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Bleomycin primes monocytes-macrophages for superoxide production

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Bleomycin primes monocytes-macrophages for superoxide production. D.O. Slosman, P.M. Costabella, M. Roth, G. Werlen, B.S. Polla.
ABSTRACT: Bleomycin (BLM) induces lung inflammation and subsequent fibrosis in humans and animal models. We hypothesized that monocytes-macrophages represent target cells for BLM toxicity and participate in the initial stages of pulmonary inflammation. We developed an animal model of early lung lesions using systemic administration of BLM (2 U·100 g^{-1} body weight over 5 days) (BLM-rats). We observed a significant decrease in body weight and in serum angiotensin converting enzyme activity in BLM-rats as compared to matched control rats, but no evidence of fibrosis was seen in optic microscopy of the lungs from BLM-rats. In contrast, electron microscopy revealed accumulation of intracapillary polymorphonuclear leucocytes and unusual presence of eosinophils. We then investigated the *in vivo* effects of BLM on the respiratory burst of monocytes-macrophages. As compared to control rats, production of superoxide (O_2^-) by alveolar macrophages from BLM-rats was increased upon stimulation with either phorbol myristate acetate (21.04±2.78 *versus* 11.45±2.26 nmol·10^6 cells·20 min^{-1}, $p<0.05$) or opsonized zymosan (9.35±0.87 *versus* 7.03±0.66 nmol·10^6 cells·20 min^{-1}, $p<0.05$). We also found in BLM-rats an increased number of circulating monocytes and an increased production of O_2^- by these cells. Monocytes-macrophages may represent a target cell in the early events of BLM toxicity *in vivo* and the increased production of O_2^- by these cells participates in tissue injury in pulmonary fibrosis.
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The antitumour agent bleomycin (BLM) is a group of glycopeptidic antibiotics with high affinity for tumour cells and deoxyribonucleic acid (DNA) binding properties used for the treatment of tumours such as lymphoma or seminoma [1]. The clinical use of BLM has been limited because of the development of a dose-dependent pulmonary fibrosis [1, 2]. BLM, intratracheally administered, has been extensively used in animal models to induce pulmonary fibrosis, but its precise target cells and pathogenic mechanisms are incompletely understood [2, 3]. In the early phase of the development of the progressive pulmonary fibrosis, endothelial cell lesions and an associated decrease of angiotensin converting enzyme activity (ACE) in serum were commonly observed [4, 5] as well as weight loss [6, 7].

^{57}Cobalt labelled BLM used for tumour imaging also concentrates in macrophage-rich tissular lesions [8, 9]. BLM, by intratracheal instillation, has been shown to alter functions of alveolar macrophages (AM), such as production of chemoattractant activity for neutrophils, macrophage-derived growth factor, interleukin-1 and interleukin-6 [10–12]. Several recent reports have also suggested a role for peripheral blood monocytes (PBM) during the early phase of lung inflammation [13, 14].

BLM has been shown to exert its cytotoxic effects through the intracellular generation of oxygen free radicals (by forming iron-BLM complexes and entering oxidation-reduction cycles) [15–17]. Reactive oxygen metabolites have been implicated in numerous lung diseases including the adult respiratory distress syndrome, emphysema, pulmonary oxygen toxicity and radiation-induced pulmonary damage [18, 19]. Monocytes-macrophages and other phagocytes generate oxygen free radicals in the extracellular milieu through the respiratory burst enzyme, reduced nicotinamide-adrenine-dinucleotide phosphate (NADPH) oxidase. In human monocytes-macrophages and neutrophils, NADPH oxidase can be activated through distinct pathways, either by phorbol esters such as phorbol myristate acetate (PMA), or by receptor-mediated events induced by opsonized zymosan or bacteria [20].

We hypothesized that monocytes-macrophages, and in particular AM, may represent target cells for BLM

toxicity *in vivo* by generating oxygen free radicals in the extracellular milieu.

We developed an animal model allowing us to investigate the early inflammatory events occurring in the lung after systemic administration of BLM. To follow the extent of BLM toxicity, we monitored weight loss, measured seric ACE activity and examined the lung using both optic and electron microscopy. We investigated the extracellular generation of superoxide anion (O_2^-) by monocytes-macrophages and found that intraperitoneal injection of BLM for 5 days primed *in vivo* monocytes-macrophages for O_2-production.

