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Response of Mouse Oral Mucosa to Repeated Doses of Bleomycin

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Bleomycin (BLM) applied at systemically tolerable doses induces denudation of tongue mucosa in the C3H-Neuherberg mouse strain. The dose–incidence curve after single injections has a sigmoid shape with an ED_{50} of 17.5 mg/kg. In contrast, the dose–response curves to repeated (two, five and 10) drug injections follow triphasic shapes and show dose–effect inversions. The effect initially increases with dose to a maximum of 70–100% at 2 × 7, 5 × 2, and 10 × 0.9 mg/kg. A marked decrease in response is observed at higher doses with a nadir of 10–30% after 2 × 11 mg/kg, 5 × 4 to 5 × 5 mg/kg and 10 × 2 mg/kg, followed by a second rise when dose is further increased. These clinical results were confirmed in a histological study. Variation of the time interval between two drug injections caused marked fluctuations in the treatment efficacy. A clear increase in drug response was induced by splitting total drug doses of 6, 14 or 22 mg/kg, the maximum effect (100%) was seen at intervals of 2 h, 0.5–1 h and 0.25 h between two injections of 3, 7 or 11 mg/kg, respectively. At longer intervals of up to 6 h, a dose-dependent decrease in drug efficacy resulted in an inverse dose–effect. Original tissue tolerance to BLM was restored only in the 2 × 3 mg/kg arm but was still elevated in the other arms after 96 h. The results can be plausibly explained by the dose-dependent induction of detoxifying processes.

Keywords: oral mucosa, BLM toxicity, time interval, dose–effect curves
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INTRODUCTION

SQUAMOUS CELL carcinomata appear to be particularly sensitive to bleomycin [1]. Therefore, this drug was recently introduced in a number of radiochemotherapy protocols for such tumours in the head and neck region [2–4]. A major side-effect during cancer treatment related to bleomycin (BLM) toxicity is the induction of chronic pulmonary fibrosis. In addition, a substantial aggravation of acute mucosal damage was observed when BLM was added to existing radiochemotherapy protocols, particularly when drug and radiation were given with close temporal proximity [3, 5]. However, the mechanisms underlying the observed increase in oral toxicity remain unclear.

Animal models have been exploited to study the mucosal response to radiochemotherapy with BLM at the tissue level. In mouse lip mucosa, an earlier and increased response to single radiation doses by single BLM injections was reported when the drug was given 4 days or less before radiation with maximum efficacy at 2 h [6]. Fractionated irradiation with increasing fraction numbers during a continuous 7-day drug infusion resulted in a decrease of the dose-enhancement factor (DEF = ED_{50} radiation alone/ED_{50} radiation plus BLM) with decreasing fraction size. The experimental results were interpreted as inhibition of the repair of sublethal radiation damage (SLD) in addition to independent cytotoxicity [7, 8]. A clear reduction of repopulation by BLM was observed in the interval between split radiation doses [9].

As an alternative animal model, the mouse tongue mucosa was established [10–12]. In the C3H-Neuherberg mouse strain, mucosal denudation similar to acute reactions induced by radiation can also be inflicted by BLM alone. In a recent study, the combined effect of single doses of BLM and radiation on mouse oral mucosa was quantified [13]. The ED_{50} after BLM alone was found to be 17.5 mg/kg. In contrast to radiation treatment, the latent times to complete denudation were dependent on the drug dose. In combination studies with BLM and single X-ray doses [13], the drug toxicity was independent of the time interval and the sequence of application, but a significant decrease in latency occurred when the drug was given 2 h or less before irradiation. These results suggested a reduction in the number of abortive divisions of sterilised cells by BLM, which after X-rays alone considerably contribute to prolongation of the latent phase [11].

Isobologram studies combining various doses of BLM and soft X-rays [13] suggested that the *in vivo* survival of keratinocyte target cells follows an upward concave dose–response curve, i.e. small drug doses were relatively more effective. Similarly shaped dose–effect curves have also been observed in *in vitro* experiments [14–16]. Provided the "negative shoulder" is repeated during fractionated drug

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treatment, repeated administration of small drug doses should result in a relative increase in mucosal toxicity as compared to larger doses.

