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INTERACTION BETWEEN BLEOMYCIN AND GLUTATHIONE IN CANCER-BEARING ANIMALS

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ABSTRACT

We performed an experiment on the anticancer effect of Bleomycin and Glutathione administered simultaneously in tumor bearing mice.

1) The radioactivity of ^{35}S-Glutathione in organs tended to be lower after the simultaneous administration of the labelled Glutathione and Bleomycin than when the labelled Glutatione was given alone.

2) The reaction of Glutathione and Bleomycin in vivo depends upon the quantitative ratio of the two substances.

3) In cancer-bearing mice a comparison was made of anticancer effectiveness between Bleomycin used alone and in combination with Glutathione. During an initial 60 day period treatment with Bleomycin alone produced an excellent anticancer effect and also provided a marked life prolonging effect when compared with control. These effects in the Bleomycin + Glutathione group were no better than in the control group.

4) Past the 60th day of treatment (after 30 doses of Bleomycin or Glutathione were given) fatalities occurred frequently in the Bleomycin group due to the drug toxicity, while in the Bleomycin + Glutathione group a greater increment of survival time was attained due to a detoxifying effect of Glutathione.

INTRODUCTION

In order to enhance the therapeutic effectiveness of cancer chemotherapy it is necessary to maintain the concentration of anticancer drugs in tumor cells at therapeutically adequate levels, to reduce the incidence and severity of side effects, if any, as much as possible and to carry out the therapy as originally planned without detriment to the defense mechanism of the body. Since effects and side-effects are more or less inseparable in every anticancer drug, controlling them adequately would be the greatest factor determinant of the therapeutic effectiveness of cancer chemotherapy.

Bleomycin (hereafter BLM in short) with pronounced anticancer activity against squamous cell carcinoma of the oral region is also liable to produce severe side effects, which has been pointed out as posing a problem of clinical importance. Lessening of adverse effects of Bleomycin by the use of Glutathione (hereafter GSH in short) is one of the solutions to this problem and we make it a rule to use GSH concomitantly with BLM in clinical patients. However, the conjoined use of BLM and GSH might possibly result in the inactivation of BLM, that is, a loss of its anticancer activity.[1~3] In contrast, Sonezaki[4] in their experiments on Ehrlich ascites carcinoma noted that the anticancer effect of BLM was enhanced by the combined use of GSH; similar findings were reported by Hoshino[5] and Hattori.[6] In view of such discrepancies in experimental results that must be

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taken up seriously in performing therapy with these agents, we have conducted a series of both in vitro and in vivo experiments to investigate possible interactions between BLM and GSH. As reported previously, the results suggested that BLM might be inactivated by GSH when the substances are present at least in a certain critical quantitative ratio.[7,8] As our previous studies were undertaken to investigate changes occurring in BLM by the concomitant use of GSH, it was felt necessary to shed more light on the subject from an opposite direction. Thus, in our present study, the kinetics of ^{35}S-GSH was investigated by radioactivity measurement in cancer-bearing mice following the concurrent administration of BLM and ^{35}S-labelled GSH. An investigation was made further of the life-prolonging effect of long-term combination therapy with these two agents in cancer-bearing mice.

METHODS AND RESULTS

EXPERIMENT 1

Effect of BLM on radioactivity of ^{35}S-GSH in vivo

In vivo interaction between BLM and GSH was investigated in cancer-bearing mice. The experiment was conducted in ddN female mice, in which squamous cell carcinoma was produced by the application to the skin of 0.3% 20-MC solution in acetone similarly as in studies reported previously.[9,10] The experiment was commenced at 17 weeks after application of MC (at age of 21 weeks) when the induced solid tumor had already grown adequately. The animals received varying combinations of BLM and GSH as indicated in Table 1. BLM and GSH were simultaneously administered by intraperitoneal injection and

Table 1. Experimental procedure (Experiment 1)

Time (min.) / group	0	15'	30'
^{14}C · BLM	BLM	→	sacrifice
^{35}S · GSH	GSH	→	sacrifice
^{14}C · BLM + GSH	BLM GSH	→	sacrifice
BLM + ^{35}S · GSH	BLM GSH	→	sacrifice

BLM: 50 mg/kg i.p.
GSH : 10 mg/kg i.p.

