In summary our study has defined the characteristics of pulmonary haemorrhage in patients with small vessel vasculitis. The results emphasise the clinical usefulness of measuring the transfer coefficient in patients suspected of having pulmonary haemorrhage.

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Hyaluronate in bronchoalveolar lavage fluid: a new marker in sarcoidosis reflecting pulmonary disease

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Abstract

Hyaluronate (hyaluronic acid) was not detectable in bronchoalveolar lavage fluid from smoking or non-smoking healthy volunteers but was present in fluid from 23 patients with sarcoidosis; the mean concentration was 16 μg/l returned fluid (range ≤5-430) or, expressed in relation to the amount of albumin recovered, 0·22 μg/mg albumin (range ≤0·05-3·6). The serum hyaluronate concentrations in the patients with sarcoidosis were normal. There was a significant inverse correlation between vital lung capacity and hyaluronate concentrations in bronchoalveolar lavage fluid ($p < 0.001$), and patients with abnormal lung volumes had hyaluronate concentrations that were on average six times higher than those in patients with normal vital capacity. Duration of disease, pulmonary radiological findings, and markers for macrophage activation (angiotensin converting enzyme) and lymphocyte activation (β_2 microglobulin) were not correlated with bronchoalveolar lavage fluid hyaluronate.

It was concluded that in sarcoidosis release of hyaluronate into the airways is related to lung volume and therefore to the course of the disease. Increased synthesis of hyaluronate in lung parenchyma may reflect activation of fibroblasts, and measurements of hyaluronate may have clinical value for prognosis and treatment.

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Introduction

Fibrotic changes in the lungs may develop in a few patients with sarcoidosis. It is a clinical problem to distinguish at an early stage between those patients who have spontaneously resolving sarcoidosis and those who develop progressive pulmonary destruction. The search for markers to identify the population at risk has been an urgent task. Measurements of serum angiotensin converting enzyme activity,[1] lung scanning with gallium-67,[2] and bronchoalveolar lavage with analyses of lymphocyte subpopulations[3] and inflammatory markers such as β_2 microglobulin[4] have been the main approaches during recent years to assess the intensity of alveolitis in sarcoidosis. It has not, however, been ascertained whether the intensity of alveolitis can predict the risk of developing irreversible lung fibrosis.

Hyaluronate (hyaluronic acid) is a glycosaminoglycan and a connective tissue element present in lung parenchyma.[5] Its release into the culture medium of growing fibroblasts has been shown and its production is stimulated by various inflammatory stimuluses,[6-8] some of which may operate in sarcoidosis.

Studies of animals have shown an increased production of hyaluronate in experimental allergic granulomatous pulmonary inflammation.[9] From such studies we have postulated an enhanced production of hyaluronate in sarcoid lungs. The recent development of a radioassay for hyaluronate[10] has enabled its determination in small tissue samples and in various body fluids.[11-14] In this study we used this method to measure hyaluronate concentrations in bronchoalveolar lavage fluid from patients with sarcoidosis and healthy controls. The data were correlated with various radiological stages, pulmonary function, and inflammatory cell markers.

Subjects and methods

Twenty three patients (17 men, six women) with sarcoidosis verified by biopsy were included in the study; their mean age was 36 years (range 26-45). No patient was treated with glucocorticoids during the study. Two patients had been treated previously with steroids. The mean duration of the disease was 3·5 years (range one month to 17 years). Six patients were smokers. Seventeen apparently healthy volunteers (six men, 11 women) with a mean age of 29 years (range 20-52) served as a control group. Ten were smokers.

The following chest radiographic criteria were used: stage I, bilateral hilar lymphadenopathy; stage II, bilateral hilar lymphadenopathy with parenchymal infiltrates; and stage III, parenchymal infiltrates without hilar lymphadenopathy. Lung volumes were measured by standard spirometry and helium dilution technique.

Before bronchoscopy patients and controls were given atropine and a narcotic intramuscularly. The upper respiratory tract was anaesthetised with topical lignocaine hydrochloride. A fibreoptic bronchoscope (Olympus model BF 4B2, Tokyo, Japan) was securely wedged in a subsegmental bronchus in the right middle lobe, and 200-300 ml sterile 0·9% sodium chloride at 37°C was infused in boluses of 50 ml. The fluid was gently aspirated immediately after each instillation and collected in a plastic bottle coated with silicon kept on ice. The lavage fluid was passed through a double layer of Dacronets (Millipore Corp, Bedford, Massachussetts). After centrifugation at 400 *g* for five minutes the supernatant was kept frozen at −70°C before analysis. The recovery of fluid instilled was 61 (SD 14)% in patients and 67(9)% in controls.