The present study was, therefore, initiated to determine the toxic effect of repeated injections of BLM on oral mucosa in C3H-Neuherberg mice. In detail, the impact of total drug dose and number of injections was investigated in functional and histological studies. The effect of the time interval between two consecutive administrations was studied at three different dose levels.

MATERIALS AND METHODS

Female mice of the inbred C3H strain from the Neuherberg colony were used in all experiments. All animals were 10–13 weeks old with a body weight between 20 and 25 g. The mice were housed five per cage in a specified pathogen-free envionment with controlled conditions of air temperature and humidity. A 12/12 h light/dark cycle was maintained with changes at 6 a.m. and 6 p.m. The animals had free access to a standard pellet diet and water.

Bleomycin (Mack, Illertissen, Germany) was dissolved in sterile 0.9% NaCl (3 mg/ml) and stored at 4°C for 4 days maximum. This stock solution was further diluted to yield injection volumes of 0.01 ml/g body weight, i.e. about 0.25 ml per animal and injection. The drug was administered intraperitoneally. The animals were weighed before each injection.

Scoring of mucosal response

Scoring of the tongue mucosa started 5 days after the single injection and on day 1 after the last injection in all other protocols. Scoring was performed daily until re-epithelialisation was complete. Pentobarbital anaesthesia [130 mg/kg intraperitoneally (i.p.)] was administered for immobilisation. The tongue was pulled out carefully by means of a forceps and examined under a cold light source with a magnifying lens (3×). The mucosal response of the central portion of the inferior tongue surface was examined in detail, although reactions at other tongue sites were observed in protocols with higher drug toxicity. Complete epithelial denudation of the lower tongue surface, here designated as tongue ulcer, was used for quantal dose–response analyses.

Histological processing

Animals were sacrificed by cervical dislocation, the mobile tongue was removed and fixed in formol–alcohol for at least 24 h. After embedding in methyl-methacrylate, 3-μm thick sections were cut in six sagittal planes, separated by 200 μm, and stained with hematoxilin and eosin. For each time point a total length of ~2.5 cm of the surface of the lower tongue was examined in each of three animals. Cell density was determined as the number of epithelial nuclei per mm tongue surface length. The relative cell density was calculated as the percentage of cells in relation to the cellularity in control epithelium, i.e. 420 per mm surface length. Bi- and multinucleate cells were assessed as percentage of the overall epithelial keratinocyte count.

Experimental design

Single injections with graded doses of BLM ranging from 4 to 22 mg/kg were applied in order to establish a dose–response curve. At least seven animals were used per dose point. The drug was administered between 9 a.m. and 11 a.m.

In a second set of experiments, graded total doses of BLM were given in 2, 5 and 10 injections with constant doses per injection. The first dose on each day was always administered between 8 a.m. and 9 a.m. In the two-fraction arm the second injection was given 24 h later. In the five-fraction protocol two injections per day were given 8 h apart, with a 16-h overnight interval, yielding an overall treatment time of 2.5 days. In the 10-fraction protocol four daily injections were separated by intervals of 4 h, also resulting in an overall treatment time of 2.5 days. At least seven animals were used per dose point. For some data points, repeated experiments were carried out which yielded similar results; hence presentation of the pooled data appeared to be justified.

An additional morphological study was carried out after treatment by 2 × 7 mg/kg, 2 × 11 mg/kg and 2 × 15 mg/kg given within 24 h. Animals were sacrificed on days 2, 4, 6, 8 and 10 after the first injection. In this experiment, three animals were used per data point.

The effect of time between two consecutive drug applications was studied with two injections of 3, 7 or 11 mg/kg. The intervals studied ranged from 0.25 to 96 h. At least seven animals were used per dose and time interval. Again, results obtained in some repeat experiments were in good aggreement and justified pooling of all data.

