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Prevention of bleomycin-induced fibrosing alveolitis with indomethacin: stereological studies on rat lungs

Gerhard Mall [1], Peter Zimmermann [1], Ingbert Siemens [1], Arne Burkhardt [2], and Herwart F. Otto [1]

[1] Department of Pathology, University of Heidelberg, Heidelberg, Federal Republic of Germany
[2] Department of Pathology, University of Bern, Bern, Switzerland

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Summary. The prevention of the pulmonary toxicity of bleomycin (BLM) has been investigated in experimental models where pulmonary damage was induced with one intra-tracheal dose of BLM. The present investigation was carried out as a pre-clinical study in which BLM was administered systemically. The non-steroid anti-inflammatory drug indomethacin (INDO) was chosen as a possible candidate for pulmonary protection. Twenty female Wistar rats were treated daily with 4 mg/kg (7.3 units) BLM intra-peritoneally for 50 days and 20 rats with BLM and with 1 mg/kg INDO subcutaneously for 62 days. There were 20 animals as controls. Histological examination revealed fibrosing alveolitis in the BLM-treated group which was markedly suppressed in the combination group. Quantitative morphological (stereological) parameters demonstrate that BLM induced alveolar wall thickening (+45%), pulmonary fibrosis (+110%), and an increase of alveolar wall nuclei and of intra-alveolar macrophages (volume densities +43% and +133%, $P<0.001$). In contrast, after combination with INDO significant differences to the control group could not be detected except for a slight increase of intra-alveolar macrophages (+62%). Thus, INDO is a highly efficient agent in the prevention of BLM-induced pulmonary damage.

Key words: Bleomycin – Indomethacin – Fibrosing alveolitis – Morphometry

Introduction

The anti-neoplastic activity of the bleomycins, a mixture of different glycopeptides obtained from *Streptomyces verticillatus*, was discovered by Umezawa (1974). Bleomycin (BLM) is used clinically, alone or in combination regimes, especially for treatment of malignant lymphomas and testicular carcinomas (Burger et al. 1981; Yagoda et al. 1972). It shows only minor toxic effects on the haematopoietic system and the immune system. However, serious pulmonary side-effects occur which lead to interstitial pneumonitis in 10% of treated patients. In 2% of patients a fatal pulmonary fibrosis develops (Sikic 1985). This may be caused by the low activity of the BLM-degrading hydrolase in lungs (Sebti and Lazo 1988).

The morphology and pathogenesis of BLM-induced pulmonary fibrosis have been established in a long series of experimental investigations in several mammalian species (Adamson and Bowden 1977; Aso et al. 1976; Jones and Reeve 1978; McCullogh et al. 1978; Thrall et al. 1979). One single dose of BLM was administered intratracheally in these studies (Snider et al. 1978). The intra-tracheal route was also used for tests on pulmoprotective drugs such as glucocorticoids, anti-fibrogenetics and anti-oxidants (Pepin and Langner 1985; Phan and Fantone 1984; Sterling et al. 1982; Zuckermann et al. 1980). However, recent investigations indicated substantial differences of pulmonary alteration when intra-tracheal administration of BLM was compared with systemic application (Costa et al. 1983; Ekimoto et al. 1983; Shen et al. 1988), and clinicans have, therefore, demanded more reliable pre-clinical tests of potentially damaging and protective effects (Scheulen 1987).

Thrall et al. (1979) reported suppression of pulmonary damage with the non-steroid anti-inflammatory drug indomethacin (INDO) after intra-tracheal instillation of BLM. INDO is an established drug in long-term treatment of rheumatic disorders (Lockie 1986) and its side-effects are well defined. Thus it seems to be an ideal candidate for clinical trials. In the present experimental study fibrosing alveolitis was induced by systemic administration of BLM in rats, and potential beneficial effects in INDO were examined using quantitative morphological methods (Weibel 1979).

Offprint requests to: G. Mall, Pathologisches Institut, Städtische Kliniken, Grafenstraße 9, W-6100 Darmstadt, Federal Republic of Germany

Materials and methods

Sixty female Wistar rats (initial body weight: 200+9 g) were purchased pathogen-free and Sendai virus-free from Ivanovas (Kißlegg, FRG). They were randomly assigned to three groups by the use of random numbers and were caged individually in isolation. Twenty animals were treated with daily intra-perioneal (i.p.) injections of 4 mg/kg (7.3 units) BLM (bleomycin sulphate; Mack, Illertissen, FRG), 20 animals with 4 mg/kg BLM i.p. in combination with 1 mg/kg indomethacin (MSD, Munich, FRG) subcutaneously (s.c.) per day, control animals received daily i.p. injections of 1 ml 0.9% saline. Body weights were recorded twice weekly and the dosage of BLM and INDO was adjusted individually to body weight changes. BLM was applied for 50 days, INDO for 62 days, the latter starting 2 days before BLM treatment. All animals were fed with tap water and a standard laboratory diet (Altromin, Lage, FRG) ad libitum. Sixty days after the start of BLM treatment the animals were sacrificed and the lungs were examined by histological and stereological investigations.

