Eur Respir J
1988, 1, 748–757

Elastase activity of bronchoalveolar cells in advanced pulmonary sarcoidosis

M. Mordelet-Dambrine*, C. Lafuma**, G. Stanislas-Leguern*, L. Robert**, J. Chrétien*, W. Hornebeck**

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ABSTRACT: Elastase activities were investigated in bronchoalveolar lavage (BAL) cells from healthy controls (C, n=8) and patients with untreated mediastinopulmonary sarcoidosis at different stages: I (n=5), II (n=7), III_A (n=3), III_B (n=4). Elastase activities (EA and ELA) were measured against a synthetic substrate, succinyl-trialanine-paranitroanilide (SLAPN) and radiolabelled ligamentum nuchae ^3H insoluble elastin (specific radioactivity 3.7 $MBq \cdot mg^{-1}$). A significant increase of ELA of BAL cells was observed in patients (S III), in particular those exhibiting fibrotic roentgenographic patterns and a higher number of polymorphonuclear cells. On the contrary, EA of BAL cells showed no variation between different stages of sarcoidosis. ELA was never detected in the macrophage culture medium and appeared to be mainly associated with non-adherent macrophages, whereas EA appeared to be preferentially associated with adherent macrophages. The inhibitory profiles of ELA and EA were investigated using general proteinase inhibitors and also compounds interacting specifically with leucocyte elastase. ELA was mainly due to a serine protease, which has a sensitivity to inhibitors similar to leucocyte elastase and is increased in advanced stages of sarcoidosis. EA appears to be only slightly modified, if at all, in sarcoidosis and is probably related to the action of both a serine protease and a metalloenzyme. Thus, only ELA yields diagnostic information concerning stage III of sarcoidosis associated with a fibrotic or bullous roentgenographic pattern.

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* INSERM U 214, Hôpital Laennec, 42, rue de Sèvres, 75007 Paris, FRANCE.
** CNRS UA 1174, Laboratoire de Biochimie du Tissu Conjonctif, Faculté de Médecine, Créteil, FRANCE.

Correspondence: W. Hornebeck, CNRS UA 1174, Laboratoire de Biochimie du Tissu Conjonctif, Faculté de Médecine, 8, rue du Général Sarrail, Créteil 94010, France.

Keywords: Bronchoalveolar lavage; elastase-like activity; elastinolytic activity; sarcoidosis.

Accepted after revision March 28, 1988.

This work was supported by funds provided by INSERM (U 214), CNRS (UA 1174) and the University of Paris Val de Marne (Paris XII).

Sarcoidosis is a systemic disorder of unknown aetiology associated with granulomatosis, which may spontaneously regress or be followed by a progressive pulmonary fibrosis with diffuse parenchymal shadows on chest X-ray. The pathogenesis of pulmonary sarcoidosis has not been completely established, although many immunological abnormalities have been observed [1–3]. The evolution of sarcoidosis into interstitial pulmonary fibrosis leads to an alteration of the lung extracellular matrix. Morphological examination of this pulmonary fibrosis showed a considerable disorganization of interstitial collagens associated with some changes in interstitial elastin structure [4]. This reference is related to idiopathic pulmonary fibrosis, which is a different disease and not the end stage of sarcoidosis.

Perpetuation of this granulomatous disease and tissue injury would require the continuous migration of monocytes in the lung and/or activation and proliferation of macrophages already present in the lung tissue [3, 5]. Phagocytic cells including polymorphonuclear leucocytes (PMN) and pulmonary alveolar macrophages (PAM) can injure lung parenchymal cells and macromolecular structural components by several mechanisms [6, 7]. One involves the secretion of a variety of potent degradative enzymes, including neutral proteinases such as elastases. Recent studies [8–13] have shown that macrophages are able to synthesize and secrete different types of elastases such as a neutral metalloproteinase and a cysteine elastinolytic proteinase. Moreover, macrophages are also able to bind and internalize neutrophil serine elastase by a receptor mediated pathway [14]. This neutrophil serine elastase can hydrolyse most of the extracellular matrix components. Several studies suggest that the secretion of lysosomal enzymes by PMNs or PAMs could act either by initiating an inflammatory response or by amplifying a pre-existing inflammatory reaction [15]. Although PMNs are usually present in small numbers in granulomatous lung inflammation, recent data also suggest that PMN or PAM elastases may contribute to the structural injury associated with granuloma formation [15].

Bronchoalveolar lavage (BAL) is considered to be the most appropriate technique to assess the inflammatory and immune effector cells, and also to estimate the levels of mediators such as elastases in the lower respiratory tract [16–18]. An important question is whether or not elastase activity associated with BAL cells, namely PAMs, may reflect the degree of severity of sarcoidosis, *i.e.* if the quantitative and qualitative evaluation of elastase activity in BAL cells might provide a means to evaluate the deterioration of pulmonary parenchyma in sarcoidosis and, therefore, if this parameter might be of clinical value in predicting the evolution of the disease.

