ± 0.011
BLM-S (4) + Gamma	0.862 ± 0.027	0.660 ± 0.010	0.900 ± 0.019
BLM-S (5) + Gamma	0.823 ± 0.029	0.809 ± 0.036	0.875 ± 0.021
BLM-S (6) + Gamma	0.826 ± 0.021	0.753 ± 0.008	0.841 ± 0.019
ACT-D (1)* ± Gamma	0.730 ± 0.019	0.582 ± 0.016	0.904 ± 0.021
ACT-D (2) ± Gamma	0.720 ± 0.038	0.579 ± 0.021	0.839 ± 0.020
ACT-D (3) ± Gamma	0.635 ± 0.016	0.752 ± 0.016	0.878 ± 0.027
ACT-D (4) ± Gamma	0.745 ± 0.040	0.611 ± 0.016	0.825 ± 0.015
ACT-D (5) ± Gamma	0.712 ± 0.031	0.698 ± 0.016	0.740 ± 0.016
ACT-D (6) ± Gamma	0.686 ± 0.034	0.700 ± 0.016	0.827 ± 0.025

* Time of injection of bleomycin sulfate and actinomycin D before irradiation.
Values are mean value of data obtained for three mice. Errors are standard error of mean values.

lated cells × PE; where PE is the number of colonies observed for untreated samples per 1000 cells inoculated. Results were statistically analyzed by one way analysis of variance and the significance of any intergroup differences in the survival fraction was evaluated.

RESULTS

Effects of low-dose radiation on CFU-S

A group of mice were exposed to single whole body irradiation at doses of 1-3 Gy at a dose rate of 195 cGy/min. 24 hours after irradiation, bone marrow transplantation was performed. Results obtained with these experiments show a decrease in SF with increasing radiation doses (p-value<0.05). SF of cells receiving radiation doses of 1 Gy and 3 Gy was 0.78 and 0.51, respectively (Fig. 3). BMC of those animals receiving radiation at different fractions showed a decrease in survival after the first dose of irradiation (SF=0. 775), but after delivering the second and third dose with 3 and 2 day time intervals respectively, the effect of radiation on clonogenicity of BMC decreased in particular after the second radiation dose (SF=0.096) (Fig. 3). However, variations in survival were found to have a p-value of 0.394 which is not statistically significant.

Effects of BLM-S alone or in combination with radiation on CFU-S

Injection of BLM-S three hours prior to irradiation at a final concentration of 0.3 mg/kg caused a uniform dose response when used in combination with single doses of radiation (Fig. 3, left panel): a response quite similar to the effect of 1 Gy gamma rays alone (SF=0.74). Similar results were seen for fractionated doses of radiation, although with a higher survival fraction (SF= 0.85, p-value= 0.765). In both cases, a similar dose response was found for irradiated mice in the presence of BLM-S (Fig. 3). BLM-S alone caused only a 15% reduction in SF when used for the first time (SF=0.85). A remarkable variation in dose response of bone marrow cells was not seen when treatment with BLM-S was repeated for the second or third time (Fig. 3, Table I).

Results of the animals receiving BLM-S 1-6 hours prior to 1 Gy irradiation are summarized in Table I and graphed in Fig. 4. These results show that the effect of combined treatment with radiation and BLM-S is similar to the effects of BLM-S alone. Animals irradiated in

