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REDOX CYCLING OF FE(III)-BLEOMYCIN BY NADPH-CYTOCHROME P-450 REDUCTASE

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The bleomycins, a family of glycopeptide antibiotics, are effective in the chemotherapy of several solid tumors and malignant lymphomas in man (1).Their cytostatic activity is due to DNA strand breaks, the number of which has been shown to correlate with the reduction of cell proliferation (2). Although the ultimate DNA-attacking molecular species is not clearly identified hitherto, it is generally accepted that it is produced by or via a ternary oxygen-Fe(II)-bleomycin complex (3), which has similarity to heme-containing oxygenases (4). Up to now, in vitro this complex has been produced by addition of Fe(II) or generated from Fe(III) and bleomycin by millimolar amounts of reducing agents such as 2-mercaptoethanol, dithiotreitol, ascorbate etc. or by a superoxide anion radical-generating system consisting of xanthine oxidase and hypoxanthine (5). NADPH-dependent enhancement of DNA chain breakage by bleomycin in microsomes has been reported (6). Nevertheless,no enzyme with NAD(P)H-Fe(III)-bleomycin reductase-activity has been identified nor used yet.

We report on the enzymatic activity of isolated NADPH-cytochrome P-450 reductase to reduce Fe(III)-bleomycin, as measured by concomitant NADPH-oxidation, and demonstrate that bleomycin degrades DNA catalysed by NADPH-cytochrome P-450 reductase in the presence of NADPH, Fe(III), and oxygen in vitro.

MATERIALS AND METHODS

^{3}H-Thymidine-labeled DNA was isolated (7) from L1210 cells harvested from the culture in RPMI 1640 medium (8) plus 15% fetal calf serum (Gibco-Bio Cult, Karlsruhe, FRG) in the late logarithmic phase 24 h after addition of (methyl-^{3}H)-thymidine (Radiochemical Centre, Amersham, UK). NADPH-cytochrome P-450 reductase (EC 1.6.2.4) was solubilised and purified from rat liver microsomes (9) to a specific activity of 25.5 U/mg protein. Commercially available bleomycin (Mack, Illertissen, FRG) was used: activity 1,400 - 2,100 µg/mg bleomycin sulfate, 55-70% bleomycin A_2, 25-32% bleomycin B_2. Catalase (EC 1.11.1.6), NADPH and reduced glutathione were purchased from Boehringer, Mannheim, FRG; superoxide dismutase (EC 1.15.1.1) from Sigma, München, FRG; $Cu(SO_4)$, $Fe(NO_3)_3$, mannitol and Tris-HCl from Merck, Darmstadt, FRG. Traces of iron were removed from all reagents by CPG/8-hydroxyquinoline chromatography (Pierce, Rockford, USA). The iron concentration in the standard reaction mixture was less than 0.002 mM before the addition of defined amounts of $Fe(NO_3)_3$, as measured by atomic absorption spectrophotometry.

Chain breakage of ^{3}H-thymidine-labeled DNA was determined by measurement of acid-soluble radioactive products (10). The standard reaction mixture contained 0.5 mM NADPH, 26.6 µg/ml ^{3}H-thymidine-labeled DNA, 0.03 mM $Fe(NO_3)_3$, 20 µg/ml bleomycin and 0.25 U/ml NADPH-cytochrome P-450 reductase in KCl-Tris-buffer, pH 7.4(11).Reactions were started by the addition of NADPH-cytochrome P-450 reductase after equilibration for 5 min in a shaking water bath at 37°. For termination of reactions 0.05 ml-aliquots were added to 0.05 ml 15% trichloroacetic acid and chilled in an ice bath. After addition of 0.05 ml 0.1 mM bovine serum albumin samples were centrifuged and radioactivity was determined in 0.1 ml supernatant by liquid scintillation counting (12). The total amount of acid-soluble radioactivity in the reaction mixture was measured after hydrolysis of the trichloroacetic acid-samples at 96° for 30 min by the same method. The equivalent for 1 µg ^{3}H-thymidine-labeled DNA, determined by the diphenylamine-method (13), was 11,600 ± 350 cpm ($\bar{x}$ ± S.D., n = 9).

