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Redox Cycling of Bleomycin-Fe(III) and DNA Degradation by Isolated NADH-Cytochrome b_5 Reductase: Involvement of Cytochrome b_5

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SUMMARY

Isolated and purified microsomal NADH-cytochrome b_5 reductase (EC 1.6.2.2) was incubated with bleomycin (BLM) and $FeCl_3$ in the presence of NADH. Only when purified cytochrome b_5 was added could an increased NADH consumption be observed indicating redox cycling of the BLM-Fe(III) complex. In the presence of DNA, BLM-Fe(III)-related NADH consumption was accompanied by malondialdehyde (MDA) formation, further evidence for BLM activation yielding oxidative DNA cleavage. BLM, $FeCl_3$, cytochrome b_5 and NADH were absolutely necessary to provide these effects. Addition of DNA changed the initial velocity (v_0) and the shape of the NADH consumption curves, both probably due to an interaction between DNA and BLM-Fe(III). Furthermore, DNA effectively protected BLM-Fe(III) from autoxidative degradation during redox cycling. BLM-Fe(III)-related, reductase-catalyzed NADH consumption and MDA formation were also dependent on oxygen, showing the involvement of oxygen in the reduction process and in the action of the drug-metal complex in attacking DNA. However, superoxide dismutase (EC 1.15.1.1) and catalase (EC 1.11.1.6) did not affect NADH consumption. Also, superoxide dismutase and catalase were almost without influence on MDA formation, suggesting that no free (or freely accessible) reactive oxygen species occurred during the redox cycle and DNA damage. The results reveal that the BLM-Fe(III) complex undergoes redox cycling by the microsomal NADH-dependent cytochrome b_5 reductase-cytochrome b_5 system. The significance of this effect for the action of BLM and the involvement of cytochrome b_5 is discussed with regard to the presence of these enzymes in the cell nucleus.

BLMs are glycopeptide antibiotics produced by *Streptomyces verticillus* (1). They are effectively used in cancer therapy. Some of their metal complexes, for example BLM-Fe(II), can activate oxygen and attack DNA, causing single or double strand breaks and base elimination (2–7). DNA damage also occurs in whole cells treated with BLM (8). Therefore, it is now generally accepted that the activity of the drug is related to DNA degradation.

However, it is still a question how the active BLM-Fe(II) complex is formed in the cell, because ferrous iron is presumably not freely available under physiological conditions. Rather, it is likely that the inactive BLM-Fe(III) is formed. Therefore, a (biological) reducing system is necessary to reduce this complex to form the active BLM-Fe(II) and maintain a redox cycle, because BLM-Fe(II) yields BLM-Fe(III) after its reaction with DNA and because the intracellular BLM concentrations expected under therapeutic conditions are presumably very low. Extensive *in vitro* studies performed with the BLM-Fe(III) complex revealed that this complex can be redox cycled by chemical reductants (9, 10), by microsomes (11) and by isolated microsomal NADPH-cytochrome P-450 reductase (EC 1.6.2.4) (12–14), also yielding DNA degradation.

In previous studies we could demonstrate that isolated rat liver nuclei were also able to redox cycle BLM-Fe(III) at the expense of NADH or NADPH, accompanied by massive damage of nuclear DNA (15, 16). We observed that NADH was much more efficient than NADPH and postulated the involvement of NADH-cytochrome b_5 reductase (16). This prompted us to isolate the microsomal NADH-cytochrome b_5 reductase, which is also present in the nuclear envelope, and to test its ability to reduce BLM-Fe(III).

In the present study, we incubated isolated microsomal NADH-cytochrome b_5 reductase with BLM, Fe(III), NADH, and DNA. We measured NADH consumption as a parameter of redox cycling and MDA formation as a parameter of DNA degradation (17), in relation to the concentrations of the components required. Surprisingly, isolated cytochrome b_5 was necessary for the redox cycling of the drug. Furthermore, the effects of oxygen and antioxidative enzymes like SOD and catalase on BLM-Fe(III) activation were examined.

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ABBREVIATIONS: BLM, bleomycin; MDA, malondialdehyde; SOD, superoxide dismutase; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid, Na salt; TBA, thiobarbituric acid.

