MECHANISM OF ACTION OF PHLEOMYCIN, A TUMOR-INHIBITORY ANTIBIOTIC

Nobuo Tanaka, Hideyo Yamaguchi and Hamao Umezawa
Institute of Applied Microbiology, University of Tokyo, Tokyo

Received December 26, 1962

Phleomycin, isolated from the culture of Streptomyces verticillus by Maeda et al (1956), is a water-soluble, copper-containing antibiotic complex, effective against a variety of bacteria. Recent investigations by Lein et al and Umezawa et al revealed that it has a significant activity against Ehrlich carcinoma, sarcoma 180 and adenocarcinoma 755 of mice. In the studies of the mode of action of phleomycin, it was observed that this antibiotic selectively inhibits the DNA synthesis in E. coli and HeLa cells, as reported by Shiba et al (1959) with mitomycin C. However, phleomycin has much less activity of depolymerizing the DNA to acid-soluble products than mitomycin C.

Selective inhibition of the DNA synthesis in E. coli K12: A culture of E. coli K12 grown in a glucose-salts medium was harvested while in the logarithmic phase of growth, washed and resuspended in a similar fresh medium. After addition of phleomycin or mitomycin C to give the minimal growth-inhibitory concentration (phleomycin 2 μg/ml and mitomycin C 1 μg/ml respectively), the bacterial suspensions were incubated with shaking at 37°. They were analyzed for nucleic acids and protein after fractionation by Schneider's procedure. As presented in Table 1, the DNA synthesis was inhibited in the presence of phleomycin, although the formation of protein and RNA was not significantly affected, as compared with that of control within 90 minutes. The same tendency was observed with mitomycin C.

Inhibition of the incorporation of tritiated thymidine into the DNA in E. coli 15T⁻: The cells of E. coli 15T⁻(thymine-less mutant) grown in a thymidine-containing glycerol-salts medium were collected during the logarithmic phase of growth, washed and resuspended in a similar fresh medium. They were incubated at 37°

for 30 minutes with graded concentrations of phleomycin or mitomycin C, and then contacted with tritiated thymidine (1 μc/ml) for 10 minutes, at which the uptake of thymidine into the DNA reached the maximal level in antibiotic-free bacterial suspensions Thymidine-n6-^{3}H with specific activity of 4.32 c/mM was obtained from the Radiochemical Centre, Amersham and used throughout the experiments. As shown in Table 2, phleomycin inhibited the incorporation of thymidine into the DNA. At the minimal growth-inhibitory concentration (10 μg/ml) 58% inhibition occurred. Stronger inhibition (90%) was observed with mitomycin C at the minimal growth-inhibitory concentration (0.5 μg/ml).

Table 1. Effect of phleomycin and mitomycin C on growth, protein and nucleic acids synthesis of E. coli K12.

Antibiotic	Time incubated min.	Turbidity E_{700}	Protein (Folin reaction) E_{700}	RNA (orcinol reaction) E_{660}	DNA (indol reaction) E_{490}
-	0	0.20	0.36	0.25	0.04
	30	0.25	0.46	0.31	0.06
	60	0.32	0.58	0.37	0.06
	90	0.38	0.72	0.48	0.08
Phleomycin	0	0.20	0.36	0.25	0.04
	30	0.25	0.46	0.31	0.04
2 μg/ml	60	0.32	0.56	0.36	0.04
	90	0.36	0.68	0.46	0.05
Mitomycin C	0	0.20	0.36	0.25	0.04
	30	0.25	0.45	0.31	0.04
1 μg/ml	60	0.31	0.56	0.36	0.04
	90	0.34	0.67	0.46	0.04

Table 2. Inhibition by phleomycin and mitomycin C of the incorporation of tritiated thymidine into the nucleic acid fraction of E. coli 15T⁻.

Antibiotic	cpm/μg eq. DNA	% Inhibition
-	364	
Phleomycin 160 μg/ml	89.1	76
40	107	71
10	154	58
Mitomycin C 8 μg/ml	3.82	99
2	12.8	96
0.5	36.0	90

The radioactivity was determined in a windowless gas-flow counter and corrected for self-absorption.

