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Acute Pulmonary Toxicity of Bleomycin: DNA Scission and Matrix Protein mRNA levels in Bleomycin-Sensitive and -Resistant Strains of Mice

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SUMMARY

The severity of bleomycin (BLM)-induced pulmonary fibrosis in mice varies markedly among several different murine strains. We have examined the DNA from lungs of sensitive (i.e., C57BL/6N) and resistant (i.e., BALB/c) strains of mice using a nucleoid sedimentation technique to detect early *in vivo* changes in the integrity of DNA after intravenous BLM. Mice received intravenous injections of BLM (80 mg/kg) or vehicle; lung nucleoids were prepared 15 min to 6 hr later. BLM produced striking decreases in nucleoid sedimentation distance versus paired controls in both strains within 15 min after injection, indicating extensive DNA scission. Repair of DNA strand breaks was complete in the resistant (BALB/c) mice by 5 hr; in contrast, only partial repair occurred in the sensitive (C57BL/6N) strain during that time. We then examined lungs for subsequent changes in steady state poly-(A)$^+$ RNA levels and mRNA levels for lung matrix proteins (type I procollagen, type III procollagen, and fibronectin). Steady state levels of poly-(A)$^+$ RNA were depressed to 50% of control 1 through 6 days after BLM injection in the lungs of sensitive mice. Resistant mice had pulmonary poly-(A)$^+$ RNA levels similar to those of C57BL/6N mice, except for a 2-fold elevation 1 day after BLM injection. BLM injection affected the steady state levels of mRNA encoding lung matrix proteins differently than total poly-(A)$^+$ RNA. Fibronectin mRNA/poly(A)$^+$ RNA was elevated 2-fold 1 day after BLM treatment only in the sensitive strain and remained elevated at 3 and 6 days. In contrast, α_2I procollagen mRNA increased in both murine strains and α_1III procollagen mRNA decreased in both strains. Thus, a 7-fold or greater increase in the type I:type III procollagen mRNA ratio was seen in both strains 3 to 6 days after BLM injection. These data demonstrate that BLM treatment rapidly produces extensive pulmonary DNA damage *in vivo*, that persistence of DNA damage rather than the initial level of strand scission is associated with sensitivity to BLM lung disease in these mice, and that changes in the levels of mRNA encoding pulmonary matrix proteins occur *in vivo* within 1 to 3 days after intravenous BLM treatment.

Pulmonary fibrosis is an infrequent but serious consequence of exposure to environmental substances and therapeutic agents. The basic pathological processes that mediate pulmonary fibrosis have not been completely established. BLM is an antitumor agent that, in clinical usage, produces pulmonary injury leading to severe progressive pulmonary fibrosis in 1–2% of patients (1). This response greatly limits the therapeutic utility of the drug. BLM also produces a dose-related pulmonary toxicity in animal models that is histologically similar to the response seen in humans (2). Analysis of the events preceding pulmonary fibrosis in these models should be useful in providing a more complete understanding of the initial pathogenesis of fibrosis.

The mechanism by which BLM produces pulmonary injury is not known. BLM does bind to double-stranded DNA in solution and initiate DNA strand scission via a mechanism requiring oxygen and metal ions (for a review, see Ref. 3). BLM also degrades DNA in cultured tumor cells in an oxygen-dependent manner, and the ensuing lethal effects of BLM are correlated both with oxygen tension and with the extent of DNA damage (4). Thus, evidence from *in vitro* and cell culture studies suggests that BLM-mediated DNA damage may underlie the antitumor and untoward effects of BLM *in vivo*. DNA damage in lung or tumor tissue, however, has not been reported after BLM treatment, primarily because of difficulties in measuring DNA strand scission *in vivo*. Methods are now available that allow detection of chemically induced pulmonary DNA damage *in vivo* using nucleoid sedimentation techniques (5).

Previously, we reported that a single intravenous dose of BLM produces pulmonary fibrosis in a susceptible strain of mice (C57BL/6N) with a histologic pattern and organ distri-

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ABBREVIATIONS: BLM, bleomycin; oligo(dT)$_{16}$, (deoxythymidine)$_{16}$; poly(A)$^+$ RNA, polyadenylated RNA.

bution of injury matching that seen in patients given the drug therapeutically (6). Resistant mice (BALB/c strain) showed no significant pulmonary fibrosis when given a similar dose of BLM intravenously (7). The differential pulmonary fibrotic response of murine strains appears to be independent of the route of BLM administration, because it has also been seen after intratracheal injections and continuous subcutaneous infusions of BLM (6, 8). In addition, murine strain-specific elevations occur in the pulmonary levels of mRNA encoding extracellular matrix proteins in C57BL/6N mice before the increased deposition of collagen when mice are treated by continuous subcutaneous infusion of BLM (9). In pharmacokinetic studies, we showed that the pulmonary content of BLM was not decreased in the resistant mice compared with the sensitive strain and proposed that murine strain sensitivity to BLM was based on a nonpharmacokinetic factor, presumably resident within the pulmonary tissues (7).

We have now used nucleoid sedimentation techniques to assess acute *in vivo* DNA scission and repair occurring in the lungs of BLM-sensitive and -resistant mice after a single dose of intravenous BLM that can cause fibrosis in sensitive strains of mice (6, 7). In this model, we have also measured the steady state levels of pulmonary mRNAs coding for extracellular matrix proteins (α_2I procollagen, α_1III procollagen, and fibronectin) 1 to 6 days after intravenous BLM injection, because these proteins increase during fibrosis.