30 minutes after administration the animals were sacrificed and determined for the radioactivity of ^{14}C-BLM and ^{35}S-GSH and the antibacterial activity of this anticancer drug in various organs (tumor, skin, lung and liver). The method of administration and the time interval from medication to killing of the animals were chosen for the experiment on the basis of the results of previous experiments[7,8] which indicated that a fall in tissue levels of BLM was most marked at 30 minutes after the simultaneous administration of BLM and GSH. The dosage of GSH was 10 mg/kg, i.e. 1/10 of 100 mg/kg employed for previous experiments, as this dose level was thought to be suitable for studying possible changes in the behavior of GSH. Radioactivity of tissue specimens was measured in a liquid scintillation counter (Packard Model 3380) while antibacterial activity was determined by the use of bioassay techniques (thin-layer disc method using B. subtilis PCI 219 as test organism).

1) Radioactivity of ^{14}C-BLM and ^{35}S-GSH in organs

^{14}C-BLM was found to permeate well into the skin and tumor and, though in somewhat smaller quantities, also into the lung liver. Amounts of radioactivity detected in various tissues following the administration of BLM alone were substantially the same as those recovered at comparable time points after the concomitant dosage of BLM and GSH. This lack of discrepancy in radioactivity measurements is assumed to be due to the presence of both compounds in tissues in a quantitative ratio considered necessary. ^{35}S-GSH, on the other hand, was shown to penetrate well into various organs, with the largest fraction being recovered from the liver, followed by the skin, tumor and lung in descending order. When BLM was administratered simultaneously with GSH, this resulted in a decline of radioactivity of ^{35}S-GSH invariably in all organs studied. The antibacterial activity of BLM was assayed with half of test samples. The results indicated that the antibacterial activity observed was generally lower than that of previous studies and tended to be diminished notably in the skin, liver and lung though no consistent tendency was seen.

Table 2. Radioactivities of ^{14}C-Bleomycin and ^{35}S-Glutathione in various organs following the intraperitoneal administration of the two compounds in varying combinations.
(all tissue specimens were taken 30 min. after injection of 50 mg/kg BLM and 10 mg/kg GSH)

organ / group	Radioactivity dpm/mg			
	Tumor	Skin	Lung	Liver
^{14}C-BLM only	320 ± 27	381 ± 34	269 ± 33	289 ± 38
^{14}C-BLM + GSH simultaneously	331 ± 58	373 ± 38	326 ± 29	265 ± 47
^{35}S-GSH only	966 ± 41	972 ± 182	873 ± 54	1828 ± 296
BLM + ^{35}S-GSH simultaneously	738 ± 59	641 ± 18	763 ± 86	1198 ± 96

mean of 5 animals ± S. E.

2) Changes in radioactivity of GSH in organs by the simultaneous use of ^{35}S-GSH and BLM

Table 3. Antibacterial activity of Bleomycin in organs following the simultaneous intraperitoneal administration of BLM and GSH in varying combinations.
(all tissue specimens were taken 30 min. after a combined i.p. dose of 50 mg/kg BLM and 10 mg/kg GSH)

organ / group	Antibacterial activity mcg/g			
	Tumor	Skin	Lung	Liver
^{14}C-BLM only	0.40 ± 0	5.08 ± 1.77	2.10 ± 0.50	1.12 ± 0.72
^{14}C-BLM + GSH simultaneously	0.40 ± 0	1.46 ± 0.49	2.32 ± 0.62	0.40 ± 0
BLM + ^{35}S-GSH simultaneously	0.40 ± 0	1.12 ± 0.20	1.52 ± 0.20	0.40 ± 0

mean of 5 animals ± S. E.

Table 4. Effect of Bleomycin on the radioactivity of ^{35}S Glutathione in organs.
(10 mg/kg GSH and 50 mg/kg BLM administered simultaneously; tissue specimens taken 30 min. after GSH dose)

organ / group	Radioactivity dpm/mg			
	Tumor	Skin	Lung	Liver
^{35}S-GSH only	966 ± 41	972 ± 182	873 ± 54	1828 ± 296
^{35}S-GSH BLM simultaneously	735 ± 59*	641 ± 18	763 ± 86	1198 ± 96
decrease ratio %	23.13	34.05	12.60	34.46

