High-dose corticosteroids during bleomycin-induced alveolitis in the rat do not suppress the accumulation of hyaluronan (hyaluronic acid) in lung tissue

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High-dose corticosteroids during bleomycin-induced alveolitis in the rat do not suppress the accumulation of hyaluronan (hyaluronic acid) in lung tissue. O. Nettelbladt, A. Tengblad, R. Hällgren.
ABSTRACT: A single intratracheal injection of bleomycin in rats induced, 4 days later, a considerable accumulation of hyaluronan (hyaluronate, hyaluronic acid) in the lung tissue. This connective tissue reaction was quantified biochemically by analysing hyaluronan (HA) in bronchoalveolar lavage fluid (BAL) and lung tissue extracts. The molecular weight of the HA recovered during lavage was $0.2–0.3 \times 10^6$ daltons. The HA accumulation was related to an increase in lung water content and associated with an increased influx of eosinophils, neutrophils and lymphocytes into BAL fluid. High-dose corticosteroid treatment (prednisolone 15 mg·kg^{-1} bw per day) to bleomycin injured rats had no effect on the lung tissue content of HA, the recovery of HA during BAL or the molecular weight of HA accumulated in the alveolar space. Furthermore, steroids did not influence the increased lung water content, or the appearance of inflammatory cells in lavage fluid. These findings indicate that the early connective tissue response to the bleomycin lung injury is mediated by a mechanism which is unaffected by systemic high-dose corticosteroids.
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Corticosteroid treatment is the main pharmacologic therapy used in most non-infectious, inflammatory interstitial lung diseases [1–3]. Although various mechanisms by which corticosteroids could modulate inflammatory or immune mechanisms in interstitial lung diseases have been investigated [4–6], their mode of action in these diseases is still not fully understood [1, 5, 7]. The rationales for treating the interstitial inflammatory process are: firstly, reduction of the acute hypoxaemia due to the interstitial inflammatory oedema [2]; secondly, prevention of the development of pulmonary fibrosis, supposed to be associated with the inflammatory process [1, 3].

In previous studies, high levels of hyaluronan (hyaluronate, hyaluronic acid by older nomenclature) have been demonstrated in bronchoalveolar lavage (BAL) in various inflammatory interstitial lung diseases [8–12]. Furthermore, increased recovery of hyaluronan (HA) during lavage may be related to the inflammatory activity in the lung and/or the severity of these diseases [9–12]. An inverse correlation was observed between BAL levels of HA and diffusion capacity in patients with farmer's lung and adult respiratory distress syndrome (ARDS) [9, 11]. Since high molecular weight HA is

extremely hydrated [13], the hypothesis was advanced that HA could be an underlying factor in alveolar interstitial oedema [9]. From studies of the bleomycin rat model [14–16], this hypothesis has gained further support.

During the inflammatory oedematous phase of bleomycin-induced lung injury, we have been able to demonstrate the morphological appearance of HA in the interstitial alveolar tissue and simultaneously a considerable increase in the concentrations of HA in BAL and lung tissue. We have also shown time-dependent relationships between the increase in HA levels in lavage and influx to the lavage fluid of polymorphonucleated granulocytes [15].

Furthermore, there is a strong correlation between the increase in extractable HA from lung tissue and the increase in the lung water content [16]. It was calculated that the increased lung concentration of HA could account for a significant part of the interstitial oedema, provided that the temporal parallel changes in extractable HA and lung water occurred in the same compartment(s).

The purpose of the present study was to investigate the influence of high daily doses of corticosteroids on the bleomycin-induced accumulation of HA in rat lung tissue

and the possible effect on the lung water content. A further aim was to study the effect of steroids on the molecular size of HA accumulated in the lung after bleomycin injury.