Materials and Methods

Animals and chemicals. Female C57BL/6N and BALB/c mice (specific pathogen-free; Charles Rivers, Kingston, NY) weighing between 17 and 20 g (6–10 weeks old) were housed in plastic cages and provided with food and water *ad libitum*. Clinical grade BLM (Blenoxane) was supplied by Bristol Myers Co. (Wallingford, CT). Tris base and sucrose (Ultrapure grade) were obtained from Schwarz/Mann Biotech (Cleveland, OH). 2-Mercaptoethanol (electrophoresis grade) was from Bio-Rad Laboratories (Richmond, CA). Hoechst 33258 was from Sigma Chemical Co. (St. Louis, MO). Other chemicals were reagent grade.

Bleomycin treatment and tissue handling. For nucleoid sedimentation analysis, mice received intravenous injections of 80 mg/kg BLM (dissolved in 0.9% NaCl) of saline alone via the tail vein (5 ml/kg injection volume). Fifteen minutes to 5 hr after tail vein injection of BLM, the mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (100 mg/kg). The body cavities were opened, the aortae were sectioned, and the lungs were perfused via the right ventricle with 2 ml of 0.9% NaCl. The bronchi were sectioned at the hilum and the lungs were removed, rinsed in 0.9% NaCl, and placed into an ice-cold glass homogenizer containing 2 ml of homogenization solution (0.34 M sucrose, 60 mM NaCl, 15 mM Tris, 10 mM EDTA, and 10 mM 2-mercaptoethanol, pH 7.4). The lungs were homogenized gently, on ice, with a loose-fitting Teflon pestle and the homogenate was filtered through 110-μm nylon mesh and maintained on ice.

Nucleoid sedimentation. Nucleoid sedimentation analysis of lung tissue was carried out as described by Romagna *et al.* (5). Sucrose gradients (12.5 to 25%, 4.7 ml volume) containing 2 M NaCl, 0.5 M EDTA, 1 M Tris (pH 8), and 2 μg/ml Hoechst 33258 were pumped at 4° into Ultracclear (Beckman) ultracentrifuge tubes using a Pharmacia (Piscataway, NJ) fast protein liquid chromatography gradient system. Lysis buffer (2 M NaCl, 10 mM EDTA, 10 mM Tris, and 0.67% Triton X-100, pH 8; 150 μl/gradient tube) was layered onto the gradients, followed immediately by 50 μl of lung homogenate. The tubes were inclined and gently rotated to mix the homogenate and lysis buffer and were allowed to stand 20 min on ice. The tubes were then placed into a precooled SW 50.1 ultracentrifuge rotor (Beckman) and centrifuged at 39,000 × *g* for 100 to 150 min. The gradients were allowed to sit 1 hr after the end of the centrifugation, and then the nucleoid bands were visualized by UV illumination at 365 nm. The sedimentation distance was evaluated by measuring the distances from the front and rear of the nucleoid band to the meniscus and averaging the two values (5).

In preliminary experiments, the nucleoid preparation was validated by centrifugation of lung nucleoids, prepared as described above from untreated mice (C57BL/6N), through sucrose gradient solutions containing ethidium bromide (0.5, 1, 10, and 30 μg/ml). To test for a direct effect on nucleoid sedimentation of BLM in the homogenization mixture, we prepared lung homogenate, as described above from untreated C57BL/6N mice; BLM or distilled water was added to 200-μl aliquots of homogenate to yield a maximum final BLM concentration of 600 nM. The homogenates were incubated on ice for 15 min, then 50-μl aliquots of homogenates were layered onto lysis buffer on sucrose gradients and centrifuged as described above. Finally, in some experiments mice received 4-nitroquinoline-1-oxide (0.26 mmol/kg, intraperitoneally) dissolved in dimethyl sulfoxide/0.9% NaCl (injection volume of 2.5 ml/kg), as described by Romagna *et al.* (5). The mice were anesthetized 1 hr after the dose, and lung nucleoids were prepared and sedimented as described above.

RNA preparation. Using four mice in each group, total lung RNA was isolated, as described by Hoyt and Lazo (9), 1, 3, and 6 days after BLM treatment. For measurement of fibronectin, α_2I procollagen, and α_1III procollagen mRNA, 1 to 10 μg of total lung RNA was applied directly to nitrocellulose by vacuum filtration (Schleicher and Schuell Minifold II, 6.0-mm^2 rectangular orifice). β-Actin mRNA was determined by subjecting 4 μg of total RNA to electrophoresis in 1.1% agarose/2.2 M formaldehyde and capillary transfer to Hybond-N filter (Amersham). RNA was then fixed to the filters by drying. Filters were stored at room temperature under vacuum until use.

Assay of poly(A)$^+$ RNA. The relative amounts of poly(A)$^+$ RNA in the lung samples were determined by the method of Harley (10), as described previously (9). Total RNA on nitrocellulose was hybridized with a 5′-^{32}P-labeled oligo(dT)$_{16}$ (Pharmacia, Piscataway, NJ). The filter was washed and an autoradiographic exposure was made.

mRNA assays. The specificity of probes used to identify mRNAs was verified previously by hybridization to total, poly(A)$^+$, and nonpolyadenylated mouse lung RNA that had been fractionated by electrophoresis in 1.1% agarose/2.2 M formaldehyde (9). α_2I and α_1III procollagen mRNAs were measured by hybridization with murine cDNA isolated from plasmids pAZ1001, restricted with *Xho*1, and pMCS-1, restricted with *Xba*. These plasmids were provided by Dr. Benoit de Crombrugghe (M.D. Anderson Hospital and Tumor Institute, Houston, TX)(11). The level of fibronectin mRNA was determined with a rat cDNA probe, provided by Dr. R. O. Hynes (Massachusetts Institute of Technology, Cambridge, MA) (12), λRLF1 restricted from pBR325 with *Eco*R1. After hybridization, the filters were washed three times in 15 mM sodium chloide/1.5 mM sodium citrate, containing 0.1% sodium dodecyl sulfate, at 60°. The levels of β-actin mRNA were assayed by Northern blot with a human cDNA isolated from *Bam*HI-digested plasmid pHFβA-1 provided by Dr. Larry Kedes (Stanford University, Palo Alto, CA) (13). The filter was washed five times in 150 mM sodium chloride/15 mM sodium citrate/35 mM sodium phosphate/0.17% sodium dodecyl sulfate, at 55°.

