

BLEOMYCIN: EFFECTS UPON MAMMALIAN EPIDERMAL DNA SYNTHESIS

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Abstract. The effects of bleomycin upon epithelial DNA synthesis have been evaluated in a keratinizing tissue culture line and following topical application on intact mouse epidermis. In both models, bleomycin inhibited epithelial DNA synthesis after prolonged exposure of the tissue to large doses of the polypeptide antibiotic. The effect is probably limited by the penetration of bleomycin through epithelial cell membranes. Inhibition of epidermal DNA synthesis was also observed in non-treated animals subjected to restraint and/or partial starvation. These conditions, which are commonly associated with studies of topical therapeutic agents, must, therefore, be carefully controlled.

Key words: Bleomycin; DNA; Epidermis; Skin; Inhibitor

A significant need exists for the development of agents which alter epidermal macromolecular metabolism and which can be used in local therapy for benign proliferative diseases such as psoriasis and in the treatment of cutaneous malignancies. This need, coupled with our awareness of certain previously observed characteristics of bleomycin (BLM), prompted the investigations which are described in this paper.

Bleomycin, a complex of several glycopeptide antibiotics, was first isolated from *Streptomyces verticillus* by Umezawa, et al. (21). Following systemic administration of radioactively labelled bleomycin, distribution studies indicated a significant accumulation of the drug in epithelial tissues, particularly in the skin and lungs (9). BLM has been used locally and systemically as a chemotherapeutic agent for various cutaneous lesions, including squamous cell carcinoma (12), xeroderma pigmentosum (17), verruca vulgaris (7), and a variety of other neoplasms. Although the mechanism of

action of the drug has not been completely clarified, BLM has been shown to inhibit cell proliferation and DNA synthesis (3, 20), to cause scission of DNA strands (11), and to alter chromosomal structure (10). In addition to specific antiepithelial action, systemic bleomycin therapy in man is associated with major cutaneous side effects (2), a fact which increased our belief that topical application of bleomycin might have a place in the therapy of proliferative epidermal disease.

The studies reported below were aimed at the elucidation of the pharmacological effects of BLM upon mammalian epidermis and upon a keratinizing tumor cell line of epithelial origin.

MATERIALS AND METHODS

Chemicals

Thymidine, (methyl-³H, 6.7 Ci/mmole), was purchased from New England Nuclear Corporation, Boston, Ma. Bleomycin (Blenoxane) and bleomycin ointment were gifts of Bristol Laboratories, Syracuse, New York. Dimethyl-sulfoxide was purchased from Fisher Chemical Co. All other chemicals were of reagent grade obtained from a variety of sources.

Animals

Male, albino, CD-1 strain mice of approximately 20 g were obtained from Charles River Laboratories, Wilmington, Ma. They were fed a high moisture-content diet obtained from the same source.

Cells

ME-180 cells, derived originally from a metastatic cervical carcinoma (19), are carried in our laboratory for a variety of studies. The cell line is characterized by the production of intercellular desmosomes and intracellular filaments which are the morphological hallmarks of keratinizing cell

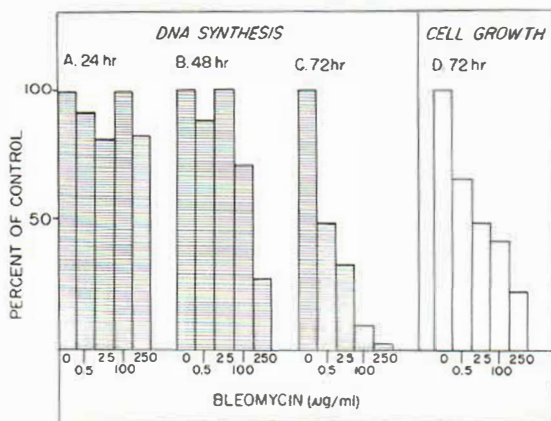


Fig. 1. Effects upon ME-180 cells of increasing concentrations of BLM. DNA synthesis and cell growth were monitored over a 72 hour period. Methods are described in the text.

lines. ME-180 cells were cultured at 37°C in a 2–3% CO₂ atmosphere as monolayers in minimal essential medium (Earle's) (Grand Island Biochemicals, Grand Island, N.Y.) supplemented with 20% fetal calf serum, 250 U of penicillin and 250 µg of streptomycin per milliliter. The cells were plated at a density of 3 × 10⁵ cells/ml of medium in scintillation vials which had been pre-incubated with culture medium for 16 hours. The culture medium was changed 48 hours after the original plating and daily thereafter. At 48 hours, BLM at concentrations of 0.5 to 250 µg/ml was added to some cultures, while controls were maintained in BLM-free medium. Cell growth was monitored daily by counting duplicate vials of control and BLM-treated cultures.