Material and methods

Animals

Adult male Sprague-Dawley inbred rats (ALAB, Sollentuna, Sweden) weighing 190–205 g were kept in separate cages and food and water provided *ad libitum*. The animals were divided into two main groups. One group was used for lung tissue extraction of HA and water content analysis. The other group was used for analysis of cells and concentration and molecular weight determination of HA in bronchoalveolar lavage (BAL). All rats were sacrificed by aortic exsanguination under chloral hydrate anaesthesia after 4 days. The Day 4 time point was chosen since previous studies, histologically and biochemically, have shown peak levels of lung HA on Days 3–7 [14–16].

Induction of bleomycin-induced pulmonary injury

Tracheostomies were performed on all animals to facilitate the intratracheal injection of 1.5 mg bleomycin sulphate (Lundbeck, Copenhagen, DK) in 0.3 ml sterile saline under chloral hydrate anaesthesia. To prevent bacterial infections, all rats received 10 mg *i.p.* sodium cefuroxime (Glaxo, Greenford, Middlesex, England), immediately before and one day after tracheostomy. One group of the animals was given an injection of sodium succinate-prednisolone (Organon, Oss, The Netherlands), 15 mg·kg^{-1} *i.p.* daily, starting at the time of administration of bleomycin. Two groups of control animals were included. One control group received only 0.3 ml of sterile saline intratracheally, whereas the other control group also received steroids as described above.

Lung tissue preparations

All preparations were made immediately after death. The lungs were removed *en bloc* and dissected free. The right lung was ligated at hilus and weighed immediately (wet-weight, w.w.) at room temperature, and then after freeze drying (dry-weight, d.w.). The dried lung was pulverized in a mortar. The homogenized lung tissue samples were kept dry and frozen at -20°C until analysed.

Extraction of lung tissue hyaluronan

Twenty mg of the pulverized dried lung was extracted with 2 ml 0.5 M NaCl for 16 h with constant shaking at 4°C. The samples were then centrifuged for 15 min at 2,000 g. The supernatants were recovered and the HA concentration analysed.

Bronchoalveolar lavage

Lavage was performed as described previously [15]. Immediately after death, the lungs were perfused by intratracheal infusion of 5 aliquots each of 5 ml phosphate-buffered saline (PBS) under gravity at a constant hydrostatic pressure of 25 cm. After 3 min the fluid was recovered by gravity. The lavage fluid was centrifuged at 400 g for 10 min. The supernatant was kept frozen at -20°C until analysed.

Analysis of BAL and lung tissue hyaluronan

The concentrations of HA in the lung tissue extracts and in the BAL fluids were determined in duplicate with a radiometric assay (HA-50-test, Pharmacia Diagnostics, Uppsala, Sweden), according to the principles outlined previously [17]. Briefly, 100 µl sample or standard is mixed with 200 µl ^{125}I-labelled protein (HABP, isolated from bovine nasal cartilage with specific affinity for HA) and incubated for 60 min at 4–7°C. 100 µl HA-Sepharose at a concentration of 1 mg·ml^{-1} is then added and the tubes incubated for a further 45 min at the same temperature. Two ml of washing solution is added and the HA-Sepharose recovered after centrifugation at 2,000 g for 10 min. Bound radioactivity in the pellet was measured in a gamma counter. Known amounts of HA were used in order to construct a standard curve plotting bound radioactivity as a function of HA concentration in the samples.

Determination of the molecular weight of hyaluronan from lavage fluid

The molecular size distribution of HA in the BAL fluid was determined as described previously [18]. Briefly, HA was isolated and concentrated by affinity chromatography to 5–10 mg·l^{-1} using an HA-binding protein-substituted agarose gel (HABP-Sepharose). The HA-binding regions from proteoglycans and the link proteins isolated from nasal cartilage were immobilized on the gel as described previously [19]. Protease inhibitors were added to BAL fluid (10–40 ml), pooled from two animals. The fluid was mixed with 1 ml of the HABP-Sepharose. After incubation for 20 h, the gel slurry was packed into a column and washed with 1 ml of 0.15 M NaCl. The HA bound to the gel was eluted from the column with 4 times 1 ml of 4 M guanidinium chloride, 0.5 M sodium acetate buffer pH 5.8. The isolated material was fractionated by calibrated gel chromatography and the molecular size distribution of HA was determined [20, 21]. The elution volume of the hyaluronan was determined by radioassay measurements of the HA concentrations in each fraction.

