Eur Respir J
1991, 4, 407–414

Alveolar accumulation of hyaluronan and alveolar cellular response in bleomycin-induced alveolitis

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ABSTRACT: Hyaluronan (HA) accumulating in the alveolar interstitial tissue of rats injured by a single intratracheal instillation of bleomycin has been visualized histologically and assayed. HA was present already by Day 1 after bleomycin treatment, increased to a maximum value on Days 3 and 7 and then declined.

A time-dependent relationship between this early connective tissue response and the invasion of inflammatory cells in the alveolar tissue was apparent. The dominating invading cells by Day 1 were granulocytes showing positive staining for the monoclonal antibody OX-42 reflecting the C3b receptor. The numbers of macrophages expressing class II antigens started to increase on Day 1, reaching a maximum on Days 3–7 and then declined. Macrophages were the dominating OX-42+ cells by Day 7. The appearance of W3/13+ cells ("pan-T-lymphocytes") showed a similar pattern to that for the class II expressing macrophages. The number of cells expressing CD4 antigen increased until Day 3 and levelled off on Day 30 whilst the largest number of cells expressing CD8 antigen was seen on Day 30. Few cells expressing B-cell phenotype outside lymph nodules were identified. Alveolar lining epithelial cells, probably epithelial type II cells, expressed class II antigens by Days 3–14.

The time-related accumulation of HA and the appearance of T-cells, macrophages and granulocytes expressing signs of activation suggests that these cells may be involved in the early connective tissue response of the lung injured by bleomycin.

Eur Respir J., 1991, 4, 407–414.

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Keywords: Alveolar epithelial cells; alveolitis; bleomycin; granulocytes; hyaluronan; immunoperoxidase staining; lung tissue extracts; lymphocyte populations; macrophages; pulmonary fibrosis.

Received: March 1990; accepted after revision October 10, 1990.

Dr A. Tengblad sadly died before the publication of this paper.

This study was supported by grants from the Swedish Heart Lung Foundation, the Swedish Cancer Society, the Pharmacia Research Foundation and AB Draco, Lund, Sweden, and the Swedish Work Environment Fund.

Hyaluronan (hyaluronate or hyaluronic acid by older nomenclature) is an important constituent of the loose connective tissue [1–3]. It is produced by all mesenchymal cells but especially by activated fibroblasts [4]. Hyaluronan (HA) is involved in processes of repair [5] and plays a central role in embryonic development [6, 7]. Hyaluronan is, apparently, also involved in the regulation of inflammatory events [8–11] and its synthesis is regulated by mechanisms associated with immune responses [12–14]. Recent clinical studies have demonstrated increased concentrations of HA in bronchoalveolar lavage fluid (BAL) from patients with various interstitial lung diseases [15–19]. Its appearance in lavage fluid has been proposed as a marker of lung disease activity but may also reflect a role in lung physiology [17–18].

In the early phase of bleomycin-induced lung injury, there is a considerable but transient accumulation of HA in the alveolar space [20–22]. Indirect support has been adduced that the unique water-binding properties of HA [23] may contribute to the interstitial/alveolar oedema in clinical [17, 18] and experimental [22] alveolitis. The mechanisms underlying the lung accumulation of HA have not been identified but previous studies have indicated that inflammatory/immunological mechanisms are involved [16–19, 21].

In the present study in rats, we have performed immunohistochemical studies of the lung tissue during the early phase of the bleomycin-induced lung alveolitis in order to characterize the kinetics of the cellular responses of lymphocytes, macrophages, granulocytes and lung epithelial cells in relation to the accumulation of HA in the alveolar tissue.

Materials and methods

Animals. Adult male Sprague-Dawley inbred rats (ALAB, Sollentuna, Sweden), weighing 190–205 g, were used in this study. Animals were kept in separate cages and food and water provided *ad libitum*.

Induction of bleomycin-induced pulmonary injury. Tracheostomies were performed on all animals to

facilitate the intratracheal injection of 1.5 mg bleomycin sulphate (Bleomycin®, Lundbeck, Copenhagen, DK) in 0.3 ml sterile saline under chloralhydrate (360 mg·kg^{-1} BW) anaesthesia. Control sham-treated animals received 0.3 ml of sterile saline intratracheally in the same manner. All animals were sacrificed by aortic exsanguination under chloralhydrate anaesthesia before isolating the lung tissue preparations. In order to minimize the risk for bacterial infections, all rats received 10 mg sodium cefuroxime (Zinacef®, Glaxo, Greenford, Middlesex, UK), intraperitoneally, immediately before and 1 day after tracheostomy.