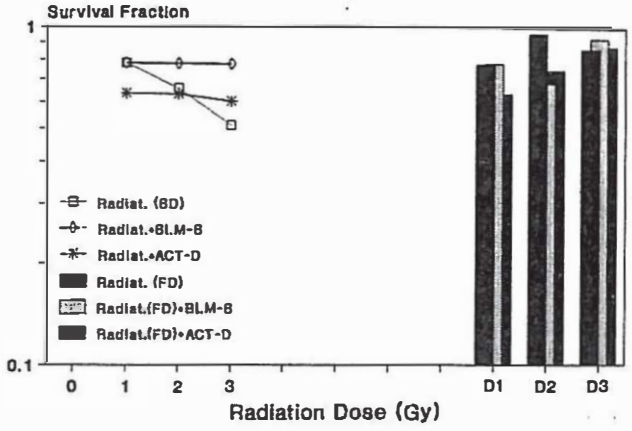

Fig. 3. Dose response curves obtained for bone marrow stem cells following single (SD) or fractionated (FD) doses of gamma rays alone or in the presence of bleomycin sulfate and actinomycin D. BLM-S at 0.3 mg/kg and ACT-D at 0.01 mg/kg body weight was injected 3 hours prior to irradiation. Each point represents the mean survival fraction calculated for data obtained from three mice. D1, D2, and D3 are indicative of the results obtained at each radiation fraction.

the presence of BLM-S in the third fraction showed a higher level of survival near control values which might be indicative of a modest protective effect of BLM-S for radiation induced damages (Fig. 4).

Injection of BLM-S at all times before irradiation had a uniform effect because mean values of calculated SF were seen to be similar for all injection times (Fig. 4) with a p-value of 0.852, which is not significant. Survival fraction of CFU-S cells following first treatment was calculated to be around 0.8 for all injection times prior to irradiation (Fig. 4), although a slight sensitivity was shown by bone marrow cells following second treatment three days later (SF around 0.7) and the survival fraction after the 3rd radiation dose was seen to be near normal (Fig. 4). This may indicate a modest adaptive response of BMC to the effects of bleomycin alone or in combination with radiation.

Effects of ACT-D alone or in combination with radiation on CFU-S

Fig. 3 shows the effects of ACT-D at a final concentration of 0.10 mg/kg on radiation induced changes in clonogenicity of CFU-S. Whole body irradiation was received by mice treated with ACT-D 3 hours prior to irradiation. As shown in Fig. 3, the effects of radiation alone on CFU-S caused a decrease in SF with increasing radiation doses (p-value<0.05). Irradiation in the presence of ACT-D resulted in a similar survival for doses up to 3 Gy so that a slope was not formed on the survival curve. In all cases the SF calculated for various doses in the presence of ACT-D corresponds to the effect of 2 Gy of gamma-rays alone (Fig. 3).

Results on the survival of CFU-S after treatment of mice with ACT-D from 1-6 hours prior to radiation therapy are summarized in Table I and graphed in Fig. 5. The effect of combined treatment on BMC at each fraction is statistically significant with a p-value<0.53, i.e. only about 50% of cells survived after ACT-D treatment alone, but after repeating experiments for the second and third times an increased rate of cell survival was observed (SF= 0.684 and 0.916, respectively; Table I). When ACT-D was administered 1-6 hours prior to receiving 1 Gy of gamma-rays, similar results were obtained for all treatment times with a p-value of 0.98, which is not statistically significant. The SF for the first treatment was calculated to be around 0.7 for all samples, which is not much different from that of ACT-D alone (Fig. 3, Table I). Results shown in Fig. 3 for mice receiving their second dose of radiation 3 days after their first exposure indicate a lower level of survival, especially when the drug is injected at one or two hours prior to irradiation. Survival fraction values after the 3rd radiation dose are shown to be near normal (Fig. 3), which might indicate adaptation of BMC to the effects of this drug.

Fig. 3 also shows a comparable mode of action of actinomycin D and bleomycin sulfate on bone marrow cells when used in combination with either single or fractionated doses of radiation. Three hours of treatment with BLM-S alone produced a uniform dose response in bone marrow cells for various injection times (Table I), whereas ACT-D produced a pronounced effect on survival when used for the first time, but the effect decreased with sequential use of ACT-D, so that for the third injection the survival fraction was near control values (Table I). The effect of ACT-D combined with radiation was also more pronounced than that of BLM-S, although a uniform effect was observed (Fig. 3, left panel). Using ACT-D and BLM-S with fractionated doses of radiation showed an adaptive response for both drugs (Fig. 3, right panel).