Experimental Procedures

Materials. BLM was obtained from Mack (Illertissen, Federal Republic of Germany). It consisted of 55 to 70% of BLM-A_2, 25 to 32% of BLM-B_2, and minor amounts of other bleomycins of the A and B group. NADH, TES, DNA, Na salt, type XIV from herring testes, Triton X-100, deoxycholic acid, Na salt, and SOD were from Sigma (Muenchen, Federal Republic of Germany). Catalase was from Boehringer (Mannheim, Federal Republic of Germany). DE-52 (DEAE-cellulose) was purchased from Serva (Heidelberg, Federal Republic of Germany). ADP-agarose and all Sephadex-type gels were from Pharmacia (Sweden). All other chemicals were obtained from Merck (Darmstadt, Federal Republic of Germany) and were of analytical grade. Optical measurements were performed in a Shimadzu spectrophotometer (model UV-240).

Preparation of microsomes. Male Wistar rats were killed by decapitation. The livers were removed, rinsed with 0.25 M sucrose, and homogenized in a Braun homogenizer in an ice bath. All subsequent steps were performed at either 4° or 0°. The homogenate was centrifuged at 960 × *g* and 8000 × *g* to remove tissue debris and large organelles. The final supernatant was centrifuged for 40 min at 105,000 × *g* and the resulting pellet was resuspended in 0.1 M Tris-acetate buffer (pH 8.1) containing 1 mM EDTA. Solid NaCl was added to a concentration of 1 M and the suspension was sonicated for 2 min with some interruptions for cooling. After centrifugation at 105,000 × *g*, the microsomal pellet was washed twice with the above buffer and the so-called "washed microsomes" thus prepared were frozen at −80° (18).

Estimation of protein. Protein contents of microsomal suspensions were determined according to the method of Lowry *et al.* (19).

Preparation of NADH-cytochrome b_5 reductase. NADH-cytochrome b_5 reductase was detergent solubilized and purified from thawed washed microsomes according to the method of Iyanagi *et al.* (20). The purified enzyme was frozen at −80° in 10 mM phosphate buffer (pH 7.7) containing 0.1% (v/v) Triton X-100, 10% (v/v) glycerol, and 0.1 mM EDTA and was thawed before use.

Examination of the reductase activity. Reductase activity was determined by the ferricyanide method according to Strittmatter and Velick (21) using a molar extinction coefficient of 1.02×10^{-3} $M^{-1}cm^{-1}$ at 420 nm. One unit of NADH-cytochrome b_5 reductase was defined as the amount of enzyme catalyzing the reduction of 1 μmol of ferricyanide/min.

Preparation of cytochrome b_5. Cytochrome b_5 was co-purified according to the method of Chiang (22) from the detergent-solubilized microsomal homogenate, which was used for the preparation of the reductase. Cytochrome b_5 that remained bound to the column after the first DE-52 step was eluted with 0.1 M Tris-acetate (pH 7.6) containing 0.2% Triton X-100 (v/v), 0.2% Na-deoxycholate (w/v), 10% glycerol (v/v), 1 mM EDTA, and 0.5 M KCl. In all subsequent steps cytochrome b_5 was detected by measuring absorption at 412 nm. The cytochrome b_5-containing fractions were pooled and concentrated by dialysis against solid sucrose. The concentrate was applied on a Sephadex G-75 column (4 × 60 cm) equilibrated with 10 mM Tris-acetate (pH 8.1) containing 0.4% Na-deoxycholate (w/v) and 0.1 mM EDTA. The cytochrome-containing fractions were pooled and concentrated by centrifugation in Centricon concentrators (Amicon, Danvers, MA). The concentrate was then applied to a Sephadex G-100 column (3.5 × 75 cm) equilibrated with the same buffer. The red fractions containing cytochrome b_5 were concentrated again by the Centricon method. The concentrate was diluted several times with 25 mM TES buffer (pH 7.5) and reconcentrated to remove the detergent-containing buffer.

Estimation of the cytochrome b_5 concentration. Cytochrome b_5 was quantified by measuring the absorption of the dithionite-reduced form at 423 nm as described by Omura and Takesue (23) using a molar absorption coefficient of 1.71×10^{-1} M^{-1} cm^{-1}.

Determination of NADH consumption and MDA formation. NADH consumption was measured at 37° in a thermostated cuvette of the spectrophotometer. The standard incubation mixture (1 ml) contained 25 mM TES (pH 7.5), 2.5 mM $MgCl_2$, 50 μM $FeCl_3$, 50 μg/ml BLM, 0.2 mg/ml DNA, 0.2 units/ml cytochrome b_5 reductase, 0.2 nmol/ml cytochrome b_5, and 0.15 mM NADH. In control experiments, the components to be omitted were replaced by aliquots of the respective solvent. At the first step, the BLM-Fe(III) complex was formed by mixing BLM and $FeCl_3$ in a cuvette and incubating the mixture for 3 min at room temperature. The other components, with the exception of NADH, were then added and the cuvette was adjusted to 37° in a water bath for 3 min. It was then placed in the photometer, the absorption was adjusted to zero, and the reaction was started by quick mixing of NADH. The time course of the absorption change was recorded at 340 nm. The initial velocity (v_0) of the NADH consumption was determined from the linear initial section of the curves.

