Accumulation of hyaluronan in bronchoalveolar lavage fluid is independent of iron-, complement- and granulocyte-depletion in bleomycin-induced alveolitis in the rat

O. Nettelblad*, K. Lundberg***, A. Tengblad***, R. Hälgren**

ABSTRACT: Previous studies on bleomycin-induced alveolitis in rats have demonstrated a transient histological accumulation of hyaluronan (hyaluronate or hyaluronic acid) in the alveolar interstitium, corresponding to increases in hyaluronan (HA) levels in bronchoalveolar lavage (BAL) fluid and lung tissue extracts. The accumulation of HA was related to the influx of inflammatory cells, especially polymorphonucleated cells (PMNs) in BAL fluid and the increase in lung water. In this study we have investigated the influence of iron, complement and PMN dependent mechanisms on the early connective response of the lung in the bleomycin rat model. Iron depletion had no effect on HA or the cellular composition of lavage fluid recovered on day 4 post bleomycin. Treatment of bleomycin-injured rats with cobra venom factor (CVF), totally neutralized complement haemolytic activity but had no effect on lavage HA or the cell invasion in BAL. Treatment with anti-neutrophil serum (ANS), reduced blood and lavage PMN by 70–80%, but had no influence on HA levels in BAL. These results suggest that regulatory mechanisms other than those dependent on iron, complement activation or PMN recruitment are responsible for HA accumulation in this fibrosing alveolitis animal model.

Clinical observations have established that hyaluronan (hyaluronate or hyaluronic acid by older nomenclature) (HA) appears in bronchoalveolar lavage fluid (BALF) from patients with various inflammatory interstitial lung diseases [1-5]. A link between the lavage recovery of hyaluronan and disease activity [1, 2, 5] and possibly also prognosis [5] has been proposed. The correlation between increased lavage concentrations of HA and the appearance of increased numbers of inflammatory cells in BAL has suggested that inflammatory mechanisms stimulate lung tissue cells to an enhanced HA synthesis [2, 3, 5]. HA is an important component of connective tissue and may be of importance not only for cell differentiation during embryonic development [6, 7] but also for tissue repair [8].

Studies on bleomycin-induced alveolitis have demonstrated that HA accumulates in the edematous alveolar septa at an early stage [9]. A single intratracheal instillation of bleomycin induces a progressive increase of HA in the lung tissue [10] and a concomitant increase in BALF [11]. The maximum HA increase is seen 4-7 days after bleomycin administration whereafter the HA levels decline [9-11]. Since the kinetics of HA accumulation parallels the inflammatory phase in this model, including an influx of polymorphonucleated cells (PMNs) in BALF [11], it is proposed that HA accumulation is a consequence of the bleomycin-induced inflammatory reaction. In vitro studies have also demonstrated that various inflammatory mediators are capable of stimulating fibroblasts to synthesise HA [12-14]. Other studies on the bleomycin model have shown that the increased collagen deposition is iron dependent and could be reduced by either iron deficiency [15] or desferroxamine treatment [16]. In the same model, complement depletion reduces both collagen deposition [17] and synthesis [18], whereas neutrophil-depleted animals develop a more severe fibrotic lesion [17] with increased rate of collagen synthesis [19]. The purpose of this study was to establish whether or not corresponding manipulations, in the same model, also influence the early connective tissue response of the lung as reflected by the appearance of HA in BALF.
Material and methods

Animals

Adult male Sprague-Dawley inbred rats (ALAB, Sollentuna, Sweden) weighing 190–205 g at the time of tracheostomy were used in this study. All rats were kept in separate cages and food and water provided ad libitum. Animals were sacrificed by aortic exsanguination under chloral hydrate anaesthesia.

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, Denmark) in 0.3 ml sterile saline under chloral hydrate anaesthesia. All rats received 10 mg sodium cefuroxim (Glaxo, Greenford, Middlesex, England) intraperitoneally immediately before and 1 day after tracheostomy in order to minimize the risk of bacterial infections. Sham treated control animals received 0.3 ml of sterile saline intratracheally in the same manner as the bleomycin treated rats.

Iron depletion

Iron deficiency was produced according to the techniques used by Chandler et al. in hamsters [15] with slight modifications. Rats (initial weight 80 g) were kept on a iron deficient diet (<15 mg iron·kg⁻¹ obtained from Ewos AB, Järna, Sweden) for 3 wks and bled twice from the retro-orbital venous sinus during a 2wk period (1.5 ml per bleeding). No bleeding or other operations were performed the week before bleomycin administration. Microhaematocrits were determined before the experiment, at each bleeding and when the rats were sacrificed. Serum iron was analysed at the time of sacrifice.

