EFFECTS OF BLEOMYCIN ON PHA-STIMULATED LYMPHOCYTES AND ISOLATED HEPATIC NUCLEI*

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Experiments were carried out in which lymphocytes stimulated by phytohemagglutinin were exposed during 3-day culture to concentrations of bleomycin varying from 0.0 to 300 μg/ml. Inhibition of one hour ^{3}H-thymidine incorporation in these 72-hour cultures increased as the concentration of bleomycin increased from 1.5 μg/ml (14.1 % inhibition) to 300 μg/ml (96.4 % inhibition), and was a linear function when dose and percent control ^{3}H-thymidine uptake were plotted logarithmically. In 3 of 9 experiments, ^{3}H-thymidine uptake at the 1.5 μg/ml dose was increased. This increase was not statistically significant, but the possibility of sublethal DNA damage followed by repair was raised. Exposure of resting lymphocytes to bleomycin in the absence of PHA showed no significant increase above baseline thymidine uptake. Addition of bleomycin to cultures at 24 and 48 hours after the addition of PHA produced the same quantitative reduction in uptake of thymidine at 70 hours as the same concentration added at zero time, suggesting that the effect of bleomycin is independent of the initial stimulatory events resulting from the addition of PHA.

Evidence of a repair-like process following damage by bleomycin was observed in a different system utilizing isolated hepatic nuclei. A two to five fold increase of thymidine tri-phosphate incorporation was obtained in response to addition of bleomycin in this isolated nuclear system (rat liver nuclei). This process was time and dose related and continued for at least one hour in the presence of bleomycin.

These results demonstrate that bleomycin causes dose-dependent reduction of DNA synthesis in PHA-stimulated lymphocytes. Furthermore, they suggest that DNA damage caused by low concentrations of bleomycin may be repaired, and that when damage exceeds the cell's ability to repair, cell death results.

Effects of bleomycin on PHA-stimulated lymphocytes

Human peripheral blood lymphocytes stimulated by phytohemagglutine *in vitro* undergo "blastogenesis"[1~4] and synthesize DNA, RNA and proteins[5~7]. This *in vitro* system of proliferating lymphoid cells has been used to evaluate the kinetics of lymphocyte proliferation[8], the immunologic functions of lymphocytes[9], and the effects of chemotherapeutic agents[10~13].

Bleomycin is a glycopeptide antibiotic derived from cultures of *Streptomyces verticillus* by UMEZAWA and co-workers[14]. The anti-neoplastic character of this agent has been demonstrated in a spectrum of animal neoplasms[15~16]. Clinical trials with bleomycin have shown it to be useful in the treatment of lymphomas and some squamous tumors[17~20].

The experiments described below were conducted in order to quantitate the effects of bleomycin

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upon proliferating lymphocytes in the PHA-stimulated system.

While studying the activity of bleomycin in this system, it became evident that another laboratory model, comprised of isolated hepatic nuclei[21~23] was also useful in studying the DNA-damaging effects of bleomycin, and experiments utilizing this system are also described.

Materials and Methods

The procedures employed in these experiments have been described in detail[21~24] and will be reviewed only briefly.

1. Lymphocyte Cultures

Venous blood samples were sedimented by means of high molecular weight dextran. The leukocyte-rich supernatant was removed and centrifuged and the cell sediment was resuspended in TC199 (8 ml) and fetal calf serum (FCS, 2.0 ml). Cell counts were adjusted by dilution with FCS to $7.5\sim15\times16^6$/ml. Each culture contained 7.8 ml of TC199, 2.25 ml of FCS, and streptomycin (100 μg/ml) and penicillin (100 units/ml). Those cultures to which PHA was added contained 0.1 ml of PHA (Burroughs Wellcome). Bleomycin complex* was dissolved in saline and added to cultures just prior to phytohemagglutinin. Each concentration was set up in triplicate with appropriate controls.

Thymidine (New England Nuclear Corporation, ^{3}H-Tdr, (sp. act. 6.7 curies/m mole) 1.0 μCi per 5×10^6 cells, was added to each culture one hour before termination of the experiment. For analysis, cells were washed twice in phosphate-buffered saline (pH 7.9) and centrifuged. They were resuspended and then precipitated and washed three times with cold 5 % trichloracetic acid. The insoluble residue was then washed in methanol, dissolved in 0.5 ml Soluene^R and mixed with 10 ml Liquifluor^R (New England Nuclear)-toluene in preparation for counting in a Packard Scintillation counter. Each sample was counted for 5 minutes and activity calculated by subtracting background counts.