³H-Thymidine incorporation into ME-180 cells

Following exposure of ME-180 cells to bleomycin as noted above, the antibiotic-containing medium was removed and replaced with medium containing 2.5 µCi ³H-thymidine per milliliter for 3 hours. The incorporation reaction was terminated by removal of the labelled medium, trypsinization of the monolayers and extraction of DNA with perchloric acid according to the procedure of Schmidt & Thannhauser (13). Aliquots of the perchloric acid extract were used for DNA assay according to the technique of Shatkin (16) and for determination of radioactivity in a Packard Tricarb Scintillation Spectrometer. Specific activities were determined as counts per minute incorporated per microgram DNA.

In vivo incorporation of ³H-thymidine into mouse epidermis

The backs of the mice were shaven; the remaining hair shafts and roots removed by wax depilation (4). In our initial studies each mouse was placed in a restraining apparatus which was designed to minimize loss of medication and cross contamination between mice. The animals were given free access to the high moisture diet noted

above. 400 mg BLM ointment containing 2 mg BLM was mixed with 60 µl of dimethyl sulfoxide (DMSO) and applied twice daily to the depilated areas of skin. One control group was treated twice daily with ointment base and DMSO, while the second control was left untreated. In later studies, the mice were not restrained, but were kept in separate cages and allowed free access to food and water.

BLM applications continued for 3 days following depilation, the total dose applied being 12 mg. On day four, the control and treated animals were injected intraperitoneally with 2.5 µCi ³H-thymidine/g body wt and were sacrificed by cervical dislocation 3 hours after injection. The dorsal skin was removed and incubated for 15 min at 37°C in 0.25% trypsin in phosphate-buffered saline, pH 7.2. The epidermis was separated from the dermis by scraping, rinsed with saline, collected by centrifugation (1000 rpm × 5 min), pressed onto a filter paper to remove excess moisture, weighed and homogenized in 4 volumes of 1 N perchloric acid. The DNA content and radioactivity of the perchloric acid extracts were determined as described above.

In vitro incorporation of ³H-thymidine into skin slices

Restrained and unrestrained mice (as described above) were divided into control, ointment base, and BLM-treated groups of 6 animals each and given food and water freely. After 3 days of treatment the animals were sacrificed, residual ointment was removed, and the skin dissected free of subcutaneous fat and cut into 1 cm² pieces. Each piece of skin was sliced on a 50 µm Stadie-Riggs microtome to remove the bulk of the dermis and the residual sheets (consisting of epidermis and papillary dermis) were incubated at 37° for 3.5 hours in minimal essential medium (Eagle's) containing 20 mM Tris-HCl, pH 7.5, and 2.5 µCi/ml ³H-thymidine.

Table 1. *In vivo* incorporation of ³H-thymidine into mouse epidermis

2.5 µCi ³H-thymidine/g body weight were injected intraperitoneally as described in Methods. Incorporation proceeded for 2.5 hours. Data represent results from 4 groups of 6 animals each. The average error as measured by the coefficient of variation seems constant within the Restrained and Unrestrained categories. Data from restrained animals exhibit an average of 4% greater error than data from unrestrained animals

	cpm/µg DNA		cpm/mg epidermis	
	Mean ± S.D.	Error	Mean ± S.D.	Error
Restrained				
Fed	11.2 ± 1.37	12%	235 ± 39.0	17%
Starved	3.7 ± 0.48	13%	84 ± 6.9	8.2%
Unrestrained				
Fed	21.9 ± 2.16	9.8%	407 ± 12	2.9%
Starved	8.2 ± 0.57	7.1%	186 ± 4.5	2.4%

Table II. Statistical analysis of data displayed in Table I

Animal group	Variable	Change in specific activity		Average change in specific activity (%)	Statistical significance (two-way ANOVA with replication)
		cpm/ μ g DNA (%)	cpm/mg epidermis (%)		
Fed	Restraint	↓49	↓42	↓46	$p < 0.01$
Starved	Restraint	↓54	↓55	↓54	$p < 0.01$
Restrained	Starvation	↓66	↓64	↓65	$p < 0.01$
Unrestrained	Starvation	↓63	↓54	↓58	$p < 0.01$
Combined effect of starvation and restraint		↓83	↓79	↓81	$p < 0.01$

Following incubation, the skin slices were washed in phosphate-buffered saline at 4°C to stop the reaction and to remove non-incorporated ^3H -thymidine. The slices were incubated for 15 min at 37° in 0.25% trypsin in phosphate-buffered saline and the epidermis was removed from the papillary dermis by scraping. The tissue subsequently was handled as described above.