Lung tissue preparations. Bleomycin treated rats (n=3 at each time point) were sacrificed after 1, 3, 7, 14 and 30 days. Sham-treated (n=3) or untreated control rats (n=2) were sacrificed after 0 (=untreated), 7 and 30 days. Sections were made from the left lung. All preparations were made immediately after death. The lungs were removed en bloc and dissected free. The right lung was ligated at the hilus and freeze dried. The dried lung was pulverized in a mortar. The homogenized lung tissue samples were kept dry and frozen at -20°C until analysed for HA content. The left lung was infused under gravity with Histocon®) (Histolab, Gothenburg, Sweden) at 4°C at a constant hydrostatic pressure of 25 cm until it reached normal inflated size. Thereafter the specimens were snap frozen in chilled isopentane and stored at -70°C.

Extraction of lung tissue hyaluronan. The HA was extracted from the pulverized dried lung with 0.5 M NaCl. Twenty mg of the lung material was extracted with 2 ml of buffer 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. We have demonstrated previously that this extraction procedure gives similar results to those obtained using extraction with guanidine chloride, trypsin digestion and heat inactivation in order to destroy any proteoglycan or link protein that might interfere with HA [22].

Analysis of hyaluronan. The concentration of HA in tissue extract was determined in duplicate with a radiometric assay (HA-50-test, Pharmacia diagnostics, Uppsala, Sweden), as described previously [22, 24, 25].

Localization of HA in lung tissue. HA was detected by using an avidin-enzyme, biotin-protein system, based upon the specific interaction between HA and the protein core of a nasal proteoglycan as described previously [20]. However, in this study frozen acetone-fixed sections, as outlined above, were used instead of cetylpyridinium-chloride buffered formalin-fixed paraffin embedded sections. The specificity of the staining was checked by pretreatment of sections with Streptomyces hyaluronidase as described previously [20].

Immunoperoxidase staining of lung sections. Longitudinal acetone-fixed cryostat sections, 6 μm thick, including apex, hilar tissue and the base of the left lung were processed for peroxidase-anti-peroxidase (PAP) staining according to the method of STERNBERGER [26]. The mouse monoclonal antibodies (MoAbs) and secondary antibodies used are given in table 1. Preformed complexes of horseradish peroxidase and monoclonal mouse anti-horseradish peroxidase antibody (diluted 1/500) were obtained from Dakopatts (Copenhagen, Denmark). The peroxidase reaction was developed with

Table 1. – Monoclonal antibodies (MoAbs) used in histological sections of rat lung tissues

MoAb	Ig type (dilution)	Specificity (Reference no.)	Secondary antibody (dilution)
OX-6*	IgG_1 (1/500)	Rat Ia (class II) antigens corresponding to I-A-coded molecules in mice [27]	Rabbit anti-mouse IgG** (1/40)
OX-8***	IgG (1/25)	Rat counterpart of the CD-8 antigen on suppressor-cytotoxic T-cells [28]	" " "
OX-33†	IgG_1 (1/40)	Rat leucocyte-common antigen present only on B-lymphocytes [29]	Goat anti-mouse IgG†† (1/40)
OX-42†	IgG_{2a} (1/320)	Membrane polypeptide (rat homologue of the human iC3b receptor?) of most macrophages (but only 35% of alveolar macrophages), granulocytes and dendritic cells [30]	" " "
W3/13***	IgG (1/50)	A 95 kDa glycoprotein present in large amounts on all T-cells and minor amounts of other cells [31]	Rabbit anti-mouse IgG (1/40)
W3/25***	IgG (1/10)	The counterpart to CD-4 antigen on "helper-inducer" T-cells and some macrophages [29]	" " "

Sources: *: Sera-Lab, Cambridge, UK; **: MIAB, Uppsala, Sweden; ***: produced by hybridomas kindly supplied by A. Williams, Oxford, UK and purified as previously described by LARSSON *et al.* [32]; †: Serotec, Oxford, UK; ††: ATAB®, Scanborough, Maine, USA. IgG: immunoglobulin G.

3-amino-9-ethylcarbazole [33] and the sections were counterstained with Mayer's haematoxylin. The dilutions of the antibodies were determined using sections from normal rat lymph nodes. Controls without the primary antibodies or with irrelevant antibodies gave no staining. Each specimen was also stained with haematoxylin and eosin.