Results were expressed as cpm/10^6 cells; values for replicate determinations were averaged and standard errors calculated.

2. Isolated Rat Liver Nuclei

Liver samples (about 1 g) were rinsed in 0.3 M sucrose and suspended in 15 to 20 volumes of 0.3 M sucrose -4×10^3 M $CaCl_2$ with one stroke in a loose Dounce homogenizer. The suspension was then homogenized with a loose fitting rubber pestle, filtered through a monofilament nylon screen (110 mesh), and centrifuged at $750\times g$ for 4 minutes. The resulting pellet was resuspended in 18 ml of 2 M sucrose -10^{-3} M $CaCl_2$, layered over 13 ml of 2.2 M sucrose, and sedimented by centrifugation for 20 minutes in a Spinco SW25.1 rotor at 20,000 rpm. Finally, the nuclei were suspended in 2.5 ml of 0.3 M sucrose, and used immediately.

Reactions were stopped with 1 ml of 1 M NaOH, and DNA was precipitated with 5 ml of a suspension of 40 mg of Celite (Johns-Manville) in ice-cold trichloracetic acid (10 %). The DNA was dissolved three times (0.3 ml of 1 M NaOH) and precipitated (8 ml of 5 % trichloracetic acid), and the final precipitate, dissolved in 1 ml of 1 M NaOH, was heated at 80°C for 15 minutes. Finally, the DNA was precipitated with trichloracetic acid on a pad of Celite and the precipitate was washed with acid, ethanol and ether.

DNA was estimated with diphenylamine[22] and radioactivity was measured in a hyamine-toluene liquid scintillation mixture, as described above.

Results

The addition of bleomycin complex to lymphoblast cultures inhibited ^{3}H-thymidine uptake progressively as the concentration was increased. The curve depicting inhibition took the form of a typical linear "cell-kill" curve in the concentration-range between 1.5 μg/ml and 300 μg/ml when

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Fig. 1. One-hour ³HTdr uptake of PHA-stimulated lymphocytes cultured with varying concentrations of bleomycin for 72 hours. Each point represents a mean of 9~27 separate determinations, plotted as percent of thymidine uptake of respective control specimens for that experiment. Standard error is indicated by barred vertical lines

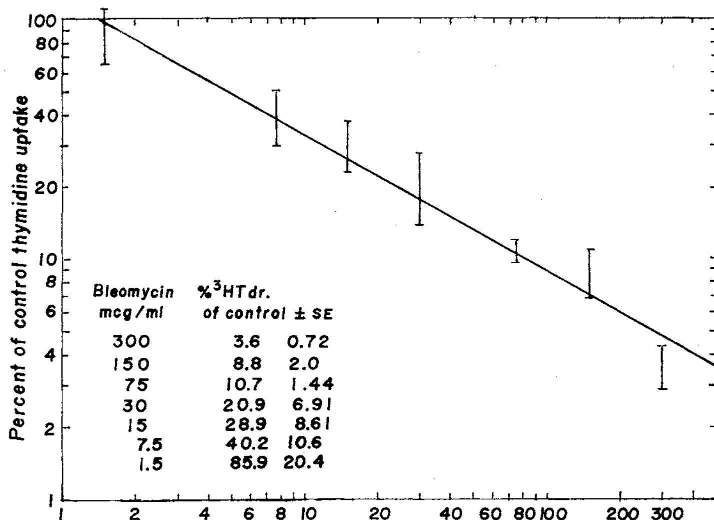


Table 1

Time after PHA bleomycin added	Total time bleomycin present	³HTdr-CPM/10⁶ cells
Zero Time	72 hours	264
24 hours	48 hours	278
48 hours	24 hours	259
70 hours	2 hours	2,010
Control	0 hour	2,001

Effect of bleomycin added at varying times after PHA during 3-day culture period: Bleomycin (150 μg/ml final concentration) was added to cultures at zero time, 24, 48 and 70 hours. Thymidine was added to all cultures 2 hours before termination, and all cultures were harvested in the usual manner at 72 hours. (All determinations were carried out in duplicate.)