RESULTS

Effects of bleomycin upon ME-180 cells

ME-180 cells, in early exponential growth, were cultured as monolayers in the presence of 0.5, 25, 100 or 250 μg of BLM per milliliter of culture medium. Control and BLM-treated cells were incubated in the presence of ^3H -thymidine and the incorporation (counts per minute/mg DNA) was measured. As is apparent in Fig. 1, during the first 24 hours no significant effect upon DNA synthesis was seen at any concentration of BLM. After 48 hours, BLM at concentrations of 100 and 250 $\mu\text{g}/\text{ml}$ caused inhibition of thymidine incorporation and by 72 hours the inhibition of DNA synthesis was evident even at a BLM concentration of 0.5 $\mu\text{g}/\text{ml}$. Higher concentrations almost completely suppressed ^3H -thymidine incorporation.

The effects of bleomycin upon cell growth and division were analysed directly. Cell growth was measured by counting trypsinized cells from duplicate vials and, 24 hours following bleomycin, significant inhibition of cell growth occurred at a dose of 250 $\mu\text{g}/\text{ml}$ medium. At concentrations below 250 $\mu\text{g}/\text{ml}$, minimal inhibition occurred at 24 hours and moderate, although variable, inhibition was evident at 48 hours. In Fig. 1 the counts following 72 hours of exposure to bleomycin are displayed. A clear dose-response curve is evident, indicating that in this keratinizing cell line, bleomycin inhibits both DNA synthesis and cell growth.

Effects of restraint and starvation upon mouse epidermal DNA synthesis

We believe that for the adequate evaluation of local antimetabolites a model system must be available in which the agents can be applied in reproducible quantities and left on the skin for a defined period of time. The possibility of systemic absorption and cross contamination must be minimized.

To meet these requirements we developed an apparatus in which mice could be restrained for prolonged periods of time. The medication could easily be applied to restricted areas of skin and cross contamination could not occur. Using this apparatus, we were able to prove that local inhibition of epidermal DNA synthesis occurred following topical bleomycin without any evidence of systemic effect (data not shown). However, unexpected metabolic consequences of the restraining procedure became apparent as is indicated by the data in Table I. Four groups of 6 animals each were studied. Two groups were not restrained but were maintained in individual containers. One group was fed, the other allowed only water. The restrained animals were similarly divided into fed and fasted groups. The fasted groups were included as we noted variable patterns of food intake among the restrained animals. Weight loss was apparent in those that did not eat.

The *in vivo* ^3H -thymidine incorporation data summarized in Table I indicate that both the restraining procedure and starvation caused inhibition of incorporation. Such inhibition was apparent when incorporation was measured as counts per minute per microgram of DNA or counts per minute per milligram of epidermis. These data were subjected to statistical analysis and the results are detailed in Table II. Starvation and restraint,

Table III. Effects of BLM on *in vivo* incorporation of ^3H -thymidine into mouse epidermis

2.5 μCi ^3H -thymidine/g body weight were injected into each animal. Data represent results from 6 groups of 6 animals each. Error expressed as coefficient of variation

Animal group	Maneuver	cpm/ μg DNA (mean \pm S.D.)	Error (%)	cpm/mg tissue (mean \pm S.D.)	Error (%)	Significance (Duncan's test)
Restrained	Control	32.6 \pm 9.10	28	—	—	BLM vs. control: $p < 0.01$
	Vaseline+ DMSO	24.8 \pm 6.53	26	—	—	Vaseline vs. control: $0.01 < p < 0.05$
	BLM+DMSO	4.5 \pm 0.22	5	—	—	BLM vs. vaseline: $p < 0.01$
Unrestrained	Control	133.9 \pm 11.20	8	55.37 \pm 14.7	26	BLM vs. control: $p < 0.01$
	Vaseline+ DMSO	58.88 \pm 4.33	7	21.72 \pm 2.6	12	Vaseline vs. control: $p < 0.01$
	BLM+DMSO	36.69 \pm 5.58	16	11.96 \pm 1.3	10	BLM vs. vaseline: $p < 0.01$

either alone or in combination, cause a statistically significant decrease in the specific activity of ^3H -thymidine incorporation in epidermis. The analysis of variance (ANOVA) indicates that starvation and restraint act independently to reduce the ^3H -thymidine incorporation. When present together, starvation and restraint further reduce specific activity by an additional 20 to 30%. The clear effect of restraint noted in these experiments obliged us to use both restrained and unrestrained animals in our further studies, although it should be emphasized that the restrained model is much more controllable from the viewpoint of cutaneous drug application.