RESULTS

BLM given both as single or repeated injections induced ulcerative lesions in the lower tongue epithelium of the C3H-Neuherberg mouse strain. First clinical symptoms, i.e. erythema and slight oedema, appeared after dose-dependent time intervals of several days after the first treatment. Increased desquamation of keratin subsequently led to mucosal erosions, i.e. complete epithelial denudation. Similar reactions were frequently recorded at the tip and margins of the tongue, but only rarely at the superior surface. However, scoring was restricted to the central portion of the lower tongue in order to achieve data comparable to previous studies on radio- and radiochemotherapy in this tissue [10, 11, 13].

The frequency of animals developing complete denudation after a single BLM application followed a steep dose–response curve, as shown in Fig. 1. The ED_{50}, i.e. the drug dose inducing

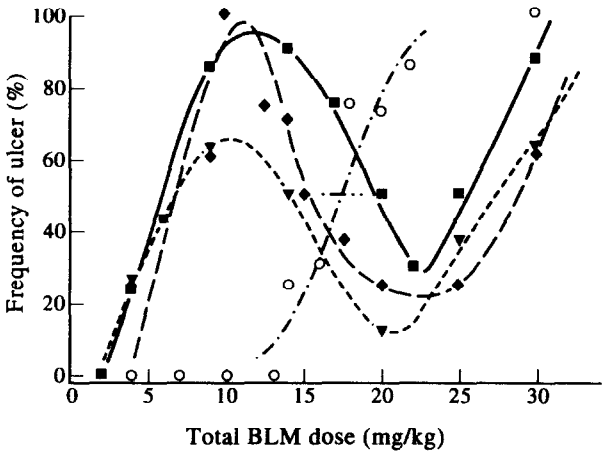

Fig 1. Frequency of animals developing tongue ulcer after BLM given in one injection (○), two injections in 24 h (■), five injections in 2.5 days (◆), or 10 injections in 2.5 days (▼). The single dose–response curve was established by probit analysis, the error bars represent the 95% confidence interval.

Table 1. Functional effects on mouse oral mucosa of graded BLM doses given in two, five and 10 injections. Latent times to complete denudation are normalised to the day of first drug injection. Statistical errors represent 1 S.E.M.

Number of injections	Total dose (mg/kg)	Number of animals	Ulcer frequency (%)	Latent time (days)	Ulcer duration (days)
1	4	8	0	—	—
	7	8	0	—	—
	10	8	0	—	—
	13	8	0	—	—
	14	8	25.0	9.5±0.5	3.0±1.0
	16	16	31.3	9.2±0.6	2.4±0.3
	18	8	75.0	8.3±0.3	3.5±0.3
	20	15	73.3	8.4±0.4	3.3±0.4
	22	7	85.7	6.6±0.4	4.0±0.5
	30	8	100.0	5.5±0.9	3.7±0.7
2	2	8	0	—	—
	4	8	25.0	7.0±0	3.3±0.3
	6	16	43.8	8.7±0.8	3.4±0.2
	9	8	85.7	6.8±0.2	2.8±0.2
	14	21	90.5	7.2±0.6	3.1±0.3
	17	8	75.0	8.0±0.3	3.0±0.3
	20	8	50.0	7.8±0.5	2.8±0.5
	22	23	30.4	9.3±0.5	3.4±0.5
	25	8	50.0	7.3±0.3	3.3±0.6
	30	8	87.5	7.4±0.2	2.9±0.4
5	4	8	25.0	8.0±0	3.0±0
	9	8	62.5	8.6±0.3	2.0±0
	10	8	100.0	8.1±0.1	2.6±0.2
	12.5	8	75.0	7.7±0.2	2.7±0.3
	14	7	71.4	8.0±0.5	2.6±0.4
	15	8	50.0	8.0±0	2.5±0.3
	17.5	8	37.5	8.3±0.3	2.3±0.3
	20	8	25.0	9.0±1.0	3.0±1.0
	25	8	25.0	8.0±1.0	2.5±0.5
	30	8	62.5	8.0±0	2.4±0.3
10	4	8	25.0	8.0±0	3.0±0
	9	8	62.5	7.8±0.2	3.0±0.3
	14	8	50.0	6.0±3.0	3.3±0.3
	20	8	12.5	6.4±0	3.0±0
	25	8	37.5	8.0±0	3.7±0.3
	30	8	62.5	7.5±0.5	2.4±0.3