mean of 5 animals ± S E.
* : significance by 5% level

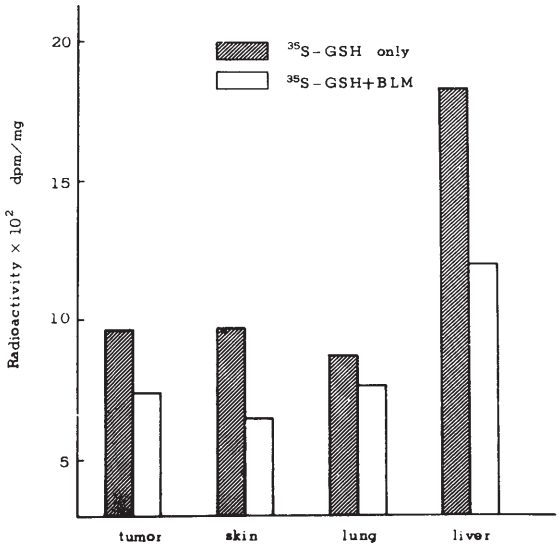

Fig. 1. Changes in radioactivity of ^{35}S-Glutathione in organs following the simultaneous administration of 10 mg/kg GSH and 50 mg/kg BLM.

A comparison was made between the radioactivity of ^{35}S-GSH in organs observed after the administration of the labelled GSH alone and that after the simultaneous administration of the labelled GSH and BLM. Following a concomitant dose of ^{35}S-GSH and BLM there was a 14 to 35% decrement of radioactivity recovered from various organs in comparison with corresponding radioactivity measurements obtained after the administration of ^{35}S-GSH alone. This decrement of radioactivity was marked notably for tumor tissues (23.13%) ($P < 0.05$).

EXPERIMENT 2

Effects of BLM and GSH in prolonging the life span of cancer-bearing mice

In an experiment conducted in parallel with Experiment 1, 30 ddN female mice with a

carcinoma produced in a similar way (23 weeks of age, after 17 weeks of application of MC to the skin) were divided into 3 groups of 10 animals each, with ample heed paid to make the groups as uniform as possible in terms of general condition of health, body weight and the degree of progression of malignancy. As indicated in Table 5, animals in the control group were injected intraperitoneally with 0.2 ml of 1/10 M phosphate buffer saline, pH 7.0; animals in another group received BLM alone at 5 mg/kg intraperitoneally, while those in a third group were given the intraperitoneal injection of 5 mg/kg BLM and 500 mg/kg GSH. Medication was continued at a frequency of 3 times a week untill recipient animals expired. During the period of treatment each animal was daily examined for general condition, estimated for the degree of regression of tumor and subjected to body weight measurement. The animals were housed in cages at a room temperature of 25 ± 1°C and a relative humidity of 55 ± 5% and fed a solid feed CE-2 (Japan Clea).

Table 5. Experimental procedure (Experiment 2)

group	medication
I	1/10 M PBS (PH7.0) 0.2 ml/mice, i.p.
II	BLM, 5 mg/kg i.p.
III	BLM (5 mg/kg) + GSH (500 mg/kg) i.p. simultaneously

admin. schedule: 3/W $\xrightarrow{\text{continuously}}$ death

1) Changes in general condition

Whereas pilo-erection was observed in none of animals in any groups throughout the entire period of treatment, diarrhea was seen at a considerably high frequency and tended to occur near the animal's death. In 2 to 3 weeks after commencement of medication (i.e. after 7 to 10 doses of BLM were given) the tumor began to show a tendency to shrink in size in both the BLM-treated and BLM + GSH-treated groups as compared with the control group and in some instances even disappeared completely. However, later at about 70 days of treatment (after 30 BLM doses were given) a tendency for the tumor to expand became evident and diarrhea ensued, eventuating in death in many of the animals. These symptoms were particularly pronounced in the control group.