Calculation of total and relative lung water content

The total lung water from the right lung was calculated as the difference between the wet and dry weights of tissue samples. The relative water content in percent was calculated according to the formula: $100 \times$ the total lung water/wet weight.

Lavage fluid cell analysis

The cell pellet was resuspended in 1 ml PBS and counted after staining in a Bürker chamber and expressed as total cell concentration. After another washing in PBS the cell suspension was adjusted to a concentration of 10^6 cells·ml^{-1}. Preparations for cytological studies were made in acytocentrifuge (Cytospin Shandon, Southern Products Ltd, Runcorn, England) at 750 rpm and were stained with May-Grünwald-Giemsa before differential counting.

Statistical analysis

Two-tailed unpaired t-test was used to analyse the data. A value of $p < 0.05$ was considered significant.

Results

Lung tissue HA analysis

No difference was seen in total extractable lung HA between sham-treated control animals with (n=4) or without (n=3) steroid treatment, the (mean±SEM) concentrations being 114±7 and 110±9 μg·g^{-1} d.w., respectively. Compared with control animals, a significant ($p < 0.001$) increase in the HA content of the lung was found at the investigation on Day 4 after bleomycin-administration (n=8; mean±SEM HA 248±63 μg·g^{-1} d.w.). Steroid treatment did not influence the bleomycin induced HA accumulation in the lung tissue; mean±SEM HA content in the steroid treated group (n=7) was 288±47 μg·g^{-1} d.w. (fig. 1).

Water content analysis

No difference could be seen in the relative water content between sham-treated control animals with (78.0±0.4%) or without (78.2±0.5%) (mean±SEM) steroid treatment. There was a significant ($p < 0.001$) increase in the relative water content in the bleomycin injured animals (82.2±0.5%). Steroid treatment did not influence the