ulcer of the lower tongue mucosa in 50% of the animals, was 17.5 mg/kg with 95% confidence limits of 16.3 mg/kg and 18.8 mg/kg, as computed by probit analysis. The latent time to ulcer after a single drug injection was dependent on the drug dose, and ranged from 9.5 to 5.5 days (Table 1). The individual ulcer duration was independent of the BLM dose; the average duration, calculated over all dose groups, was 3.4±0.3 days ($n=27$; ± S.E.M.). No weight loss was observed after BLM doses up to 22 mg/kg.

The histological changes following a single BLM dose of 22 mg/kg have recently been reported [13]. Briefly, abnormal mitotic figures and abnormal nuclei appeared by day 2 and culminated on days 4–6, in combination with distinct enlargement of the individual cells. On day 6, bi- and multinucleate cells in non-denuded areas reached a maximum of 10 and 6%, respectively. Complete epithelial cell loss occurred in foci after 6 days and became confluent after 8 days; denuded areas were covered by a pseudomembrane of keratin, fibrin and cell detritus.

In contrast to the monophasic sigmoid dose–effect relationship after single injections of BLM, repeated application of the drug resulted in triphasic dose–response curves, as illustrated in Fig. 1. The detailed results of these experiments are summarised in Table 1. The frequency of responding animals increased with dose in the low-dose region, reaching a peak of 62.5–100% after total drug doses between 9 and 14 mg/kg, i.e. in a dose range where no response to single injections was observed. Further dose escalation, however, did not result in saturation. Rather, a decreasing efficacy of the drug led to a nadir of 12.5–25% responders after total doses of 20–25 mg/kg. This low toxicity of fractionated total doses hence coincided with a high efficacy of single drug injections. Beyond this dose range the frequency of responders increased again with the drug dose.

In contrast to single dose treatment, the latency to complete denudation was independent of the number of injections and the drug dose used. The average latent time, calculated for all responders ($n=122$), was 7.9±0.1 days (± S.E.M.) after the first injection. The individual ulcer duration on average was 3.2±0.1 days, being independent of both treatment protocol and BLM dose.

Weight loss of up to 5% of the pretreatment body weight

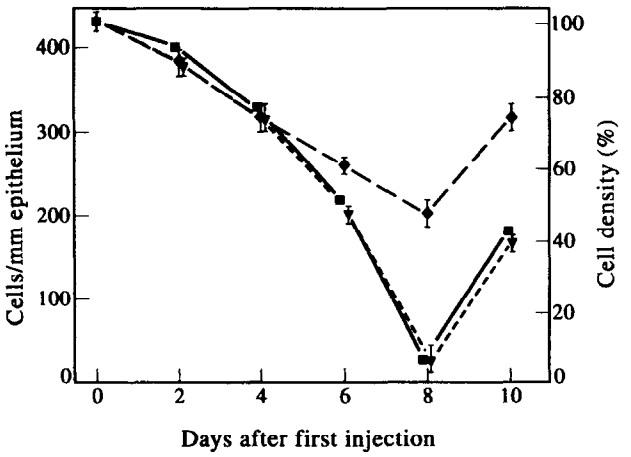

Fig 2. Changes in cell density in mouse oral epithelium after administration of BLM. The relative cell density was calculated based on the cellularity in control epithelium, i.e. 420 cells per mm surface length. The doses were 2 × 7 mg/kg (■), 2 × 11 mg/kg (◆) and 2 × 15 mg/kg (▼). Error bars depict 1 S.E.M.