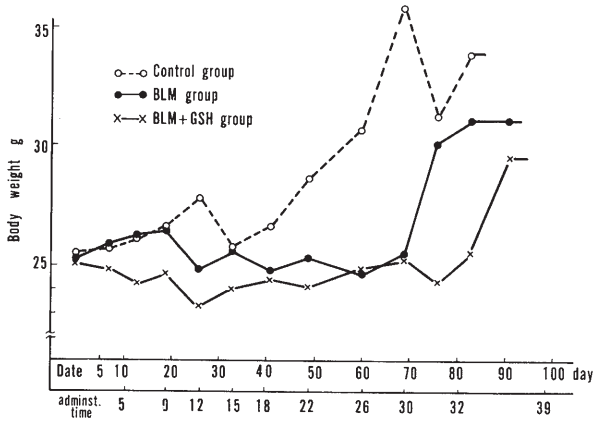

Fig. 2. Changes in body weight of recipient animals

2) Changes in body weight

The average weight of mice was about 25 g for the 3 animal groups (control 25.5 g, BLM group 25.3 g, BLM + GSH group 25.2 g) initially. Subsequent changes in body weight in each treatment group during the course of treatment are as represented graphically in Fig. 2. As can be seen, there was a distinct upward tendency of body weight noted in the control group, apparently as a result of day-to-day growth of the tumor. In the BLM group the body weight curve followed a similar course as in the control group for the initial 3 weeks of experiment (until 9 BLM doses were given). Thereafter the body weight rapidly declined due to the inhibition of tumor growth by BLM and then bacame nearly stationary till the 8th to 9th week. After day 60 (after 26 BLM doses were given) as a demarcation date, surviving mice tended to show a progressive weight gain due to tumor growth.

Body weight changes in the BLM + GSH group were essentially the same as in the BLM-treated group but were slightest in degree of all the three treatment groups. It should be noted, however, that an upward trend of body weight stemming from tumor growth became clear-cut past day 60 as was the case with the BLM-treated group.

3) Effects of BLM and GSH on survival of cancer-bearing mice

Survival curves for the 3 groups are represented in Fig. 3. In the control group fatalities began to occur at the third week, one or two animals died every day during the ensuing periods and all the animals died by the 121th day. In contrast, both in the BLM and BLM + GSH groups none of the animals died of malignancy during the initial 4 weeks of treatment. Notably in the BLM group even a tendency for the tumor to shrink in size became noticeable after 2 to 3 weeks of treatment. The longest survival time in the experimental groups was 126 days with BLM alone and 131 days with BLM + GSH.

The number of survivors during a period from the 30th to 60th day was larger by one in the BLM group than in the BLM + GSH group. At the 60th day after initiating treatment 90%, 60% and 30%, respectively, of animals in the BLM, BLM + GSH and control

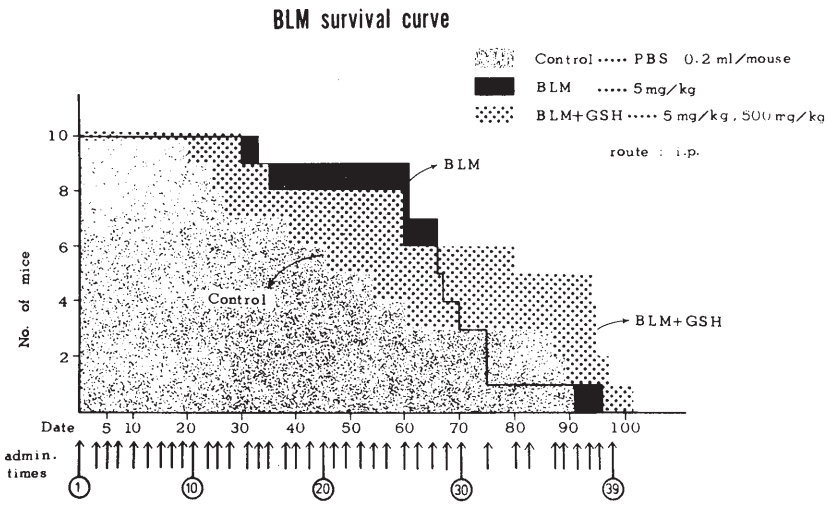

Fig. 3. Effect of Glutathione on the anticancer action of Bleomycin in cancer-bearing mice

groups were surviving, thus the survival rate was highest for the group treated with BLM alone ($P < 0.01$) but there was no significant difference between the two experimental groups in this respcet. From the 60th day on the number of deaths rapidly increased in the BLM group, with fatalities occurring day after day. Since 26 to 30 doses of BLM were given by this point of time, it seems logical to interprete this abrupt increase in the number of deaths as due to toxicity of BLM. In the BLM + GSH group, on the other hand, the survival rate was higher probably due to inactivation and detoxification of BLM by GSH. Thus, the mean length of survival was 70.7 days for the BLM + GSH group as against 53.4 and 67 days, respectively, for the control and BLM groups. Consequently, the mean increment of length of survival (ILS %) amounted to 35.1% for the BLM + GSH group as against the corresponding figure for the BLM group of 25.5%.