For MDA determination, samples of 50 μl were taken at given times and were transferred into 0.5 ml of ice-cold trichloroacetic acid (10%). The samples were centrifuged and the MDA was determined by the TBA assay (24) by using 1 ml of TBA (1%) for 0.5-ml sample. MDA concentrations were calculated on the basis of a molar absorption coefficient of 1.56×10^5 M^{-1} cm^{-1} at 532 nm. In some experiments the sensitivity of the TBA assay was increased by using 100-μl samples, 200 μl of trichloroacetic acid and 0.5 ml of TBA (increase by a factor of 3.7).

Anaerobic incubations were carried out in a sealed quartz cuvette with two inlets. Incubation mixture (2 ml) minus NADH, containing 1 μl of octanol to prevent foam generation, was put into the cuvette and gassed with helium in a water bath for 5 min at 37°. As shown in control experiments, octanol did not affect the reactions examined and was almost completely evaporated after the treatment with helium. The measurement of NADH consumption was performed as described for the aerobic experiment with the exception that the reaction was started by addition of deoxygenated NADH with the aid of a syringe, via the inlet tube. Samples for MDA determination were also carefully taken via the tube and treated as described above. After 5 min, the inlets were opened and air was forced through the solution. The NADH consumption was followed for another 5 min and again MDA samples were taken. For control, incubation mixtures deoxygenated as described above were aerated at $t = 0$.

Results

In the presence of BLM, $FeCl_3$, and cytochrome b_5, NADH consumption catalyzed by NADH-cytochrome b_5 reductase was greatly increased compared with controls (Table 1). Addition of DNA to the incubation mixture decreased the initial velocity of NADH consumption. The thiobarbituric acid assay of the latter mixture revealed that MDA was formed (Table 1). Control experiments presented in Table 1 showed that both NADH consumption and MDA formation were strongly dependent on BLM, $FeCl_3$, cytochrome b_5, and cytochrome b_5 reductase. MDA

TABLE 1

NADH consumption and MDA formation catalyzed by NADH-cytochrome b_5 reductase (0.2 units/ml) at 37° in the presence of BLM (50 μg/ml), $FeCl_3$ (50 μM), DNA (0.2 mg/ml), cytochrome b_5 (0.2 nmol/ml), and NADH (0.15 mM).

Incubation modus	NADH consumption (v_0)	MDA formation
	nmol/ml/min	*nmol/ml/5 min*
Complete system	17.8 ± 0.9[a]	39.24 ± 1.8
−BLM/Fe(III)	0.02 ± 0.02	0.11 ± 0
−BLM	0.06 ± 0.02	0.11 ± 0
−Fe(III)	0.34 ± 0.08	0.53 ± 0.35
−DNA	34.3 ± 2.0	1.46 ± 1.2
−Cytochrome b_5	0.11 ± 0.09	0.18 ± 0.25
−Reductase	0.58 ± 0.29	0.74 ± 0.26
−NADH	NA[b]	0.11 ± 0

[a] Mean value of five experiments ± standard deviation; all other values are mean values of three experiments ± standard deviation.
[b] Not applicable.

formation was also absolutely dependent on NADH. The residual amount of MDA produced in the absence of DNA may be due to lipid or detergent contaminants associated with the enzyme protein.

Fig. 1A shows the effect of cytochrome b_5 on the activation of BLM-Fe(III) by NADH-cytochrome b_5 reductase. BLM-Fe(III)-related NADH consumption increases with increasing cytochrome b_5 concentrations, reaches a plateau at 0.2 nmol/ml, and decreases at concentrations higher than 0.3 nmol/ml. This finding correlates with MDA formation (Fig. 1B) for which a similar dependence on cytochrome b_5 concentration was observed. The reason for this phenomenon is not clear. On the other hand, there was a linear relationship between the reductase concentration (up to 0.5 units/ml) and the BLM-Fe(III)-dependent NADH consumption and MDA formation (Fig. 2). Both effects also increased parallel to BLM or $FeCl_3$ concentrations (at a fixed concentration of the counterpart), being linear within the concentration ranges used in this study (results not shown).