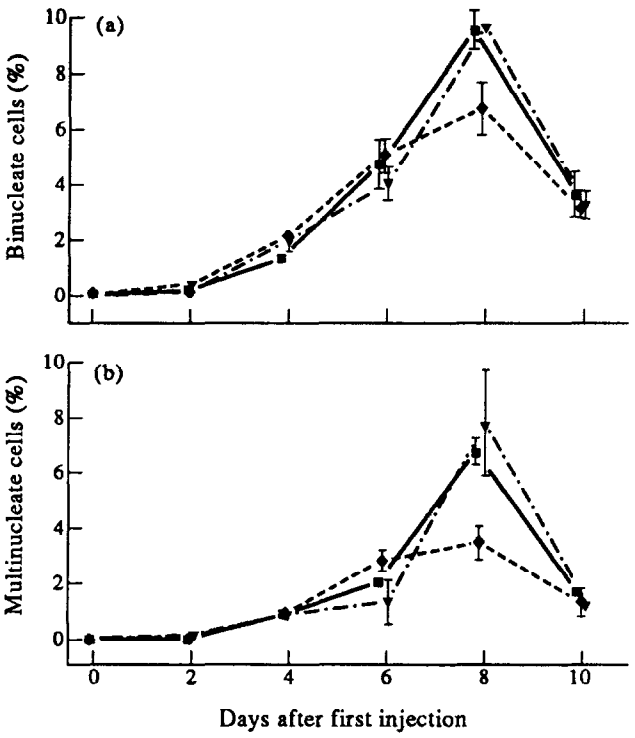

Fig 3. Frequency, i.e. percentage of overall keratinocyte counts, of binucleate cells (a) and multinucleate cells (b) in mouse tongue epithelium after treatment with 2 × 7 mg/kg (■), 2 × 11 mg/kg (◆) and 2 × 15 mg/kg (▼) of BLM. Error bars depict 1 S.E.M.

was observed only in the dose groups with maximum response and was independent of the number of injections, suggesting some systemic toxicity of the treatment.

Histological changes were assessed between days 2 and 10 after 2 × 7, 2 × 11 and 2 × 15 mg/kg. Cellular damage, presenting as vacuolisation and nuclear fragmentation in individual cells, was observed as early as day 2 and the number of cells damaged progressed to a maximum on day 8. In accordance, increasing areas with low cell density were observed, resulting in foci with complete denudation on day 8, which were covered by a mixture of keratin, cell debris and fibrin. These changes were qualitatively similar at all doses, but were less pronounced in the 2 × 11 mg/kg arm.

The fluctuations in cell density after BLM treatment are illustrated in Fig. 2. Cellularity after 2 × 7 mg/kg and 2 × 15 mg/kg dropped with increasing rates to reach a nadir of about 5% on day 8. Rapid regeneration, equivalent to a net increase of 75 cells/day, followed immediately. After the intermediate dose of 2 × 11 mg/kg, cell counts on days 2 and 4 were comparable to the other dose arms, but subsequently dropped only to a nadir of 47%, also reached on day 8. Again, a steep rise in cell density of ~60 cells/day was observed between days 8 and 10.

Bi- and multinucleate cells, not present in control epithelium, appeared by day 2. As shown in Fig. 3, their frequency increased continuously to a peak on day 8 and subsequently declined rapidly during the phase of regeneration. Again, the effect was less pronounced in the 2 × 11 mg/kg arm, resulting in only 7% binucleate and 3.5% multinucleate cells, as compared to 9.5 and 7% in the remaining two dose arms, respectively. Thus, in accordance with the functional findings shown in Fig. 1, the histological results demonstrated that a dose of 2 × 11 mg/kg was less toxic than the lower dose of 2 × 7 mg/kg or the higher dose of 2 × 15 mg/kg.

