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BLEOMYCIN INHIBITION OF DNA SYNTHESIS IN ISOLATED ENZYME SYSTEMS AND IN INTACT CELL SYSTEMS

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Abstract—Bleomycin (BLM) inhibits DNA and RNA synthesis in different isolated enzyme systems. The inhibition effect can be reduced by adding RNA to the reaction mixture. The activity of the RNA dependent DNA polymerase and of a cell-free protein synthesizing system is not affected by BLM. The antibiotic reduces cell proliferation (L5178y mouse lymphoma cells) *in vitro* at low concentrations by cytostatis and at higher concentrations by cytotoxicity. In BLM-treated L5178y cells DNA synthesis is strongly reduced, while RNA and protein synthesis are not affected. *In vivo*, using growing quail oviducts, cell proliferation and cytodifferentiation are markedly inhibited after BLM treatment. This is attributed to the observed inhibition of DNA synthesis. RNA and protein synthesis as well as gene expression are not influenced by BLM under the conditions used.

Bleomycins (BLM), antibiotics discovered by Umezawa *et al.* [1], are a group of basic glycopeptides with known structures [2]. BLM exhibits antibacterial and antitumor activity *in vitro* and *in vivo* [1, 3]. It shows a specific antineoplastic activity against squamous cell carcinomas [4, 5] and squamous cell tumors [6]. Several features distinguish this antibiotic from other cytostatic agents: (1) it accumulates in special tissues [7], (2) it is inactivated at a low rate in organs with a high BLM concentration [7], and (3) it preferentially affects such cells, whose enzyme pattern is modified by oncogenic RNA viruses [8, 9].

Previous experiments with HeLa cells *in vitro* showed that BLM in low concentrations inhibited ^{3}H-thymidine incorporation into DNA, while the incorporation of leucine and uridine into protein or RNA was unimpaired [10]. In isolated enzyme systems, BLM strongly inhibits the reactions of DNA-dependent DNA polymerase and DNA-dependent RNA polymerase [review, 11].

In this paper, experiments showing that DNA synthesis is the main target of BLM action, *in vitro* as well as *in vivo* are reported.

MATERIALS AND METHODS

Chemicals. Unlabeled ribo- and deoxyribonucleoside-triphosphates, creatine phosphate, creatine phosphokinase, DNA dependent DNA polymerase I (Kornberg polymerase, fraction VII), isolated from *E. coli* according to the methods of Jovin *et al.* [12] were obtained from Boehringer Mannheim, Tutzing, Germany. ^{3}H-dCTP (sp. act. 9·3 Ci/m-mole), ^{3}H-UTP (sp. act. 2·02 Ci/m-mole), ^{3}H-L-tryptophan (sp. act. 2·0 Ci/m-mole), ^{3}H-thymidine (sp. act. 5·0 Ci/m-mole), ^{3}H-uridine (sp. act. 8·0 Ci/m-mole), ^{14}C-D-biotin (sp. act. 46 mCi/m-mole), ^{14}C-L-phenylalanine (sp. act. 310 mCi/m-mole) were obtained from The Radiochemical Centre, Amersham, England. Clealand's reagent (dithiothreitol) was from Calbiochem, Los Angeles, U.S.A., RNA (yeast) from Worthington, Biochemical Corp., Freehold, U.S.A., diethylstilbestrol, progesterone and actinomycin D from Serva, Heidelberg, Germany, DNA dependent RNA polymerase from *E. coli*, isolated according to Burgess [13] from Miles, Kankakee, U.S.A. Bleomycin (55–70% A_2, 25–32% B_2, $<7\%$ A'_2 and $<1\%$ B_4) and herring sperm DNA, isolated according to Zahn *et al.* [14], were a gift of H. Mack, Illertissen, Bayern, Germany.

Enzyme preparations. The DNA dependent DNA polymerase (sp. act. $1{\cdot}9 \times 10^{-9}$ mole ^{3}H-dCTP incorporated per mg protein in 30 min) and the DNA dependent RNA polymerase (α-amanitin sensitive enzyme corresponding to form II with sp. act. $2{\cdot}3 \times 10^{-12}$ mole ^{3}H-UTP incorporated per mg protein in 30 min) were isolated from L5178y mouse lymphoma cells, as described [15, 16]. Viral DNA polymerase (sp. act. of the RNA dependent DNA polymerase activity $9{\cdot}4 \times 10^{-13}$ mole ^{3}H-dCTP incorporated per mg protein in 20 min; sp. act. of the DNA dependent DNA polymerase activity $1{\cdot}2 \times 10^{-12}$ mole ^{3}H-dCTP incorporated per mg protein in 20 min) was isolated from purified Rauscher murine leukaemia virus (RML virus) from infected Balb/c mice spleens [17]. The components for the protein synthesizing cell free system were obtained from L5178y cells. The pH 5 fraction was isolated according to Stavy *et al.* [18] and the microsomes according to Munro *et al.* [19] and Korner [20].