For fixation of the lungs, the animals were anaesthetized by i.p. injections of 10 ml 10% chloral hydrate/kg. The vascular system was flushed by vascular perfusion with a dextran solution (Rheomacrodex, Glandorf, FRG) for 5 min in order to remove the intravascular blood cells. Then the lungs were carefully removed and fixed with 3% glutaraldehyde in 0.2 mol phosphate buffer at a pressure of 20 cm water for 3 h and post-fixed by immersion with glutaraldehyde for 72 h. Glutaraldehyde was chosen as fixative to prevent collapse of lungs after intra-tracheal pressure fixation. Subsequently, the lung volumes were determined according to Scherle (1970). The whole lungs were then temporarily embedded in agar and dissected by means of a tissue sectioner into a random set of equidistant (1 mm) parallel slices. On average, 30 pulmonary slices were obtained per animal. A point grid was used for area-weighted sampling of probes for morphological investigations (Weibel 1979). Ten specimens ($10 \times 10 \times 1$ mm) per animal were embedded in Paraplast, and ten specimens ($2 \times 2 \times 1$ mm) per animal in Epon-Araldite. For histological and stereological investigations, 4-µm paraffin sections were stained with haematoxylin and eosin and Ladewig's stain, and 1-µm (semi-thin) sections with methylene blue and basic fuchsin (Di Sant'Agnese and De Mesy Jensen 1984).

Volume densities (V_V) and surface densities (S_V), were derived according to the basic stereological equations (Weibel 1979) from point densities (P_P) and densities of intersection points (I_L).

For synoptical description of the stereological evaluations the following abbreviations are introduced: V(l), total volume of both lungs [=V(ref)]; V(p), total volume of pulmonary parenchyma (alveoli, alveolar ducts, small vessels); V(np), total volume of non-parenchymatous components (bronchi, arteries, veins, connective tissue sheaths, interlobular septa, pleura, scars); V(s), total volume of alveolar septa; S(s), total alveolar surface of septa; V(npf), total volume of fibrous tissue in V(np) (including scars); V(sf), total volume of fibrous tissue in V(s); V(sn), total volume of septal nuclei; V(m), total volume of intra-alveolar macrophages.

The stereological analysis was performed as a multi-stage sampling procedure at four stages of magnification which increases the efficiency of evaluation (Weibel 1979). Quadrats for counting were obtained by random systematic sub-sampling (Weibel 1979). Counting was performed with a Zeiss eyepiece containing 100 test points and 10 test lines.

Stage 1 (magnification 28:1): The ten paraffin sections per animal were used for determination of V(p)/V(l) and V(np)/V(l).

Stage 2 (magnification 160:1): The ten semi-thin sections per animal (20 test quadrats) were used for determination of V(s)/V(p).

Stage 3 (magnification 400:1): Furthermore, the ten semi-thin sections per animal (40 test quadrats) were also used for determination of S(s)/V(s).

Stage 4 (magnification 1000:1): Finally, the ten semithin sections (40 test quadrats) were used for determination of V(sn)/V(s), V(sf)/V(s), V(m)/V(s) and V(npf)/V(np).

Stereological densities [reference volume: V(l)] were obtained from the ratios of stages 1–4 by multiplication, and the stereological densities times V(l) revealed total volumes and surface areas, e.g.:

V_V of septal nuclei:

$$V(\mathrm{sn})/V(\mathrm{l}) = V(\mathrm{sn})/V(\mathrm{s}) \times V(\mathrm{s})/V(\mathrm{p}) \times V(\mathrm{p})/V(\mathrm{l})$$

Total volume of septal nuclei:

$$V(\mathrm{sn}) = V(\mathrm{sn})/V(\mathrm{s}) \times V(\mathrm{s})/V(\mathrm{p}) \times V(\mathrm{p})/V(\mathrm{l}) \times V(\mathrm{l}).$$

For estimation of alveolar wall thickness, we introduced the volume-to-surface ratio of alveolar septa (V_S) according to:

$$V_V/S_V = V_S$$

The volume-to-surface ratio is an estimator of the arithmetic mean thickness of biological barriers provided that the barrier separates completely two spaces. Those barriers, for example, are the glomerular filtrating membrane in the kidney and the air-blood barrier in the lung (Weibel 1979). If we suppose that all alveolar walls are honeycomb-like infinite sheets which completely separate the air space of the alveoli the volume-to-surface ratio would correspond to the half arithmetic mean thickness (T/2) of the alveolar walls:

$$T = 2 \times V_S$$

Though a small part of the alveolar surface is attached to the pleura, bronchi, major blood vessels etc., and wall thickness cannot be defined to those sites we preferred the term "mean alveolar wall thickness" to the abstract stereological term $2 \times V_S$.

The arithmetic means and standard errors were calculated in each group. In order to obtain stable variances the data were logarithmically transformed. Variances from transformed data were tested for stability with the Cochran test. One way analysis of variance was used to compare the arithmetic means of the three groups. Scheffe's test was employed to detect divergences between the two groups. A result was considered to be significant if $P < 0.05$ (Sachs 1974).

Results

BLM treatment resulted in a continuous reduction of body weights and lung volumes in both treated groups (Table 1). Mortality amounted to 7 of 20 rats in the BLM group, 7 of 20 rats in the BLM-INDO group, and 0 of 20 rats in the control group. Animals dying during the last 15 days were not included in the statistical evaluation. Increasing mortality forced us to stop the BLM injections after 50 days.