The influence on mucosal toxicity of the time between consecutive administrations of the drug was studied with two injections of 3, 7 or 11 mg/kg. The results of this investigation are summarised in Table 2 and Fig. 4. As illustrated there, splitting a total dose of 6 mg/kg into two injections progressively raised the frequency of responders from 0 to 100% when the time interval between injections was extended to 2 h. A decrease in drug efficacy was observed with longer intervals and eventually no mucosal toxicity was observed when the second dose was applied after 96 h. A total BLM dose of 14 mg/kg, given as a single injection, induced denudation in 25% of the animals. Application of the same dose in two injections, separated by 0.5–1 h, increased the response frequency to 100%. A plateau of 62.5–75% responders was observed with intervals ranging from 1.5 to 6 h, and marked fluctuations between 31 and 90% were observed with longer intervals. However, the efficacy of the two injections was higher than the single dose even with a 4-day interval. A BLM dose of 22 mg/kg, which as a single dose inflicted ulcer in 85% of the animals, resulted in 100% responders when given in two injections separated by 15 min. Longer intervals from 0.5 to 12 h resulted in almost constant response frequencies between 50 and 62%, whereas marked fluctuations between 30 and 81% were seen with intervals between 24 and 96 h.

In general, splitting a defined BLM dose resulted in an increase in mucosal toxicity with maximum efficacy at time intervals which were clearly dose-dependent. A plateau in drug response was observed at intermediate intervals; the level of the plateau was also dose-dependent and resulted in an inversion of the dose response. Marked fluctuations occurred when the second dose was applied between 6 and 96 h after the first dose. However, a general trend to a decrease in response rate with increasing interval was observed. Original tissue sensitivity to 3 mg/kg was restored after 96 h. A second injection of 7 and 11 mg/kg, however, still caused a significant response at this time, whereas single doses between 4 and 13 mg/kg do not induce any ulcer (Table 1), indicating that part of the damage induced by the first dose was still remembered after 4 days in the higher dose arms.

Table 2. Functional response of mouse tongue epithelium to two doses of BLM given with graded intervals between injections. Latency of ulcer is normalised to the day of the first drug injection. Errors depict 1 S.E.M.

Total dose (mg/kg)	Time interval (h)	Number of animals	Ulcer frequency (%)	Latency (days)	Ulcer duration (days)
6	0	8	0	—	—
	0.5	8	75.0	8.3±0.2	3.0±0.3
	1	8	87.5	8.9±0.5	3.4±0.2
	2	8	100.0	8.8±0.4	3.5±0.3
	6	8	87.5	7.6±0.7	4.0±0.4
	12	7	28.6	13.0±1.0	2.5±0.5
	24	16	43.8	8.7±0.8	3.4±0.2
	48	7	14.3	12.0±0	2.0±0
	72	8	62.5	10.2±0.6	4.0±0.3
	96	7	0	—	—
14	0	8	25.0	9.5±0.5	3.0±1.0
	0.5	8	100.0	6.8±0.4	3.1±0.3
	1	8	100.0	6.4±0.3	3.1±0.2
	1.5	8	75.0	7.2±0.3	3.8±0.3
	2	8	75.0	6.3±0.4	3.3±0.4
	4	8	75.0	7.8±0.6	3.8±0.5
	6	8	62.5	7.8±0.2	3.8±0.6
	12	16	31.3	9.6±0.9	3.6±0.6
	24	21	90.4	7.2±0.6	3.3±0.3
	48	16	81.3	10.2±0.6	3.2±0.3
	72	16	68.8	10.9±0.6	3.5±0.2
	96	7	42.9	14.3±0.3	3.0±0.6
22	0	7	85.7	6.6±0.4	4.0±0.5
	0.25	8	100.0	6.6±0.5	4.0±0.6
	0.5	8	87.5	6.4±0.4	3.9±0.4
	1	8	62.5	6.2±0.6	3.8±0.4
	2	8	5.0	7.3±0.8	3.3±0.3
	4	8	62.5	7.8±0.4	4.2±0.7
	6	8	50.0	8.0±0.6	3.8±0.6
	12	8	62.5	9.0±0.6	3.6±0.4
	24	23	30.4	9.3±0.5	3.4±0.5
	48	8	62.5	10.2±0.6	2.8±0.4
	72	16	81.3	10.4±0.2	3.3±0.2
	96	15	28.6	12.3±0.7	2.8±0.3