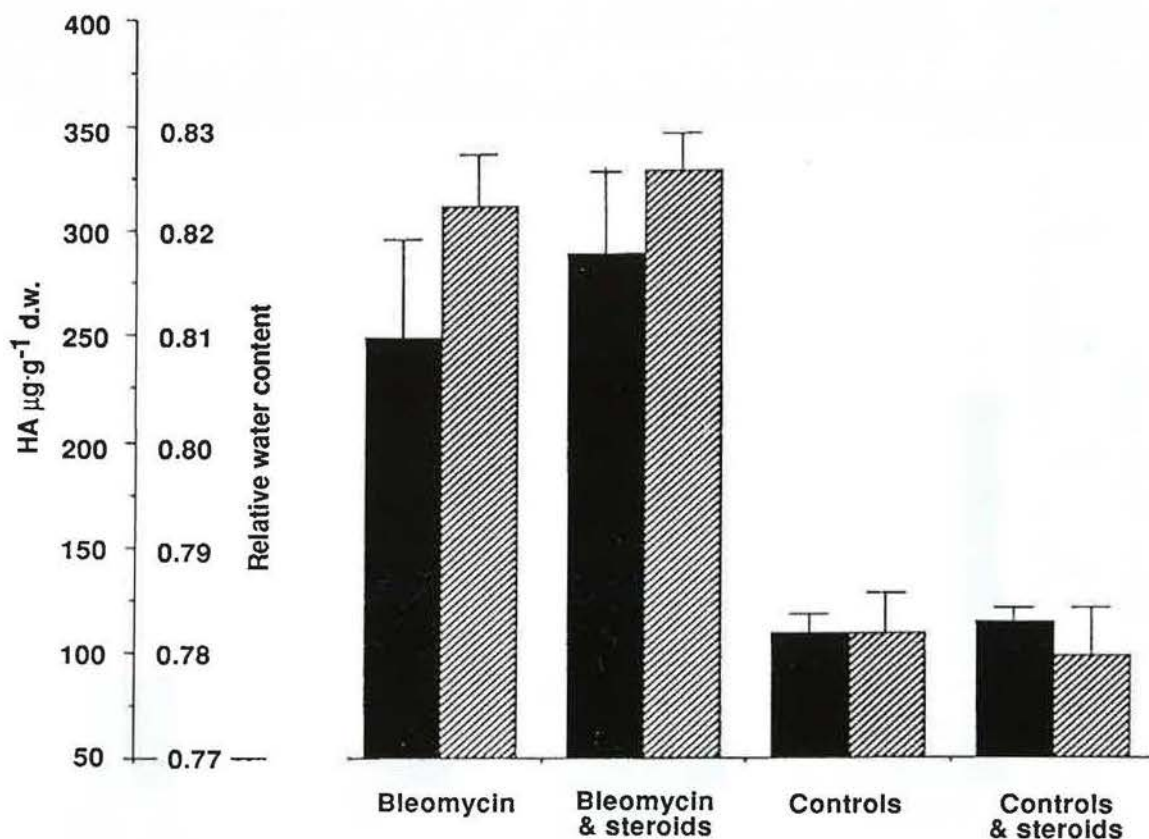Fig. 1. – The amount of extracted lung tissue hyaluronan (black, mean±SEM) and the relative water content (crosshatching, mean±SEM) from the right lung of rats investigated 4 days after a single intratracheal instillation of bleomycin. The first group (n=8) received bleomycin only. The second group (n=7) was also given prednisolone, 15 mg·kg^{-1} bw daily. Control groups, without (n=3) or with prednisolone (n=4) are also illustrated. There were significant differences in both hyaluronan (HA) ($p < 0.001$) and relative water content ($p < 0.001$) values between bleomycin treated rats and control rats, but corticosteroid treatment did not influence either lung tissue HA or the relative water content in bleomycin injured or control rats.
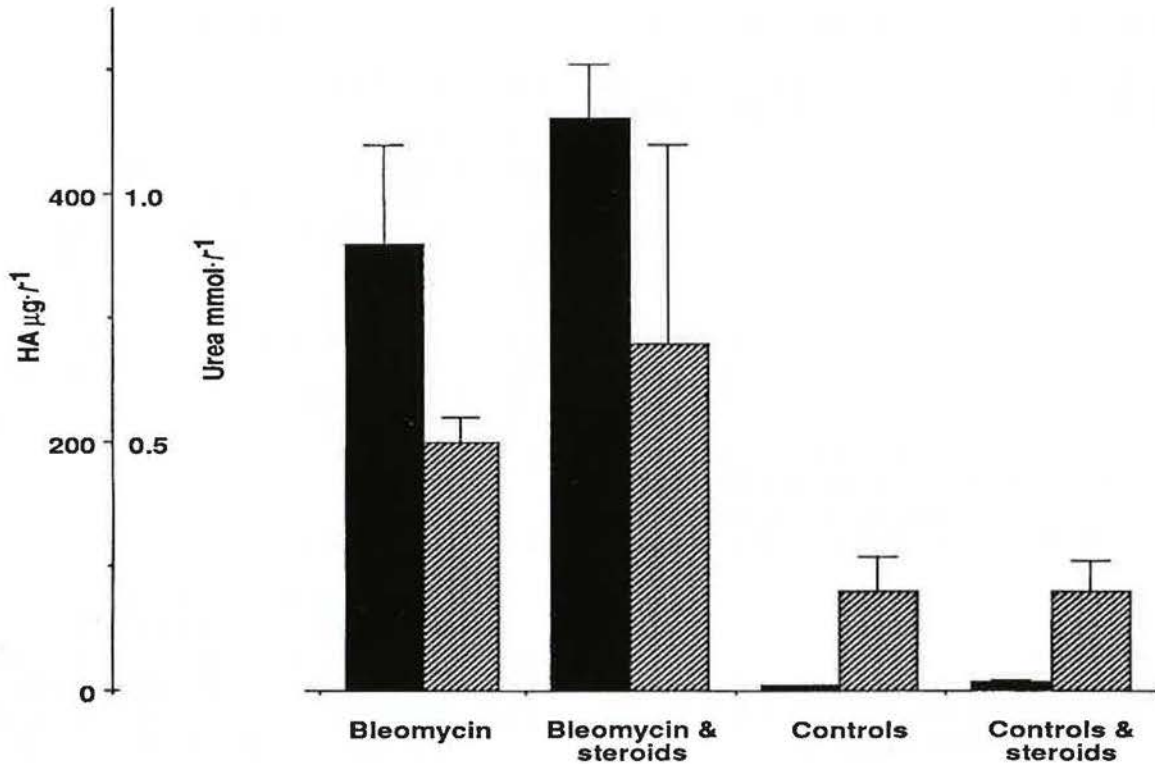
Fig. 2. – The concentration of hyaluronan (HA) (black, mean±SEM) and urea (crosshatching, mean±SEM) in bronchoalveolar lavage (BAL) from rats investigated 4 days after a single intratracheal instillation of bleomycin or NaCl. The first group (n=8) received only bleomycin. The second group (n=7) was also given prednisolone, 15 mg·kg⁻¹ bw daily. Control groups, without (n=4) or with prednisolone (n=4) are also illustrated. HA increased significantly (p<0.001) 70–75 times in animals that had received bleomycin, with or without additional steroids, compared to controls whereas the urea concentration only increased 2.5–3.5 times (p<0.001) but corticosteroid treatment did not significantly influence either lavage HA or urea in bleomycin injured or control rats.