Qualitative histological examination revealed a normal pulmonary structure in control animals, and spontaneous respiratory disease was not observed. In the BLM group, fibrosing alveolitis occurred (Fig. 1), which has been previously described in detail (Adamson and Bowden 1977; Aso et al. 1976; Burkhardt and Gebbers 1983; Jones and Reeve 1978; McCullough et al. 1978). In the BLM-INDO group the extent of pulmonary damage was considerably suppressed (Figs. 2–4). Hallmarks of BLM induced pulmonary damage were observed including diffuse interstitial and perivascular oedema, hypercellularity of alveolar walls, accumulation of macrophages with foamy cytoplasm in the intra-alveolar space, and septal, perivascular and peribronchial fibrosis with pronounced subpleural collagen deposition.

In order to substantiate the morphological observations, morphometric methods were employed. The vol-

Table 1. Absolute volumes and surface areas

Parameter	Control group ($n=12$)	Bleomycin-treated group group ($n=12$)	Bleomycin/indomethacin ($n=13$)	Statistics: one-way analysis of variance
Body weight (g)	297 ± 7	157 ± 7	143 ± 4	$P<0.001$
Total lung volume (cm^3)	5.78 ± 0.3	4.17 ± 0.2	3.96 ± 0.1	$P<0.001$
Alveolar septal volume (mm^3)	658 ±19	626 ±30**	455 ±18	$P<0.001$
Alveolar surface (m^2)	0.273± 0.010	0.178± 0.006	0.183± 0.005	$P<0.001$
Volume of septal nuclei (mm^3)	74.0 ± 2.3	77.3 ± 6.3**	50.2 ± 2.6	$P<0.001$
Alveolar macrophage volume (mm^3)	56.0 ± 3.4	91.8 ± 6.0**	63.4 ± 5.9	$P<0.001$
Septal fibrosis volume (mm^3)	29.9 ± 1.2	44.2 ± 3.5**	25.9 ± 2.1	$P<0.001$
Total fibrosis volume (mm^3)	45.7 ± 2.4	71.2 ± 6.1**	43.4 ± 3.5	$P<0.001$

Mean ± standard errors (SEM). The means of the three groups were compared by one-way analysis of variance. A result was considered to be significant if $P<0.01$ (statistics). Divergences (Scheffe's test) between the different treatments were indicated as ** ($P<0.01$)