Radiolabeling of probes. Oligo(dT)$_{16}$ was labeled with [γ-^{32}P] adenosine triphosphate and T4-polynucleotide kinase at 37°, as previously described (10), and the radioactive probe was purified by ion exchange column chromatography on DEAE-Sephacel (Pharmacia). cDNA probes were isolated from plasmids by electrophoresis in low-melt agarose (Bio-Rad) followed by excision of the appropriate band. cDNAs in agarose were radiolabeled, as described previously (9), with [α-^{32}P]deoxycytidine triphosphate by random primer extension with Klenow fragment to a specific activity greater than 10^9 dpm/μg.

Hybridization signal. The hybridization signal with total lung RNA was determined by scanning densitometry (LKB Ultroscan XL;

Pharmacia LBK) of autoradiographic exposures (Kodak X-Omat AR film plus DuPont Cronex intensifying screen; −70°). The signal area associated with 2 μg of RNA in slot blots was calculated from the peak height and peak width at half the height on a chart recording. The signal area for 4 μg of electrophoresed RNA was determined by integration with LKB 2400 Gel Scan XL software. The pulmonary mRNA content (total signal area/lung set) was calculated by multiplying the signal area/μg RNA analyzed by the total number of μg RNA isolated from the lung set. The ratio of mRNA signal to the poly(A)$^+$ RNA signal in the sample was also calculated.

Data presentation and analysis. Results from the hybridization assays were expressed as the mean signal area ± standard error, relative to saline-treated control C57BL/6N mice. Significant differences between the hybridization signals at $p < 0.05$ were determined by analysis of variance and contrasting for multiple comparisons, as described by Snedecor and Cochran (14).

Results

Validation of the nucleoid preparation. Intact nucleoids, which are residual nuclear structures containing nuclear matrix protein and attached supercoiled DNA, show a biphasic response in their sedimentation rate through sucrose gradients if increasing concentrations of intercalating agents such as ethidium bromide are included in the gradient solutions (15). This response is characterized by a marked decrease in sedimentation rate at low concentrations of the intercalator followed by increasing sedimentation rates as the ethidium bromide concentration is increased. The biphasic response is eliminated if nucleoid DNA is degraded, e.g., by nucleases or ionizing radiation (16), and thereby released from constraints. We employed this feature of nucleoids to validate our mouse lung nucleoid preparation (Fig. 1A). Over an ethidium bromide concentration range of 0 to 75 μM, there was an initial reduction in the sedimentation distance relative to control (maximum of 50% reduction of sedimentation distance at 3μM ethidium bromide) followed by a return of sedimentation distance to control values at higher concentrations (Fig. 1A). Both the concentration range of the response and its overall profile are similar to results seen with intact nucleoids in other laboratories (5, 17, 18). Thus, the presence of the expected biphasic response to ethidium bromide suggests that the DNA within our nucleoid preparation is intact and supercoiled (16).

To test whether our nucleoid preparation was sensitive to DNA damage produced *in vivo*, we treated mice (C57BL/6N) with 4-nitroquinoline-1-oxide (0.26 mmol/kg, intraperitoneally) or vehicle, as described by Romagna *et al.* (5) and prepared lung nucleoids 1 hr later. Nucleoid sedimentation distances in treated specimens were 57% of control (data not shown), a value similar to that obtained previously (5).

Effect of BLM *in vitro* on lung nucleoid sedimentation. BLM present in the lungs *in vivo* potentially might degrade or associate with nucleoid DNA during the preparation of lung nucleoids and alter nucleoid sedimentation characteristics directly. To test for this possibility, we incubated lung homogenates with BLM (40 to 600 nM) and 10 mM EDTA for 15 min on ice before the preparation and sedimentation of the lung nucleoids. As shown in Fig. 1B, BLM had no effect on the sedimentation rates of the nucleoids. These results are consistent with previous studies that indicate that 5 mM EDTA markedly inhibits BLM-induced DNA scission *in vitro* (19) and they demonstrate that BLM does not directly alter DNA supercoiling (e.g., by intercalation) at these concentrations.