DISCUSSION

Exposure to ionizing radiation causes damage in all tissues of the body including bone marrow stem cells (BMC). For cancer treatment, chemotherapeutics are used alone or in combination with radiation for better efficacy of cell killing. However, both of these agents produce damage to some extent in normal tissues. Pluri-

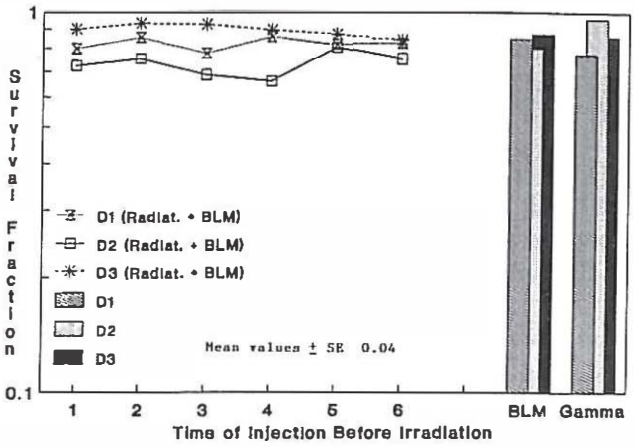

Fig. 4. Mean survival of bone marrow stem cells following treatment with bleomycin sulfate injected 1-6 hours prior to irradiation. Data shows the survival fraction calculated for mice irradiated in the presence of BLM-S at three different fractions. Histograms indicate the mean values obtained for radiation and BLM-S alone.

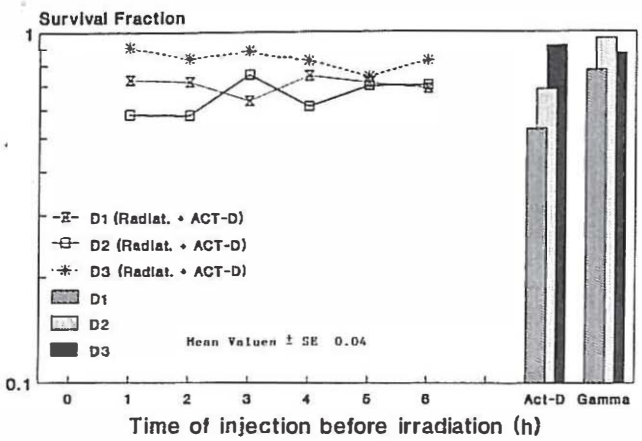

Fig. 5. Mean survival of bone marrow stem cells following treatment with 0.01 mg/kg ACT-D injected 1-6 hours prior to gamma irradiation. Mice received radiation doses at three different fractions with time intervals. Bars indicate mean values obtained with either radiation or ACT-D alone.

potent BM stem cells are the most radioresistant cells in the body with a dose of 95 cGy as shown by spleen colony assay *in vivo*.[35,36] In the present study, a survival curve was obtained using this technique after whole body irradiation of mice with doses of 1, 2, and 3 Gy (Fig. 3). The difference between the SF calculated for each dose was statistically significant with a $p<0.05$. This curve is shallower than that obtained by Till and McCulloch [35] because they performed BM transplantation shortly after irradiation while we have done BM transplantation about 24 hours after treatments (Fig. 3). This difference might be due to repair of sublethal damage induced by radiation as shown by Elkind [29] by leaving hamster cells at room temperature between irradiation fractions. These results are also in agreement with those reported for recovery of mouse bone marrow cells *in vivo* [37] and L-cells *in vitro*.[38] For animals receiving a fractionated radiation dose of 1 Gy at each fraction (Fig. 3), a three and two days repair time between irradiations, compared to an 11 hour cell cycle time for mouse BM is apparent. Therefore, most of the SLD and PLD induced in CFU-S would have enough time for repair.