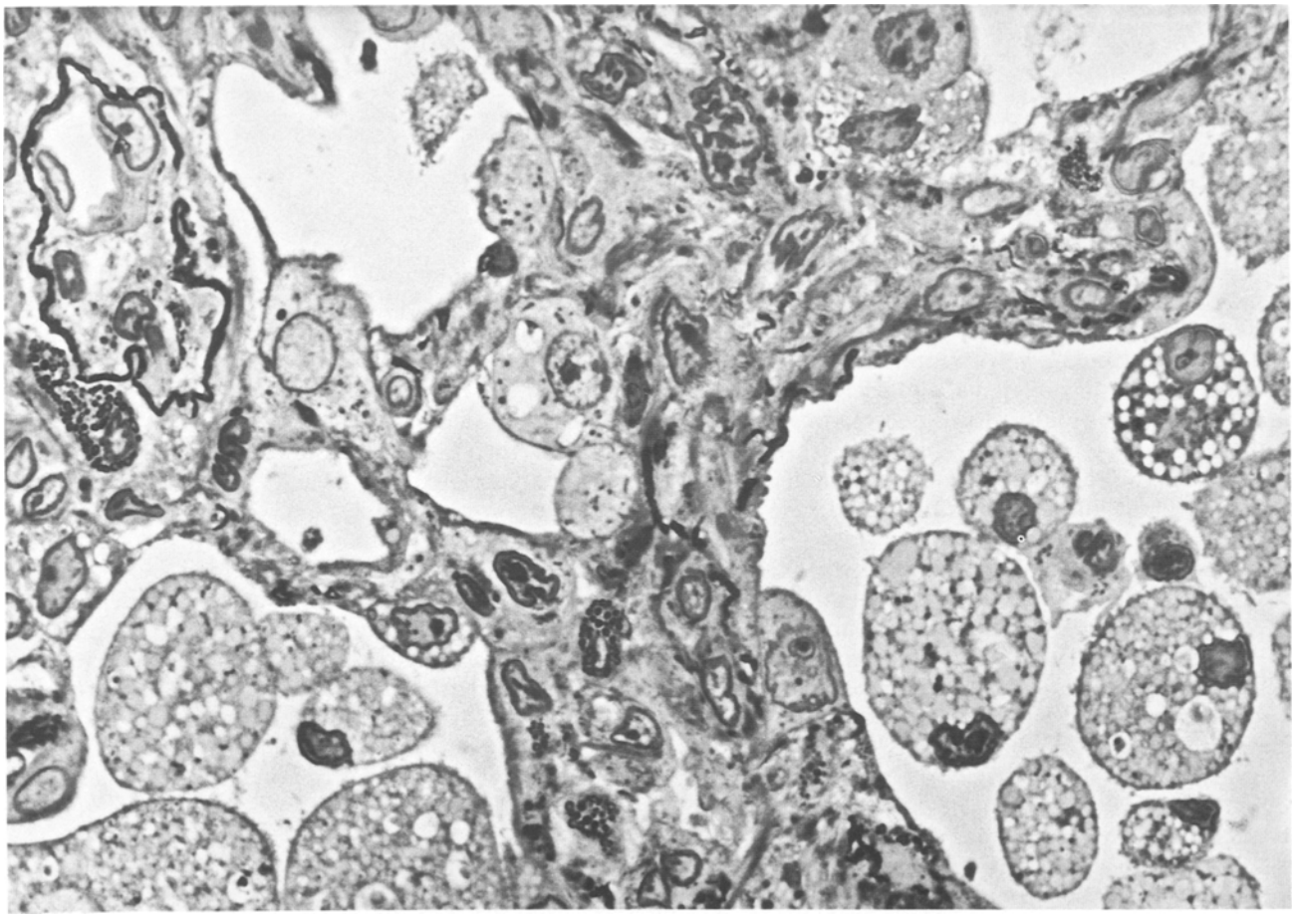


Fig. 1. Light microscopy, semi-thin section. Marked thickening of alveolar walls with hypercellularity, septal fibrosis and a large number of alveolar macrophages after bleomycin (BLM) treatment. The severe pulmonary changes which are demonstrated in this micrograph showed a multifocal distribution within both lungs. ×1250

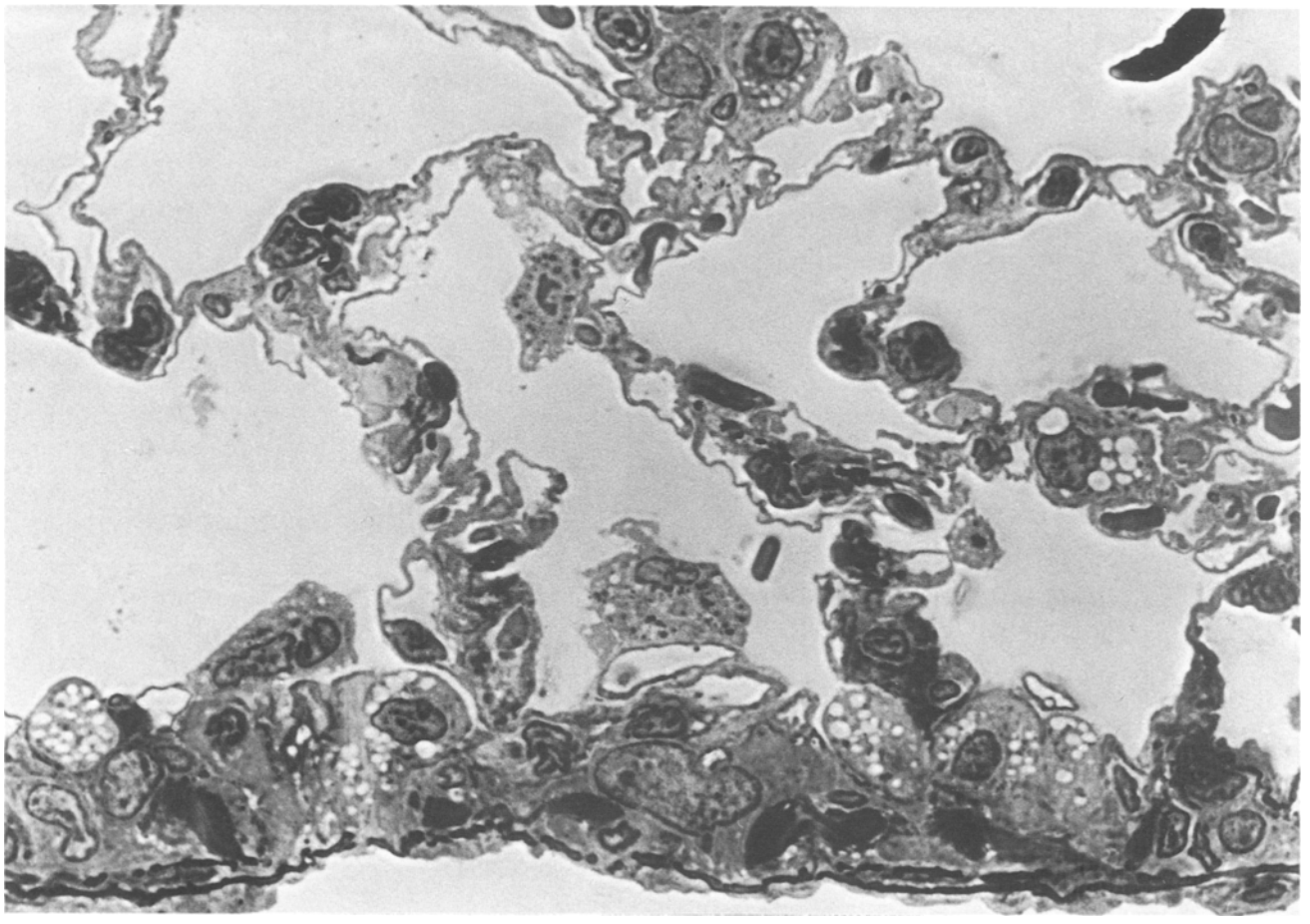


Fig. 2. Light microscopy, semi-thin section. Besides the foci with severe changes (cf. Fig. 1), diffuse damage of pulmonary parenchyma was observed after BLM treatment. The typical morphological changes in the subpleural space are shown: alveolar walls as well as visceral pleura are thickened when compared with controls (Fig. 4). ×1250

ume-to-surface ratio (V_S ratio) of aveolar walls was introduced as an estimator of "mean alveolar wall thickness" ($2 \times V_S$ ratio). In addition, volume densities and total volumes of alveolar walls, septal nuclei, alveolar macrophages, septal fibrous tissue, and total fibrous tissue were determined as indicators of oedema, inflammation and fibrosis. Surface density and total surface of alveolar walls were used as a marker of pulmonary destruction, atelectatic induration (Burkhardt 1986) and scarring.