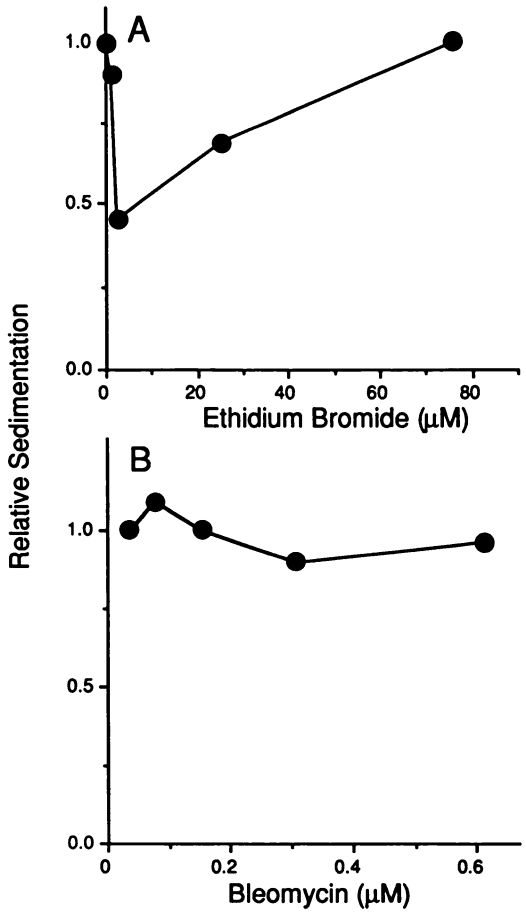

Fig. 1. Effect of ethidium bromide on mouse lung nucleoid sedimentation. Lung nucleoids were prepared from an untreated mouse, as described in Materials and Methods, and centrifuged through linear sucrose gradients (12.5–25%) containing 2 μg/ml Hoechst 33258 (controls) or the indicated concentrations of ethidium bromide. Nucleoid bands were located and sedimentation distance from the meniscus was measured directly. B, Effect of BLM *in vitro* on mouse lung nucleoid sedimentation. Lung homogenates were prepared, as described in Materials and Methods, from an untreated mouse and incubated at 4° for 15 min with the indicated concentrations of BLM. Sedimentation distance was then determined for the nucleoids prepared from the homogenate.

Effect of BLM *in vivo* on lung nucleoid sedimentation. BLM (80 mg/kg) injected intravenously into BLM-sensitive (C57BL/6N) and -resistant (BALB/c) mice rapidly and markedly reduced the sedimentation distance of lung nucleoids relative to control (Fig. 2). A maximal reduction of sedimentation distance to 25 to 35% of control ($p < 0.05$) occurred within 15 min after BLM injection; this initial effect was of comparable magnitude in both the sensitive and resistant mice. Sedimentation distance increased transiently toward control values in both strains of mice at 30 min ($p < 0.05$, compared with 15-min values). After 1 hr, the sedimentation distance increased in the resistant (BALB/c) mice, such that by 5 hr after BLM treatment the sedimentation distance was equal to that seen with untreated control lungs (Fig. 2). In contrast, the sedimentation distance in the sensitive mice showed only a mild increase over this period and remained significantly depressed compared with control 5 hr after BLM treatment. Thus, the difference in relative sedimentation values between the resistant and sensitive mice at 3 and 5 hr after BLM was significantly different ($p < 0.05$) Six hours after BLM treatment, the sedi-

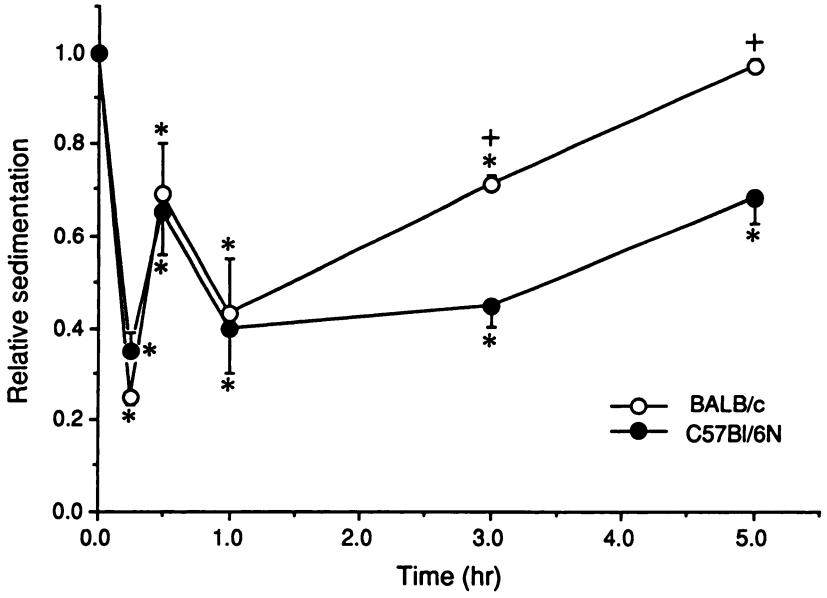

Fig. 2. Effect of BLM *in vivo* (80 mg/kg intravenously) on lung nucleoid sedimentation in BLM-sensitive (C57BL/6N) and -resistant (BALB/c) mice. Pairs of mice (treated and control) received BLM or vehicle intravenously via the tail vein. The lungs were removed and nucleoids were prepared. The relative sedimentation is the ratio of the sedimentation distance from the meniscus of treated versus control for each pair of mice; each *data point* represents the mean for three pairs of mice and *error bars* represent standard error (*error bars* not visible are contained within *data points*) $^*p < 0.05$ compared with the saline-treated control; $+p < 0.05$ compared with the other strain at that time point.

mentation distances of nucleoids from a single C57BL/6N and BALB/c mouse were 1.07 and 1.00, respectively, and, thus, were similar to that of the corresponding saline-treated mice, suggesting DNA integrity can be restored in both strains by 6 hr.

Effect of BLM on pulmonary RNA. We next analyzed pulmonary steady state levels of poly(A)$^+$ RNA and mRNAs for fibronectin, α_2I and α_1III procollagens, and β-actin, after BLM treatment. We also assessed the effect of BLM (80 mg/kg, intravenously) on lung weight and lung RNA content 1 to 6 days after BLM injection. With both strains, lung weight was unaffected except in BALB/c mice 1 day after BLM treatment, when a transient decrease (25%) in wet lung weight was noted. Minor increases (<20%) in lung RNA content were seen 1 and 3 days after BLM treatment in C57BL/6N mice (Fig. 3A). BLM caused substantial changes, however, in lung poly(A)$^+$ RNA content in both strains (Fig. 3B). In C57BL/6N mice, lung poly(A)$^+$ RNA was decreased to 50–60% of control within 1 day after treatment and was maintained at this level over the remainder of the time course (Fig. 3B). In contrast, BLM treatment induced a sharp 2-fold increase in lung poly(A)$^+$ RNA content in BALB/c mice 1 day after BLM injection. Thereafter, poly(A)$^+$ RNA dropped to about 55% of control, similar to the change measured in C57BL/6N mice at days 3 and 6 (Fig. 3B). Northern blot analyses as well as acridine orange staining of gels demonstrated that RNA degradation did not occur, although variation in the amount of pulmonary β-actin mRNA among individual mice was sometimes seen. Thus, the poly(A)$^+$ RNA signal from each mouse lung was used to normalize for the content of specific mRNA species (9, 10). Fig. 4 is a representative Northern blot with a human β-actin cDNA probe, showing the appropriate 2.1-kilobase mRNA in both untreated and BLM-treated C57BL/6N and BALB/c mice.