This study was primarily designed to investigate the relationships of BAL cell elastase activity in patients with pulmonary sarcoidosis with: 1) pulmonary radiologic stage; 2) luminal PMNs, PAMs and lymphocytes as assessed by BAL and 3) serum angiotensin converting enzyme (SACE). The aim of this study was also to investigate the contribution of PAMs to the expression of elastase activity during the different stages of sarcoidosis and to define the inhibition profile of this enzyme activity. Indeed, PAMs represent the major cellular type in BAL, and their elastolytic activity may contribute to the evolution of pulmonary sarcoidosis.

Methods

Patients

Twenty seven patients (twelve men and fifteen women, mean age 34±2 yr) were studied. The group of healthy patients (seven non-smokers and one smoker) showed no pulmonary or extra-pulmonary abnormality. Written informed consent was obtained from each subject. As previously described [19], the patients with untreated mediastino-pulmonary sarcoidosis were identified on the basis of roentgenographic changes, using the following criteria. Stage I: bilateral hilar adenopathy (five patients); Stage II: hilar adenopathy associated with parenchymal shadows (seven patients); Stage III: more extensive parenchymal shadows without adenopathy (seven patients). Four of these patients (Stage III_B) exhibited fibrotic or bullous roentgenographic patterns, whereas three (Stage III_A) presented only parenchymal shadows without adenopathy. All sarcoidosis patients were non-smokers. The study was approved by the Ethical Committee.

Bronchoalveolar lavage (BAL)

BAL was performed after the administration of local anaesthesia. A total volume of 300 ml of sterile normal saline solution (0.15 M NaCl) at 37°C was injected in 100 ml fractions via a fibreoptic bronchoscope (Olympus Model BF B3; Olympus Corp. of America, New Hyde Park, N.Y.). Each fraction was recovered immediately after instillation initiated by the application of a pressure of 5–10 cm of water. The lavage fluids were collected in a sterile, siliconized glass flask in order to reduce the adherence of cells to the walls of the flask. An aliquot was removed for cell numeration in a Malassez haemocytometer and differential cells counts on cytocentrifugation preparations (Cytospin, Shandon, Paris, France) with May-Grunwald Giemsa stain. The viability of the cells was determined by the dye exclusion technique using trypan blue.

Isolation of BAL cells

The remaining volume of BAL fluid was centrifuged at 500 g for 10 min at 4°C. The cell pellet was washed once with Hanks' balanced salt solution (HBS Gibco) without Ca^{++} and Mg^{++} and centrifuged at 500 g for 10 min. The cells were resuspended in Hanks' balanced salt solution with Ca^{++} and Mg^{++} in order to obtain a cellular suspension with a final concentration of $1x10^6$ PAM·ml^{-1}: a second numeration was then performed in order to differentiate large, neutral, red-stained PAMs from unstained lymphocytes or PMNs. An aliquot of $3–5x10^6$ PAMs was kept and centrifuged. The cell pellet was resuspended in 1 ml of Triton X-100 (Sigma Chemical Ca solution (0.5%)).

Alveolar macrophage cultures

PAMs obtained from five selected patients: (one healthy non-smoking patient, one healthy smoking patient, and one patient at each stage of sarcoidosis), were cultured.

Aliquots of cell pellets resuspended in Hank's balanced salt solution as described above, were plated in Falcon plastic tissue culture dishes (Falcon Labware, Div., Becton, Dickinson and Co, Oxnard, CA) and incubated at 37°C for 1 h in humidified air and 10% carbon dioxide. After incubation, the culture medium and non-adherent cells were discarded. In one Falcon culture dish, adherent cells were stained by the May-Grunwald Giemsa method in order to determine the homogeneity of the macrophage cell population and its extent of contamination by PMNs. The other cultured macrophages were incubated in Eagle's minimum medium in the presence of 0.32% bovine serum albumin, for 1, 2, 3, or 24 h, at 37°C, in 10% carbon dioxide/air v/v. At the end of the culture period, the non-adherent cells were counted, centrifuged at 500 g for 10 min, and the pellet resuspended in 1 ml of 0.5% Triton X-100 solution; this suspension of non-adherent macrophages as well as its supernatant were stored at -20°C. The adherent cells were lysed with 1 ml of 0.5% Triton X-100, scraped from the plates with a rubber policeman, and stored at -20° C. The release of lactate dehydroge-nase was estimated in the culture medium (Test-Combination LDH

opt. Boehringer Manheim GmbH) and no difference between the viability of alveolar macrophages from healthy patients and from sarcoidosis patients was detected.