Materials and methods

Animals and experimental design

Age- and weight-matched male Sprague-Dawley rats from the same breeding lot were divided into three groups: 1) a group receiving BLM (Lundbeck, Holland) by intraperitoneal injection (2 U of BLM·100 g^{-1} body weight per day) for five days (BLM-rats); 2) a control-fed (CF) group in which animals received an equal volume of the vehicle (0.9% saline) intraperitoneally (animals in these groups had access to food *ad libitum*, they were weighed and the amount of food consumed was determined every day) (CF-rats); and 3) a pair-fed (PF) group which did not receive any injection and was offered the same amount of food as consumed on the day before by the BLM-rats (PF-rats). This group of PF-rats was used to investigate the potential role of a decreased food intake in the weight-loss observed in BLM-rats. For each experiment, 4–6 CF- and BLM-rats were simultaneously injected.

Rats were anaesthetized with sodium pentobarbital (50–70 mg·kg^{-1}, *i.p.*) and sacrified on day 6 after the start of BLM or saline administration. Blood was collected by intracardiac puncture, and was used to determine the total and differential leucocyte counts (13 animals) or the seric ACE activity (45 animals). For leucocyte counts and differentials, 2.5 ml of blood was collected with a syringe containing ethylenediaminetetra-acetic acid, and samples were stained with May-Grünwald and Giemsa. The trachea was canulated with a 22-gauge plastic catheter and the lungs were dissected and used for bronchoalveolar lavage (20 animals), for microscopy (6 animals), or for determination of dry-to-wet weight ratio (percent of total lung weight remaining after a 24 h exposure to 75°C) (20 animals). Previous data have shown that the normal lung dry-to-wet weight ratio is 19.4% and that a ratio lower than 17.0% indicates presence of lung oedema [21].

Determination of ACE activity in serum

ACE is a sensitive marker of endothelial cell lesions occurring when rats are exposed to BLM [4–7]. Because our goal was to develop a sub-acute model of BLM toxicity where there would be no evidence of macroscopic fibrosis, ACE activity in serum was measured as a control index of toxicity and was determined as previously described using a spectrophotometric technique [22]. The enzyme was assayed on a centrifugal analyser with furylacryloyl-phenylalanyl-glycyl-glycine as the substrate. The reaction rate remained linear throughout the incubation time for all sera.

Light and electron microscopy

For microscopy, the lungs of 6 BLM-rats and 3 CF-rats were removed and fixed by intratracheal instillation with 2.5% glutaraldehyde buffered with cacodylate (instillation pressure 15 cmH_2O). The lungs were immersed in the same fixative for 6 h, rinsed in cacodylate, post-fixed in osmium tetroxide, coloured with uralyl acetate, dehydrated in alcohol and propylene oxide and then embedded in Epon 812. Semi-thin sections were cut on an LKB ultratome and stained with 1% toluidine blue. Ultrathin sections were examined on a Philips 400 electron microscope.

Peripheral blood monocytes (PBM) of the rat

Six to 9 ml of blood was withdrawn in a syringe containing 1.5 ml citrate-phosphate-dextrose-adenine-anticoagulant (CPDA) (Travenol Lab, Deerfield, IL), centrifuged at 5,000 rpm for 8 min at 4°C. Buffy coats were isolated, diluted in Hank's buffered saline solution (HBSS) (Gibco, Paisley, Scotland) (v/v) and centrifuged over Ficoll-Hypaque (Pharmacia Fine Chemicals, Dübendorf, CH). Mononuclear cells were recovered, centrifuged, resuspended in 0.84% NH_4Cl to lyse remaining erythrocytes and washed twice in Hank's buffered saline solution (HBSS). Cells were counted and resuspended at 1.0×10^6 cells·ml^{-1} in RPMI-1640 (Gibco), with 10% foetal calf serum (FCS) (Gibco), 1% glutamine (Gibco) and incubated (in 10 cm tissue culture dishes) (Falcon, Becton Dickinson, Cockeysville, MD) for 60 min at 37°C. Non-adherent cells were then removed, adherent cells washed three times with phosphate buffered saline (PBS), gently scraped in the same buffer and counted again. PBM viability was assessed by trypan blue exclusion and was superior to 95%. After centrifugation (1,200 rpm, 10 min), PBMs were resuspended at 1.0×10^6 cells·ml^{-1} for measurement of O_2^- production. O_2^- production was determined in 2–6 samples depending on the number of PBMs.