Effect on the E. coli DNA containing tritiated thymidine: Reich et al (1960) demonstrated that mitomycin C caused E. coli to

break down the DNA, acid-soluble products being formed. Following the method of Reich et al (1960), the effect of phleomycin was investigated. The cells of E. coli 15T$^-$ grown in a thymidine-supplemented synthetic medium were shaken with thymidine-n6-^{3}H (0.5 μc/ml) in a thymidine-containing or thymidine-free medium at 37° for 40 minutes and washed with thymidine-containing medium. They were incubated with phleomycin or mitomycin C (20 μg/ml or 1 μg/ml respectively, which is twice as much as the minimal growth-inhibitory concentration) at 37° for 4 hours, and were extracted with 5% trichloroacetic acid at 0° for 2 hours. The results are summarized in Table 3. Phleomycin yielded much less labelled acid-soluble degraded products than did mitomycin C. By the method employed, approximately 20% of the DNA originally present became acid-soluble in the presence of mitomycin C, but less than 1% with phleomycin. These results indicated that phleomycin has much less activity of breaking down the DNA than mitomycin C.

Table 3. Effect of phleomycin and mitomycin C on appearance of tritiated thymidine in the acid-soluble fraction in E. coli 15T$^-$.

Medium	with thymidine	without thymidine
Control	160	160
Phleomycin 20 μg/ml	600	220
Mitomycin C 1 μg/ml	9,580	9,120

The numbers represent cpm/ml, determined in a windowless gas-flow counter and corrected for self-absorption.

Effect on the uptake of tritiated thymidine into the nuclei of HeLa cells: The 2 day culture of HeLa cells during the logarithmic phase of growth was incubated at 37° for 4 hours in a lactalbumin calf serum medium containing graded concentrations of phleomycin, and then the cells were contacted with thymidine-n6-^{3}H (1 μc/ml) for an hour. They were fixed with ethanol, stained with acetic orcein and processed for autoradiography with Kodak AR10 films. Preparations were exposed for 5 days. The cells were microscopically observed for nuclear grains, indicative of thymidine incorporation. Radiographic background in all preparations averaged less than one grain per 25 μ^2. Cells showing more than 10 nuclear grains were considered specifically labelled. As presented in Table 4, phleomycin inhibited the incorporation of thymidine into the nuclei of HeLa cells. At the concentration of 1 μg/ml,

54% inhibition was observed and 92% inhibition at 2 μg/ml. As observed by Umezawa et al, phleomycin significantly inhibited the mitosis of HeLa cells at the concentration of 1 μg/ml and completely at 2 μg/ml.

Table 4. Effect of phleomycin on the incorporation of tritiated thymidine into the nuclei of HeLa cells.

Phleomycin μg/ml	Number of cells containing nuclear grains in 1,000 cells	Inhibition %
0	406	
1	186	54
2	33	92
4	2	99

Phleomycin, at the minimal growth-inhibitory concentration, did not show any significant influence on the incorporation of ^{14}C-amino acids into the protein fraction nor on the ^{32}P incorporation into the RNA fraction in E. coli K12. The oxidation of glucose and lactose was not significantly affected in the presence of phleomycin. Microscopic observations revealed that phleomycin caused the elongation of E. coli but did not produce spheroplasts in a magnesium-sucrose broth.

It was concluded from the above results that phleomycin selectively inhibits the DNA synthesis in E. coli and HeLa cells. It has much less activity of breaking down the DNA to acid-soluble products than mitomycin C.

References

Lein, J. (Bristol Laboratories) Personal communication.
Maeda,K., Kosaka,H., Yagishita,K. & Umezawa,H., J. Antibiotics (Japan), 9A, 82 (1956)
Reich,E., Shatkin,A.J. & Tatum,E.L., Biochim. Biophys. Acta 45, 608 (1960)
Shiba,S., Terawaki,A., Taguchi,T. & Kawamata,J., Nature, 183, 1056 (1959)
Umezawa,H., Hori,M., Ishizuka,M. & Takeuchi,T., J. Antibiotics (Japan), (in press)