Under anaerobic conditions no or very little NADH was consumed, as presented in Fig. 3. The low initial NADH consumption was probably due to traces of oxygen remaining in the incubation mixture. NADH consumption was raised to the normal velocity when air was introduced. In the complete system, the amount of MDA formed after a 5-min incubation in the absence of oxygen was 1.8 ± 0.5 nmol/ml. After aeration and an additional 5-min incubation, this amount reached a value of 29.1 ± 2.6 nmol/ml (mean values ± standard deviation, three experiments).

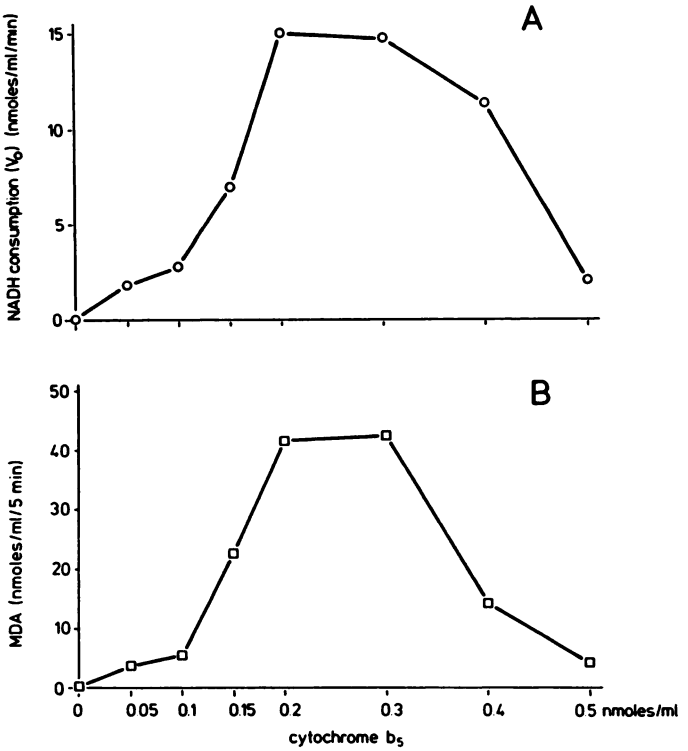

Fig. 1. The effect of cytochrome b_5 upon the initial velocity of NADH consumption (A) and MDA formation (B) catalyzed by NADH-cytochrome b_5 reductase (0.2 units/ml) at 37° in the presence of BLM (50 μg/ml), $FeCl_3$ (50 μM), DNA (0.2 mg/ml), and NADH (0.15 mM). Measurement of the parameters and the evaluation of the data were performed as described for standard conditions under Experimental Procedures, with the exception that the concentration of cytochrome b_5 was varied.

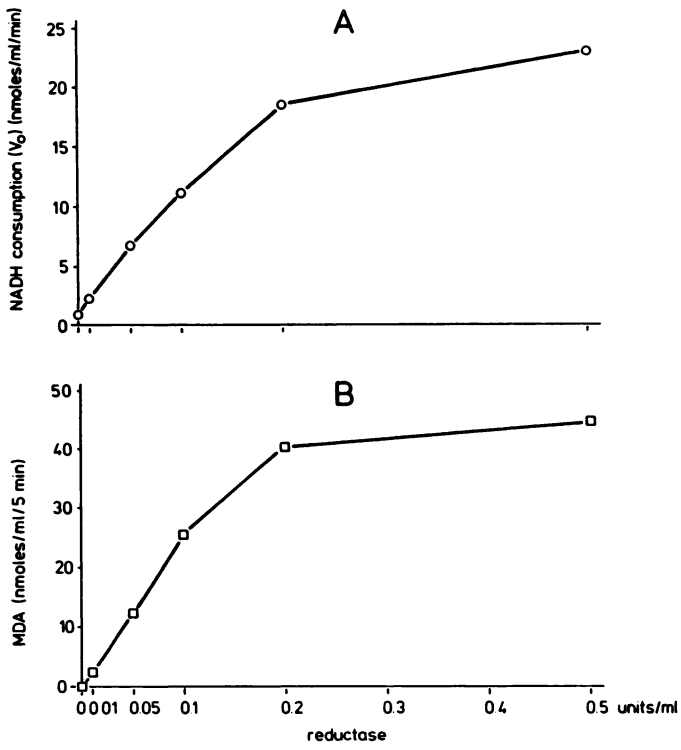

Fig. 2. The effect of microsomal NADH-cytochrome b_5 reductase upon the v_0 of NADH consumption (A) and MDA formation (B) at 37° in the presence of BLM (50 μg/ml), $FeCl_3$ (50 μM), DNA (0.2 mg/ml), cytochrome b_5 (0.2 nmol/ml), and NADH (0.15 mM). Measurement of the parameters and the evaluation of the data were performed as described for standard conditions under Experimental Procedures, with the exception that the concentration of the reductase was varied.