Cytokeratin. The rabbit antibody denoted tissue polypeptide antigen (TPA) (diluted 1/2) reactive with cytokeratins 8, 18 and 19 [34, 35], was obtained from Sangtec Medical, Bromma, Stockholm, Sweden. A swine anti-rabbit immunoglobulin G (IgG) (Dakopatts, Copenhagen, Denmark) diluted 1/200 was used as secondary antibody. In the next step preformed complexes of horseradish peroxidase and rabbit anti-horseradish peroxidase antibody (diluted 1/80, Dakopatts) were used and the peroxidase reaction was developed as described above. A highly differentiated colon adenocarcinoma was used as a positive control for TPA cytokeratin antibodies. Controls with irrelevant antibodies gave no staining.

Evaluation of HA and immunohistochemical staining. Twenty serial consecutive sections were made from the left lung of each animal. Sections with the same serial numbers were used for each MoAb. All sections stained with MoAbs were evaluated using a subjective visual assessment based on a 5 grade semiquantative scale, where - means no detectable staining, (+) only occasional, + small, ++ moderate and +++ large number of positive cells. HA staining was graded as: - = no staining, (x) = small in certain areas, x = positive, xx = positive and strong staining. The histological distribution of positive cells and HA were also described for each section. The general histological pattern was assessed from the haematoxylin and eosin stained sections.

Statistical analysis. Non-parametric Mann-Whitney U test was used to analyse biochemical data. A value of $p<0.05$ was considered as the level of significance.

Results

Histopathology. In control animals there were no signs of septal fibrosis and only a few areas with slight increases in inflammatory cells. In bleomycin-treated animals, interstitial inflammation appeared on Day 1 reaching a maximum between Days 3–7, whereas septal fibrosis developed between Days 14 and 30. Increased numbers of polymorphonucleated granulocytes (PMNs), lymphocytes, eosinophils and macrophages within sparsely distributed areas with septal oedema were apparent already on Day 1. The number of interstitial macrophages further increased, whereas the number of PMNs decreased on Day 3. By Day 7 most of the inflammatory cells were macrophages. Newly developed bundles of proliferating fibroblasts in relation to eosinophils appeared between Day 14 and 30, whereas the numbers of other inflammatory cells, especially PMNs and lymphocytes, declined.

Determination and localization of HA in lung tissue. The extracted amounts of HA from the control lung (n=5) were on average 102±4 (SEM) $\mu g \cdot g^{-1}$ d.w. In bleomycin treated animals the HA lung content increased significantly ($p<0.05$) on Day 1 (139±4; n=3) and reached peak levels by Days 3 (195±25; n=3) and 7 (190±20; n=3). The HA content then declined and was, by Days 14 and 30, 136±23 (n=3) and 116±20 (n=3) $\mu g \cdot g^{-1}$, respectively.

In normal lung sections positive staining for HA was seen in the connective tissues surrounding blood vessels and bronchi. No staining for HA was present in the alveolar structures. After bleomycin injury, faint positive staining for HA was seen by Day 1 in the alveolar interstitium of inflamed alveolar tissue. The intensity and distribution of the HA staining increased on Days 3 and 7 in parallel with the increase in invading inflammatory cells in the swollen interstitial tissue. By Day 14 positive HA staining was seen around the proliferating fibroblasts. No positive HA staining was seen in the alveolar tissue on Day 30. The distribution of HA in normal and injured lung tissue, as well as the quantitative increase seen on Days 1–14 post bleomycin, agreed with previous findings [20, 22].

Immunohistochemical stainings. The data obtained from the immunoperoxidase and HA staining of cryostat rat lung sections are summarized in table 2.

Distribution of antigens recognized by MoAb OX-6. A small number of macrophage and lymphocyte-like cells were expressing class II antigens (OX-6+) in the healthy alveolar tissue (fig. 1a). The staining pattern was not influenced by sham-treatment. In bleomycin injured lungs, OX-6+ interstitial and intra-alveolar cells had increased between Days 1–30. The maximum class II antigen expression was seen on Days 3–7. In areas of intense inflammation and thickened alveolar walls, alveolar lining cells with characteristics of type II pneumocytes (see below) were also reactive with OX-6 antibodies (fig. 1b). This staining pattern was seen on Days 3–14, with maximum on Day 7.