Alveolar macrophages of the rat

Bronchoalveolar lavage was performed in isolated lungs by instilling 100 ml 0.9% NaCl. An average volume of 70 ml was recovered. Cells were centrifuged (1,200 rpm 10 min) at 4°C, resuspended in 0.84% NH_4Cl to lyse erythrocytes, and washed twice in PBS. Cells were

counted and resuspended at 1.0×10^6 cells·ml^{-1} in RPMI-1640 with 10% FCS and 1% glutamine and incubated in 10 cm culture dishes for 60 min at 37°C. Non-adherent cells were then removed, adherent cells washed twice with PBS, gently scraped in the same buffer and counted again. AM viability, assessed by trypan blue exclusion, was superior to 95%. The cells were pooled for each group. After centrifugation (1200 rpm, 10 min), AMs were resuspended at 1.0×10^6 cells·ml^{-1} for measurement of O_2^- production.

Measurement of superoxide production

O_2^- production was measured by the superoxide dismutase inhibitable reduction of ferricytochrome c as previously described [23]. Briefly, 10^6 AMs or PBMs in suspension were added to ferricytochrome c (1.24 mg, Sigma Chemical, St Louis, MO) with or without superoxide dismutase (Sigma, 30 µg), and stimulated with PMA (100 ng·ml^{-1}, Sigma) or opsonized zymosan (3 mg·ml^{-1}, Sigma). The suspensions were incubated for 20 min at 37°C then centrifuged at 4°C, and the optical absorbance of the supernatant was determined at 550 nm in a spectrophotometer. The amount of O_2^- generated is expressed as nmoles $O_2^- \cdot 10^{-6}$ cells·20 min^{-1}. Two to six replicate samples were measured in each experiment.

Statistical analysis

Unpaired two-tailed Student's t-test was used to compare the percentage of monocytes and ACE activity and, to compare the O_2^- production of PBM and AM of BLM-rats and CF-rats either in control condition or after stimulation.

To compare the variation of weight over the six day period between BLM-rats, CF-rats and PF-rats, and to analyse the modifications of human PBM superoxide production with exposure to increasing concentrations of BLM, one-way analysis of variance was used.

Results

Model characterization

Systemic and pulmonary effects of BLM. At day 6, we observed a 4.5±0.8% (mean±SEM, n=30) increase in body weight of CF-rats and a 10.6±0.4% (n=36) decrease in body weight of BLM-rats. In the experiments in which PF-rats were studied simultaneously to the BLM- and CF-rats (n=20 in each group), CF-rats gained at day six 9.1±2.7% of body weight, BLM-rats lost 13.7±1.2% of body weight whereas PF-rats lost 8.9±1.1% of body weight ($p<0.05$ when compared to CF-rats as well as to BLM-rats) (fig. 1). ACE activity was decreased in BLM-rats (62.6±4.2 U·l^{-1}) as compared to CF-rats (78.3±4.6 U·l^{-1}, n=45, $p<0.05$). At day 6, there was no evidence of pulmonary oedema: no significant difference in dry-to-wet lung weight ratio was observed between BLM- and CF-rats (21.6±8% *vs* 20.9±8%, n=20). There was no difference between BLM- and CF-rats in the total number of alveolar cells obtained by bronchoalveolar lavage. There was a significant monocytosis in BLM-rats: 18±2% as compared to 8±2% in CF-rats (n=13, $p<0.05$), without leucocytosis or other significant alterations in differential counts.