Enzyme assays. The assay conditions (containing 100 μg/ml native DNA, isolated from herring sperm and 2 mM dithiothreitol) for determination of polymerase activities from Rauscher murine leukaemia viruses as well as from bacterial and mammalian cells were the same as described previously [11]. The

enzyme reaction was started by addition of 0·02 ml enzyme preparation to 0·08 ml of the assay mixture. For the determination of RNA dependent DNA polymerase activity the assay conditions were the same except that endogenous RNA from the virions replaced the DNA [17]. Incubation was performed at 37° for 20 min. The acid precipitable radioactivity was collected as described previously [16].

The standard reaction mixture for assaying protein synthesis *in vitro* was essentially as described [21]. The reaction mixture containing 2 mM dithiothreitol was incubated for 30 min at 37°. The acid precipitable radioactivity was collected on nitrocellulose filters [21].

Lymphoma cells. L5178y cells were grown in Fisher's medium for leukaemic cells (Grand Island Biological Co., Grand Island) in suspension cultures [22]. Dose response experiments (inoculation 5×10^3 cells/ml) were performed as described previously [23]. Cell concentrations and volume distributions were determined with a Coulter Counter (model B) with a Size Distribution Plotter (Coulter Electronics, Hialeah, U.S.A.). The viability of the L5178y cells was checked by the exclusion of the vital stain trypan blue.

For the determination of DNA, RNA and protein synthesis, suspensions of exponentially growing cells at 3×10^5 cells/ml were supplemented with BLM 1 hr prior to the addition of the labeled precursors ^{3}H-thymidine (1·2 μCi/ml), ^{3}H-uridine (1·9 μCi/ml) and ^{3}H-L-tryptophan (50 nCi/ml). The incubation was continued for 1 hr. Samples of 5 ml were analyzed for acid-insoluble radioactivity [24].

Quails. Immature female Japanese quails (*Coturnix japonica*), with an average weight of 100 g and an age of 35 days were obtained from J. Bökamp, Schloß Holte, Germany. To stimulate the oviducts the quails were injected subcutaneously with 5 mg diethylstilbestrol in 0·25 ml sesame oil for eight consecutive days. Within this period the wet weight of the oviducts increased from 0·1 g to 2·8 g.

In some experiments avidin synthesis was induced in quail oviducts, stimulated for 8 consecutive days with diethylstilbestrol by one intramuscular injection of 5 mg progesterone (dissolved in 0·25 ml sesame oil). Oviducts were removed 24 hr after injection with progesterone.

The isolation of DNA from the oviducts of ^{3}H-thymidine treated quails and the determination of its specific radioactivity were performed as described previously [25].

For preparation of the fresh oviduct tissue homogenates and for the isolation of polysomes on sucrose gradients (0·5 to 1·5 M) the described procedures were followed [26]. One ml of homogenate (0·01 mg DNA/ml; 1·2 mg RNA/ml; 3·4 mg protein/ml) was layered on top of the gradient. This was then centrifuged at 2° in a SW 40 rotor of a Spinco L2-50 B at 40,000 rev/min for 70 min. Fractions of 0·5 ml were collected for absorbance measurements at 260 nm and for radioactivity assays [26].

Miscellaneous methods. Determination of avidin was performed as described [27]. Protein was determined by biuret reaction [28]. RNA was measured by the method of San-Lin *et al.* [29], DNA by Kissane *et al.* [30]. Radioactivity was measured in a Packard liquid scintillation spectrometer with the solvent system described previously [31].