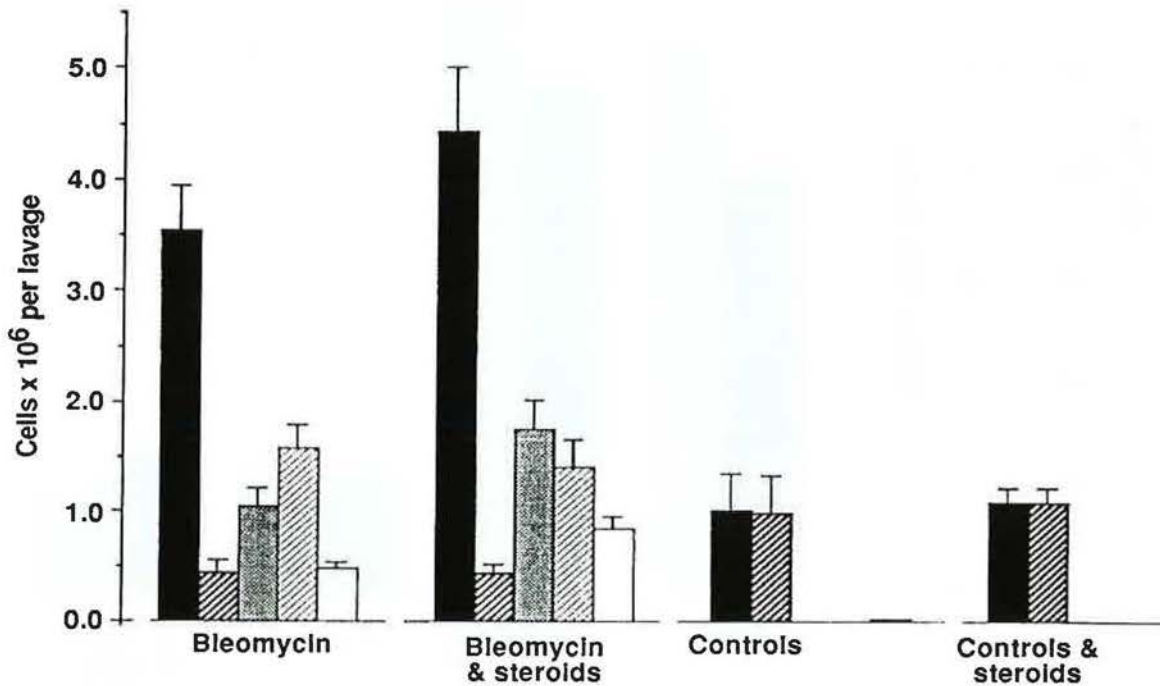Fig. 3. – Total cell counts (mean±SEM, black) and absolute numbers of macrophages (dark crosshatching), eosinophils (grey), neutrophils (light crosshatching) and lymphocytes (unfilled) in bronchoalveolar lavage (BAL) fluid from rats investigated 4 days after a single intratracheal instillation of bleomycin and from control rats. The first group (n=8) received only bleomycin. The second group (n=7) was also given prednisolone, 15 mg·kg⁻¹ bw daily. Control groups, without (n=4) or with prednisolone (n=4) are also illustrated.