Hyaluronate was analysed in duplicate in serum and bronchoalveolar lavage fluid according to principles previously outlined.[10 14] In short, protein with specific affinity for hyaluronate was prepared from cartilage by affinity chromatography and labelled with iodine-125. The radioactive protein was incubated with varying amounts of free hyaluronic acid and a fixed amount bound to a Sepharose gel. The protein was allowed to partition between free and bound polysaccharides. The amount of radioactivity pelleted with the gel is related to the amount of free hyaluronate in the system. Of the test sample, 500 μl was incubated with the following reagents for 20 hours at 4°C during slow rotation: (1) 400 μl of 1·5M sodium chloride, 0·025M phosphate buffer (pH 7) containing 33mM ethylenediaminetetra-acetic acid (Merck, Darmstadt, Federal Republic of Germany), 3 mg/l soybean trypsin inhibitor (Worthington, Freehold, New Jersey), 1mM iodoacetic acid (Sigma, St Louis, Missouri), 3mM phenylmethylsulfonyl fluoride (Sigma), 17mM benzamidine (Sigma), 3 mg/l pepstatin (Sigma), and 0·33M ε-amino-*N*-caproic acid (Sigma); (2) 0·5 μl hyaluronate substituted gel suspended in 400 μl 0·55M sodium chloride and 0·05M sodium borate (pH 7); and (3) 100 μl ^{125}I-labelled cartilage protein in 4M guanidine hydrochloride (about 20 000 cpm). After the incubation the gel was collected by centrifugation and washed twice. Standard curves for hyaluronate were obtained by using sodium hyaluronate (Healon, Pharmacia, Uppsala, Sweden) dissolved in saline containing bovine serum albumin (Sigma).

The mean serum hyaluronate concentration in a healthy control group matched for age and sex was 30 (SD 14) μg/l.

β_2 Microglobulin was measured in duplicate in serum and bronchoalveolar lavage fluid with a commercial kit (Phadebas β_2-microtest, Pharmacia). The range in serum was 1·2-2·4 mg/l in healthy people with a similar age distribution to that of the patient group.

Serum angiotensin converting enzyme activity was measured by spectrophotometry according to the principles outlined by Liebermann *et al.*[2] To minimise the contribution of unspecific absorbance each sample of extracted hippuric acid was filtered through a Durapore filter (0·25 μm) (Millipore). The normal range (8-32 kU/l) was based on measurements in 104 healthy controls aged 25-50 years.

This study was performed according to the Declaration of Helsinki and with the free and informed consent of all volunteers and patients. The study was approved by the local ethical committee.

Results

In bronchoalveolar lavage fluid from patients with sarcoidosis the geometric mean hyaluronate concentration was 16 (range ⩽5-430) μg/l returned. Hyaluronate in bronchoalveolar lavage fluid from all healthy controls was below the limit of detection (5 μg/l). The patients had significantly higher albumin concentrations ($p < 0·001$) in bronchoalveolar lavage fluid (mean 74 (SD 51) mg/l) than the controls (27 (13) mg/l). To compensate for differences in leakage from the capillary bed the hyaluronate concentrations were corrected for albumin (fig 1); the geometric mean hyaluronate concentration in the patients was 0·22 (range ⩽0·05-3·6) μg/mg albumin recovered. The mean serum hyaluronate concentration in the patients was 39 (10) μg/l, which was not different from that in healthy controls.

To assess the extent to which hyaluronate in bronchoalveolar lavage reflects pulmonary disease we measured vital capacity. The inverse correlation between bronchoalveolar lavage fluid hyaluronate corrected for albumin and vital capacity was significant ($r = -0·67$, $p < 0·001$) (fig 2). Eight patients with abnormally low vital capacity—