Interaction of BLM-S and radiation on CFU-S

Investigations have been carried out to assess possible side-effects of combined therapy with BLM-S and radiation on normal CFU-S by means of spleen colony assay technique in mice.[35] Experimental evidence has indicated that hemopoietic stem cells are sensitive to ionizing radiation and have little capacity to repair sublethal damage.[39, 40] Previous reports have provided evidence of *in vivo* cytotoxic and cytogenetic effects of BLM-S against a broad spectrum of solid experimental and human tumors as well as lymphocytic cells.[6,14,16-18,41] Both laboratory and clinical studies have suggested that BLM-S could be potentially valuable when used in combination with radiotherapy, [19, 42] because of a degree of synergism demonstrated by Jorgensen. [43] However, results obtained in this study show neither a synergistic nor an additive effect for combined treatment of radiation and BLM-S (Figs. 3 and 4).

The dose response curve obtained for single doses of radiation alone is shallower than that reported previously, [35] because we performed bone marrow transplantation about 24 hours after irradiation. This difference in dose response might be due to the repair of sublethal damage induced by radiation, as shown by Elkind. [29] Data obtained for fractionated doses of radiation does not show much difference from the effect of radiation at each fraction (Fig. 3). Survival of BMC after BLM-S treatment was not also much different for various BLM-S injection times ($p=0.765$) so that the survival fraction calculated for the three experiments form a straight line rather than a curve (Fig. 3) These observations for both radiation and BLM-S when used alone are indicative of a functioning repair mechanism [37] and lesions produced by injection of BLM-S alone or irradiation can effectively be repaired during two or three days post-treatment. Although x-ray and BLM-S induced lesions are repaired,[13,44] different repair pathways exist.[13] Not only is the SF calculated for the effects of radiation doses of 1-3 Gy in the presence of BLM-S not indicative of an additive effect, but also it shows that BLM-S prevents the expression of some radiation induced lesions (Fig.

3). This observation is in contrast with those reported by Jorgensen [45] and the recent study of Hansen and Sorensen [46] who found a synergistic effect for combined treatment with BLM-S and radiation. The effect of BLM-S on radiation induced lesions observed in this study was similar for all radiation doses used (Fig. 3). This might be due to the direct effect of BLM-S on DNA. [21]

Various injection times of BLM-S prior to radiotherapy caused a uniform effect with a p-value of 0.852, indicating a similar effect of BLM-S on bone marrow cells for each treatment time. When BLM-S was applied 1-6 hours prior to irradiation, the SF calculated for the second fraction 3 days after the first treatment was less than the results obtained for the first irradiation fraction (Fig. 4). These effects seem to be subadditive as shown by Begg et al.[22] and Kimler et al.[47] Some investigators also found an additive effect for BLM-S.[19-21, 48]

These authors used synchronized or epithelial cells in which BLM-S produced a high degree of cytotoxicity, while it had a slight effect on BMC. [15, 24, 25] On the other hand, in experiments with mouse spermatogonial stem cells, Hansen and Sorensen [46] showed an apparent sensitizing effect for BLM-S when applied 1 hour before or after 9 Gy of irradiation. These observations indicate different modes of action of BLM-S on different cell systems. Bleehen et al. [19] studied the effects of BLM-S in combination with radiation on the survival of three bacterial strains and two mammalian cell lines. These authors suggested that the results for mammalian cells show no potentiating effect to be produced by BLM-S and radiation therapy in clinical practice. Mechanisms proposed for the mode of action of BLM-S indicate that in the presence of Fe(II), BLM-S induces DNA double strand breaks by first connecting a single strand of DNA between a guanine and pyrimidine.[49] Another factor that may be significant in the intercellular as well as interspecies variations in BLM-S induced damage[17] is the cellular concentration of enzymes that may inactivate BLM-S or some of its active intermediates.

However, results presented in the present paper clearly show that BLM-S did not enhance radiation induced cell killing of CFU-S at all pre-irradiation injection times but rather decreased radiation effects. We therefore propose that using BLM-S in combination with radiation induces some kind of lesion that make cells resistant to radiation induced damage; in other words, a modest protective effect may be induced by BLM-S treatment before irradiation.