The stereological parameters (Table 2) strikingly indicate the BLM effects and the protection with INDO (Figs. 5, 6). In the BLM group, mean alveolar wall thickness ($2 \times V_S$ ratio of alveolar walls) was significantly increased (+45%) as well as the volume density (V_V) of alveolar septa (+30%), V_V of septal nuclei (+43%), V_V of intra-alveolar macrophages (+133%), V_V of septal fibrous tissue (+103%), and V_V of total fibrous tissue (+110%). The surface density (S_V) of alveoles was slightly decreased after BLM treatment (−10%). In the BLM-INDO group, significant divergences to the controls were not detected except a mild increase in V_V of intraalveolar macrophages (+61%).

Total volumes and surface areas are demonstrated in Table 2. In the BLM group, volumes of alveolar septa (+38%), septal nuclei (+54%), intra-alveolar macrophages (+45%), septal fibrous tissue (+71%) as well as total fibrous tissue (+67%) were significantly higher when compared with the parameters of the combination group.

It should be emphasized that the stereological parameters are affected by the state of lung inflation and the technique of fixation (Weibel et al. 1973). We kept these factors as constant as possible. A glass tube system was constructed which allowed simultaneous fixation of ten lungs at identical pressures. In order to avoid systematic errors the sequence of fixation was determined by the use of random numbers. Furthermore, glutaraldehyde was used as fixative which prevents completely the collapse of lungs after intra-tracheal pressure fixation.

For quantitative morphological analysis of fibrosis staining was performed according to Di Sant'Agnese and De Mesy Jensen (1984), which shows a sky-blue appearance of collagen.

Discussion

In the present pre-clinical study various pulmonary alterations after induction of BLM toxicity by systemic ad-

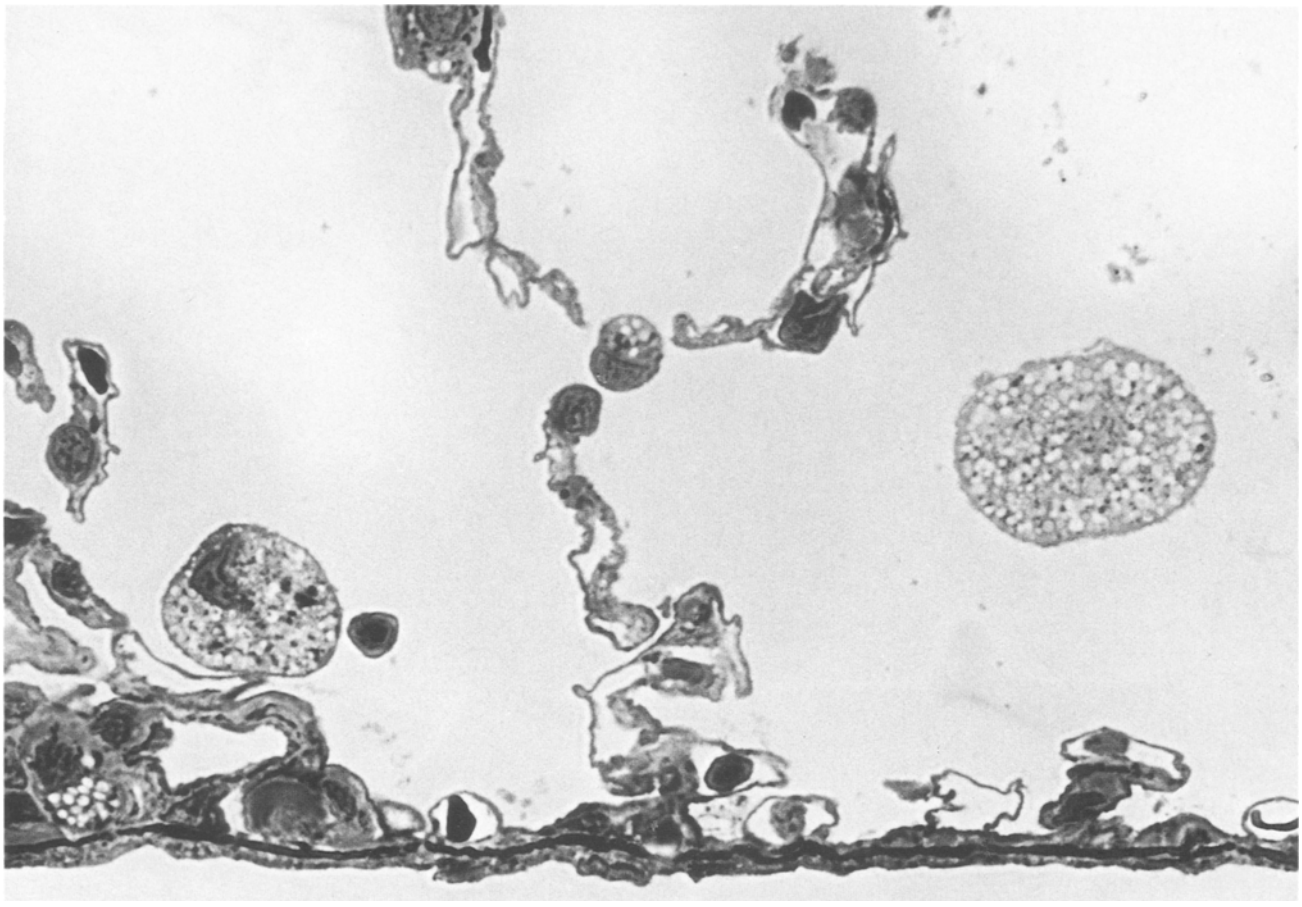


Fig. 3. Light microscopy, semi-thin section. Appearance of the subpleural pulmonary tissue after combined BLM-indomethacin (INDO) treatment. Alveolar walls and visceral pleura do not show significant BLM effects when compared with non-treated controls (Fig. 4). The slight increase in intra-alveolar macrophages in the combination group which was confirmed by the stereological evaluations is shown. ×1250

ministration were quantified and the prevention to toxicity using INDO were also measured by means of morphometric methods.