Table 2. ³H-Thymidine uptake of cultures containing 1.5 μg/ml bleomycin for 72 hours

Specimen	Mean CPM ³HTdr/10⁶ cells* (±S.E.)	Mean control ³HTdr/10⁶ cells (±S.E.)	³HTdr uptake % of control (±S.E. of ratio)
MA	319±122	975±195	32.8±12.9
TBL	672±303	1,567±393	42.9±23.1
AW	1,090± 90	2,640	41.3±34.0
GS	664±101	623± 53	107 ±18.6
JW	433±134	761±332	56.9±36.4
JM	452± 49	698±340	64.8±42.1
RM	1,021± 79	736±207	138.7±43.7
SA	1,450±244	970	149.5±25.1
MW	1,373± 71	804±195	168 ±43.2

* Each mean value represents 3 separate determinations.

concentration of bleomycin and percent ³H-thymidine uptake were plotted logarithmically (Fig. 1). In order to determine whether or not bleomycin affected the events necessary for PHA stimulation of the lymphocytes, the agent was added at various times after addition of PHA. The addition of bleomycin (150 μg/ml) to cultures 24 and 48 hours after the addition of PHA produced the same quantitative reduction in uptake of thymidine at 70 hours as this concentration added at zero time (Table 1). Exposure of unstimulated, freshly isolated lymphocytes to concentrations of bleomycin varying from 1.5 to 1,500 μg/ml showed no significant alteration in baseline thymidine uptake.

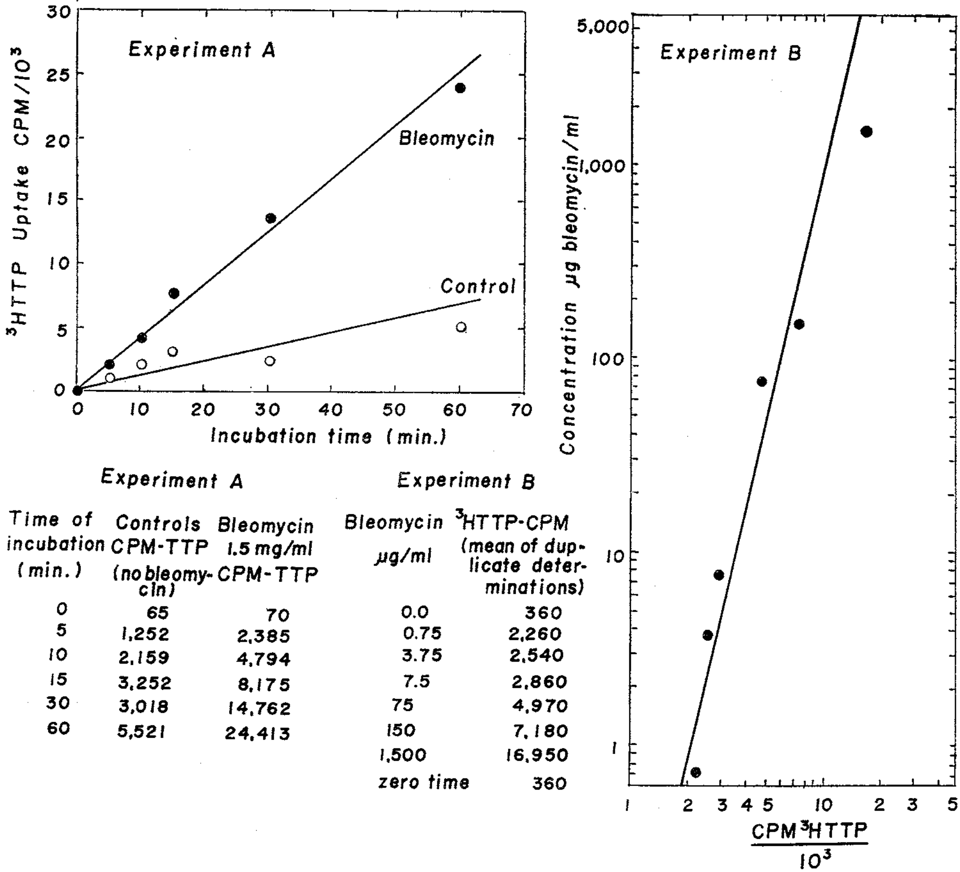
At the lowest concentrations of bleomycin used in 72-hour cultures (1.5 μg/ml), inhibition of ³H-thymidine incorporation into lymphoblasts was not seen in four of nine experiments. In these experiments there was either no effect or apparent stimulation of incorporation (up to 168 % of the control value) (Table 2). Although these determinations were not significantly different from 100 %