Interaction of ACT-D and radiation on CFU-S

Injection of ACT-D at 3 hours prior to irradiation with doses of 1, 2, and 3 Gy of gamma-rays caused an additive effect (Fig. 3). This effect is more pronounced at a dose of 1 Gy but not for higher doses of up to 3 Gy. The combined effect of 1 Gy of radiation and ACT-D was similar to the effects of 2 Gy of radiation (Fig. 3). This observation might be due to the cell cycle delay induced by ACT-D, because the presence of this drug prevents cell growth and produces cell cycle delay.[29, 50] If the sampling time exceeds 24 hours, it might be possible to obtain a survival curve with a steeper slope, since irradiation causes cell killing at G_2 phase while G_2 cells are resistant to ACT-D.[29]

ACT-D in combination with radiation was seen to have a subadditive effect at various time intervals because ACT-D prevents repair of PLD and SLD induced by radiation. [51] Administration of this drug at 1-6 hours prior to irradiation produced almost similar effects (p-value = 0.98) (Fig. 5). It was shown that using ACT-D shortly before or after irradiation produced a lower SF compared to that obtained by each of these agents alone.[29] This observation might be due to the cumulative effect of ACT-D, which is consistent with our findings and that of Twentyman et al.[30] For studying the effects of the time of injection prior to irradiation, survival fractions were obtained for various time intervals (p-value= 0.001). Although the time interval between the 2nd and 3rd fraction was shorter (2 days), the SF calculated was similar to that obtained after the first fraction (Fig. 3, Table I). This process might be due to either repair of the SLD and PLD induced by radiation and ACT-D or induction of resistance in BMC by ACT-D administration.[30] Damage induced in the 2nd fraction was more pronounced, which might be due to the direct effect of ACT-D on cells.[50]

Based on the data presented here, although the effect of ACT-D after the first injection is remarkable, we conclude that repeated administration of ACT-D alone or in combination with radiation for solid tumors might not cause severe bone marrow depression. This process might be due to the resistance of bone marrow stem cells to the additional effects of ACT-D. Similar to the report of Twentyman et al.[30] for solid tumors, we also could not find a specific time of ACT-D injection for obtaining a greater effect on bone marrow cells.

Comparison of the effects of ACT-D and BLM-S

The results presented here for the effects of ACT-D and BLM-S on radiation induced lesions are indicative of a modest adaptive response of CFU-S. Although the dose of ACT-D and BLM used in these studies was in the therapeutic dose range usually used in clinical practice, ACT-D caused a more pronounced effect on survival of CFU-S than BLM-S, especially when used alone (Table I). The dose of ACT-D was much less than

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However, results presented in the present paper clearly show that BLM-S did not enhance radiation induced cell killing of CFU-S at all pre-irradiation injection times but rather decreased radiation effects. We therefore propose that using BLM-S in combination with radiation induces some kind of lesion that make cells resistant to radiation induced damage; in other words, a modest protective effect may be induced by BLM-S treatment before irradiation.

Interaction of ACT-D and radiation on CFU-S

Injection of ACT-D at 3 hours prior to irradiation with doses of 1, 2, and 3 Gy of gamma-rays caused an additive effect (Fig. 3). This effect is more pronounced at a dose of 1 Gy but not for higher doses of up to 3 Gy. The combined effect of 1 Gy of radiation and ACT-D was similar to the effects of 2 Gy of radiation (Fig. 3). This observation might be due to the cell cycle delay induced by ACT-D, because the presence of this drug prevents cell growth and produces cell cycle delay.[29, 50] If the sampling time exceeds 24 hours, it might be possible to obtain a survival curve with a steeper slope, since irradiation causes cell killing at G_2 phase while G_2 cells are resistant to ACT-D.[29]