Table 1. Quantitative comparison of BLM action on different polymerases. The BLM concentrations, causing a 50 per cent reduction of the precursor incorporation rate into acid insoluble polymer from six experimental series per average value with their standard deviations (S.D.) are given. The assay conditions have been standardized for the various polymerase reactions as described in Materials and Methods

Enzyme	Source of enzyme	BLM concentration causing 50% inhibition (±S.D.) (μg/ml)
DNA dependent DNA polymerase	mouse lymphoma cells L5178y	4·2 ± 0·5
	E. coli	4·9 ± 0·5
	RML virus	1·2 ± 0·3
DNA dependent RNA polymerase	mouse lymphoma cells L5178y	5·3 ± 0·7
	E. coli	4·8 ± 0·6
RNA dependent DNA polymerase	RML virus	> 100

RESULTS

Experiments with isolated enzyme systems. BLM reduces the activity of the enzymes which catalyze the DNA dependent nucleic acid synthesis (Table 1). The DNA dependent DNA polymerases as well as DNA dependent RNA polymerases isolated from mouse lymphoma cells and *E. coli* are inhibited by BLM to a similar extent. The activity of DNA dependent DNA polymerase from RML virus is significantly more reduced than other DNA dependent DNA or RNA polymerases, whereas the RNA dependent DNA polymerase reaction of RML virus enzyme is insensitive towards the antibiotic up to very high BLM concentrations. The BLM inhibition of the isolated DNA dependent RNA polymerase can be counteracted by RNA (Table 2). An RNA concentration of 23 μg/ml reduces the BLM potency to 50 per cent at a template concentration of 100 μg native DNA/ml and 8 μg BLM/ml in the presence of 2 mM dithiothreitol.

BLM does not reduce the incorporation rate of ^{14}C-L-phenylalanine into protein in a cell-free system from L5178y cells up to concentrations of 100 μg BLM/ml in the presence of 2 mM dithiothreitol.

Influence on macromolecular synthesis of cells in vitro. BLM inhibits cell proliferation of L5178y cells in suspension by 50 per cent of at a concentration of 1·4 μg/ml, and it alters the average volume. Upon incubation for 72 hr with the ED_{50} concentration, the cell volume increases from 1436 μm^3 to 1828 μm^3. The viability of cells is not changed significantly when treated

Table 2. The influence of RNA on BLM inhibition of the DNA dependent RNA polymerase (form II) from L5178y cells. The results of five experimental series are given with their standard deviations (S.D.)

BLM concentration (μg/ml)	RNA concentration (μg/ml)	Incorporation in per cent (±S.D.)
0·0	0	100 ± 9
8·0	0	42 ± 5
8·0	5	46 ± 5
8·0	10	61 ± 7
8·0	50	79 ± 8
8·0	100	82 ± 9
8·0	200	87 ± 8

Table 3. Effect of BLM on synthesis of DNA, RNA and protein in mouse lymphoma cells. In parallel assays without the precursors of macromolecular synthesises the inhibition of cell proliferation has been determined. Details are given in Methods. The mean values of 5 experimental series are given. The standard deviations are less than 19 per cent

BLM concentration (μg/ml)	DNA fraction ^{3}H-thymidine incorporation (10^6 cells)	(%)	RNA fraction ^{3}H-uridine incorporation (10^6 cells)	(%)	Protein fraction ^{3}H-tryptophan incorporation (10^6 cells)	(%)	Inhibition of cell proliferation (%)
0	59,348	100·0	48,720	100·0	33,356	100·0	0
1·3	49,556	83·5	52,423	107·6	30,689	92·8	50
13·0	33,888	57·1	45,943	94·3	31,913	95·4	100
50·0	15,668	26·4	47,386	97·0	31,563	94·5	100

for 72 hr even with twice the ED_{50} concentration. At higher concentrations BLM acts cytotoxically; at 3 × ED_{50} concentration the treated cells show a cell viability of 88 ± 5 per cent. The controls show a viability of 99 ± 1 per cent.

The effect of BLM on the synthesis of DNA, RNA and protein with L5178y cells growing exponentially in suspension culture is shown in Table 3. At the ED_{50} concentration BLM causes a slight decrease in the rate of incorporation of labeled thymidine (83·5 ± 14% of the control; control = 100 ± 17%) into acid-insoluble material; the incorporation of ^{3}H-uridine (107·6 ± 19%) and ^{3}H-tryptophan (92·8 ± 14%) does not seem to be influenced. After incubation with 13·0 μg BLM/ml (= 10 × ED_{50}) the rate of DNA synthesis is significantly reduced, while RNA- and protein synthesis remain unchanged.