Complement depletion

Cobra venom factor (CVF) from Naja naja kaoutia was prepared as described by Egerstsen et al. [20]. Approximately 50 units of CVF (180 µg) was given as a single intravenous injection, 2 h prior to bleomycin administration. The total complement haemolytic activity (CH50) [21], was measured on blood collected immediately before the administration of CVF (-2 h), at the time for bleomycin administration (0 h) and at sacrifice on day 4.

PMN depletion

Rabbit anti-rat neutrophil serum (ANS) was prepared as described by Sandler et al. [22], according to the principles outlined by Simpson and Ross [23, 24]. In short, rabbits were injected with rat neutrophils every eight weeks and blood was collected twice between each booster dose, whereafter sera from different rabbits were pooled. The ANS obtained was precipitated three times with NH₄SO₄ and repeatedly dialyzed against saline. The ANS was not absorbed with red cells, serum or lymphocytes as this procedure has been shown to reduce anti-neutrophil activity as much as anti-lymphocyte activity [22]. Rats were given 0.2 ml·100 g⁻¹ body weight (BW) of ANS intraperitoneally 12 h prior to bleomycin administration and 0, 24 and 48 h after bleomycin. The numbers of circulating leucocytes, polymorphonucleated cells (PMNs) and mononucleated cells (MNs) were counted in a Bürker chamber (Türks staining), before each injection and at death.

Experimental design

The animals were divided into 10 groups (tables 1 and 2). Bleomycin-injured rats and sham treated controls were investigated 3 and 4 days after a single intratracheal instillation of bleomycin and saline, respectively. Previous studies have established that the maximum HA response of the lung injured by bleomycin occurs on days 3–5. The effect of iron-depletion and treatment with cobra venom factor was studied in bleomycin and sham treated animals on day 4. The effect of treatment with antineutrophil serum was studied in bleomycin and sham treated rats on day 3 in order to avoid immune reactions to ANS, which started to appear on day 4 in our preliminary studies.

Bronchoalveolar lavage

BAL was performed as described previously [11, 25]. 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 hydrostatic pressure of 25 cm. After 3 min the fluid was recovered by gravity. The recovery was on average 21.2±0.3 (SEM) ml (84.8±1%). The lavage fluid was centrifuged at 400 g for 10 min. The supernatant was kept frozen at -20°C until analysed.

Lavage cell analysis

The cell pellet was resuspended in 1 ml PBS and counted after staining in a Bürker chamber and total cells were counted. After another washing in PBS the cell suspension was adjusted to a concentration of 10⁶ cells·ml⁻¹. Preparations for cytoclogic studies were made in a cytospin centrifuge (Cytospin Shandon, Southern Products Ltd, Runcorn, England) at 750 rpm and were stained with May-Grünwald-Giemsa before differential counting.
Table 1. — The effects of iron deficiency and complement depletion (cobra venom factor CVF treatment) on different BAL variables* 4 days after an intratracheal injection of bleomycin in rats. Control rats were given only NaCl intratracheally.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>HA (µg/l)</th>
<th>Urea (mmol/l)</th>
<th>Total cells (x10⁶/lavage)</th>
<th>Macrophages (x10⁶/lavage) (%)</th>
<th>Eosinophils (x10⁶/lavage) (%)</th>
<th>Neutrophils (x10⁶/lavage) (%)</th>
<th>Lymphocytes (x10⁶/lavage) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleomycin</td>
<td>11</td>
<td>277±52</td>
<td>0.50±0.05</td>
<td>3.1±0.4</td>
<td>0.40±0.1**</td>
<td>0.57±0.1*</td>
<td>1.3±0.2**</td>
<td>0.86±0.1***</td>
</tr>
<tr>
<td>Bleomycin iron-deplete</td>
<td>15</td>
<td>228±40</td>
<td>0.55±0.1</td>
<td>2.8±0.3***</td>
<td>0.44±0.1</td>
<td>0.39±0.1*</td>
<td>0.92±0.1***</td>
<td>1.1±0.1***</td>
</tr>
<tr>
<td>Bleomycin+ CVF</td>
<td>10</td>
<td>216±57</td>
<td>0.38±0.05</td>
<td>2.8±0.4***</td>
<td>0.28±0.04***</td>
<td>0.60±0.2**</td>
<td>0.85±1***</td>
<td>0.98±2**</td>
</tr>
<tr>
<td>NaCl</td>
<td>3</td>
<td>5±2</td>
<td>0.40±0.2</td>
<td>0.8±0.1</td>
<td>0.38±0.1</td>
<td>0.01±0.01</td>
<td>0.02±0.02</td>
<td>0.02±0.01</td>
</tr>
<tr>
<td>NaCl iron-deplete</td>
<td>3</td>
<td>5±0.5</td>
<td>0.23±0.1</td>
<td>0.8±0.2</td>
<td>0.38±0.1</td>
<td>0.01±0.01</td>
<td>0.04±0.02</td>
<td>0.02±0.01</td>
</tr>
<tr>
<td>NaCl+ CVF</td>
<td>3</td>
<td>7±1</td>
<td>0.30±0.06</td>
<td>1.1±0.2</td>
<td>0.50±0.2</td>
<td>0.02±0.01</td>
<td>0.04±0.01</td>
<td>0.02±0.01</td>
</tr>
</tbody>
</table>