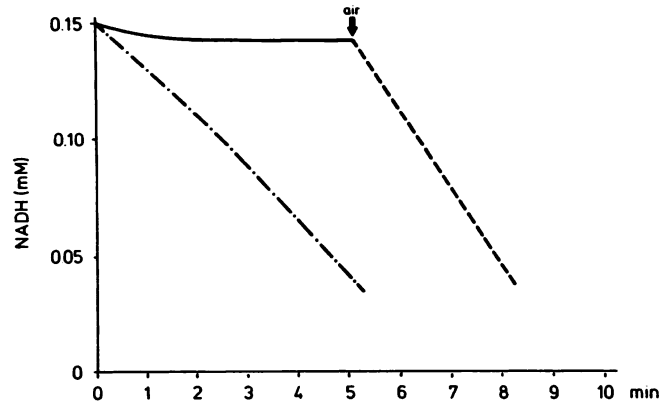

Fig. 3. The effect of oxygen upon NADH consumption catalyzed by NADH-cytochrome b_5 reductase (0.2 units/ml) at 37° in the presence of BLM (50 μg/ml), $FeCl_3$ (50 μM), DNA (0.2 mg/ml) cytochrome b_5 (0.2 nmol/ml), and NADH (0.15 mM). NADH consumption under anaerobic conditions (——); after re-aeration of the anaerobic sample (– – –) and in the control experiment, re-aerated at $t = 0$ (–·–·–) was measured. The experiments were carried out as described under Experimental Procedures. The diagrams were drawn from original tracings by setting the initial absorption value at 0.15 mM NADH.

SOD (250 units/ml), catalase (250 units/ml), or a combination of both did not significantly affect NADH consumption or MDA formation; NADH consumption amounted to 90 ± 7.8% of the control in the presence of SOD, 96 ± 8.3% in the presence of catalase and 102 ± 2.8% in the presence of both. Compared with the control, the amount of MDA formed was 84.3 ± 2.5%

with SOD, 107 ± 13.6% with catalase, and 96 ± 7.4% with both (mean values ± standard deviation, three experiments).

Fig. 4 demonstrates the dependence of BLM-Fe(III)-induced NADH consumption on the concentration of DNA. With increasing DNA concentrations the shape of the NADH consumption curves changed from hyperbolic to sigmoidal. The initial velocity of NADH oxidation decreased with increasing DNA concentrations. On the other hand, the velocity increased parallel to DNA degradation. Because the BLM-Fe(III) complex absorbs at 340 nm, which might have interfered with the NADH measurement, NADH consumption in the presence and the absence of DNA was also measured by fluorescence spectroscopy, using an excitation wavelength of 350 nm and an emission wavelength of 465 nm. The data confirmed the results obtained by spectrophotometry (results not shown). The dependence of MDA formation on DNA concentration was almost linear in the range of 0–200 μg/ml, as presented in Fig. 5.

When the sensitivity of the TBA-assay was increased (see Experimental Procedures), significant amounts of MDA could be detected in complete incubation mixtures containing BLM concentrations below 1 μg/ml (at corresponding low iron concentrations), which are expected to occur under therapeutical conditions (Fig. 6). On the other hand, the DNA cleavage potential of the drug-metal complex rapidly decreased when preincubated in the absence of DNA, as demonstrated in Fig. 7.

In general one redox cycle is defined as

$$\text{BLM-Fe(III)} \xrightarrow{\text{NADH-enzyme}} \text{BLM-Fe(II)} \qquad (1)$$

$$\text{BLM-Fe(II)} \xrightarrow{O_2} \text{BLM-Fe(III)} \qquad (2)$$

Kuramochi *et al.* (3) proposed that "activated BLM" is formed as follows:

$$\text{BLM-Fe(II)} + O_2 \rightarrow \text{BLM-Fe(II)-}O_2 \qquad (3)$$

$$\text{BLM-Fe(II)-}O_2 + \text{BLM-Fe(II)} \rightarrow \text{BLM-Fe(III)} + \text{activated BLM} \qquad (4)$$

Assuming that one molecule of activated BLM is needed for the oxidative cleavage of one deoxyribose (yielding one MDA molecule) we propose that two redox cycles would result in the formation of one MDA molecule. According to the data in Fig. 6, 1 μg of BLM (≙0.67 nmol, based on a molecular mass of 1500 Da for the BLM mixture used) formed in 30 min about 2.4 nmol of MDA. This corresponds to about 3.6 molecules of MDA or 7.2 redox cycles per BLM molecule, not counting the redox cycles that do not lead to oxidative DNA cleavage.