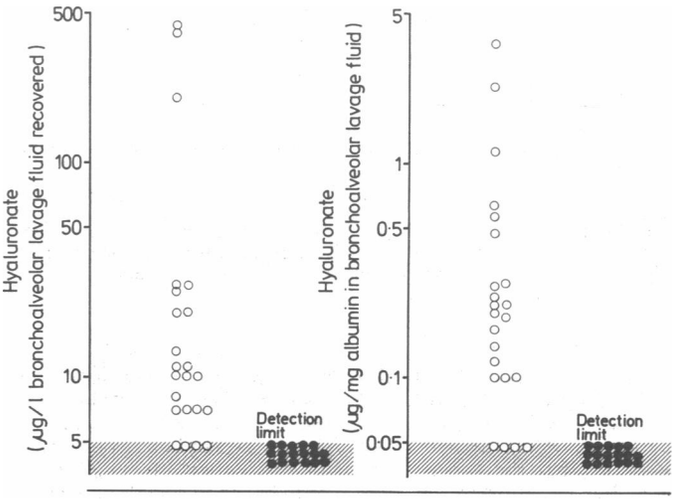

FIG 1—Hyaluronate concentrations in bronchoalveolar lavage fluid from patients with sarcoidosis (○) and healthy controls (●). Concentrations are given in relation to both volume of bronchoalveolar lavage fluid returned and amount of albumin recovered.

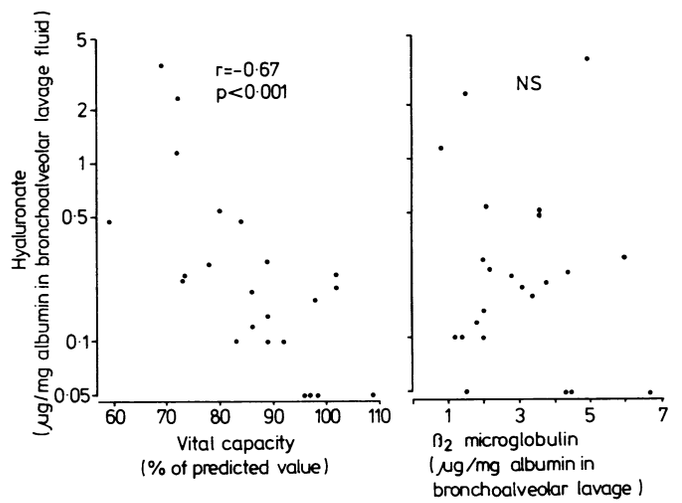

FIG 2—Hyaluronate concentrations corrected for albumin in the bronchoalveolar lavage fluid from patients with sarcoidosis plotted against (left) their vital lung capacity and (right) bronchoalveolar lavage fluid concentrations of β_2 microglobulin.

that is, less than 80% of the predicted value—had hyaluronate concentrations that were on average six times higher than those in 15 patients with normal vital capacity. To assess the possible relation between hyaluronate in bronchoalveolar lavage fluid and local pulmonary or systemic inflammatory activity we measured β_2 microglobulin and angiotensin converting enzyme activity. The hyaluronate concentrations were not related to the bronchoalveolar lavage fluid concentrations of β_2 microglobulin (fig 2) or the serum β_2 microglobulin concentration or angiotensin converting enzyme activity (fig 3). Three patients, however, who had extraordinarily high bronchoalveolar lavage fluid hyaluronate concentrations also had raised serum β_2 microglobulin concentrations.

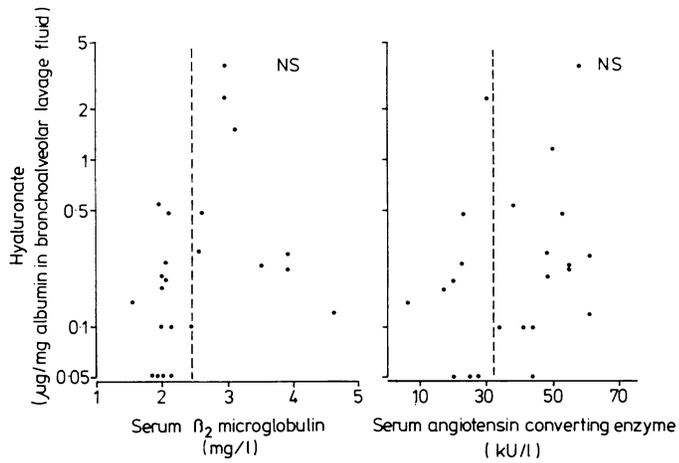

FIG 3—Bronchoalveolar lavage fluid hyaluronate in patients with sarcoidosis plotted against (left) their serum β_2 microglobulin concentrations and (right) angiotensin converting enzyme activity. Dotted lines indicate upper limits of normal ranges.