Distribution of antigens recognized by MoAb OX-42. Occasionally single cells in the control lung sections were reactive with the OX-42 antibodies (fig. 2a). After bleomycin administration, an increased number of interstitial OX-42+ cells were seen. By Day 1 the increase of OX-42+ staining was mainly confined to invading granulocytes but also some macrophages showed positive staining at this time. The relative number in OX-42+ macrophages increased progressively during Days 3–7 and was the dominating OX-42+ cell by Day 7. The majority of the OX-42+ macrophages and granulocytes had infiltrated the thickened alveolar septa (fig. 2b). The number of OX-42+ cells decreased after Day 7.

Table 2. – Semiquantitative evaluation of the tissue accumulation of hyaluronan and cells labelled with different monoclonal antibodies in the alveolar tissue

	Semiquantitative grade† of staining†† on Days						Distribution
	0††† (n=5)	1 (n=3)	3 (n=3)	7 (n=3)	14 (n=3)	30 (n=3)	
Hyaluronan staining	-	(x)	xx	xx	x	-	Septal oedematous tissue
Monoclonal antibody							
OX-6	+	++	+++	+++	++	++	Interstitial cells + alveolar macrophages
	-	-	+	++	+	-	Alveolar lining cells
OX-42	-	+++	++	+	(+)	-	Interstitial granulocytes
	(+)	+	++	+++	+	+	Interstitial and alveolar macrophages
W3/13	(+)	+	++	++	+	+	Interstitial cells
	+++	+++	+++	+++	+++	+++	Central cells in lymph nodes
W3/25	(+)	+	++	++	++	+	Interstitial cells
	+++	+++	+++	+++	+++	+++	Central cells in lymph nodes
OX-8	(+)	+	+	+	+	++	Interstitial cells
	+	+	+	+	+	+	Central cells in lymph nodes
OX-33	-	-	(+)	(+)	(+)	-	Interstitial cells
	++	++	++	++	++	++	Cortical cells in lymph nodes

The investigations were performed on frozen sections of rat lung at various days after an intratracheal injection of 1.5 mg bleomycin. †: hyaluronan was visualized by using an avidin-enzyme, biotin-protein system and graded as: - = no staining, (x) = small in certain areas, x = positive, xx = positive and strong staining. Expression of immunoperoxidase staining was graded as: - = no staining, (+) = only occasional positive cells, + = small, ++ = moderate, +++ = large number of positive cells. ††: subjective visual assessment; †††: untreated and sham-treated rats.