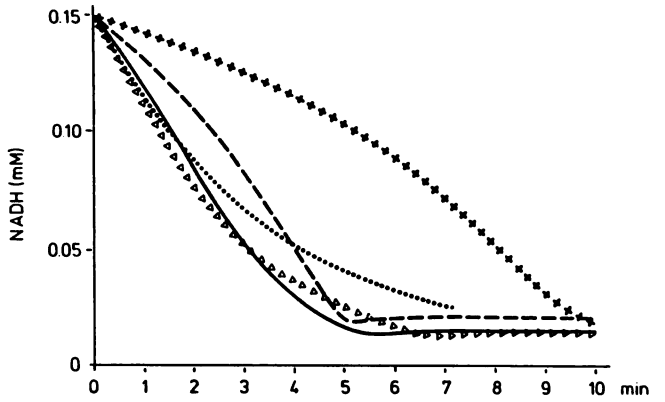

Fig. 4. The effect of DNA upon NADH consumption catalyzed by NADH-cytochrome b_5 reductase (0.2 units/ml) at 37° in the presence of BLM (50 μg/ml), $FeCl_3$ (50 μM), cytochrome b_5 (0.2 nmol/ml), and NADH (0.15 mM). Estimation of NADH consumption was carried out as described under Experimental Procedures with the exception that the concentration of DNA was varied as follows: ·····, 0 μg/ml, ▷▷▷, 50 μg/ml, ——, 100 μg/ml, – – –, 200 μg/ml, +++, 500 μg/ml. The diagrams were drawn from original tracings by setting the initial absorption value at 0.15 mM NADH.

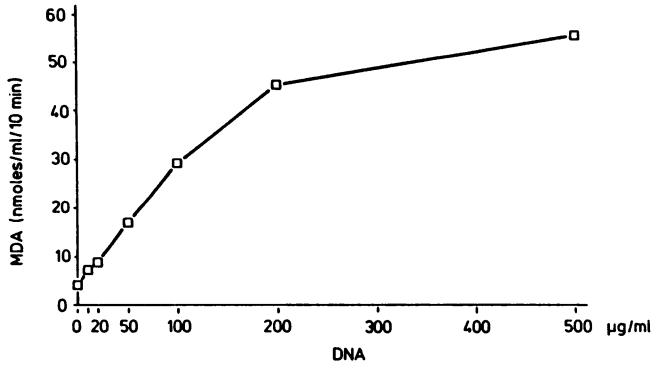

Fig. 5. The effect of DNA upon formation of MDA at 37° in the presence of NADH-cytochrome b_5 reductase (0.2 units/ml), BLM (50 μg/ml), $FeCl_3$ (50 μM), cytochrome b_5 (0.2 nmol/ml), and NADH (0.15 mM). MDA formation was determined as described for standard procedures, with the exception that the DNA concentration was varied and the incubation time was prolonged to 10 min to assure complete DNA degradation.