ACT-D in combination with radiation was seen to have a subadditive effect at various time intervals because ACT-D prevents repair of PLD and SLD induced by radiation. [51] Administration of this drug at 1-6 hours prior to irradiation produced almost similar effects (p-value = 0.98) (Fig. 5). It was shown that using ACT-D shortly before or after irradiation produced a lower SF compared to that obtained by each of these agents alone.[29] This observation might be due to the cumulative effect of ACT-D, which is consistent with our findings and that of Twentyman et al.[30] For studying the effects of the time of injection prior to irradiation, survival fractions were obtained for various time intervals (p-value= 0.001). Although the time interval between the 2nd and 3rd fraction was shorter (2 days), the SF calculated was similar to that obtained after the first fraction (Fig. 3, Table I). This process might be due to either repair of the SLD and PLD induced by radiation and ACT-D or induction of resistance in BMC by ACT-D administration.[30] Damage induced in the 2nd fraction was more pronounced, which might be due to the direct effect of ACT-D on cells.[50]

Based on the data presented here, although the effect of ACT-D after the first injection is remarkable, we conclude that repeated administration of ACT-D alone or in combination with radiation for solid tumors might not cause severe bone marrow depression. This process might be due to the resistance of bone marrow stem cells to the additional effects of ACT-D. Similar to the report of Twentyman et al.[30] for solid tumors, we also could not find a specific time of ACT-D injection for obtaining a greater effect on bone marrow cells.

Comparison of the effects of ACT-D and BLM-S

The results presented here for the effects of ACT-D and BLM-S on radiation induced lesions are indicative of a modest adaptive response of CFU-S. Although the dose of ACT-D and BLM used in these studies was in the therapeutic dose range usually used in clinical practice, ACT-D caused a more pronounced effect on survival of CFU-S than BLM-S, especially when used alone (Table I). The dose of ACT-D was much less than

that of BLM-S. Repeating ACT-D treatment with time intervals between injections did not enhance cell killing, but produced a protective effect in bone marrow cells. This is clearly demonstrated in Fig. 3, where ACT-D and BLM-S were used in combination with fractionated doses of radiation. The results suggest that no potentiating effect is likely to be produced by the combination of BLM-S or ACT-D with radiation therapy in clinical practice in bone marrow cells. It is clear from the above results that the presence of BLM-S or ACT-D before irradiation in the mammalian system investigated here produces a modest adaptive response by a mechanism which is not clearly understood.