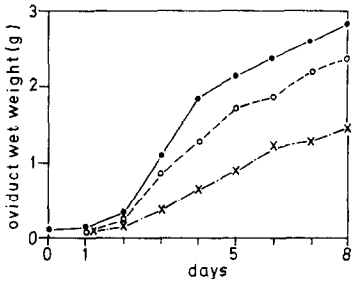

Fig. 1. The influence of BLM on the wet weight increase of oviducts from immature quails. The animals were treated with diethylstilbestrol for 8 days. Groups of 7 quails each received intraperitoneally 0 (●—— ●), 3 (○– – ○) or 10 mg BLM/kg (×–·–×) simultaneously with diethylstilbestrol. Each point represents the average weight from 7 quail oviducts; the standard deviation is less than 20 per cent.

Interference with cell proliferation and cytodifferentiation in vivo. The induction of cell proliferation and cytodifferentiation in juvenile reproductive tissue can be brought about by specific hormones. In this study oviducts of immature quails have been used as a biological model. After administration of diethylstilbestrol, a dramatic onset of cell proliferation and cytodifferentiation in the cells of the magnum portion occurs [32]. During 8 days the wet weight of oviducts increases from 0·1 g to 2·8 g. This growth can be slowed down by BLM (Fig. 1). This effect may be caused by an inhibition of DNA synthesis, as indicated by reduction of the incorporation rate of ^{3}H-thymidine into acid insoluble fractions (Table 4); RNA- and protein synthesis are not changed markedly under the conditions used. In addition the specific activity of DNA, extracted from the oviducts of BLM-treated quails, was found to be 27 per cent lower than in untreated animals (Table 5).

Table 5. Dependence of the specific radioactivity of DNA isolated from quail oviducts, stimulated for 8 days with diethylstilbestrol, from BLM, 6 hr prior to the injection of labeled percursors, the quails were injected with 30 mg BLM/kg body weight intraperitoneally. For labelling, animals were given 100 μCi thymidine intraperitoneally 120 min before decapitation

BLM concentration (mg/kg)	Specific radioactivity of DNA (dis/min per mg)	(%)
0	6420	100
30	4970	77

Influence on gene expression in vivo. Treatment of immature birds, previously stimulated with diethylstilbestrol, with progesterone causes an induction of avidin synthesis [32]. Avidin induction is linked to stimulation of RNA and protein synthesis, while progesterone does not stimulate DNA synthesis [32]. In quails, progesterone-induced synthesis of avidin can be blocked by treatment with actinomycin D (Table 6). However varied administrations of BLM, even at concentrations which have been shown to reduce the speci-

Table 4. Influence of BLM on ^{3}H-thymidine, ^{3}H-uridine and ^{3}H-L-tryptophan incorporation into oviducts, stimulated for 4 days by diethylstilbestrol, 6 hr prior to the injection of labeled precursors, the quails were treated intraperitoneally with 30 mg BLM/kg body weight. For short labelling, animals were given intraperitoneally the isotopes (50 μCi thymidine, 50 μCi uridine and 50 μCi L-tryptophan) 60 min before decapitation. Acid-insoluble radioactivity was determined as described [33]. It is expressed as dis/min per g magnum wet weight

BLM concentration (mg/kg)	DNA synthesis ^{3}H-thymidine incorporation (dis/min per g)	(%)	RNA synthesis ^{3}H-uridine incorporation (dis/min per g)	(%)	Protein synthesis ^{3}H-tryptophan incorporation (dis/min per g)	(%)
0	12,470	100	115,280	100	27,535	100
30	4115	33	119,890	104	25,330	92

Table 6. Influence of BLM on avidin synthesis in oviducts of quails treated for 8 consecutive days with diethylstilbestrol. On the ninth day 5 mg progesterone (PROG), dissolved in 0·25 ml sesame oil were injected into the muscle mass below the knee. Oviducts were removed 24 hr after progesterone injection. At different times prior to decapitation 3 or 30 mg BLM/kg in 0·25 ml saline were given either intramuscularly (i.m.) or intraperitoneally (i.p.), as indicated in the table. In one experiment actinomycin D (ACT) was administered. The avidin content in the oviducts from 8 animals was determined; the average results are given with their standard deviations (S.D.)