The steady state mRNA levels before BLM treatment for fibronectin, α_2I procollagen, and β-actin, normalized to poly(A)$^+$ RNA, were similar in both murine strains (Figs. 5–7); initial α_1III procollagen/poly(A)$^+$ RNA levels were approximately 2-fold higher in C57BL/6N mice compared with BALB/c mice. The overall pattern of change in the steady state mRNA

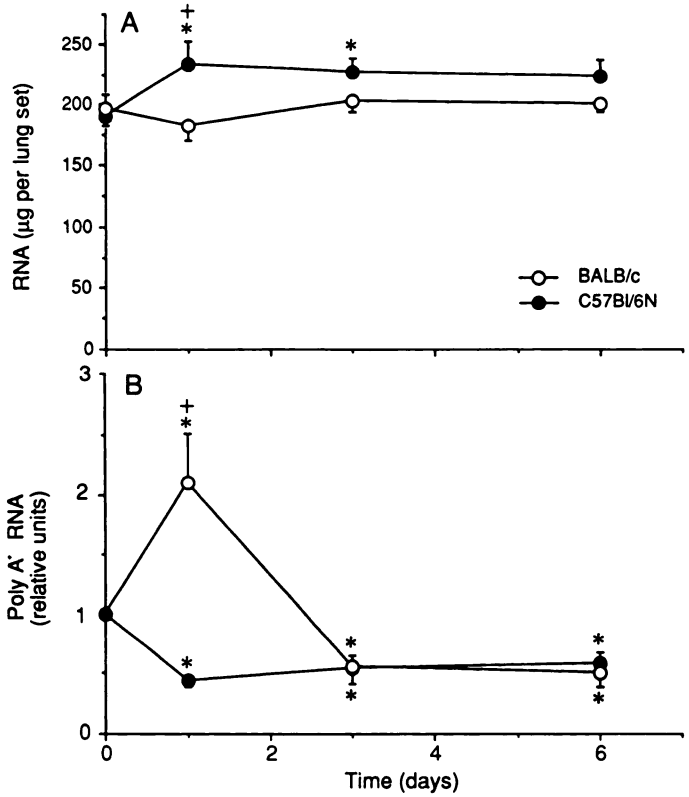

Fig. 3. Effect of BLM (80 mg/kg intravenously) on pulmonary RNA content (A) and pulmonary poly(A)$^+$ RNA content (B) of mice. Perfused lungs were removed from mice at the times indicated after BLM injection. For poly(A)$^+$ RNA analysis, total lung RNA was applied to nitrocellulose and hybridized with [5′-^{32}P]oligo(dT)$_{16}$ and the hybridization signal was determined by scanning densitometry of an autoradiograph. Data are presented as means ± standard errors either for the individual group values (A) or relative to the mean value of untreated C57BL/6N mice (B) (n = 8 for controls and 4 for experimental groups; *error bars* not visible are contained within *data points*). *Symbols* are as in Fig. 2.

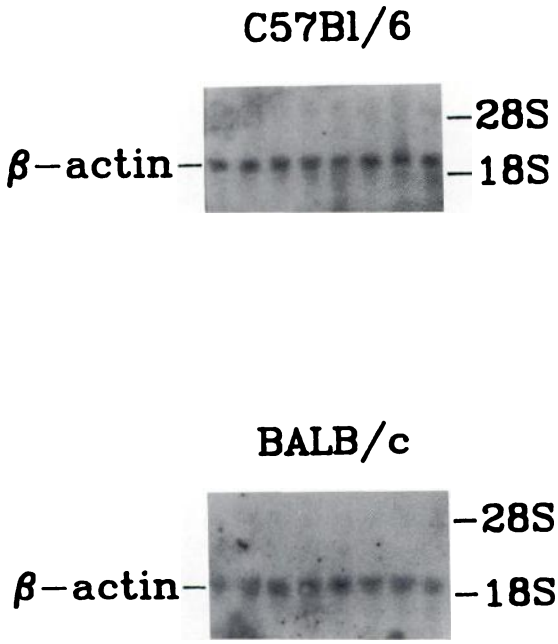

Fig. 4. Visualization of β-actin mRNA by Northern blot in C57BL/6N and BALB/c mice. *Upper*, C57BL/6N mice; *lower*, BALB/c mice. The *four lanes* on the *left* are from saline-treated mice and the *four lanes* on the *right* are from mice 1 day after treatment with 80 mg/kg BLM (intravenously).