NADPH-oxidation was monitored spectrophotometrically at 366 nm using an extinction coefficient of 3.4×10^6 cm^2 Mol^{-1}(14) at 37° in a standard reaction mixture containing 0.5 mM NADPH, up to 0.3 mM $Fe(NO_3)_3$, up to 200 µg/ml bleomycin and 0.25 U/ml NADPH-cytochrome P-450 reductase in KCl-Tris-buffer, pH 7.4. Reactions were started by addition of NADPH-cytochrome P-450 reductase after equilibration for 5 min in thermoregulated cuvettes.

RESULTS AND DISCUSSION

We have studied the influence of bleomycin on the NADPH-oxidation caused by ferric ions in a system with NADPH-cytochrome P-450 reductase in vitro. Figure 1 shows that NADPH-oxidation is significantly augmented by the addition of bleomycin. This finding may be due to the high susceptibility of the Fe(III)-bleomycin complex for enzymatic reduction by NADPH-cytochrome P-450 reductase. The reduction of Fe(III) by NADPH-cytochrome P-450 reductase in the presence of bleomycin follows classical Michaelis-Menten-kinetics with $K_M = 5.2 \times 10^{-5}$ M bleomycin and $V_{max} = 2.0 \times 10^{-5}$ M/min as measured by NADPH-oxidation (Fig.2). Thus, our results favour the direct reduction of Fe(III)-bleomycin by one-electron transfer and suggest that NADPH-cytochrome P-450 reductase has NADPH-Fe(III)-bleomycin reductase-activity. As NADPH-cytochrome P-450 reductase has been shown to generate superoxide anion radicals (15), a mechanism of the reduction of Fe(III)-bleomycin via superoxide anion radicals is not completely ruled out, however.

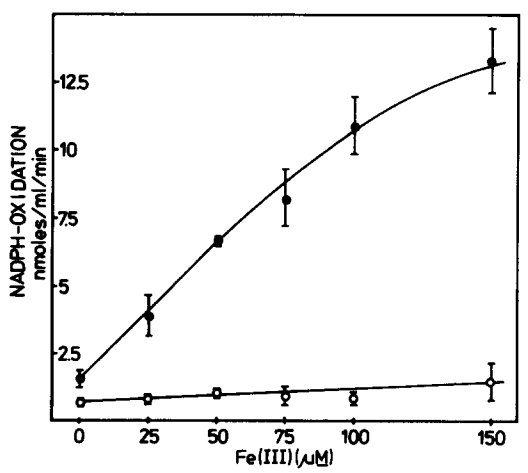


FIGURE 1: NADPH-oxidation caused by $Fe(NO_3)_3$ alone (○) and in combination with 200µg/ml bleomycin (●) in the standard reaction mixture. ($\bar{x}$ ± S.D., n = 4)

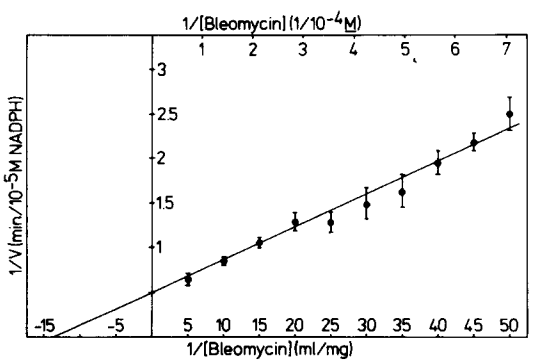


FIGURE 2: Lineweaver-Burk-plot for the reduction of Fe(III)-bleomycin as measured by NADPH-oxidation in the standard reaction mixture containing 0.3 mM $fe(NO_3)_3$ (16). ($\bar{x}$ ± S.D., n = 4)

$K_M = 5.2 \times 10^{-5}$ M bleomycin

$V_{max} = 2.0 \times 10^{-5}$ M/min

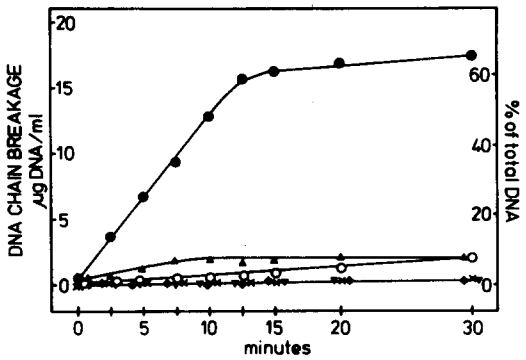


FIGURE 3: Kinetics of DNA chain breakage in the standard reaction mixture.