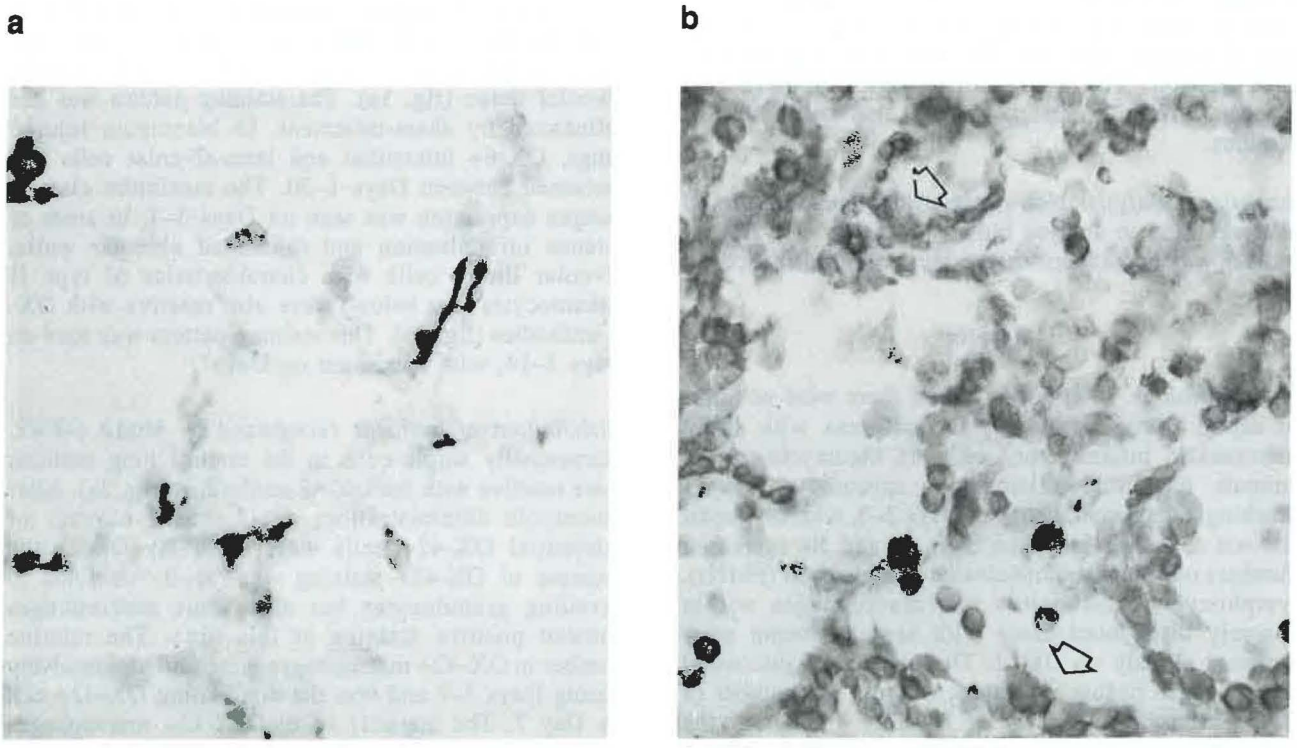

Fig. 1. – Immunoperoxidase staining of OX-6 reactive cells (darkly stained) on cryostat sections of rat lungs from: a) an untreated rat; and b) from a rat 7 days after an intratracheal injection of 1.5 mg bleomycin. Note positive staining of type II pneumocytes in b (indicated by arrows). The sections were counterstained with Mayer's haematoxylin.