bleomycin-induced water increase (fig. 1). In the bleomycin injured animals the relationship between increases in total lung HA and the relative water content, with (r=0.87) or without steroid treatment (r=0.89) were similar.

BAL levels and molecular weight of HA

There was no difference in lavage levels of HA between sham-treated control animals with (n=4) or without (n=4) steroid treatment, the (mean±SEM) concentrations being 6.9±2.6 and 4.6±0.7 $\mu g \cdot l^{-1}$, respectively (fig. 2). Four days after bleomycin administration the BAL-recovery of HA was analysed. The lavage HA levels levels increased significantly (p<0.001), compared to control animals, in bleomycin injured animals with (n=7; 462±43 $\mu g \cdot l^{-1}$) or without (n=8; 360±79 $\mu g \cdot l^{-1}$) steroid treatment (fig. 2). No significant difference between the two bleomycin groups was found. Lavage urea levels, as a measurement of plasma leakage into the alveolar space, increased 2.5–3.5 times in bleomycin treated animals compared to control animals, irrespective of steroid treatment (mean±SEM urea levels were 0.2±0.06 and 0.2±0.07 $mmol \cdot l^{-1}$ in control animals with and without steroid treatment, respectively; 0.7±0.4 and 0.5±0.05 $mmol \cdot l^{-1}$ in bleomycin injured animals with and without steroid treatment, respectively) (fig. 2). The molecular weight of HA recovered during lavage was on average $0.26 \cdot 10^6$ daltons in bleomycin treated animals and was not influenced by corticosteroid therapy (MW $0.27 \cdot 10^6$ daltons).

BAL cell populations

Compared with control rats, the total cell counts as well as the relative and absolute numbers of eosinophils, neutrophils and lymphocyte cells in BAL fluid were significantly (p<0.001) increased in bleomycin injured rats. High percentages of eosinophils, 23–46%, were observed in all bleomycin treated animals. The relative and absolute numbers of macrophages decreased significantly (p<0.001) after bleomycin administration. Steroid treatment of bleomycin injured rats had no significant effect on any of the cell variables in BAL fluid (fig. 3).

Discussion

In this study we have shown that high-dose corticosteroid treatment does not influence the considerable accumulation of HA in the rat lung tissue following experimental alveolitis induced by bleomycin. The lung damage observed after a single intratracheal dose of bleomycin is characterized by an inflammatory interstitial reaction within days and a gradual fibrotic repair that is apparent within the first two weeks [22, 23]. Recently we were able to demonstrate histologically that large amounts of HA accumulate in the alveolar interstitial

tissue during the alveolitis phase in the bleomycin model [14]. We have also shown that the HA content in BAL and lung tissue, determined biochemically, increases from Day 1 after bleomycin administration to peak concentrations on Days 3–7. Thereafter it declines progressively to normal concentrations by Days 21–30 [16]. Thus, HA accumulation precedes collagen deposition that does not occur until the second week and further increases during the first 30–60 days [22, 23].