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REFERENCES

1. Umezawa H, Maeda K, Takeuchi T, Okami Y: New antibiotics, bleomycin A & B. Journal of Antibiotics 19: 200-209, 1966.
2. Umezawa H: Bleomycin. In: Corcoanand W, Hahn FE (eds.), Antibiotics. Vol. 3, Berlin, Springer-Verlag, pp. 21-33, 1975.
3. Terasima T, Takabe Y, Katsumata T, Watanabe M, Umezawa H: Effects of bleomycin on mammalian cell survival. J Nat Cancer Inst 49: 1093, 1972.
4. Watanabe M, Tobnika Y, Katsumata T, Terasima T: The effects of bleomycin on progression through the cell cycle of mouse L-cells. Cancer Research 34: 878-881, 1974.
5. Vig BK, Lewis R: Genetic toxicology of bleomycin. Mutation Research 55: 121-145, 1978.
6. Aghamohammadi SZ, Henderson L, Cole RJ: The human lymphocytes micronuclei assay, response of cord blood lymphocytes to gamma irradiation and bleomycin. Mutation Research 30: 395-401, 1984.
7. Povirk LF, Austin MJF: Genotoxicity of bleomycin. Mutation Research 257: 127-143, 1991.
8. O.E.R.T.C. Clinical Screening Group: Study of the chemical efficacy of bleomycin in human cancer, preliminary communication.British Medical Journal 2: 643-645, 1970.
9. Matsuda A, Miyamoto K, Tsubozaki M, Ishibashi H, Ota K, Shimada M, Ito K, Yoshioka O, Yamaguchi Y, Sakamoto K, Tanaka T: Subacute and chronic toxicities of bleomycin. Nipon Kayaku Internal Report, 1968.
10. Umezawa H: Bleomycin: discovery, chemistry and action. Cancer Research 19: 3-36, 1979.
11. Barranco SC, Humphrey RM: The effect of bleomycin on survival and cell progression in Chinese hamster cells *in vitro*. Cancer Research 31: 1218-1223, 1971.
12. Tobby RA: Arrest of Chinese hamster cells in G_2 following treatment with the antitumor drug bleomycin. Journal of Cell Physiology 79: 259-266, 1972.
13. Byfield JE, Lee YC, Tu L, Kulhunian F: Molecular interactions of the combined effects of bleomycin and x-rays on mammalian cell survival. Cancer Research 36: 1138-1143, 1976.
14. Povirk LF: Bleomycin. In: Neidle S, Waring M (eds.), Molecular Aspects of Anticancer Drug Action. Berlin, Springer-Verlag, pp. 157-181, 1983.
15. Hittelman WN, Rao PN: Bleomycin induced damage in prenatally condensed chromosomes and its relationship to the cell cycle progression in CHO cells. Cancer Research 34: 3449, 1974.
16. Ostling O, Johanson KJ: Bleomycin, in contrast to gamma irradiation, induces extreme variation of DNA strand breakage from cell to cell. Int J Radiat Biol 52: 683-691, 1987.
17. Miller K: Clastogenic effects of bleomycin, cyclophosphamide and ethyl methanesulfonate on resting and proliferating human B- and T-lymphocytes. Mutation Research 251: 241-251, 1991.
18. Kligerman AD, Bryant MF, Doerr CL, Halperin EC, Kwanyuen P, Sontag MR, Erexon GL: Interspecies cytogenetic comparison: studies with X-radiation and bleomycin sulfate. Environmental and Molecular Mutagenesis 19: 235-243, 1992.
19. Bleehen NM, Gillies NF, Twentyman PR: The effects of bleomycin and radiation in combination on bacteria and mammalian cells in culture. British Journal of Radiology 47: 346-351, 1974.
20. Roizin-Towle L, Hall EJ: The effect of bleomycin on aerated and hypoxic cells *in vitro*, in combination with irradiation. Int J Radiat Oncol Biol Phys 5 (9): 1491-1494, 1979.
21. Crooke ST, Bradner WT: Bleomycin: a review. J Med 7: 335-378, 1976.
22. Begg AC, Kane LY, Fu KK, Philips TL: The effects of combined bleomycin and irradiation on solid murine tumours. Int J Radiat Oncol Biol Phys 5 (9): 1503-1507, 1979.
23. Twentyman PR, Bleehen NM: The sensitivity to bleomycin of spleen colony forming units in the mouse. Br J Cancer 28 (1): 66-70, 1973.
24. Maier P, Schmid W: Ten model mutagens evaluated by the micronucleus test. Mutation Research 40: 325-338, 1976.
25. Kurten S, Obe G: Premature chromosome condensation in the bone marrow of Chinese hamsters after application of