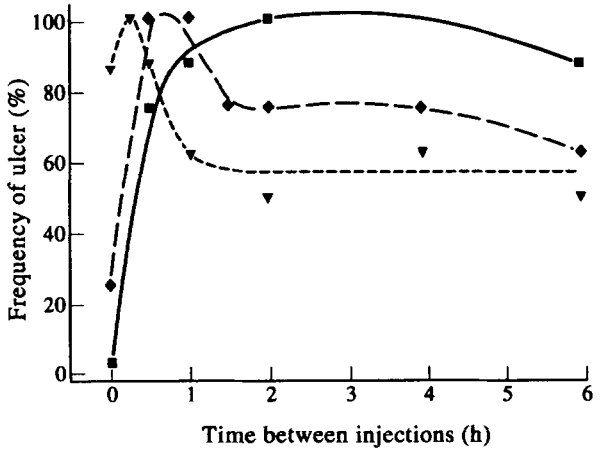

Fig 4. Frequency of animals presenting with tongue ulcer after two injections of BLM separated by graded time intervals ranging between 0.25 and 6 h. Drug doses were 2 × 3 mg/kg (■), 2 × 7 mg/kg (◆) and 2 × 11 mg/kg (▼).

The ulcer duration in these experiments was again independent of treatment schedule and dose. The latent time to denudation, if normalised to the day of the first injection, increased with increasing time interval between the two injections, but was independent of the schedule when related to the day of the second injection.

DISCUSSION

Clinical trials have shown that BLM considerably enhanced mucosal toxicity in the oral cavity when the drug was added to established radiochemotherapy protocols for head and neck cancer [2–5]. The biological basis of this increase in treatment toxicity, however, remains unclear. Simple additivity of the effects of both agents, i.e. drug and radiation, cannot be excluded, but more complex interactions may also play a role. Impairment of the repair of sublethal radiation damage has been proposed by both *in vitro* studies [17, 18] and *in vivo* investigations in oral mucosa [7, 8]. Furthermore, repopulation, i.e. the regenerative response of the epithelium during fractionated radiotherapy, could possibly be impaired by the drug, also inflicting increased toxicity.

The response of oral mucosa to both radiation and cytostatic drugs is based on the sterilisation of stem cells in the germinal layer of the epithelium. Consequential reduction in the cellular

supply to the differentiated layers in the presence of continuing superficial cell loss causes hypoplasia and eventually complete denudation or ulceration. Therefore, stem cell kill by the cytotoxic treatment must be considered the major factor of mucosal drug toxicity.

Some of the molecular mechanisms have been identified. BLM induces DNA single and double strand breaks. The DNA strand scission is inflicted by a BLM–ferrous ion chelate complex, which by reduction of molecular oxygen leads to hydrogen abstraction from deoxyribose [19].

At the cellular level, *in vitro* experiments investigating the response to single doses of BLM have demonstrated an increase in sensitivity by cell cycle synchronisation [20]. The *in vitro* cell survival curve was found to have an upward concave shape [14–16, 21]. Isobologram analysis of results from the *in vivo* combination of various BLM and X-ray doses in mouse tongue epithelium [13] also suggested that the shape of the *in vivo* survival curve of mucosal stem cells is upwardly concave.

Provided that the repeated application of small drug doses leads to repetition of the steep initial part of the survival curve, fractioned BLM treatment should be more effective than a single injection of identical total doses. In fact, a higher toxicity of two BLM treatments as compared to single BLM exposure has been observed in *in vitro* investigatons [15].