The qualitative morphological alterations in the animals treated with BLM were similar to pulmonary changes in man after BLM treatment (Bedrossian et al. 1973; Burkhardt et al. 1977; Tom and Montgomery 1980). Pulmonary histology supports the rat model as an adequate one for BLM-induced pulmonary damage in man.

Determination of pulmonary volumes and surface areas by stereological parameters revealed significantly higher values for volumes of alveolar septa, septa nuclei, intra-alveolar macrophages, septal fibrous tissue and total fibrous tissue in the BLM group when compared with the parameters of the combination group. Since total lung volumes in the treated groups are substantially influenced by the suppressed growth after treatment, differences in absolute values do not necessarily correspond to drug effects. However, divergences between the BLM group and the BLM-INDO group demonstrate the protective INDO effects.

Parallel investigations of animals that died during the last 15 days of treatment with drugs show that the deaths were correlated with the greatest loss in body weight, but not with the severity of pulmonary alterations. Thus, the general toxicity of BLM inducing anorexia and metabolic disorder must be considered as major cause of death. Tom and Montgomery (1980) found 80% mortality after 6 weeks BLM treatment with a cumulative dose of 180 units.

In recent studies on BLM-induced pulmonary fibrosis hydroxyproline levels were determined as a marker of pulmonary collagen content (Sikic et al. 1978). It has been shown, however, that relative and absolute collagen contents may be misleading if used as only markers of pulmonary injury. Fulmer et al. (1980) found no correlation between collagen concentration and the degree of idiopathic pulmonary fibrosis in humans. Therefore, in our study we determined not only the fibrosis content by morphometric methods, but also the mean alveolar wall thickness and cellularity as estimators of inflammation. Fibrosis can be easily measured on sections stained according to Di Sant'Agnese and De Mesy Jensen (1984).

Quantitative morphological analysis does not elucidate the pharmacokinetic interplay between INDO and BLM. INDO inhibits cyclooxygenase and decreases the synthesis of prostaglandins (Vane 1971). In BLM-induced injury in hamsters, Chandler and Giri (1983)