bleomycin *in vivo*. Mutation Research 27: 285-294, 1975.
26. Merck, Sharp and Dohme International: Lyovac Cosmegen (MSD), Division of Merck and Co. Inc. Rahway, NY 07065, USA, 1987.
27. Raynolds EF: "Martindale" The Extra Pharmacopeoia, 29th edition, The Pharmaceutical Press, London, 1989.
28. Muller WEG, Zahn RK: Biochemistry of antivirals, mechanism of action and pharmacology. Research in Molecular Biology 9: 46-65, 1978.
29. Elkind MM: Fundamental questions in the combined use of radiation and chemicals in the treatment of cancer. Int J Radiat Oncol Biol Phys 5 (9): 1711-1720, 1979.
30. Twentyman PR, Kallman RF, Brawn JM: The effect of time between X-irradiation and chemotherapy on the growth of three solid mouse tumours- IV: Actinomycin D. Int J Radiat Oncol Biol Phy 5 (9): 1601-1603, 1979.
31. Von der Masse H: Experimental studies on interactions of radiation and cancer chemotherapeutic drugs in normal tissue and solid tumours. Radiother Oncol 7: 47-68, 1986.
32. Herman TS, Teicher BA, Holden SA: Trimodality therapy (drug/hyperthermia/radiation) with BCNU or mitomycin C. Int J Radiat Oncol Biol Phys 18 (2): 375-382, 1990.
33. Hassen HT, Rees JKH: The effect of human recombinant granulocyte-macrophage colony stimulating factor on the proliferation and differentiation of myeloid progenitors in congenital agranulocytosis marrow cells. Haematologica 22 (4): 233-236, 1989.
34. Hassen HT, Rees JKH: Low concentrations of cytosine arabinoside, 6-thioguanine, actinomycin D and actinomycin A stimulates the differentiation of normal human marrow myeloid progenitor cells. Med Oncol and Tumor Pharmacother 6 (3): 213-217, 1989.
35. Till JE, McCulloch EA: A direct measurement of radiation sensitivity of normal mouse bone marrow cells. Radiation Research 14: 213-222, 1961.
36. McCulloch EA, Till JE: The sensitivity of cells from mouse bone marrow to gamma radiation *in vitro* and *in vivo*. Radiation Research 16: 822-832, 1962.
37. Till JE, McCulloch EA: Early repair processes in marrow cells irradiated and proliferating *in vivo*. Radiation Research 18: 96-105. 1963.
38. Han A, Miletic B, Petrovic D, Jovic D: Survival properties and repair of radiation damage in L-cells after X-irradiation. Int J Radiat Biol 8 (3): 201-211, 1964.
39. Senn JS, McCulloch EA: Radiation sensitivity of human marrow cells measured by a cell culture method. Blood 35: 56-60, 1970.
40. Grilli G, Nothdurft W, Flidner TM: Radiation sensitivity of human erythopoietic and granulopoietic progenitor cells in the blood and in the bone marrow. Int J Radiat Biol 41: 685-687, 1982.
41. Weissenborn U, Obe G: Modification of bleomycin induced chromosome aberrations by hyperthermia and under energy depleting conditions in human peripheral lymphocytes. Int J Radiat Biol 62 (30): 289-296, 1992.
42. Halnan KE, Bleehen NM, Brewin TB, Deeley TJ, Harrison DFN, Howland C, Kimbler PB, Ritchie GL, Wiltshaw E, Todd IDM: Early clinical experience with bleomycin in the United Kingdom in a series of 105 patients. Br Med J 2: 635-638, 1972.
43. Jorgensen SJ: Dose schedules and combination with radiotherapy in bleomycin treatment. Presented at the 7th International Congress of Chemotherapy, Prague, 1971.
44. Kuo MT, Haidle CW: Characterization of chain breakage in DNA induced by bleomycin. Biochemica et Biophisica Acta 335: 109-114, 1973.
45. Jorgensen SJ: Time-dose relationships in combined bleomycin treatment and radiotherapy. Europ J Cancer 8: 531-534, 1972.
46. Hansen PV, Sorensen D: Effects of vincristin or bleomycin on radiation induced cell killing of mice spermatogonial stem cells: the importance of sequence and time interval. Int J Radiat Oncol Biol Phys 20: 339-341, 1991.
47. Kimler BF, Martin DF, Evans RG: Combination of radiation therapy and intracranial bleomycin in the 9L rat brain tumour model. Int J Radiat Oncol Biol Phys 18 (5): 1115-1121, 1990.
48. Sherive PC, Harris JW: Effects of bleomycin and irradiation on euoxic and hypoxic cells. Int J Radiat Oncol Biol Phys 5 (9): 1495-1498, 1979.
49. Povirk LF, Han Y-H, Steighner RJ: Structure of bleomycin induced DNA double strand breaks. Predominance of blunt ends and single base 5′ extension. Biochemistry 28: 5808-5814, 1989.
50. Hassen HT: Differentiation induction therapy of acute myelogenous leukemias. Haematologica 21: 141, 1988.
51. Dritschilo A, Piro AJ, Kelman AD: The effect of cisplatinum on the repair of radiation damage in plateau-phase Chinese hamster (V-79) cells. Int J Radiat Oncol Biol Phys 5 (8): 1345-1349, 1979.