Table 6. Effect of Glutathione on the anticancer action of Bleomycin in cancer-bearing mice. (average length of survival and average increment of length of survival)

1 medicate. group	Dose	Average survival days	ILS (%)	survivors on 60th day
P. B. S. (control)	0.2 ml/mice	53.4		3/10
BLM	5 mg/kg	67.0	25.5	9/10*
BLM GSH simultaneously	5 mg/kg 500 mg/kg	70.7	35.1	6/10

ILS (%) = (T/C) × 100 − 100

*: Significance by 1% level

DISCUSSION AND SUMMARY

It is well known that GSH is a substance which is ubiquitous throughout the animal and vegetable kingdoms including microorganisms and has a wide variety of physiological actions. A recent study reported GSH to be able to inactivate viruses.[11] Clinical evidence indicates that GSH is useful in the prevention of side effects of BLM and 5FU.[12~15] GSH owes this action to the presence of the reactive SH group in its molecule, similarly as do many other SH compounds.[5,16] As mentioned earlier, the reaction between BLM and GSH in vivo is debatable at the present time and is probably explained by the fact that the subject has been approached by various investigators from the only single facets. BLM requires the presence of SH for exerting its anticancer activity in vivo, nevertheless the anticancer potency of BLM might possibly be lessened by SH. Contradictory as they appear at a glance, these interactions actually occur in the living body and by no means are contradicting, we think. It would be reasonable to assume that lessening (by inactivation or detoxification) of the deleterious action of BLM on normal cells, among other cytotoxic actions of this agent (including anticancer action), is related to nothing else than preventing or lessening the side effects, which in turn is expressed on the whole as enhancement of anticamcer effect. It can therefore be asserted that BLM is inactivated by GSH and this causes lessening of the side effects, resulting in enhancement of anticancer action. To be more specific, both are arguing on the same matter, but since their expressions differ, such debate is being understood in a contradictory manner and thus, it appears to us, is resulting in the confusion of the arguments temselves. In using BLM and GSH concomitantly full

consideration should therefore be paid to the method of administration including dosage and time of administration.

It is postulated that the reaction between BLM and GSH requires for its occurrence their presence at least in a certain definite quantitative ratio. This particular quantitative ratio was reported by Fujita[2] to be obtained with 5 μg/ml BLM and 250 μg/ml GSH in vitro and by Sonezakie et al.[4] to be achieved with 5 mg/kg BLM and 500 mg/kg GSH in vivo. In other words, BLM is inactivated by GSH when the two substances are present in a quantitative ratio of BLM : GSH = 1 : 50 – 100. At such a ratio, which signifies an overwhelming predominance of GSH over BLM in comparison with what was employed by us, inactivation of BLM is quite likely to occur. Our previous in vivo experiments with 50 mg/kg BLM and 100 mg/kg GSH (BLM : GSH = 1 : 2) yielded results that were suggestive of inactivation of BLM.[3,7,8]

Table 7. Radioactivity and antibacterial activity of ^{14}C-Bleomycin in organs (50 mg/kg BLM and 100 mg/kg GSH administered simultaneously; tissue specimens taken 30 min. after BLM dose)

activity / group	Radioactivity dpm/mg			Antibacterial activity mcg/g		
	lung	skin	tumor	lung	skin	tumor
BLM only	225	2331	269	7.4	68.0	5.1
BLM + GSH (simultaneously)	110	892	53	3.7	7.3	1.1
decrease ratio (%)	51.1	61.7	80.3	50.0	89.3	78.4

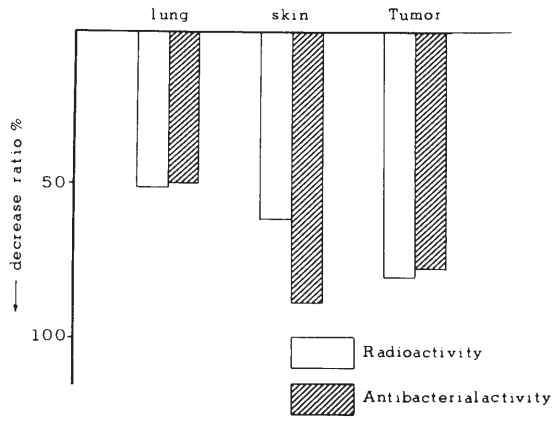

Fig. 4. Per cent decrease of Bleomycin in organs following the simultaneous administration of 50 mg/kg BLM and 100 mg/kg GSH. (tissue specimens taken 30 min. after BLM dose)