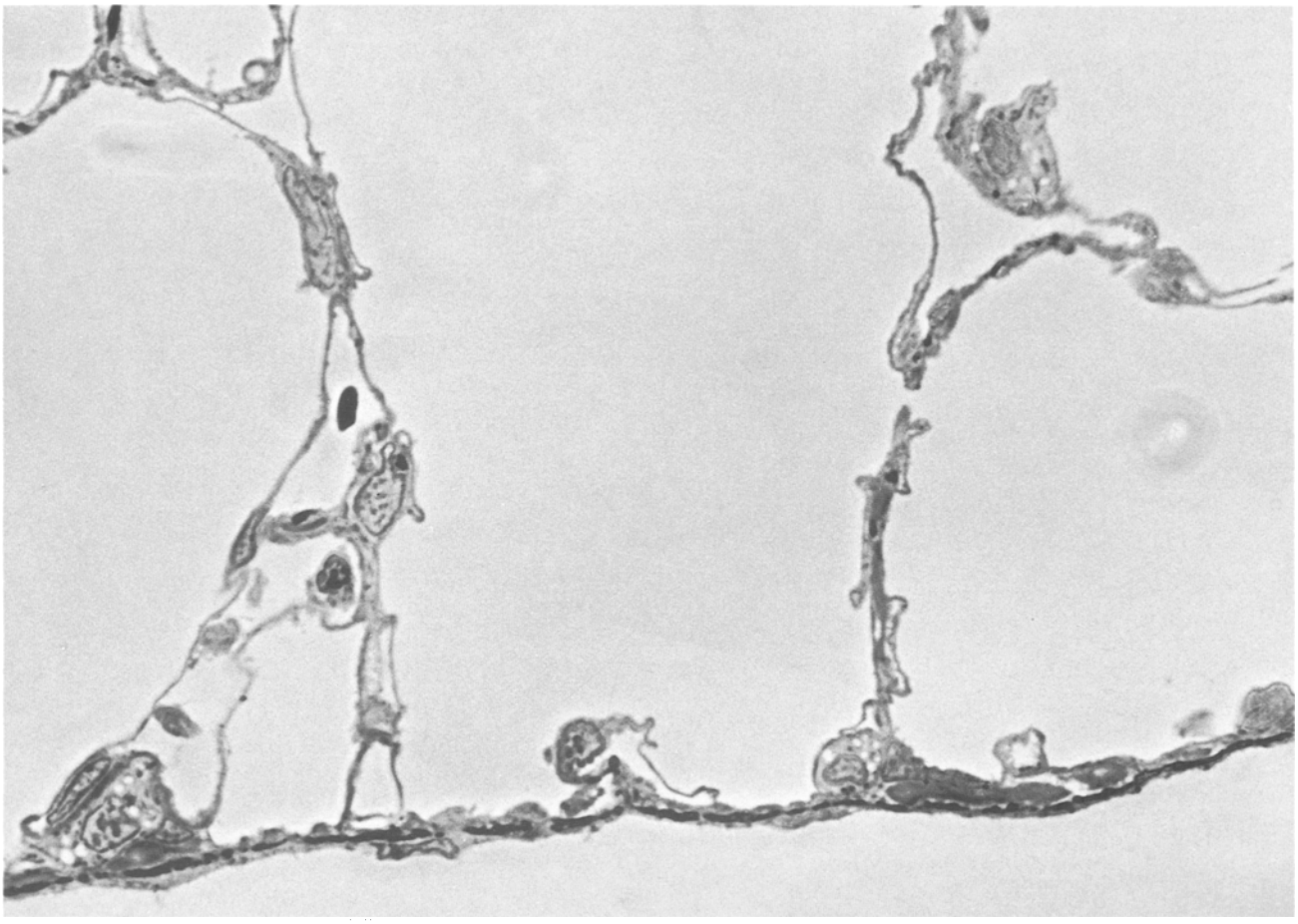


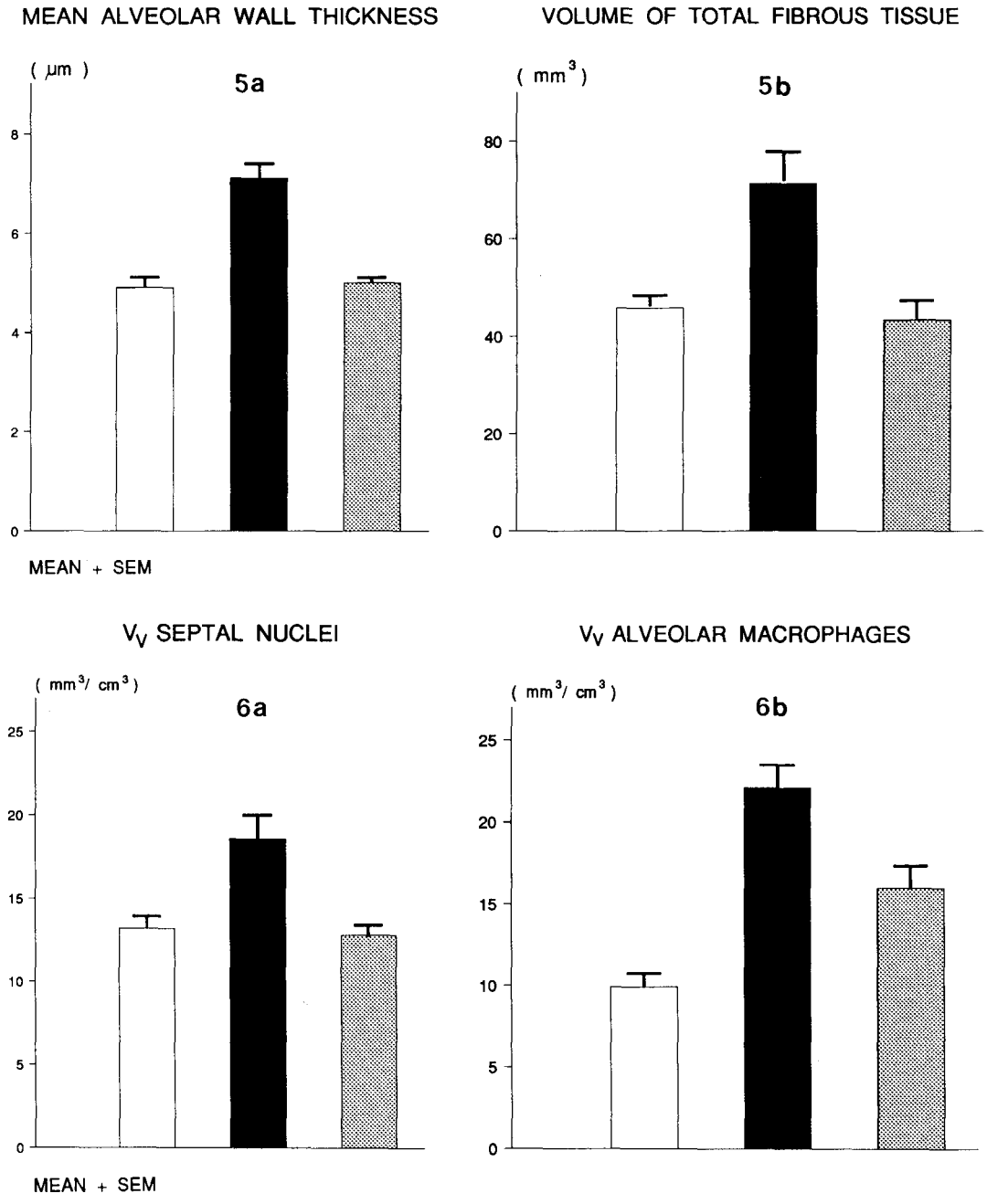
Fig. 4. Light microscopy, semi-thin section. Representative area of pulmonary tissue of a control animal. Compare the appearance of alveolar walls and visceral pleura with Figs. 2, 3. ×1250