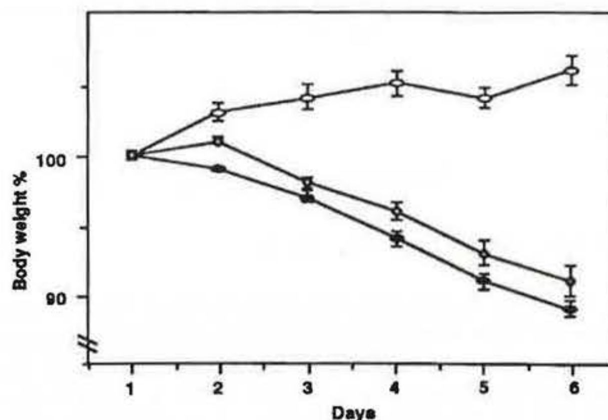


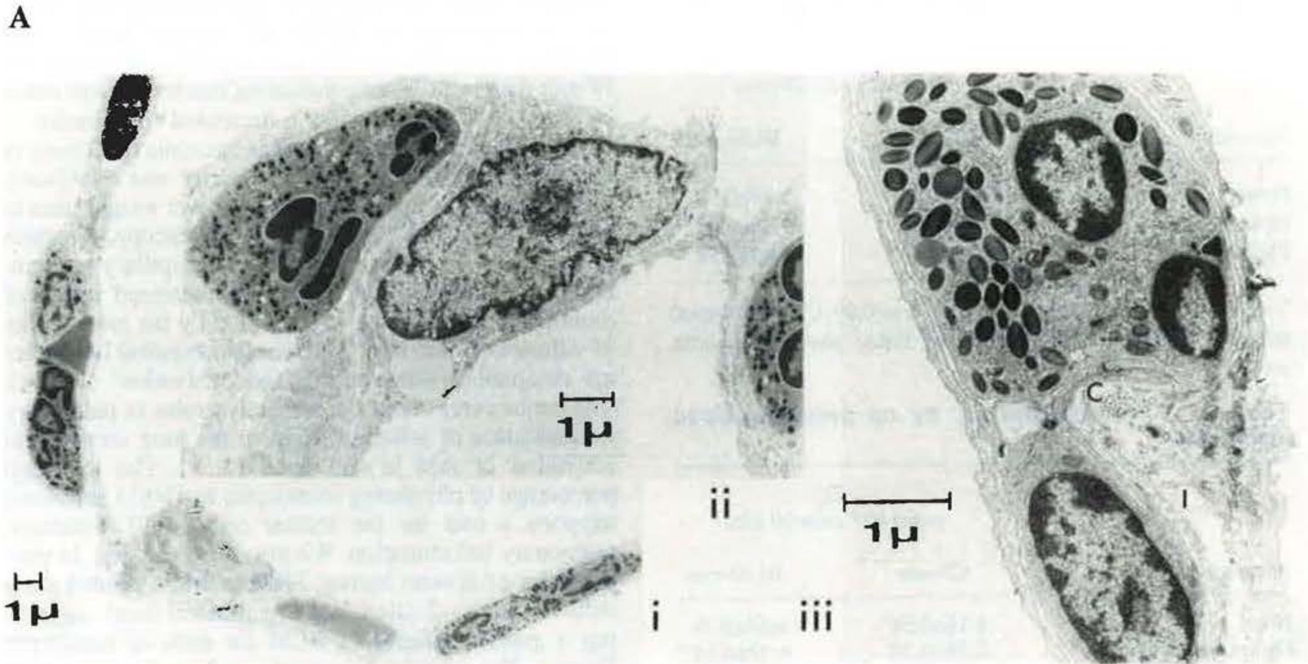
Fig. 1. – Effects of BLM on rat body weight. Rat body weight was monitored over 6 days for the 3 groups: 36 BLM-rats (O), 30 control-fed rats (animals receiving an equal volume of the vehicle, *i.e.* 0.9% saline intrapertioneally that had access to food *ad libitum*) (CF-rats, ●) and 20 pair-fed rats (receiving no injection but having the same amount of food as consumed on the day before by the BLM-rats) (PF- rats, ◊). BLM was injected at Day 1. Body weight was expressed as a percentage of initial weight. Each point was expressed as the mean±SD.