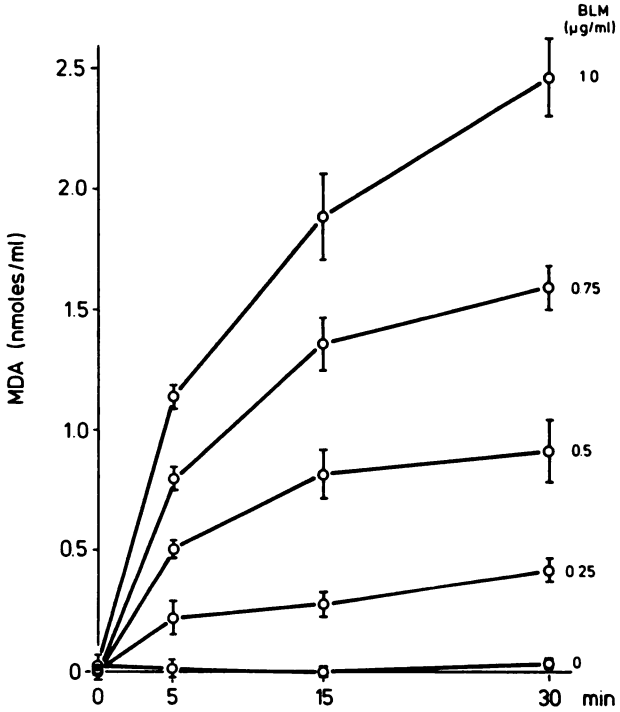

Fig. 6. MDA formation at BLM concentrations of 0–1.0 μg/ml (as indicated in the figure) at 37° in the presence of NADH-cytochrome b_5 reductase (0.2 units/ml), DNA (0.2 mg/ml), cytochrome b_5 (0.2 nmol/ml), and NADH (0.15 mM). $FeCl_3$ concentrations were 0, 0.25, 0.5, 0.75, and 1.0 μM in the respective experiments. MDA formation was determined by the modified standard procedure as described under Experimental Procedures.

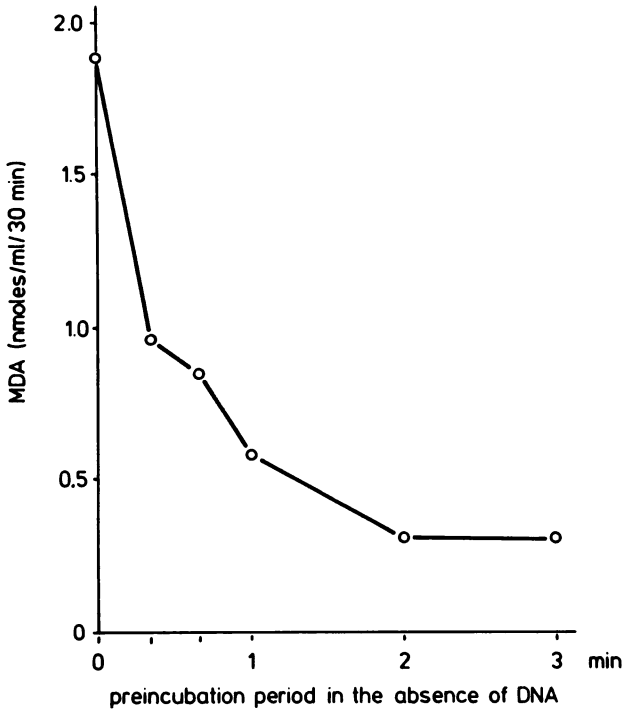

Fig. 7. The effect of preincubation of BLM (1 μg/ml, complexed with 1 μM $FeCl_3$) with NADH-cytochrome b_5 reductase (0.2 units/ml), cytochrome b_5 (0.2 nmol/ml), and NADH (0.15 mM) at 37°, in the absence of DNA, upon the ability of the drug to cleave DNA, BLM-Fe(III) was incubated in the absence of DNA for the time period indicated on the *abcissa* of the diagram. DNA (0.2 mg/ml) was then added and the incubations were continued. After 30 min, samples were taken and MDA was estimated by the modified standard procedure as described under Experimental Procedures.

Discussion

It has been shown that incubation of BLM and ferric ions with microsomes (11) or isolated microsomal NADPH-cytochrome P-450 reductase (12–14) in the presence of NADPH (NADH) and DNA results in redox cycling of the drug, with concominant DNA cleavage. Formation of MDA in these systems is now commonly accepted as an indicator of oxidative DNA degradation mediated by activated BLM (4–7, 17, 25, 26). Recently, we reported that these processes also take place in isolated rat liver nuclei (15, 16) where, with regard to BLM activation and MDA formation, NADH has been found to be much more favorable than NADPH (16). We have suggested that cytochrome b_5 reductase, which is now generally accepted as a constitutive enzyme of the nuclear membrane (for a recent summarizing article, see Ref. 27), is responsible for the NADH-dependent activation of the drug.

In this paper we are presenting data that show redox cycling of BLM-Fe(III) catalyzed by isolated microsomal NADH-cytochrome b_5 reductase. Surprisingly, cytochrome b_5 is essentially required for this reaction, suggesting that the reduction of BLM-Fe(III) to BLM-Fe(II) takes place by a mechanism similar to that of the reduction of cytochrome *c* by cytochrome b_5 reductase, for which cytochrome b_5 is also necessary (28). Redox cycling of BLM-Fe(III) by NADH-cytochrome b_5 reductase and cytochrome b_5 in the presence of DNA leads to MDA formation, indicating oxidative DNA cleavage.