*mean±SEM; **p<0.05; ***p<0.01; ****p<0.001 compared to sham treated controls; t: p<0.05 compared to rats receiving only bleomycin; HA: hyaluronan.

Table 2. — The effects of neutrophil-depletion by treatment with antineutrophil serum (ANS) on different BAL variables* 3 days after an intratracheal injection of bleomycin in rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>HA (µg/l)</th>
<th>Urea (mmol/l)</th>
<th>Total cells (x10⁶/lavage)</th>
<th>Macrophages (x10⁶/lavage) (%)</th>
<th>Eosinophils (x10⁶/lavage) (%)</th>
<th>Neutrophils (x10⁶/lavage) (%)</th>
<th>Lymphocytes (x10⁶/lavage) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleomycin</td>
<td>10</td>
<td>136±27</td>
<td>0.51±0.07</td>
<td>3.0±0.5</td>
<td>0.26±0.04***</td>
<td>0.20±0.06*</td>
<td>1.2±0.2***</td>
<td>1.4±0.2***</td>
</tr>
<tr>
<td>Bleomycin+ ANS</td>
<td>10</td>
<td>135±35</td>
<td>0.50±0.07</td>
<td>1.2±0.1**</td>
<td>0.20±0.03***</td>
<td>0.026±0.04***</td>
<td>0.352±0.04***</td>
<td>0.75±0.06***</td>
</tr>
<tr>
<td>NaCl</td>
<td>3</td>
<td>12.7±1</td>
<td>0.27±0.03</td>
<td>0.8±0.1</td>
<td>0.8±0.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NaCl+ ANS</td>
<td>3</td>
<td>9.1±0.1</td>
<td>0.30±0.06</td>
<td>1.1±0.2</td>
<td>1.0±0.2</td>
<td>0.02±0.01</td>
<td>0.04±0.01</td>
<td>0.02±0.01</td>
</tr>
</tbody>
</table>

Differential cell counts given both as total and relative (%) numbers. Control rats were given only NaCl intratracheally. *: mean±SEM; **p<0.05; ***p<0.01; ****p<0.001 compared to sham treated controls; t: p<0.05 compared to rats receiving only bleomycin; HA: hyaluronan.

**Determination of HA**

HA was analysed in duplicate in lavage fluid and serum according to the principles outlined previously [26] using a slightly modified technique [11, 25], developed by Pharmacia Diagnostics, Uppsala, Sweden.

**Statistical analysis**

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

**Results**

The BALF levels of HA in all sham treated control animals were equal to or below the detection limit (5 µg/l). Iron depletion or treatment with cobra venom factor or ANS had no influence on the lavage recovery of HA in controls (tables 1 and 2). In bleomycin-injured animals (without additional treatment), lavage HA values were 25–30 times higher than those in the sham-treated rats on day 3 (p<0.001) and reached even higher levels on day 4 (tables 1 and 2).

The total recovery of cells increased significantly after bleomycin treatment (tables 1 and 2). BAL fluid from the sham treated controls consisted mainly of alveolar macrophages (93–98%). Less than 1% of the cells were neutrophils or eosinophils. The distribution of lavage cells in the controls was not influenced by iron, complement or granulocyte depletion. After bleomycin treatment, the relative numbers of macrophages decreased whereas a marked increase in the numbers of neutrophils, eosinophils and lymphocytes was seen (tables 1 and 2).
The effect of iron depletion on bleomycin-injured lung

The haematocrit of the sham-treated controls was 38±0.5 (SEM)% and in bleomycin treated controls was 45±1%. The corresponding values in iron-depleted, sham-treated controls and bleomycin-injured rats were 19±0.9% and 29±1%, respectively. Serum iron was 42.5±2.4 (SEM) mmol·l⁻¹ in untreated controls (n=3) and 31.9±2 mmol·l⁻¹ in rats receiving only bleomycin. In iron depleted rats given saline (n=3) or bleomycin (n=15) intratracheally, serum iron was significantly reduced to 6.2±1 (p<0.001) and 5.9±4 mmol·l⁻¹ (p<0.001) respectively.