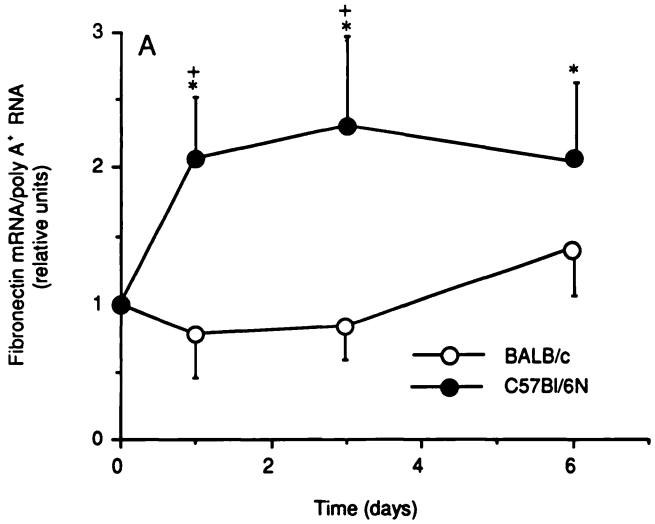

Fig. 5. Effect of BLM (80 mg/kg, intravenously) on pulmonary fibronectin mRNA/poly(A)$^+$ RNA in mice. The signal area was determined as in Fig. 3 after hybridization of filters with a ^{32}P-labeled rat fibronectin cDNA. The data are expressed relative to the mean value of untreated C57BL/6N mice. *Bars* represent standard errors and *symbols* are as in Fig. 2.

levels of each of the extracellular matrix species examined 1 to 6 days after BLM was distinct. For example, in the sensitive (C57BL/6N) mice, fibronectin mRNA was selectively increased (2-fold) relative to other lung mRNAs 1 day after BLM and this increased ratio was maintained for at least 6 days (Fig. 5). BALB/c mice, in contrast, showed no change from the control fibronectin mRNA/poly(A)$^+$ RNA ratio for at least 6 days after BLM treatment (Fig. 5). The ratio of α_2I procollagen mRNA to poly(A)$^+$ RNA increased in both strains with time after BLM treatment, 4-fold by day 3 in the lungs of C57BL/6N mice and more than 5-fold in the lungs of BALB/c mice at day 6 (Fig.

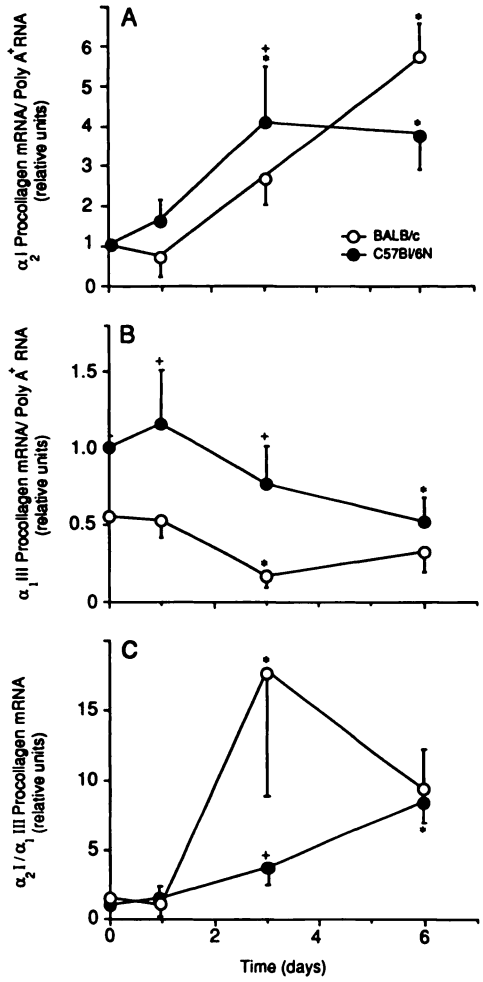

Fig. 6. Effect of BLM (80 mg/kg intravenously) on murine pulmonary α_2I procollagen mRNA/poly(A)$^+$ RNA (A), α_1III procollagen mRNA/poly(A)$^+$ RNA (B), and α_2I/α_1III (C). The signal area was determined as in Fig. 3 after hybridization of filters with a ^{32}P-labeled murine cDNA. Data are expressed relative to values from the lungs of saline-treated C57BL/6N mice. *Symbols* are as in Fig. 2.

6A). The ratio of pulmonary α_1III procollagen mRNA to poly(A)$^+$ RNA, on the other hand, was diminished in both strains over the 6-day time course (Fig. 6B). In C57BL/6N mice, pulmonary α_1III mRNA/poly(A)$^+$ RNA was significantly depressed at 6 days, reaching a value near 50% of control (Fig. 6B). In BALB/c mice, the α_1III procollagen/poly(A)$^+$ RNA ratio was significantly depressed at 3 days, to less than 50% of control (Fig. 6B). As illustrated in Fig. 6C, in both strains the relative elevation in α_2I and depression in α_1III procollagen mRNA yielded 7- to 17-fold increases in the ratio of pulmonary α_2I/α_1III procollagen mRNA by days 3 to 6 after BLM treatment. The steady state levels of β-actin mRNA/poly(A)$^+$ RNA increased more than 2-fold 1 day after BLM treatment in C57BL/6N mice and remained elevated over the 6-day time course (Fig. 7). In BALB/c mice, no increase was noted 1 day after BLM treatment but a 2-fold increase in the β-actin/poly(A)$^+$ RNA ratio was seen at 3 and 6 days. Thus, β-actin mRNA levels in lungs were affected by BLM treatment and each mRNA species exhibited a distinct pattern of change.