Effects of bleomycin upon mouse epidermal DNA synthesis

For these studies, 6 animals were used in each treatment group and all were fed the high moisture diet. The mean specific activities of thymidine incorporation for control, ointment base, and BLM treated mice are presented in Table III. The error as

measured by the coefficient of variation is not uniform within categories, suggesting non-uniform experimental conditions. However, for both restrained and unrestrained animals the decrease in thymidine incorporation following treatment with either BLM or ointment base differs significantly from the control. In addition, the BLM effect is statistically significantly different from that of the ointment base control.

The experiments detailed in Table IV were undertaken in order to analyse the effects of bleomycin upon epidermal DNA synthesis in a more direct manner. The incorporation of ^3H -thymidine into epidermal DNA was measured in an *in vitro* slice system which avoids many of the variables which are present in an *in vivo* experiment. As the data indicate, the decrease in DNA synthesis following application of BLM or ointment base is statistically significantly different from control values. In addition the decrease from BLM is significantly different from that due to vaseline and DMSO.

Table IV. Effect of BLM on *in vitro* incorporation of ^3H -thymidine into epidermis in skin slice incubation

Methods used are described in text. Data represent 4 groups of 4 mice each with skin divided into six samples. The average error, as measured by coefficient variation, is approximately constant for the series suggesting uniform experimental conditions

Animal group	Maneuver	Specific activity, cpm/mg: epidermis		Significance (Duncan's test)
		Mean \pm S.D.	Error (%)	
Restrained	BLM+DMSO	1.70 \pm 0.36	21	BLM vs. control: $p < 0.01$ Vaseline+DMSO vs. control: $p < 0.01$ BLM vs. vaseline+DMSO: $p < 0.01$
	Vaseline+DMSO	3.40 \pm 0.44	13	
	Control	4.28 \pm 0.61	14	
Unrestrained	Control	6.22 \pm 0.13	2	

DISCUSSION

Our studies of bleomycin had two major aims: first, to determine whether topical bleomycin inhibited DNA synthesis in mammalian epidermis, and second, to determine whether such inhibition, if present, could be used in clinical therapeutic situations. Bleomycin does inhibit epidermal DNA synthesis following topical application. Although the drug could, therefore, be useful clinically, we believe that because of the high concentration required for inhibition and the expense of the drug, other locally active inhibitory agents should be sought.

The reason for the relative insensitivity of epidermal and ME-180 cells to bleomycin inhibition following topical application has not been clarified by our studies. A variety of studies have shown that bleomycin affects different cell lines in different ways. HeLa cells are extremely sensitive to the agent and exposure to low doses of the antibiotic for short periods of time leads to marked inhibition of DNA synthesis (18). Other cell lines show little or no effect of bleomycin treatment, even after prolonged exposure to high concentrations of the antibiotic. This resistance may involve at least two factors. In RFL-T cells (6), a transformed fibroblast cell line, the major problem is entry of bleomycin into the cell and resistance is overcome by use of agents which alter the structure of the cell membrane. Bleomycin-resistant rat ascites hepatoma cells (8) have been shown to possess enzyme systems which inactivate bleomycin and in this way the cells become insensitive to the drug effect.

Since keratinizing epithelial cells are among the resistant types of tissue, and since it is known that epithelial cell membranes are characterized by resistance to the transport of highly charged molecules (14) such as bleomycin, we believe that the major problem is one of entry of the material into the cells. The fact that we saw no effects of bleomycin unless the agent was used in the presence of dimethyl sulfoxide, a solvent known to disrupt the membranes of epidermal cells (15), supports this conclusion. We do not have any information on bleomycin metabolizing enzymes in these cells.

The observations that starvation and restraint inhibited DNA synthesis in mouse epidermis, while not the primary goal of this investigation, may prove to be of major importance to future studies in

this area. It is well known that restraint and starvation inhibit metabolic processes in various tissues (1, 5) but we have been unable to find any reports of similar effects in epidermis. The mechanism of this inhibition remains an area for future study.

ACKNOWLEDGEMENTS

We gratefully acknowledge the assistance of Dr Bernard J. Ransil in the statistical analysis of the data.

This work was supported by Grant AM 16262 from the National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health; and by Grant 7 MO1 RR01032 from the General Clinical Research Centers Program of the Division of Research Resources, National Institutes of Health.

Data analysis was performed on PROPHET, sponsored by the Chemical/Biological Information Handling Program, National Institutes of Health, Bethesda, Maryland, USA.

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Received August 28, 1977

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