The bleomycin-induced increase in the HA concentrations in BAL fluid was not influenced by iron depletion (table 1). The recovery of cells by lavage was not influenced by iron deficiency (table 1).

The effect of ANS on bleomycin-injured lung

Treatment with ANS initially induced (12 h after the first injection) a 95% reduction in the circulating PMN counts. On the following days the PMNs started to increase and by day 3 were, on average, 25% of the control values. For the whole observation period during treatment with ANS (days 0–3), the average PMN counts were 0.32±0.05 per cell (17% of the control value). Blood mononuclear leucocytes were also slightly reduced by ANS and were 88% of the control values by day 3. ANS induced a significant reduction (p<0.001) in the recovery of total cells in bleomycin-injured lung. The numbers of neutrophils and eosinophils were reduced by 71 (p<0.01) and 87% (p<0.05), respectively (table 2, fig. 1). A reduction in recovered lymphocytes was also seen. However, ANS treatment did not affect HA levels in BAL (table 2, fig. 1).

Lavage urea levels were increased from 0.2-0.3 mmol·l⁻¹ in the control groups to 0.38-0.55 in the groups that received bleomycin (tables 1 and 2). The difference between the relative increases in lavage HA and urea thereby excludes plasma leakage as a main source of lavage HA.

Discussion

Bleomycin-induced lung damage in the rat offers a model of acute alveolitis accomplished by interstitial oedema and a repair phase with fibrosis [27, 28]. This model shows similarities with the development of fibrotic lesions in humans. Therefore, the pathophysiological mechanisms regulating the connective tissue response after bleomycin injury have been extensively studied. The majority of studies have focused on the deposition of collagen in the lung, while the early remodelling of the extracellular matrix in injured lung has been less studied. However, recently we reported the early accumulation of HA in the alveolar interstitial tissue [9] after bleomycin injury and described the kinetics of the significant, but transient, appearance of HA in lung tissue extracts [10] and lavage fluid [11]. In an earlier paper we have proposed that the observed accumulation of HA, a glycosaminoglycan with unique water-binding capacity, is related to the interstitial and alveolar oedema during the acute alveolitis phase of the bleomycin-injured lung damage [10]. In this study we have elucidated the influence of certain inflammatory mechanisms on the early connective tissue response of the bleomycin-injured rat lung.

It has been proposed that bleomycin-induced pulmonary fibrosis is dependent on peroxidation of lung tissue by iron-dependent hydroxyl radicals [29]. In our study we found that severe iron depletion had no effect on the bleomycin-induced accumulation of HA in the alveolar space. In contrast, Chandler et al. have shown that mild depletion of iron [15] or treatment with the iron-chelator desferoxamine [16] induced a reduction in collagen accumulation in bleomycin-injured lungs of the hamster. They also reported, in accordance with our
The demonstration of eosinophil granular proteins in BAL fluid [46] has provided further support for the involvement of eosinophils in IPF. Eosinophils may induce damage by releasing cytotoxic granular proteins and also induce fibroblast proliferation [46]. We have previously observed in bleomycin treated rats a strong time-dependent correlation between the appearance of polymorphonuclear cells in BAL fluid and the increased recovery of HA during lavage [11]. In this study we observed a significant depletion of neutrophil as well as eosinophil granulocytes by an antineutrophil serum. Nevertheless, the recovery of HA was unchanged. In the bleomycin model, Thrall et al. have demonstrated that PMN depletion induces a transient increase in the total lung collagen content [17] and Clark and Kuhn have extended this finding by demonstrating that PMN depletion increases the lung collagen synthesis rate [19]. Thus, PMNs may regulate the fibrotic response by interference with collagen synthesis as well as by a possible reduction in PMN collagenase activity.