Discussion

BLM is used widely in experimental models of interstitial lung disease but the molecular basis of the drug-induced pul-

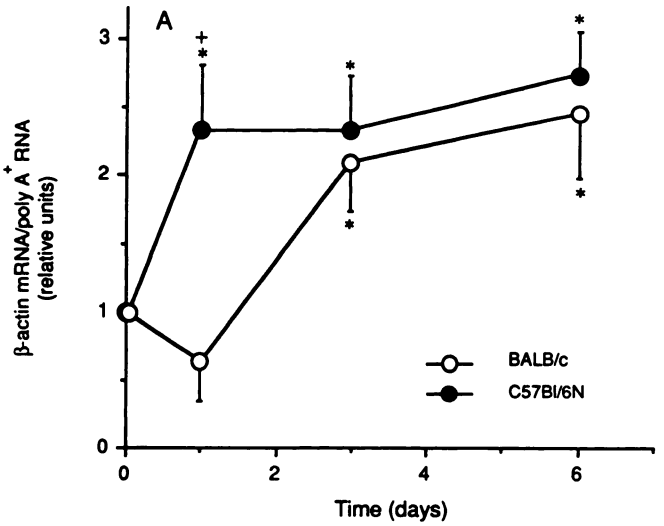

Fig. 7. Effect of BLM (80 mg/kg intravenously) on murine β-actin mRNA/poly(A)$^+$ RNA. Total RNA was electrophoresed on agrose gels and transferred to Hybond-N (Northern blot). After hybridization with a ^{32}P-labeled human β-actin probe, the signal area was determined as in Fig. 3. Data are expressed relative to saline-treated C57BL/6N mice. *Symbols* are as in Fig. 2.

monary fibrosis is not understood. BLM cleaves DNA in cultured cells (4, 19, 20) and this effect is presumed to be the mechanism by which BLM kills cells in culture and to be the basis of its antitumor activity. There has, however, been no direct evidence of BLM-induced DNA damage *in vivo* because technical limitations have prevented assessment of DNA damage after BLM treatment *in vivo*. Recently, however, Romagna *et al.* (5) developed a method for measuring DNA damage produced by alkylating agents *in vivo*, using the sedimentation rate of lung nucleoids, which are partially deproteinized nuclei containing nuclear matrix and supercoiled DNA (15). We have now adopted this method to measure acute DNA damage in the lungs of mice treated with single intravenous doses of BLM.

Our experiments were performed in parallel in two strains of mice, C57BL/6N and BALB/c, which differ in their sensitivity to BLM-induced pulmonary fibrosis (6, 7). C57BL/6N mice develop histologically and biochemically demonstrable pulmonary fibrosis after a single intravenous injection of BLM (80 mg/kg), whereas BALB/c mice do not (7). Both strains showed extensive DNA scission within 15 min after intravenous injections of BLM (Fig. 2), consistent with a direct clastogenic effect of the drug. This finding is the earliest effect of BLM described in the lung, occurring 3 days before the first pulmonary morphologic changes reported after intravenous injection of BLM [i.e., increased intravascular leukocytes (21)] and suggests that destruction of DNA is at least partially involved in BLM-induced pulmonary fibrosis. Interestingly, the sensitive and resistant strains of mice showed similar degrees of DNA damage and a similar time course of DNA repair over 1 hr after BLM treatment (Fig. 2). This finding is, in fact, consistent with our previous data that indicate that the resistant mice do not have decreased pulmonary levels of BLM or more rapid elimination of the drug from the lung (7). The present experiments further support the proposition that the variation in murine strain sensitivity to BLM does not occur on the basis of differences in initial cellular uptake of BLM, transport of BLM to the nucleus, or conversion of BLM to the active form; the accessibility of DNA to active BLM appears similar in both strains over the first hour after drug treatment (Fig. 2). It is notable that the greatest apparent DNA damage occured at the earliest measured time (15 min) and it is quite possible that greater DNA damage may have existed before the 15-min time point. The repair of at least some forms of BLM-induced DNA damage have been reported to be extremely rapid (20).

In contrast to the similar levels of DNA damage seen during the initial hour after BLM treatment, the restoration of normal sedimentation rate differed markedly in the two strains of mice (Fig. 2). Beginning 3 hr after BLM injection, the resistant BALB/c mice show progressive apparent repair of DNA that is essentially complete by 5 hr after treatment (Fig. 2). In contrast, significant levels of DNA damage remain 5 hr after BLM treatment in the sensitive C57BL/6N mice (Fig. 2). Thus, mice that are destined to develop pulmonary fibrosis after a single intravenous injection of BLM show a deficit in net apparent repair of BLM-induced acute DNA damage. Several mechanisms could account for the observed difference in the pulmonary DNA damage profiles; the lungs of sensitive mice could have 1) a decreased capacity to repair some types of DNA damage produced by BLM, 2) a higher proportion of intractable lesions, such as double-strand breaks in DNA, or 3) either a decreased inactivation of intracellular BLM or an increased retention of nuclear drug, which could lead to persistent production of new DNA damage 5 hr after BLM injection. Further studies will be necessary to define whether any of these mechanisms contribute to the observed strain variation in net DNA damage.

Previous studies using several model systems for BLM-induced pulmonary fibrosis have demonstrated that the chronic accumulation of lung collagen occurs subsequent to overexpression of pulmonary mRNAs encoding procollagens, suggesting that the excess collagen deposition is at least partially transcriptionally regulated (9, 22, 23). It is, therefore, possible that persistent pulmonary DNA damage as a result of BLM treatment in the sensitive mice might increase collagen content by directly altering the regulation of procollagen gene transcription in lung cells (e.g., via damage either to *cis* or *trans*-acting regulatory elements affecting the transcription of procollagen genes) or the factors controlling mRNA stability. The inability of BLM to cleave RNA (24) allowed us to determine such secondary effects of BLM damage. We measured the pulmonary mRNA content 1 to 6 days after BLM treatment (Figs. 3–7), a period in which there is no marked inflammation (21). These experiments yielded four major observations.