Optic and electron microscopy. On light microscopy, there was no evidence of haemorrhage, oedema or fibrosis (not shown). Electron microscopy did, however, reveal an increased number of intracapillary polymorphonuclear leucocytes as well as the presence of eosinophils (fig. 2A), which are not found in normal rodent lung. AMs presented morphological criteria of activated macrophages such as abundant lysosomes and large cytoplasmic inclusions suggestive of neutrophil ingestion (fig. 2B) [20].

Effects of BLM on rat AM and PBM in vivo

Effects of BLM on O_2^- generation by AM of rat. O_2^- generation by AM from BLM- and CF-rats was measured under basal conditions and after *in vitro* stimulation with either PMA, opsonized zymosan, or BLM. Basal O_2^- production by unstimulated AM was no different in BLM- and CF-rats (table 1). When BLM was used as a direct potential agonist of NADPH oxidase, there was no increase in O_2^- production by AM from either BLM-or CF-rats: 0.1±0.1 and 0.4±0.6 nmol·10^{-6} cells·20 min^{-1}, respectively. After stimulation of AM with PMA or opsonized zymosan, we observed, however, that O_2^- production was significantly higher in BLM-rats as compared to CF-rats (table 1).

A



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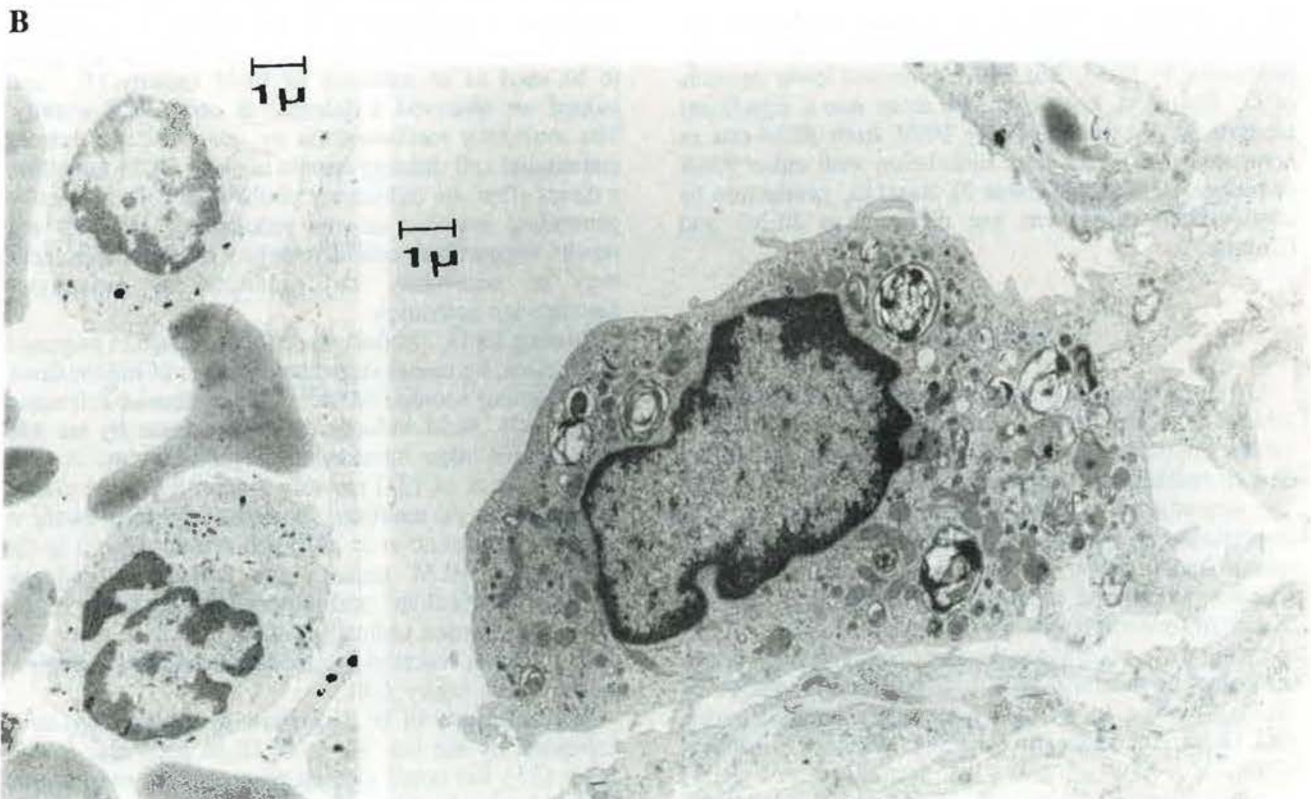