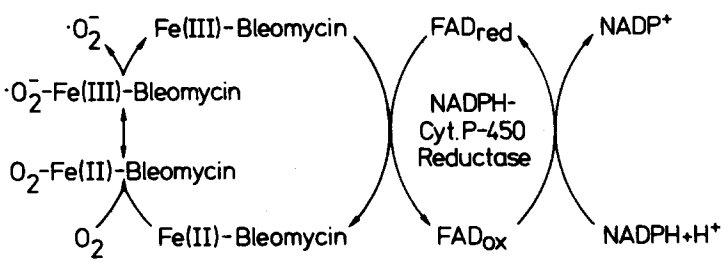
- ● complete system,
- ◆ minus bleomycin (20 µg/ml),
- O minus $Fe(NO_3)_3$ (0.03 mM),
- ▼ minus NADPH (0.5 mM),
- ▲ nitrogen atmosphere, and
- ✕ boiled NADPH-cytochrome P-450 reductase.

TABLE I: Influence of various factors on DNA chain breakage in the standard reaction mixture after 10 min. ($\bar{x} \pm$ S.D., $n \geq 4$)

	% of total DNA	%
Complete system	51.5 ± 1.9	100
- 20 µg/ml Bleomycin	0.1 ± 0.1	0.2
- 0.03 mM $Fe(NO_3)_3$	2.1 ± 0.4	4.1
- 0.5 mM NADPH	0.3 ± 0.1	0.6
Boiled NADPH-cyt.P-450 red.	1.5 ± 0.3	2.9
Nitrogen atmosphere	5.6 ± 1.0	11
+ 0.03 mM $CuSO_4$ (- $Fe(NO_3)_3$)	0.4 ± 0.2	0.8
+ 50 mM Mannitol	49.2 ± 2.3	96
+ 1 mM GSH	48.4 ± 2.3	94
+ 5 U/ml SOD	14.7 ± 1.0	29
+ Boiled SOD	47.4 ± 2.3	92
+ 26 U/ml Catalase	64.3 ± 2.8	125
+ Boiled catalase	53.3 ± 2.4	103

To evaluate the role of NADPH-cytochrome P-450 reductase in the activation of bleomycin in the presence of Fe(III), the formation of acid-soluble radioactive products from ^{3}H-thymidine-labeled DNA was determined dependent on various factors in vitro. The kinetics of this reaction demonstrates that each, active enzyme, oxygen, NADPH, bleomycin and ferric ions are essential for the degradation of DNA (Fig.3). The dependence of DNA chain breakage on oxygen agrees with previous reports (3), indicating that the formation of oxygen-Fe(II)-bleomycin is a prerequisite for the production of DNA strand breaks. To elucidate the mechanism of DNA chain breakage by enzymatically generated oxygen-Fe(II)-bleomycin, the influence of other factors was examined. The results in Table I demonstrate that DNA chain breakage by oxygen-Fe(II)-bleomycin, generated by NADPH-cytochrome P-450 reductase under aerobic conditions, is inhibited by superoxide dismutase and augmented by catalase, while reduced glutathione and mannitol, a scavenger of hydroxyl radicals, have no significant influence. Cupric ions cannot replace ferric ions. Our results favour the direct action of the superoxide anion radical on DNA (17, 18), released by the oxygen-Fe(II)-bleomycin complex. Although mannitol is not inhibitory,the involvement of the hydroxyl radical (19, 20) cannot be excluded.

According to our findings, we propose the following model for the mechanism of activation of Fe(III)-bleomycin by NADPH-cytochrome P-450 reductase:



This Fe(III)-bleomycin-redox cycle is similar to the well-known cycle of quinone anticancer drugs (21) which is maintained by NADPH-cytochrome P-450 reductase as well.

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