In this study we measured the BAL and lung tissue content of HA on Day 4 after bleomycin administration and could confirm the increase in HA concentrations observed previously. The lack of effect of corticosteroids on this early connective tissue response is surprising for a number of reasons. Firstly, it is well established that various inflammatory mediators *in vitro* may stimulate HA synthesis by mesenchymal cells [24–27]. Secondly, there is a distinct correlation and similar time dependence between the HA accumulation in lung tissue and the inflammatory reaction induced by bleomycin [15]. Thirdly, *in vitro* studies have shown that corticosteroids decrease HA production by mesenchymal cells [28, 29] but, in order to suppress HA production *in vitro*, 100 times higher corticosteroid concentrations are needed for human lung fibroblast-like cells than for skin fibroblast-like cells [30]. However, there is so far no direct evidence that the observed accumulation of HA is of mesenchymal cell origin, also rat lung endothelial cells are capable of synthesizing HA [31] and the appearance of HA in high concentrations in the alveolar space might indicate an epithelial (type II cell) origin.

The present findings suggest that the inflammatory process, related to the enhanced HA synthesis in this model, is not influenced by corticosteroids. Although we used quite high steroid doses, the inflammatory activity of the lung as reflected by the increased recovery of eosinophils, neutrophils and lymphocytes was not influenced by the treatment. Thus, the early intensive alveolitis phase of bleomycin lung injury may represent an inflammatory lung tissue reaction that is not influenced by steroid sensitive regulatory mechanisms. Using the same model, previous investigators have shown that collagen synthesis and total collagen deposition in bleomycin injured lung are reduced by steroids given in doses similar to ours [32]. These different effects of steroids suggest separate regulatory mechanisms for enhanced HA and collagen synthesis, as also indicated by the different kinetics for HA and collagen accumulation in the alveolar interstitium. Further support for the existence of different regulatory mechanisms of the inflammatory and fibrotic part of the bleomycin injury was recently demonstrated by CHANDLER *et al.* [33], who showed that the production of iron-catalysed oxygen-radicals by bleomycin is related to fibrosis in this model. Increased collagen deposition could be inhibited by making hamsters iron deficient prior to bleomycin instillation. However, iron deficiency did not affect the inflammatory component of the lung injury, as measured by morphometric analysis of lung sections or as the lung deoxyribonucleic acid (DNA) content, though no analysis of HA or lung water was performed in this study.

Thus, HA accumulation could be independent of development of fibrosis, since most patients with single episodes of farmer's lung and ARDS and high lavage HA levels [9, 11] do not develop fibrosis and in the bleomycin model corticosteroids have no effect on HA accumulation but influence the later development of fibrosis. Conversely, it is not yet clear what role early accumulation of HA has for the subsequent development of fibrosis.

In wound healing [34] and embryonic development [35–38] early accumulation of highly-hydrated HA is supposed to form a milieu that enhances cell migration and the formation of matrix essential for tissue repair or development. Thus, an accumulation of HA may in these situations be a normal physiological reaction. However, such an accumulation in the fragile alveolar wall may contribute to the interstitial oedema and, thereby, to impaired gas diffusion [14, 16]. We have previously demonstrated a close relationship between accumulation of HA in the alveolar tissue and an increase in lung water [16]. The increases in both HA and the relative lung water content are of the same magnitude, compared to controls, in this study and the administration of corticosteroids did not influence the increase in lung water in lungs injured by bleomycin.