Fig. 2. – Electron microscopy of rat lung after BLM administration. A: An increased number of intracapillary neutrophils (i: ×6,000 and ii: ×12,000) and the unusual presence of eosinophils (iii: ×25,000) are observed. Note the presence of collagen fibers (C) and of a discrete increase in interstitial width (I); B: Intravascular monocytes (left panel) (×13,000) and alveolar macrophage migrating through a Cohn's pore (right panel). The alveolar macrophage displays features of an activated macrophage such as abundant lysosmes (L) and intracytoplasmic inclusions suggestive of neutrophil ingestion (N).

Table 1. – Production of O_2^- by rat alveolar macrophage

	O_2^- nmol·10⁻⁶ cells·20 min⁻¹	
Stimulus	CF-rats	BLM-rats
None	1.04±0.23*	1.49±0.30
Opsonized zymosan	7.03±0.66	9.35±0.87**
PMA	11.45±2.26	21.04±2.78**

*: expressed as mean±SEM from n=36; **: $p<0.05$; CF-rats: control fed rats; BLM-rats: bleomycin rats; PMA: phorbol myrisate acetate.

Table 2. – Production of O_2^- by rat peripheral blood monocytes

	O_2^- nmol·10⁻⁶ cells·20 min⁻¹	
Stimulus	CF-rats	BLM-rats
None	1.18±0.34*	0.66±0.18
Opsonized zymosan	2.24±0.23	6.22±0.64**
PMA	4.16±0.45	8.21±1.23**

*: expressed as mean±SEM from n=26; **: $p<0.05$; abbreviations as in table 1.

Effects of BLM on PBM of rat. We also investigated the effects of *in vivo* systemic administration of BLM on O_2^- production by PBM. Rat PBM generated lower amounts of O_2^- than AM, but as in AM, there was a significant increase in O_2^- production by PBM from BLM-rats as compared to CF-rats after stimulation with either PMA or opsonized zymosan (table 2). Basal O_2^- production by unstimulated PBM was not different in BLM- and CF-rats.

Discussion

A role for oxygen free radicals in BLM-induced lung toxicity has long been recognized [2, 3, 16] and is further supported by the cumulative toxicity of hyperoxia or radiotherapy in patients receiving BLM [24, 25]. The potential role of monocytes-macrophages and AM in particular in the local generation of reactive oxygen species had, however, not been appreciated before in this situation. In order to study the *in vivo* effects of BLM on monocytes-macrophages, we developed an animal model allowing us to investigate the early inflammatory events occurring in the lung after systemic administration of BLM. Systemic rather than intratracheal administration was chosen in order to be more relevant to clinical situations. After 5 days of intraperitoneal BLM administration, we found typical evidence for BLM toxicity, such as weight loss, decreased ACE activity and lung inflammation, together with priming of both AM and PBM for O_2^- production. Reduction of body weight associated with a marked decrease in food intake has previously been reported in the hamster after intratracheal administration of BLM [6]. In our studies, the decrease in body weight was significantly lower in PF-rats than in BLM-rats, indicating that weight loss could be only partly explained by a decreased food intake.

The lack of pulmonary oedema. necrosis or fibrosis in our model of pulmonary BLM toxicity was established by the absence of alteration in dry-to-wet weight ratio as well as by the normal optical microscopy. Electron microscopy, however, revealed intracapillary accumulation of inflammatory cells. An increased influx of monocytes into the lung is suggested by the low number of villosities of the AM, whereas the abundant lysosomes are compatible with macrophage "activation" [13, 14]. The controversy about the respective roles in pulmonary inflammation of influx of PBM in the lung *versus* local activation of AM is still open [2, 3]. The increased percentage of circulating monocytes and PBM activation supports a role for the former cells in BLM-induced pulmonary inflammation. We also observed that *in vitro* exposure of normal human PBM to BLM primed these cells for O_2^- production (our unpublished data), suggesting a general affinity of BLM for cells of monocytic lineage. The increased generation of O_2^- by these cells may then mediate, at least in part, the effects of BLM.