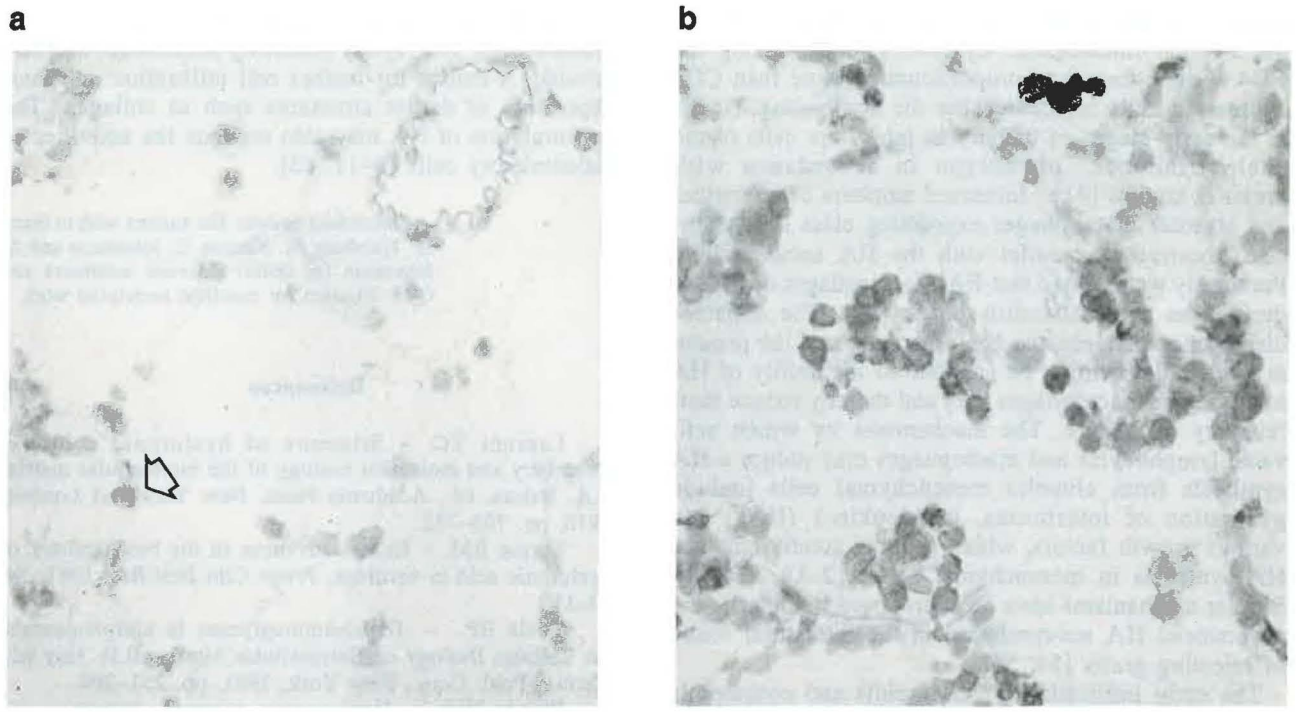

Fig. 2. – Immunoperoxidase staining of OX-42-reactive cells (darkly stained) on cryostat sections of rat lungs from: a) an untreated rat (a positive cell is indicated by an arrow); and b) from a rat 3 days after an intratracheal injection of 1.5 mg bleomycin. The sections were counterstained with Mayer's haematoxylin.

Distribution of antigens recognized by MoAbs OX-33, W3/13, W3/25 and OX-8. Cells reactive with OX-33 were present in lymph nodes from controls and bleomycin treated animals on all days investigated. Cells reactive with the "pan T" antibody W3/13 and the T-cell subclass antigens CD4 (W3/25+) and CD8 (OX-8+) were also present in central parts of the lymph nodes in all animals irrespective of treatment. Within the lymphatic tissues, the number of W3/25+ cells clearly exceeded the number of OX-8+ cells. In the interstitial lung tissue, the expression of different T-cell phenotypes was absent in control lung sections except for a few isolated cells. In bleomycin-injured lungs, W3/13+ cells appeared in the inflamed alveolar interstitium. This staining was present already by Day 1 and became more prominent between Days 3–7 and then declined. Interstitial lymphocytes expressing the CD4 antigen (W3/25+ cells) started to appear already on Day 1, increasing thereafter up to Day 3 and then levelling off on Day 30. Cells expressing the CD8 antigen (OX-8+ cells) also appeared soon after bleomycin injury and increased by Day 30. The number of cells labelling with OX-8 was generally fewer than those labelling with W3/25.

Distribution of antigens recognized by TPA. In control lung sections the epithelial cells lining the bronchi and bronchioli stained positively for cytokeratin (TPA+ cells) while the alveolar cells showed negative staining. TPA+ alveolar lining cells and some desquamated intraalveolar cells appeared between Days 3 and 7 after bleomycin administration. The number of TPA+ alveolar cells decreased thereafter but remained present throughout the observation period. The TPA+ alveolar lining cells showed characteristics of type II epithelial cells and matched the OX-6+ alveolar lining cells. The TPA+ cells in the airspaces were considered desquamated epithelial type II cells.

Discussion

The bleomycin animal model produces an acute alveolitis followed by a fibrotic repair phase [36–40]. The model has been widely used for studies of mechanisms involved in development of pulmonary fibrosis. In this model, lymphocytes [41–46], macrophages [47, 48] and neutrophils [49, 50] have been suggested to be involved in the fibrotic response. In our own studies of the same model, we have demonstrated that the enhanced collagen deposition in alveolar structures is preceded by a considerable but transient accumulation of the glycosaminoglycan HA [20–22]. The mechanisms underlying this early connective tissue response have not been identified but may be mediated by inflammatory cells, since the accumulation of HA parallels the appearance of inflammatory cells in BAL fluid [21]. The major aim of the present study was, therefore, to relate in time the alveolar accumulation of HA with the early cellular response of the bleomycin-injured lung tissue.

Lymphocytes and macrophages have been demonstrated to be implicated in collagen accumulation in the bleomycin model [42–48]. In this study we observed that the early T-cell recruitment to the inflamed alveolar

tissue is related in time with the appearance of HA in the alveolar interstitium. Lymphocytes expressing the CD4 antigen increased proportionately more than CD8 expressing cells indicating that the dominating T-cells in the early stages of bleomycin injury are cells of the "helper/inducer" phenotype in accordance with previous studies [41]. Increased numbers of interstitial and alveolar macrophages expressing class II antigens also occurred in parallel with the HA accumulation. Previously we reported that BAL macrophages decreased during the early alveolitis phase [21]. The apparent discrepancy between the BAL recovery and the present *in situ* findings might be ascribed to the ability of HA to aggregate macrophages [51] and thereby reduce their recovery by lavage. The mechanisms by which activated lymphocytes and macrophages may induce a HA synthesis from alveolar mesenchymal cells include generation of interferons, interleukin-1 (IL-1) and various growth factors, which *in vitro* greatly enhance HA synthesis in mesenchymal cells [12–14, 52, 53]. Similar mechanisms have been proposed to underlie the pronounced HA accumulation in the interstitial tissue of rejecting grafts [54, 55].