Radiological stages II and III predominated (table). No significant relation was seen between bronchoalveolar lavage fluid hyaluronate and radiological criteria, and no overall correlation between the duration of the disease and bronchoalveolar lavage fluid hyaluronate was found, but the two patients with the highest bronchoalveolar lavage fluid hyaluronate concentrations (430 and 400 μg/l) (see fig 1) had had known disease for less than six months. Six of the patients were smokers, but smoking habit did not seem to influence the hyaluronate concentrations. In addition, most of the controls were smokers and had no detectable hyaluronate in bronchoalveolar lavage fluid.

Geometric mean hyaluronate concentrations (and SEM) in bronchoalveolar lavage fluid from patients with sarcoidosis subgrouped with respect to pulmonary radiological criteria and duration of disease

	No	Hyaluronate	
		μg/l recovered fluid	μg/mg albumin
Radiological stage:			
I	2	63	0·3
II	12	19 (12-29)	0·24 (0·16-0·36)
III	9	9·6 (8·1-11)	0·18 (0·15-0·23)
Duration of disease (years):			
<0·5	8	24 (13-45)	0·27 (0·16-0·46)
0·5-2	7	9·3 (7-12)	0·13 (0·09-0·19)
>2	8	17 (12-25)	0·30 (0·22-0·39)

Discussion

This study shows that bronchoalveolar lavage fluid from most patients with sarcoidosis contains hyaluronate at measurable and, compared with normal circulating values, sometimes high concentrations. In bronchoalveolar lavage fluid from healthy controls, irrespective of their smoking habits, we were unable to detect appreciable amounts of hyaluronate. These findings indicate an increased synthesis of hyaluronate in sarcoid lung parenchyma. This postulated increased production of glycosaminoglycan in sarcoidosis is not reflected by increased circulating hyaluronate concentrations. Other inflammatory diseases that affect the connective tissue, such as rheumatoid arthritis and scleroderma, are characterised not only by an increased production of hyaluronate in synovial membranes or skin but also by raised serum concentrations of hyaluronate.[13 15]

The source of increased hyaluronate synthesis in the lung was not identified. Hyaluronate and other glycosaminoglycans, except keratan sulphate, are normal constituents of animal lung parenchyma.[5] Although the glycosaminoglycans constitute a small portion of the lung on the basis of weight, they are thought to play an important part in the function and development of the lungs.[16] There are no data on the turnover of these interstitial components in health or disease, but in theory an increased turnover rate has to be considered when they appear in increased amounts in the alveolar space. Increased synthesis of hyaluronate in the lung of patients with sarcoidosis may reflect activated interstitial fibroblasts or an expanded fibroblast mass associated with interstitial fibrosis. This hypothesis, which should be tested in patients with diseases such as fibrosing alveolitis, was partly supported by the finding that patients with sarcoidosis and signs of pulmonary dysfunction, as tested by vital capacity, also had hyaluronate concentrations in bronchoalveolar lavage fluid that were on average over six times higher than those in patients without impaired lung function. We were not, however, able to show a difference in bronchoalveolar concentrations between patients with radiological stages II and III of the disease.

The mechanisms underlying the accumulation and expansion of fibroblasts in sarcoid lungs are largely unknown but supposed to be a consequence of alveolitis induced by activated alveolar T lymphocyte and macrophage populations.[3] Alveolar macrophages release a fibroblast growth factor.[17] Whether this factor may stimulate the production of fibroblast hyaluronate like the fibroblast growth factors derived from platelets[15] has not yet been tested, but seems possible. In this study, however, we found no evidence that increased synthesis of hyaluronate was linked to activation of macrophages, as reflected by serum angiotensin converting enzyme activities,[18 19] and of T cells, as reflected by serum and bronchoalveolar lavage fluid β_2 microglobulin concentrations.[4] Unfortunately, we did not have the opportunity to compare our results with those of ^{67}Ga scanning or of a study of bronchoalveolar lymphocyte subpopulations.

Previous studies in animals may have provided some insight not only into the mechanisms but also into the pathophysiological role of increased production of hyaluronate in the lung parenchyma. Love *et al*[9] showed that a macrophage agglutinating factor, which was later identified as a complex of hyaluronate and protein,[20] was obtained from lavages from rabbits' lungs during an allergic granulomatous response to heat killed BCG. Increased synthesis of hyaluronate was already evident during the first days of the delayed hypersensitivity reaction and thus hardly suggested fibrosis but might have reflected activation of fibroblasts. Love *et al* speculated on the ability of hyaluronate to agglutinate alveolar macrophages and suggested a role in the organisation of the granuloma. In vitro studies have also shown effects of hyaluronate on the functions and activities of lymphocytes,[20] monocytes and macrophages,[21 22] and neutrophils.[23-25] Thus the possibility of a modulatory role of hyaluronate in sarcoid alveolitis cannot be excluded.