Fig. 2. Effect of bleomycin on ^{3}HTTP incorporation by normal isolated rat liver nuclei

Experiment A: Effect of 1.5 mg/ml bleomycin on ^{3}HTTP uptake as a function of time; 9.7×10^6 nuclei/tube, incubation times 0~60 minutes.

Experiment B: Effect of varying concentrations of bleomycin (0~1,500 μg/ml) on ^{3}HTTP uptake with incubation time fixed at 1 hour; 8.0×10^6 nuclei/tube.

Reaction mixture (0.5 ml): 0.1 M Tris (pH 7.4), 4×10^{-3} M $MgCl_2$, 1.6×10^{-2} M KCl, 2×10^{-3} M ATP, d GTP, d CTP, d ATP (each 8×10^{-5} M), 1.6×10^{-5} M ^{3}H-TTP (0.5 Ci per m mole), and the suspension of intact nuclei (containing 50~100 mcg of DNA).



Experiment A

Time of incubation (min.)	Controls CPM-TTP (no bleomycin)	Bleomycin 1.5 mg/ml CPM-TTP
0	65	70
5	1,252	2,385
10	2,159	4,794
15	3,252	8,175
30	3,018	14,762
60	5,521	24,413

Experiment B

Bleomycin µg/ml	³HTTP-CPM (mean of duplicate determinations)
0.0	360
0.75	2,260
3.75	2,540
7.5	2,860
75	4,970
150	7,180
1,500	16,950
zero time	360

as determined by analysis of variance of the ratio, they opened the question as to whether or not repair of DNA following damage due to bleomycin might be occurring.

Since one mechanism of action of bleomycin appears to be through direct scission of single stranded DNA[25] it seemed likely that the readily quantitated isolated hepatic nuclear system might provide a useful tool for evaluating these effects. Therefore, the effect of bleomycin in varying doses (1.5~1,500 μg/ml) and over varying time spans (up to 1 hour) upon isolated hepatic nuclei, were studied. The addition of bleomycin to isolated rat liver nuclei caused an approximately 2~5 fold increase of ^{3}H-thymidine triphosphate (^{3}HTTP) incorporation. At a concentration of 1,500 μg/ml, incorporation increased linearly over a one hour period of incubation (Fig. 2A), producing a five-fold increment over control values at the end of the incubation period. One hour incorporation of ^{3}HTTP was dose-related in the range of concentrations between 0.75 μg/ml and 1,500 μg/ml (Fig. 2B).

Table 3

Reference	Cell type	Bleomycin fraction	Conc. μg/ml	Length of incubation	^{3}H-Tdr % of control uptake
KUNIMOTO, *et al.*[26]	HeLa S-3	Bleomycin complex	0.5 1.0 10.0 100.0	9 hours	90.5 62.9 33.0 10.2
SUZUKI, *et al.*[25]	EHRLICH ascites	A_2	40	30 minutes	70
	HeLa S-3	A_2	40	4 hours 28 hours	79 43
UMEZAWA, *et al.*[17]	*E. coli* B	A_2	2 10 50		73.2 70.6 51.7
	HeLa cells	A_2	50		39.5

Discussion

The present studies demonstrate that bleomycin is an effective inhibitor of ^{3}H-thymidine uptake in normal human lymphocytes undergoing blastogenesis. The amount of inhibition is dose-related and takes a linear form when both variables are plotted logarithmically.