Treatment	Progesterone	Dose of BLM or ACT (mg/kg body weight)	Method of injection	Avidin (μg/g oviduct $\pm$ S.D.)	(%)
No BLM no ACT	–		–	0	0
No BLM no ACT	+	–	–	1170 $\pm$ 250	100 $\pm$ 21
BLM 24 hr before and simultaneously with PROG	+	3	i.m.	1250 $\pm$ 380	107 $\pm$ 30
		30	i.m.	1160 $\pm$ 280	99 $\pm$ 24
BLM 24 hr and 6 hr before and simultaneously with PROG	+	30	i.m.	1100 $\pm$ 260	94 $\pm$ 24
BLM 6 hr before and sinultaneously with PROG	+	30	i.p.	1220 $\pm$ 380	104 $\pm$ 31
ACT 6 hr before and simultaneously with PROG	+	0·5	i.p.	630 $\pm$ 190	54 $\pm$ 16

fic radioactivity of the DNA after a pulse with ^{3}H-thymidine (Table 5), do not influence the induction process of avidin (Table 6). In another set of experiments it could be shown, that BLM does not effect the appearance of rapidly-labeled polyribosomal RNA components (Fig. 2). The parallelism between UV-absorbing material and the sedimentation profiles of radioactivity, as shown in Fig. 2, indicates that the determined radioactivity is closely associated with the polyribosomes.

DISCUSSION

Recently it has been shown [8, 35] that BLM exerts its effect primarily on double-stranded DNA by a decrease in melting temperature, a release of thymine, and by strand scissions. Single-stranded DNA and RNA are not affected by this antibiotic. In isolated enzyme systems BLM causes inhibition of DNA as well as RNA synthesis [34, 9, 11]. In this paper we demonstrate a reduction of inhibition by co-incubation of the DNA dependent RNA polymerase BLM reaction mixture with RNA. Among the DNA dependent DNA polymerases tested, the enzyme isolated from oncogenic RNA viruses is inhibited significantly more than the others. At present only one explanation for this fact is available. Previous experiments showed that the BLM-modified DNA causes a non-competitive inhibition in the reaction with the DNA dependent DNA polymerase from *E. coli* [11] as well as from oncogenic RNA viruses [9]; possibly this influence on the enzyme proteins exerted by the BLM-modified DNA is different in the strength. The ineffectiveness of BLM to cause inhibition of the RNA dependent DNA polymerase activity and of the incorporation of amino acids into protein in a cell-free system must be due to the failure of BLM to modify the RNA template in both enzyme reactions.

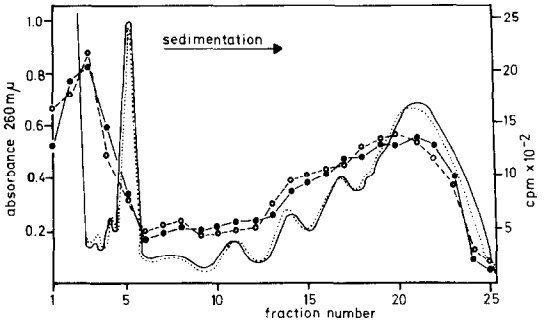

Fig 2. Effect of BLM on polysome formation in progesterone stimulated oviduct cells. The animals were injected intraperitoneally with saline or 30 mg BLM/kg (dissolved in 0·25 ml saline) 2 hr prior to decapitation. The RNA was labelled by intraperitoneal injection of 100 μCi ^{3}H-uridine 1 hr before. Recorder tracing of absorbance at 260 mμ: polysome profiles from quails treated with 0 mg BLM/kg (– – –) or 30 mg BLM/kg (· · · · · · · · · ·). The distribution of acid-insoluble radioactivity on the polysomes of oviducts from quails injected with saline (●——●) or 30 mg BLM/kg (○– – –○) is indicated.

With suspensions of mouse lymphoma L5178y cells *in vitro* as well as quail oviducts *in vivo*, BLM causes inhibition of thymidine incorporation, while uridine and tryptophan incorporation remains unchanged. No clear-cut data are available on the mode of action of BLM in whole cells. From the experiments with isolated DNA dependent DNA polymerase one might draw the conclusion that BLM interferes with the DNA synthesis during DNA replication and DNA repair. Indeed Terasima *et al.* [36] determined the S-phase to be the step most sensitive to BLM in HeLa cells; in other cell lines BLM exerted the largest cell killing effect in the G_2 phase and in mitosis [review, 37]. Therefore the inhibition of the two DNA synthesizing systems might cause arrest of cell proliferation as well as parasynchronization of the cells without affecting cellular RNA- and protein synthesis.

The discrepancy between the effect of BLM to cause inhibition of RNA synthesis in isolated enzyme systems and in intact cells, where no short term inhibition can be found, remains unanswered. Whether the described competition effect between RNA in the BLM inhibited polymerase system may have some biological significance is the subject of a further investigation.

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