Endothelial cells have also been proposed as an initial site of BLM-induced lung injury [7, 26, 27]. ACE is a zinc-containing dipeptidyl carboxy-peptidase present on the luminal membrane of lung endothelial cells and is suggested to be the source of circulating ACE. Serum ACE activity has therefore been proposed to be used as an indicator of BLM toxicity [4], and indeed we observed a decrease in seric ACE activity. The molecular mechanism(s) by which BLM produces endothelial cell damage remain unclear. BLM may have a direct effect on pulmonary endothelial cells by locally generating reactive oxygen metabolites [15–17]; our results suggest that oxidative injury of endothelial cells may be secondary and mediated by monocyte-macrophage activation.

Priming for O_2^- production could be related to increased production, by monocytes-macrophages, of interleukin-1 [28], tumour necrosis factor α [29] or platelet-activating factor [30]. BLM induces cytokine release by rat AM both *in vivo* (after intratracheal instillation) and *in vitro* [12]. Clark *et al.* [31] recently proposed that the effects of BLM may be mediated by tumour necrosis factor α whereas Dinarello *et al.* [32] had already shown in the 1970's that BLM induces the release of endogen pyrogen. Interleukin-1 and tumour necrosis factor α, by enhancing oxygen radical production by AM, may also play a role in macrophage-mediated immune complex-induced lung injury [28].

The selectivity of BLM toxicity to the lung has been explained by the low levels of BLM hydrolase in this organ [33], but could also be related to the even higher O_2^- production by AM and to the presence, in this organ, of potential agonists of NADPH oxidase: inhaled particles, bacteria, complement, or platelet-activating factor locally released by inflammatory lung cells. Mineral dust has recently also been shown to prime sheep

lung cells for superoxide release [34]. Increased production of reactive oxygen species by inflammatory lung cells may therefore represent a common mechanism for lung injury in various types of pulmonary inflammation.

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La bleomycine amorce la production de superoxide par les monocytes-macrophages. D.O. Slosman, P.M. Costabella, M. Roth, G. Werlen, B.S. Polla.
RÉSUMÉ: La bléomycine (BLM) peut induire une inflammation pulmonaire suivie par la suite d'une fibrose. Nous avons émis l'hypothèse que les monocytes-macrophages représentent des cellules cibles pour la toxicité à la BLM et participent à la phase initiale de l'inflammation. Nous avons développé un modèle animal nous permettant d'étudier les lésions pulmonaires précoces induites par une administration systémique de BLM (2 U·100 g^{-1} poids corporel sur 5 jours) (rats-BLM). Nous avons observé une réduction significative du poids corporel et de l'activité sérique de l'enzyme de conversion chez les rats-BLM par rapport aux animaux contrôles alors que la microscopie optique ne révélait aucune évidence de fibrose dans les poumons de rats-BLM. En microscopie électronique, on observe une accumulation intracapillaire de cellules polymorphonucléaires et la présence inhabituelle de cellules éosinophiles. Nous avons alors étudié les effets de la BLM *in vivo* sur le métabolisme oxydatif des monocytes-macrophages. Comparé aux contrôles, la production de superoxide (O_2^-) par les macrophages alvéolaires des rats-BLM était augmentée après stimulation soit par le phorbol myristate acetate (21.04±2.78 *versus* 11.45±2.26 nmol·10^{-6} cells·20 min^{-1}, $p<0.05$), soit par le zymosan opsonisé (9.35±0.87 *versus* 7.03±0.66 nmol·10^{-6} cells·20 min^{-1}, $p<0.05$). Nous avons aussi trouvé, chez les rats-BLM, un nombre augmenté de monocytes circulants et une augmentation de la production d'O_2^- par ces cellules. Les monocytes-macrophages pourraient représenter une cellule cible dans les évènements précoces de la toxicité à la BLM *in vivo* et l'augmentation de production d'O_2^- par ces cellules pourrait participer aux lésions tissulaires dans la fibrose pulmonaire.
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