These results are consistent with the findings of other investigations studying the effects of bleomycin complex and its sub-fractions upon several cell systems *in vitro*, as summarized in Table 3. KUNIMOTO[26] performed a quantitative study in which the effects of bleomycin on DNA synthesis in HeLa S-3 cells yielded results similar to the present experiment (Table 3, line a). SUZUKI and co-workers[25] observed "significant inhibition" of growth of HeLa S-3 cells and *Escherichia coli* B at concentrations of bleomycin as low as 1.0 μg/ml, and described inhibition of ^{3}H-thymidine uptake in both EHRLICH ascites cells and HeLa cells incubated with bleomycin at a concentration of 40 μg/ml for varying lengths of time (Table 3, line b). Similarly, UMEZAWA and co-workers[17] reported inhibition of ^{3}H-thymidine incorporation into DNA of intact *E. coli* B and HeLa cells at concentrations of bleomycin varying from 2 to 50 μg/ml (Table 3, line c).

Working with DNA isolated from *E. coli* $15T^-$ cells and HeLa cells, and with whole *E. coli* $15T^-$, SUZUKI *et al.* demonstrated that bleomycin produces scission of single-stranded DNA, both *in vitro* and *in vivo*[25]. TOBEY[27] demonstrated that in Chinese hamster cells exposed to bleomycin at a concentration of 100 μg/ml *in vitro*, initiation and termination of DNA synthesis occur at normal rates, but cells accumulate in G_2. NAGATSU *et al.*[28] similarly demonstrated that a large fraction of transplanted VX-2 carcinoma cells treated with bleomycin ended up with a G_2 content of DNA.

The presently reported results with lymphocytes are similar to those reported by UMEZAWA[17], SUZUKI[25], and KUNIMOTO[26], in that there was dose-dependent and time-dependent inhibition of DNA synthesis following exposure of PHA-stimulated lymphocytes to bleomycin for 72 hours *in vitro*. Exposure of unstimulated, freshly isolated lymphocytes to concentrations of bleomycin varying from 1.5 to 1,500 μg/ml showed no significant alteration in baseline thymidine uptake. (Baseline measurements in the system are very low, and *small* increments or decrements may not have been detectable; however, no evidence of increased thymidine uptake suggesting repair was noted.) Since the recorded reduction in thymidine uptake by PHA-stimulated lymphocytes was the same at 72 hours for cultures to which bleomycin (150 μg/ml) was added at zero time, 24 hours and 48 hours, the effect of bleomycin seems to be independent of the early events of entry into the cell cycle stimulated by PHA and occurring in the first 24~48 hours. No reduction in incorporation was seen when bleomycin was added 2 hours before ^{3}H-thymidine on day 3 of this experiment. The explicit point of action of bleomycin in the cell cycle was not further determined by these experiments.

In attempting to further delineate the significance of the apparently increased uptake of thymidine noted at low dose (1.5 μg/ml) levels of bleomycin in several experiments, studies were carried out in

which the effects of bleomycin upon isolated hepatic nuclei were investigated. A linear, dose-related and time-related increase in incorporation of thymidine tri-phosphate into DNA ("unscheduled" DNA synthesis) was demonstrated. Since nuclei in this system do not exhibit significant replicative DNA synthesis *in vitro* unless the cells have been previously perturbed in the experimental animal, the increased incorporation resulting on incubation with bleomycin is not attributed to stimulation of replicative DNA synthesis.

These results suggest that this system can simultaneously sustain and repair DNA damage caused by bleomycin. Since one action of bleomycin appears to be scission of single-stranded DNA[25] it is suggested that stimulation of thymidine uptake seen in isolated rat liver nuclei represents repair of single-stranded DNA breaks.

Stimulation of thymidine incorporation into DNA as a result of repair ef DNA damage seems to be a general cellular function, and has been reported to result from exposure of bacteria and lymphocytes to ultraviolet radiation[29~31], carcinogens and alkylating agents[32]. It is therefore not surprising that an agent such as bleomycin, which apparently causes single-stranded DNA breaks as one mode of action, might produce damage which would secondarily result in DNA repair. These data also suggest that cell death may ensue as the result of bleomycin-induced DNA damage when the cell's capacity to repair this damage is exceeded.

The ability or lack of ability to repair DNA damage may, therefore, in part, determine the sensitivity of a given cell to bleomycin. Bleomycin has been shown to have minimal toxic effects on non-lymphoid cell lines of the hematopoietic system. This could be related to a superior ability of non-lymphoid cells to repair single-stranded DNA breaks.

The present studies suggest that the investigation of sensitivity of neoplastic lymphoid cell lines to bleomycin *in vitro* may assist in evaluating the potential response to bleomycin.

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