The early infiltration of neutrophils and eosinophils in the injured alveolar tissue in the bleomycin model is well recognized [37, 49, 50, 56, 57]. Granulocytes may attack the tissue by the release of proteolytic enzymes and the generation of free radicals. Eosinophils are also reported to stimulate fibroblasts *in vitro* [58]. We have previously reported that the appearance of granulocytes in lavage fluid after bleomycin treatment is related to the lavage recovery of HA [21]. In this study we have shown that infiltrating granulocytes expressed the antigen OX-42, probably reflecting the increased presence of the complement receptor type 3 [30]. Previous studies have shown that complement depletion [49, 59] inhibits, but depletion of granulocytes augments [49, 50] the deposition of collagen in the bleomycin-injured lung. However, our recent studies indicate that the HA lung accumulation in this model occurs independently of neutrophil/eosinophil or complement depletion [60].

In this study we also found that the alveolar lining cells, presumably type II cells, expressed class II antigens on Days 3–14. Expression of class II antigens on non-lymphoid cells occurs in different organs during a variety of inflammatory conditions [61] and it has been shown that injection of interferon-gamma in mice induces a class II antigen expression in both lung macrophages and type II epithelial cells [62]. However, the bleomycin-induced accumulation of HA starts before the class II expression of the alveolar lining cells, suggesting that these cells are not involved in the appearance of HA.

In summary, the early accumulation of HA in the bleomycin injured lung is paralleled by an increased infiltration in the alveolar tissue of C3 receptor expressing granulocytes, CD4+ T-cells and activated macrophages. This study has not identified the inflammatory stimuli which may induce the mesenchymal cells to synthesize high amounts of HA, but available data suggest that they are generated by activated macrophages/lymphocytes rather than by granulocytes. The rapidly occurring accumulation of HA provides a matrix for further cell infiltration and later deposition of denser structures such as collagen. The accumulation of HA may also regulate the activities of inflammatory cells [8–11, 63].

Acknowledgements: The authors wish to thank M. Tjernberg, H. Nilsson, C. Johansson and A. Johansson for skilful technical assistance and G-M. Ekström for excellent secretarial work.

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Accumulation alvéolaire d'hyaluronan et réponse cellulaire alvéolaire dans l'alvéolite induite par la bleomycine. O. Nettelbladt, A. Scheynius, J. Bergh, A. Tengblad, R. Hällgren.

RÉSUMÉ: L'accumulation d'hyaluronan (HA) dans le tissu interstitiel alvéolaire de rats soumis à une seule instillation intra-trachéale de bleomycine, a été mise en évidence à l'examen histologique, et expérimentée. HA est présent dès le jour 1 après le traitment à la bleomycine; il arrive à des valeurs maximales aux jours 3 et 7, pour diminuer ensuite. Une relation temps-dépendante entre cette réponse précoce du tissu conjonctif et l'irruption de cellules inflammatoires dans le tissu alvéolaire est évidente. Les cellules invasives dominantes au jour 1 sont des granulocytes avec une coloration positive pour l'anticorps monoclonal OX-42, qui répond au récepteur C3b. Le nombre du macrophages et frimant des antigènes de classe II commence à augmenter le jour 1, atteint un maximum les jours 3 à 7, pour diminuer ensuite. Les macrophages sont les cellules OX-42 positives dominantes au jour 7. La forme des cellules W3/13+ ("pan-T lymphocytes") a un type similaire à celui des macrophages exprimant la classe II. Le nombre de cellules exprimant un antigène CD4 augmente jusqu'au jour 3 et se stabilise au niveau inférieur au jour 30, alors que le plus grand nombre de cellules exprimant l'antigène CD8 est observé au jour 30. Peu de cellules exprimant un phénotype de cellule B ont été identifiées en dehors des nodules lymphoïdes. Les cellules épithéliales du revêtement alvéolaire, et probablement les cellules épithéliales de type II, ont experimé des antigènes de class II aux jours 3–14.
L'accumulation d'hyaluronan en rapport avec le temps et l'apparition de cellules T, de macrophages et de granulocytes exprimant des signes d'activation, suggèrent que ces cellules pourraient être impliquées dans la réponse tissulaire conjonctive précoce du poumon agressé par le bleomycine.
Eur Respir J, 1991, 4, 407–414.