Previous observations on the bleomycin rat model, have shown differences in the kinetics of the appearance of HA and collagen in the alveolar interstitial tissue [9-11, 26, 27], as well as a disparity in the response to high dose steroids which decrease lung collagen [47] but not lung or lavage HA concentrations [48]. In this study, further differences between mechanisms regulating the lung accumulation of HA and collagen have emerged. These findings are compatible with different regulatory mechanisms for the early and late connective tissue responses induced by bleomycin. Such a difference is also indicated by previous clinical observations, where the appearance of increased concentrations of HA or procollagen III aminopeptide, supposedly fibroblast products, in BAL fluid, seemed to reflect a transient fibroblast activation rather than an increased fibroblast mass [2, 48]. Thus, the early accumulation of HA in lavage fluid during the acute phase of the bleomycin injury may reflect a transient fibroblast activation, independent of later collagen deposition, in the interstitial tissue. This may explain why agents known to alter the collagen deposition in the lung have no effects on early fibroblast activity following acute injury. At present the mechanisms responsible for the HA accumulation in the alveolar tissue in the bleomycin model are unknown. Although the lack of effects of iron, complement or PMN depletion, as performed in this study, does not fully exclude the possibility that these principles are involved in the increase in BAL fluid HA seen after bleomycin injury, the results suggest that other regulatory mechanisms are operative. It is also conceivable that bleomycin itself induces lung cells to synthesize HA [49] whereas collagen synthesis is more dependent on the local inflammatory activity.

findings, that iron depletion did not prevent the invasion of inflammatory cells in the BAL fluid. These authors suggest that the effect of iron-depletion on lung collagen may be due to a reduction in the production of free radicals. If so, the results available indicate that the early connective tissue response of the lung as reflected by hyaluronan accumulation is independent of radical formation unlike the later collagen response. However, iron deficiency may alter collagen metabolism by mechanisms other than mediated by free radicals. Iron is a cofactor essential for the prolyl hydroxylase [30] and iron depletion might result in insufficient hydroxylolation of collagen with subsequent instability of the triple helical structure [31] and decreased net production of collagen [32]. Furthermore, lymphocyte depletion affects collagen accumulation in the bleomycin model [33]. The proliferation of lymphocytes could be regulated by iron in vitro [34] and inhibited by hydroxyl radical scavengers [35].

The complement system may be involved in many inflammatory processes both as a mediator of the inflammatory process and by direct cytolytic activity. In the bleomycin model, activated complement components may be of importance in recruiting e.g. granulocytes and macrophages to the inflammatory site in the lung. Certain complement components are also reported to display an activity that resembles the action of the family of insulin-like growth factors [36]. Thus, activated complement components may directly or indirectly influence the connective tissue response. There is a paucity of information concerning the possible role of complement in human interstitial fibrosis. However, in adult respiratory distress disease the activation of complement is a prominent finding [37]. Furthermore, the degree of complement activation in this disease correlates with the increased HA levels in BAL fluid [4].

In the present bleomycin model, complement depletion by cobra venom factor had no effect on the HA accumulation in the alveolar space. In contrast, it was reported earlier that complement-depletion in bleomycin-injured animals transiently reduced the deposition of collagen [17], possibly by reducing collagen synthesis [18]. Furthermore, in our study, complement depletion had no effect on the lavage recovery of inflammatory cells suggesting that the recruitment of these cells in the initial inflammatory process as well as the early connective tissue response are complement independent in the bleomycin model. However, in this context the possibility that active complement components may in fact be generated by cobra venom factor has to be considered [38].

Neutrophils appear in increasing numbers in the alveolar interstitial tissue and in BAL in both human idiopathic pulmonary fibrosis (IPF) [5, 39, 40] and in the bleomycin model [11, 17, 19]. The neutrophils may damage the alveolar wall by releasing tissue-damaging substances, such as oxygen radicals [41, 42] and proteolytic enzymes [43]. The appearance of increased numbers of eosinophils is also seen in lavage fluid in IPF [44-46] and may reflect progressive lung disease. The demonstration of eosinophil granular proteins in BAL fluid [46] has provided further support for the involvement of eosinophils in IPF. Eosinophils may induce damage by releasing cytotoxic granular proteins and also induce fibroblast proliferation [46]. We have previously observed in bleomycin treated rats a strong time-dependent correlation between the appearance of polymorphonuclear cells in BAL fluid and the increased recovery of HA during lavage [11]. In this study we observed a significant depletion of neutrophil as well as eosinophil granulocytes by an antineutrophil serum. Nevertheless, the recovery of HA was unchanged. In the bleomycin model, Thrall et al. have demonstrated that PMN depletion induces a transient increase in the total lung collagen content [17] and Clark and Kuhn have extended this finding by demonstrating that PMN depletion increases the lung collagen synthesis rate [19]. Thus, PMNs may regulate the fibrotic response by interference with collagen synthesis as well as by a possible reduction in PMN collagenase activity.
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