The present investigation demonstrated a triphasic dose–response curve of oral mucosa to repeated (two, five or 10) injections of BLM, with an initial increase in tissue sensitivity, an intermediate dose region with decreasing toxiciy, followed by a second increase in mucosal sensitivity at high drug doses. The triphasic dose response has also been confirmed in our histological study, where the changes in cell density and in the frequency of abnormal, i.e. multinucleate cells were less pronounced after an intermediate total dose as compared to lower and higher doses.

The increase in drug toxicity at low repeated doses in comparison to single injections of similar total doses can plausibly be explained by the upward concave shape of the stem cell survival curve, yielding an increase in efficacy by dose fractionation. However, the cell survival curve does not provide a rational basis for the decrease in drug effect at intermediate total doses and hence other mechanisms must be involved.

Cell cycle effects could account for the declining drug effect after the first dose, as BLM has been shown *in vitro* to induce a block at the S/G2 and/or at the G2/M transition point [20, 22, 23]. Assuming a dose-dependent duration of the block, a second dose applied after a constant interval of 24 h (two injection arm) could meet stem cell populations with varying overall sensitivity. Yet, different shapes of the dose–effect curves of the five and 10 injection protocols would then be expected. Furthermore, proliferation in the epithelium of lower mouse tongue proceeds in a highly synchronised pattern [11] with only $\sim 30\%$ of the germinal cells undergoing DNA synthesis and mitosis per day and $>70\%$ residing in G1. Hence, cell cycle effects of the first dose would affect only a minority of the stem cells.

The induction of BLM metabolising systems with increasing drug dose, and an overproduction of these systems at intermediate doses followed by saturation at high doses, could represent an alternative explanation of the experimental findings. The intracellular degradation of BLM is based on two detoxifying systems. Firstly, BLM can be hydrolised by a specific BLM hydrolase, probably a cysteine protease [24, 25], which has been demonstrated in a number of tissues [26–29]. The sensitivity of the particular tissue has been shown to correlate with its BLM hydrolase content [26, 30, 31]. However, so far, no experimental evidence for the inducibility of this enzyme is given.

Alternatively, non-specific antioxidants, such as catalase or glutathione, may protect tissues from the activated BLM–iron–oxygen complex that confers drug toxicity. It has been shown that these enzyme complexes can be induced in lung by chronic drug administration [32], and a similar process may occur in oral mucosa on a shorter time scale.

Splitting defined drug doses with graded time intervals resulted in an initial increase in mucosal toxicity, which can readily be explained by the shape of the cell survival curve. However, the time to achieve the maximum response was clearly longer with the lower dose. This suggests that the damage induced by the first dose becomes effective at an earlier time at higher dose levels, possibly based on dose-dependent changes in the intracellular BLM concentration during the initial postinjection period. The initial phase of increasing toxicity with increasing interval was followed by a dose-dependent decrease in drug efficacy with longer intervals, leading to an inverse effect of drug dose. This decrease in drug toxicity occurred earlier and at a higher rate with higher drug doses. Assuming the involvement of detoxifying processes, this suggests that the time to induction of the detoxifying process, as well as the effectiveness of detoxification are drug dose-dependent. The biological basis of the fluctuations in treatment efficacy at >6-h intervals remains unclear, but cell kinetic effects which cannot easily be identified may be involved at these time points.

The results from this "time line" study indicate that the triphasic dose–response curve to repeated drug injections, discussed above, is dependent on the treatment protocols, i.e. the time interval between injections. A shift of the dose–effect curves would be expected if the time between injections was changed, but the general triphasic shape would be conserved.

In conclusion, repeated injections of BLM induce a highly complex response pattern in mouse oral mucosa dependent on both drug dose and time between injections. A plausible explanation for the experimental results may be that the first injection induces a detoxifying process in a dose-dependent manner, which, by overshoot response, causes a decrease in drug toxicity at intermediate doses. Higher drug doses may then lead to saturation of the detoxifying system and thus to a final increase in toxicity.

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