First, the time course of changes in total lung poly(A)$^+$ RNA differed dramatically between resistant (BALB/c) and sensitive (C57BL/6N) mice over the first day after BLM treatment (Fig. 3). The resistant mice showed a 2-fold increase in lung steady state poly(A)$^+$ RNA levels, whereas sensitive mice showed a depression to 50% of control values. The significance of these distinct changes is not clear, although the strain difference in total poly(A)$^+$ RNA levels might be related to the strain difference in net DNA repair (Fig. 2) and this hypothesis certainly warrants further investigation. Three to 6 days after BLM treatment, lung poly(A)$^+$ RNA in the resistant mice declined to levels below those of untreated control mice and comparable to those of sensitive mice (Fig. 3). This depression in lung mRNA coincides with the reported depressed indexes of lung cell proliferation occurring during the first week after intrave-

nous BLM treatment (21) and may reflect a general inhibition of lung cell metabolism.

Second, mRNA encoding α_2I procollagen was progressively increased by more than 3.5-fold compared with control by day 6 in both strains (Fig. 6). Increases in lung α_2I procollagen mRNA have been reported previously 7 days after intratracheal instillation of BLM in rats or hamsters (22, 23). In contrast to the α_2I procollagen response, levels of mRNA for α_1III procollagen were depressed in both strains 3 and 6 days after BLM treatment (Fig. 6). These results differ from those of Kelly *et al.* (22), who found increased α_1III procollagen mRNA over the first week after intratracheal instillation of BLM in rats. Different animal species or routes of drug administration could account for the discrepancy. The 7- to 17-fold increases in α_2I/α_1III procollagen mRNA ratios at 3 and 6 days occur before inflammation or observable morphologic changes within the lung tissue after intravenous BLM treatment (21) and in the absence of changes in lung weight, an index of pulmonary edema. Whether the shift in the procollagen mRNA ratio occurs as a result of a direct effect of BLM on the DNA of lung fibroblasts [as suggested previously (25)], as a response of resident pulmonary cells to effects of BLM on other pulmonary cell types, or as a result of an early and inconspicuous infiltrate of nonpulmonary cells remains to be determined.

Our procollagen mRNA data are compatible with previous experimental results analyzing lung collagen synthesis and deposition after BLM injury. These studies have shown that 1) there is relative elevation in the ratio of type I to type III collagen synthesis rates in lung tissue 6 days after BLM treatment in rats (26), 2) fibrotic lungs contain an elevated ratio of type I to type III procollagen (26, 27), and 3) fibroblasts isolated from rat lungs after BLM treatment contain an elevated ratio of type I to type III collagen (28). Thus, our data indicate that an elevation in the ratio of type I to type III procollagen mRNA occurs concomitantly with or precedes the relative elevation of type I procollagen synthesis in BLM lung disease and, therefore, may be an important early response in the development of pulmonary fibrosis. It is obvious, however, that other factors are necessary to allow the mRNA response to be expressed as excess type I collagen synthesis and deposition, because BLM-resistant mice also showed an elevated α_2I/α_1III procollagen mRNA ratio (Fig. 6C).

Third, we saw a marked increase in the pulmonary level of fibronectin mRNA, relative to other mRNA species, in the lungs of sensitive mice but not in the resistant mice (Fig. 5). This response was clearly evident as early as 1 day after BLM treatment, thus preceding changes in procollagen mRNA levels, and persisted for the full 6 days after BLM treatment. Interestingly, the pulmonary content of fibronectin mRNA showed no major changes (data not shown), indicating that pulmonary fibronectin mRNA levels in sensitive mice were selectively maintained in a setting of decreased total pulmonary poly(A)$^+$ RNA (Figs. 3B and 6). In contrast to our results, Raghow *et al.* (23) found in hamsters a 2.5- to 3-fold increase in total pulmonary fibronectin mRNA content beginning 4 days after intratracheal instillation of BLM and a 6-fold increase by 7 days after treatment. This may reflect the greater inflammatory response occurring acutely after BLM treatment in the intratracheal instillation model, compared with intravenous administration or an enhanced sensitivity of hamsters. Nevertheless, both our data and these previous studies (9, 22, 23) point to changes in the regulation of fibronectin expression as an early event in BLM-induced lung disease. Such increases in mRNA might lead to elevated pulmonary levels of fibronectin, which could be biologically important as a growth factor for fibroblasts (29) and can serve as a binding site for the attachment of pulmonary epithelial cells to matrix (30).

Finally, BLM treatment also appears to increase the mRNA levels of β-actin relative to poly(A)$^+$ RNA (Fig. 7). The increase in β-actin mRNA occurred more rapidly in the lungs of C57BL/6N mice, compared with BALB/c mice. Although the functional significance of the elevated β-actin mRNA is not known, it is interesting that Mitchell *et al.* (31) have observed an increase in α smooth muscle actin in rat lung parenchymal cells after BLM treatment.

Our results represent the first direct demonstration of DNA damage *in vivo* by BLM. It is interesting that, although the two murine strains studied differ substantially in their sensitivity to the chronic effects of BLM, such as collagen deposition, after an intravenous injection of BLM, the initial DNA damage was similar. Therefore, our data are consistent with the hypothesis that DNA damage is necessary but not sufficient for pulmonary fibrosis. It is persistence of DNA damage rather than initial damage that is more closely associated with sensitivity to BLM-induced fibrosis. The complex pattern of change in steady state mRNA levels after BLM does not reveal a causal relation between DNA damage and altered mRNA metabolism at this time. Nevertheless, the finding of an elevated ratio of type I to type III procollagen mRNA in the resistant as well as the sensitive mice confirms that resistant mice are susceptible to at least some of the acute effects of BLM in the lungs. It is possible that, as with DNA damage, the lungs of resistant mice are capable of responding more effectively to this challenge than the lungs of sensitive mice and, thus, avoid excessive collagen deposition.

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