In a series of experiments conducted thus far, the reaction between BLM and GSH was investigated exclusively from the aspect of BLM. Interaction between BLM and GSH, if it really exists, would have an effect on GSH as a matter of course. In our previous in vitro experiment with a BLM-GSH (1000γ : 100 mg) mixture, we recognized alterations in BLM but without any concurrent changes in GSH.[9] Thereupon, a study was made of the stability of GSH in a mixture of 15 mg BLM and 3 mg GSH (5 : 1). The results clearly

indicated a decrese in the remaining ratio of GSH.[9] In the present in vivo experiment BLM and ^{35}S-GSH were administered simultaneously to mice in the same dose ratio (i.e. 50 mg/kg and 10 mg/kg, respectively). As was expected,there was a tendency for tissue levels of GSH to decrease as compared with those observed following the administration of GSH alone and this lowering of GSH level was particularly distinct in tumor ($P < 0.05$).

Table 8. Stability of Glutathione in mixtures with Bleomycin. (alloxan 305 method, Yamanouchi)

Specimens	37°C 180 min. incubate		
	305 mμE	GSH %	Remaining ratio %
GSH + Aq. dest	0.392	89.3	100
(BLM + GSH) + Aq. dest	0.132	30.1	33.7
(BLM + GSH) + pH7.2 phosphate buffer saline	0.138	31.4	35.2

* BLM 15 mg: GSH 3 mg

All these experiments demonstrated that there is an interaction between BLM and GSH, with its occurrence depending upon the quantitative ratio of the two compounds.

In subsequent experiments the anticancer effect of the simultaneous administration of BLM and GSH was investigated in cancer-bearing animals. The number of 60-day survivors was significantly greater ($P < 0.01$) in the BLM group than in the control group but there were no significant differences between the BLM and BLM + GSH groups and likewise between the BLM + GSH and control groups. However, past the 60th day of treatment a life-prolonging effect became distinct in the group treated with BLM + GSH, both the average length of survival and the average increment of length of survival being greater with BLM + GSH than with BLM alone. These findings may reasonably be substantiated as a result of prevention or lessening of the cytotoxic effect of BLM by GSH and also provide ample justification for concluding that the anticancer effect of BLM is enhanced by the concomitant use of GSH.

We have pursued studies of the reaction between BLM and extrinsic GSH in vivo and the anticancer effect of the conjoined use of these agents in cancer-bearing animals. However, as these experimental studies were all carried out with organs, it is earnestly hoped that further in-depth investigations will be made to elucidate the reaction between the two substances at the cellular level.

CONCLUSION

The in vivo interaction between ^{35}S-GSH and BLM and the anticancer effect of BLM and GSH administered simultaneously were investigated in cancer-bearing mice.

1) ^{35}S-GSH and BLM were administered intraperitoneally to cancer-bearing mice and 30 minutes the animals were sacrificed and determined for the ^{35}S-GSH content of various organs. The radioactivity of ^{35}S-GSH in organs tended to be lower after the simultaneous administration of the labelled GSH and BLM than when the labelled GSH was given alone. This tendency was prominent, notably with the radioactivity content of tumor tissue.

2) GSH and BLM interact in vivo and this reaction depends for its occurrence upon the quantitative ratio of the two substances.

3) In using GSH for the prophylaxis of adverse reactions to BLM it is important to pay full consideration to the dosage of GSH and the time of its administration. Otherwise the

anticancer action of BLM may be seriously impaired.

4) In cancer-bearing mice a comparison was made of anticancer effectiveness between BLM used alone and in combination with GSH. During an initial 60-day period treatement with BLM alone produced an excellent anticancer effect and also provided a marked life-prolonging effect when compared with the control. These effects in the BLM + GSH group were no better than those in the control group.

5) Past the 60th day of treatment (after 30 doses of BLM or GSH were given) fatalities occurred frequently in the BLM group due to the drug toxicity, while in the BLM + GSH group a greater increment of survival time was attained owing to a detoxifying effect of GSH. The results of our present experiments may be interpreted, on the whole, as indicating that the anticancer effect of BLM is enhanced by the concomitant use of GSH.

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