Table 2. Relative volumes and surface areas

Parameter	Control group ($n=12$)	Bleomycin-treated group ($n=12$)	Bleomycin/indomethacin group ($n=13$)	Statistics: one-way analysis of variance
Mean alveolar wall thickness (μm)	4.9± 0.2	7.1± 0.3**	5.0±0.1	$P<0.001$
V_V alveolar septa (mm^3/cm^3)	116 ± 5	151 ± 6**	115 ±4	$P<0.001$
S_V alveolar surface (cm^2/cm^3)	476 ±11	431 ±15	462 ±7	NS
V_V septal nuclei (mm^3/cm^3)	13.2± 0.8	18.6± 1.5**	12.8±0.7	$P<0.01$
V_V alveolar macrophages (mm^3/cm^3)	9.9± 0.8	22.1± 1.4**	16.0±1.3*	$P<0.001$
V_V septal fibrosis (mm^3/cm^3)	5.3± 0.3	10.6± 0.7**	6.5±0.4	$P<0.001$
V_V total fibrosis (mm^3/cm^3)	8.2± 0.6	17.2± 1.5**	10.9±0.8	$P<0.001$

Means ± standard errors (SEM). The means of the three groups were compared by one-way analysis of variance. A result was considered to be significant if $P<0.01$ (statistics). Divergences (Scheffe's test) between the different treatments were indicated as ** ($P<0.01$), divergences between the BLM-INDO group and the control group as * ($P<0.01$)

found increased circulating plasma levels of products of the cyclo-oxygenase pathway of arachidonic acid metabolism (prostaglandin-E_2, thromboxane-B_2 and 6-keto-prostaglandin-F_{1a}). This indicates an important role of prostaglandins in the pathogenesis of BLM-induced lung fibrosis. Prostaglandins and other soluble factors (e.g. macrophage derived growth factor) may also be involved in macrophage-dependent modulation



Figs. 5, 6. The graphs show the stereological parameters which indicate alveolitis as well as fibrosis in the BLM-treated group and the protection with INDO. The BLM-induced increase in mean thickness of alveolar walls, total fibrous tissue of both lungs and volume densities of septal nuclei ($P<0.001$) was completely inhibited with INDO, and the increase in volume densities of alveolar macrophages was considerably suppressed. □, control group; ▨, BLM-INDO group; ■, BLM group

of fibroblast collagen production and proliferation (Clark and Greenberg 1987). In vitro, INDO suppresses fibroblast proliferation and improves collagen abnormalities of diabetic rats (Neupert and Müller 1975; Yue et al. 1985). At an early stage of pulmonary damage, INDO may protect from endothelial damage, septal oedema and microthrombosis since it reduces vascular permeability in vivo (Ohuchi and Sugimura 1987; Thrall et al. 1979) and abolishes collagen-induced aggregation of platelets (Honey et al. 1986; Krishnamurthi et al. 1984; Leytin et al. 1984).

Recently published data on non-steroid drugs revealed effects on the immune system which have been shown, for example, in patients with rheumatoid arthritis (Goodwin 1984). In vitro, INDO decreases production of the rheumatoid immunoglobulin (Ceuppens et al. 1982). However, the immune system is involved in the pathogenesis of BLM-induced pulmonary fibrosis (Schrier et al. 1983; Schrier and Phan 1984) and BLM treatment can be associated with exacerbation of rheumatoid arthritis.

BLM-induced disturbances of oxidant and anti-oxi-

dant systems in the lung may also contribute to pulmonary damage. BLM acts as mini-enzyme and generates superoxide anions in the presence of iron and oxygen in vitro. Free radicals peroxidize arachidonic acid in vitro and enhance production of chemotactic leucotrienes (Passero and Disanto 1988). Furthermore, BLM-stimulated macrophages generate chemotactic activity in vitro and BLM-exposed pulmonary tissue stimulates granulocytes to produce superoxides (Moseley et al. 1984; Weselius et al. 1984). Polymorphonuclear leucocytes are involved in early BLM-induced pulmonary alterations (Thrall et al. 1982). INDO may act beneficially on the reactions of polymorphonuclear leucocytes by inhibition of lipo-oxygenase, leucotaxis and superoxide anion production.

With regard to possible clinical trials of BLM-INDO treatment it should be emphasized that not only the inflammatory response, but also the pulmonary fibrosis was markedly suppressed. Questions arise on how INDO may affect tumour progression, since prostaglandins are known to be one of the regulators of tumour growth and spread. In small colon carcinoma transplants on mice, INDO suppresses tumour growth. This may be related to increase in macrophage/NK cytotoxicity caused by a reduction of tumour-associated prostaglandins (Eisenthal 1990; Schrier and Phan 1984). In contrast, with large burdens of colon carcinoma transplants, INDO facilitated tumour growth despite inhibition of tumour-associated prostaglandin production (Tanaka et al. 1989). More important are potential anti-neoplastic interactions between BLM and INDO. Stöhr and Goerttler (German Institute of Cancer Research, Heidelberg, FRG) studied the growth of tumour cell lines using variable concentrations of BLM and INDO but did not find any effects of INDO on the anti-tumour activity of BLM in vitro (personal communication).

It is concluded that experimental fibrosing alveolitis induced by systemic BLM administration is markedly suppressed when BLM therapy is combined with INDO. One may suggest from our results that clinical application of the combination therapy will also decrease pulmonary toxicity in man.

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