NADH-cytochrome b_5 reductase, up to 0.5 units/ml, shows a linear relationship to NADH consumption and MDA formation. Contrarily, cytochrome b_5 shows unusual behavior; after an increase up to 0.2 nmol/ml, these parameters reach a plateau and decrease above 0.3 nmol/ml. At the present time there is no explanation for this phenomenon. Probably, cytochrome b_5 acts as a competitor for BLM-Fe(III) at higher concentrations. Extensive studies are needed to understand the kinetics and mechanisms of the electron transport from these enzymes to BLM-Fe(III).

The obvious dependence of NADH consumption on oxygen suggests a direct involvement of oxygen in the activation process, i.e., the so called activated BLM (7), which virtually attacks DNA and which is a complex of BLM, iron, and oxygen, may be formed directly at the active site of the enzyme. However, more systematic studies are needed. The strong dependence of MDA formation on oxygen once more confirms the direct involvement of O_2 in the cleavage of the deoxyribose moiety of DNA as previously proposed by several groups (4–7, 17, 25, 26). The minor effects of SOD and catalase on the BLM-Fe(III)-related NADH consumption and MDA formation confirm previous observations, indicating that no free active oxygen species are involved in DNA degradation by the activated BLM (2, 3, 7). However, the findings with SOD are in contrast to previous results obtained with NADPH-cytochrome P-450 reductase (12), indicating that different mechanisms of activation of BLM-Fe(III) by these two enzyme systems may exist.

In the absence of DNA, the initial velocity of NADH consumption is higher than in the presence of DNA. However, in the DNA-free sample the velocity decreases rapidly and only the addition of fresh BLM-Fe(III) leads to recovery of NADH consumption, as observed at BLM concentrations in the range of 10 μg/ml. On the other hand, NADH consumption occurs much longer when DNA is present (results not shown). This can be explained by a protection of BLM-Fe(III) by DNA, which is a target for activated BLM and thus inhibits self-degradation of the drug. There may be different reasons for the low initial velocity of NADH consumption in the presence of DNA. It may be due to DNA-dependent stabilization of BLM-Fe(II) formed, accompanied by inhibition of activation. There is considerable data regarding this phenomenon, which particularly occurs in the presence of higher DNA concentrations (29, 30). On the other hand, DNA may also simply decrease the accessibility of the BLM-Fe(III) complex to the enzyme, due to steric protection. NADH consumption is accelerated parallel with DNA degradation, favoring this last explanation. Up to 200 μg/ml, there is a linear relationship between MDA formation and DNA reaching a plateau at higher concentrations (Fig. 5). This may be due to the inhibitory effect of DNA mentioned above. But it is also possible that the concentrations of BLM-Fe(III) or NADH (which were kept constant during the whole experiment) became limiting at higher DNA concentrations.

DNA-related protection of the drug from autoxidative degradation is more plausibly demonstrated at low BLM concentrations, at which significant amounts of MDA could be measured, indicating numerous strand breaks, whereas preincubation of the drug in the absence of DNA leads to a sharp decline of the DNA cleavage potential of BLM. This phenomenon has also been observed with other BLM-activating systems (31, 32). It could explain the particular activity of BLM in the cell nucleus, which is the only place where it can be redox cycled several times under the "protection" of DNA.

According to the data presented in this study, we suggest

that the iron complex of BLM mimics a cytochrome molecule, which is readily reduced by enzymes of the nuclear electron transport chain. After activation, the free diffusible BLM-iron-oxygen-complex may leave the reducing enzyme system and attack DNA. According to Burger *et al.* (33), at 4° the decay of activated BLM takes minutes, so it might be stable enough at 37° to diffuse within the nucleus towards DNA. However, it is also possible that activation takes place while the drug is bound to DNA, inasmuch as DNA has a profound effect on NADH consumption. Furthermore, nuclear enzymes may come into contact with BLM bound to DNA during mitosis, when the nuclear membrane becomes disintegrated for a short time period, so that redox cycling would occur very close to DNA. This may also explain the increased sensitivity of dividing cells to BLM during mitosis. More studies are needed to answer all these questions.

On the basis of the results presented in this study, we propose that NADH-cytochrome b_5 reductase together with cytochrome b_5 may responsible for the NADH-dependent activation of BLM-Fe(III) and the related degradation of DNA observed in isolated rat liver nuclei (16). Furthermore, we suggest that this enzyme system may play a key role in the mechanism of action of this anticancer drug.

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