The present observations merit some comments on the clinical experience of corticosteroids in interstitial lung diseases. Up to 10% of patients treated with high doses of bleomycin for neoplastic disorders develop a severe interstitial pneumonia with a high mortality rate [39–42]. Although the condition is sometimes reversible, spontaneously or during corticosteroid treatment, those who survive the initial pneumonitis often subsequently develop pulmonary fibrosis and late respiratory death. WHITE and STOVER [42] have demonstrated that treatment with high doses of corticosteroids resulted in significant clinical and radiographic improvement, but pulmonary function tests remained abnormal and it was necessary to prolong therapy over many months to avoid relapses. The only patient with bleomycin-induced pneumonitis in the acute stage, unsuccessfully treated with high doses of steroids, studied with BAL in our hospital had a very high lavage HA level (unpublished data). Steroids are also recommended in other conditions with severe interstitial inflammatory oedema, such as ARDS, sarcoidosis and allergic alveolitis [1, 2, 43, 44]. The general concept is that steroids have an effect in these disorders by reducing the inflammatory processes of the lung. It is uncertain whether steroids influence the lung synthesis of HA in these conditions. However, we have reported that BAL fluids from patients with ARDS and farmer's lung contain high concentrations of HA, linked to impaired gas diffusion [9, 11]. In patients with idiopathic pulmonary fibrosis (IPF) we have also found elevated levels of HA in BAL [12], although not of the same magnitude as observed in ARDS and allergic alveolitis. Patients with active and progressive IPF are characterized by significantly higher HA levels than those who have a stable disease [12]. High doses of corticosteroids are also recommended in IPF but only around one third of the patients seems to respond to this therapy [1–3,

45–49]. Thus, the clinical experience of interstitial fibrotic lung diseases would suggest that the effects of corticosteroids on the inflammatory connective tissue response are highly variable and new improved, therapeutic principles are urgently needed [50].

We suggest, that the lack of effect of steroids on the accumulation of HA in the lung in bleomycin-induced alveolitis might reflect an inflammatory mechanism resistant to systemic high-dose corticosteroids. This could also be the case in certain interstitial lung diseases in man. We propose that HA accumulation in the alveolar tissue is a major factor behind interstitial alveolar oedema and possibly also of importance for the subsequent development of fibrosis. In order to test these hypotheses, other therapeutic principles which may reduce the accumulation of lung HA, either by influencing its synthesis or elimination, are needed.

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Les corticostéroïdes à haute dose, au cours d'une alvéolite induite par la bléomycine chez le rat, ne suppriment pas l'accumulation d'hyaluronan (acide hyaluronique) dans le tissu pulmonaire. O. Nettelbladt, A. Tengblad, R. Hällgren.

RÉSUMÉ: Une injection intra-trachéale unique de bléomycine chez les rats, a provoqué, 4 jours après, une accumulation considérable de hyaluronan (hyaluronate, acide hyaluronique) dans le tissu pulmonaire. Cette réaction du tissu conjonctif a été quantifiée biochimiquement par l'analyse de l'hyaluronan (HA) dans le liquide de lavage broncho-alvéolaire (BAL) et dans les extraits de tissu pulmonaire. Le poids moléculaire de HA recueilli pendant le lavage est de 0.2–0.3×10^6 daltons. L'accumulation de HA est en relation avec une augmentation du contenu d'eau pulmonaire et est associée à un influx accru d'éosinophiles, de neutrophiles et de lymphocytes dans le liquide du BAL. Un traitement par corticostéroïdes à haute dose (15 mg·kg⁻¹ de prednisolone par jour), donné aux rats soumis à la bléomycine, n'a pas d'effet sur le contenu du tissu pulmonaire en HA, sur la récolte de HA au cours du lavage alvéolaire, ou sur le poids moléculaire du HA accumulé dans l'espace alvéolaire. De plus, les stéroïdes n'ont pas eu d'influence sur l'augmentation du contenu aqueux du poumon, ni sur l'apparition de cellules inflammatoires dans le liquide de lavage. Ces observations indiquent que la réponse précoce du tissu conjonctif à l'agression par la bléomycine est médiée par un mécanisme que n'est pas influencé par les fortes doses de corticostéroïdes par voie systémique.
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