Irrespective of the mechanisms behind increased production of hyaluronate in lung parenchyma in sarcoidosis, our study has suggested a practical, clinical use of measurements of hyaluronate in bronchoalveolar lavage fluid. No single variable will be infallible in the assessment of the activity and prognosis of sarcoidosis, but hyaluronate may be a new complementary marker, reflecting the link between the immune system, the activation of fibroblasts, and the development of lung fibrosis.

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SHORT REPORTS

Failure of random zero sphygmomanometer in general practice

The main cause of inaccuracy in measuring blood pressure is systematic bias between observers,[1] a major factor being preference for a particular final digit.[2] Use of a random zero sphygmomanometer (Gelman Hawksley Ltd) can reduce this inaccuracy.[3] The device has a random zero position (between 0 and 60 mm Hg), which is changed blind by the observer before each measurement. The true blood pressure can thus be determined only by means of subtraction once the column has come to rest. I assessed terminal digit preference when this instrument was used.

Patients, methods, and results

Doctors from four general practices participated in a screening programme for hypertension. The technique for measuring blood pressure was standardised, each doctor being provided with a random zero sphygmomanometer and instructed in its use. Blood pressure was measured after a rest of five minutes. The cuff was inflated above the systolic pressure estimated from palpation of the radial artery and the constant relief valve set at 2 mm Hg/s. The level of the column was read to the nearest 2 mm Hg at Korotkoff sounds I and V and at the resting zero point. The systolic and diastolic pressures were then recorded. The pressures recorded in 2096 patients over three years from 1 January 1980 were used to assess terminal digit preference and to examine differences both between practices and over time.

Terminal digit preference was evident with both systolic and diastolic recordings. Fifty four per cent of the values given for systolic pressure and 49% of those for diastolic pressure ended in zero, with a random distribution between the four other even digits (table). Despite instructions to record to the nearest 2 mm Hg, 12% of systolic and 15% of diastolic readings ended in an odd number, principally 5. All four practices showed the preference for zero, though the proportion of readings ending in zero ranged from 44% to 75% for systolic and 40% to 67% for diastolic pressures. In the practice with the greatest bias (C) a subsequent review of 200 random blood pressure readings taken before the study showed a distribution of final digits that was not significantly different from that recorded during the study with the random zero sphygmomanometer. In the other practices 36% of systolic and 38% of diastolic pressures ended in an even digit other than zero compared with less than 5% of the pressures measured before the study.

Terminal digit preference in each practice (figures are numbers (%) of readings)

Practice	No of readings	Final digit						
		0	2	4	6	8	5	Other odd
				Systolic				
A	781	480 (61·5)	51 (6·5)	43 (5·5)	45 (5·8)	45 (5·7)	87 (11·2)	29 (3·7)
B	842	370 (44·0)	105 (12·5)	114 (13·5)	71 (8·4)	66 (7·8)	66 (7·8)	51 (6·1)
C	171	128 (74·9)	5 (2·8)	6 (3·4)	7 (3·9)	10 (5·6)	15 (8·9)	1 (0·6)
D	302	150 (49·6)	50 (16·7)	38 (12·7)	30 (9·8)	27 (9·1)	7 (2·2)	
				Diastolic				
A	781	437 (55·9)	52 (6·6)	40 (5·1)	52 (6·6)	60 (7·7)	112 (14·4)	28 (3·6)
B	842	340 (40·4)	104 (12·4)	107 (12·7)	77 (9·1)	86 (10·2)	73 (8·7)	56 (6·7)
C	171	115 (67·0)	7 (3·9)	4 (2·2)	4 (2·2)	7 (3·9)	32 (19·0)	3 (1·7)
D	302	129 (42·8)	53 (17·4)	31 (10·1)	40 (13·4)	35 (11·6)	11 (3·6)	3 (1·1)

In the first year of the study 55% of systolic values ended in zero, in the second year 55%, and in the third year 51%; the corresponding figures for diastolic pressures were 51%, 48%, and 52%. Thus the bias was consistent throughout the study.

Comment

Using the random zero sphygmomanometer did not abolish terminal digit preference, though it probably reduced its magnitude in three of the four practices. This degree of bias is unacceptable