Determination of angiotensin converting enzyme in serum (SACE)

The determination of the SACE concentration was performed by the spectrophotometric method of CUSHMAN and CHEUNG [20], as modified by LIEBERMAN [21]. The concentration of sodium hippurate was measured spectrophotometrically at 228 nm. The ACE-assay was performed using 100 µl of serum and the results were expressed as units per ml of serum (1 SACE unit corresponds to the release of 1 nanomole of sodium hippurate in 1 min at 37°C). Storage of individual sera at -20°C for up to one week did not quantitatively modify their SACE concentration.

Elastase activity

Elastase-like activity (EA) measured in Triton X-100 extracts of BAL cells, adherent and non-adherent culture PAMs, and in the culture medium, was determined using succinyltrialanine-paranitroanilide as substrate (SLAPN, from Choay Laboratories, France) [22]. For assaying enzyme activity, 50–100 µl of cell suspension or culture media were added to 20 µl of 125 mM SLAPN in 1-methyl-2-pyrrolidone, and incubated in 0.1 M Tris HCl (pH 8.2), 5 mM calcium chloride, 0.01% BRIJ 35 and 0.02% sodium azide, at 37°C for 24 h. Standards of purified porcine pancreatic elastase (SIGMA, St Louis, USA) were assayed in parallel. Changes in absorbance were measured spectrophotometrically at 410 nm. The data were expressed in n moles of hydrolysed substrate·h^{-1}·10^6 cells (ϵ nitroaniline=8,800 M^{-1} cm^{-1}).

Elastase activity against insoluble ^3H elastin (ELA) was determined on identical samples. Insoluble elastin from bovine ligamentum nuchae was purified according to the method of LANSING *et al.* [23]. Its amino acid composition, hexose and hexosamine contents were consistent with the presence of only trace amounts of collagen and structural glycoprotein contaminants [24]. Pretreatment of ^3H insoluble elastin with trypsin or chymotrypsin allowed us to confirm the purity of the ^3H insoluble elastin used.

Insoluble elastin was radiolabelled with $Na\,B^3H_4$ by the Nuclear Research Centre (Saclay-France) as described by STONE *et al.* [25]: specific activity 3.7 MBq·mg^{-1}. This method allowed us to detect elastinolytic activity associated with 1 ng of purified pancreatic-elastase (60 IU·mg^{-1}), and to obtain a linear response of elastinolytic activity exhibited by BAL cells from sarcoidosis patients, as a function of BAL cell numbers. The insoluble ^3H elastin (200 µg) was suspended in the same buffer used for SLAPN assays and together with enzyme samples (final volume=1 ml) was incubated for 24 h at 37°C. After centrifugation at 4,500 g for 1 min, the radioactivity present in aliquots of the supernatants was determined using a 7500 Beckman liquid scintillation counter. The results were expressed in µg elastin hydrolysed·$24h^{-1}$·10^6 cells.

In order to determine the inhibition profile of elastase activity (EA or ELA), samples were preincubated (for 30 min, at 20°C in 100 mM Tris HCl, 0.1% BRIJ, pH 8.4) with different inhibitors: metal chelators such as EDTA (5 mM), serine protease inhibitors such as phenylmethyl sulphonyl fluoride (4 mM PMSF), oleoyl-(bis-alanyl)-proline-valine-CH_2Cl (10 µM Ol-$(Ala)_2$-Pro-Val-CH_2Cl) and Eglin C (5 µM). Eglin C is a peptide extracted from leeches with about a 7 kDa molecular weight, which inhibits human leucocyte elastase with a high k_{ass}. [26, 27]. Ol-$(Ala)_2$-Pro-Val-CH_2Cl is also a potent inhibitor of leucocyte elastase [28]. Bestatin (from SIGMA Chemicals) was tested as an inhibitor of aminopeptidase. Leupeptin, pepstatin (from SIGMA Chemicals) and N-ethyl maleimide were used as inhibitors of cathepsin B, aspartic and cysteine proteinases, respectively.

Statistical methods

The data were analysed using the least squares linear regression to determine the correlation between two variables, and the unpaired t-test to compare the results between different groups. Results were expressed as means±SEM.

Results

Lavage fluid composition

No significant difference was found in the percentage BAL fluid recovery in the four groups (table 1). The number and percentage of lymphocytes were increased in all stages of sarcoidosis as compared to the healthy group but there was no significant difference between lymphocyte percentage in the different sarcoidosis groups. The number of PAMs appeared constant, whereas the relative percentage of PAMs diminished in Stage II and III of sarcoidosis. Alveolar PMN number and relative percentage were found to increase only in Stage III. The apparent decrease of PMN numbers in Stage I and II sarcoidosis compared to control was not significant.

Serum angiotensin converting enzyme (SACE)

SACE concentrations (U·ml^{-1}) were enhanced at all stages of sarcoidosis (SI: 43.3±8.7 (NS): SII 54.2±6.7 ($p<0.05$); SIII: 59.8±12.6 ($p<0.05$)) as compared to the healthy control group (30.5±5.3). Maximal increase

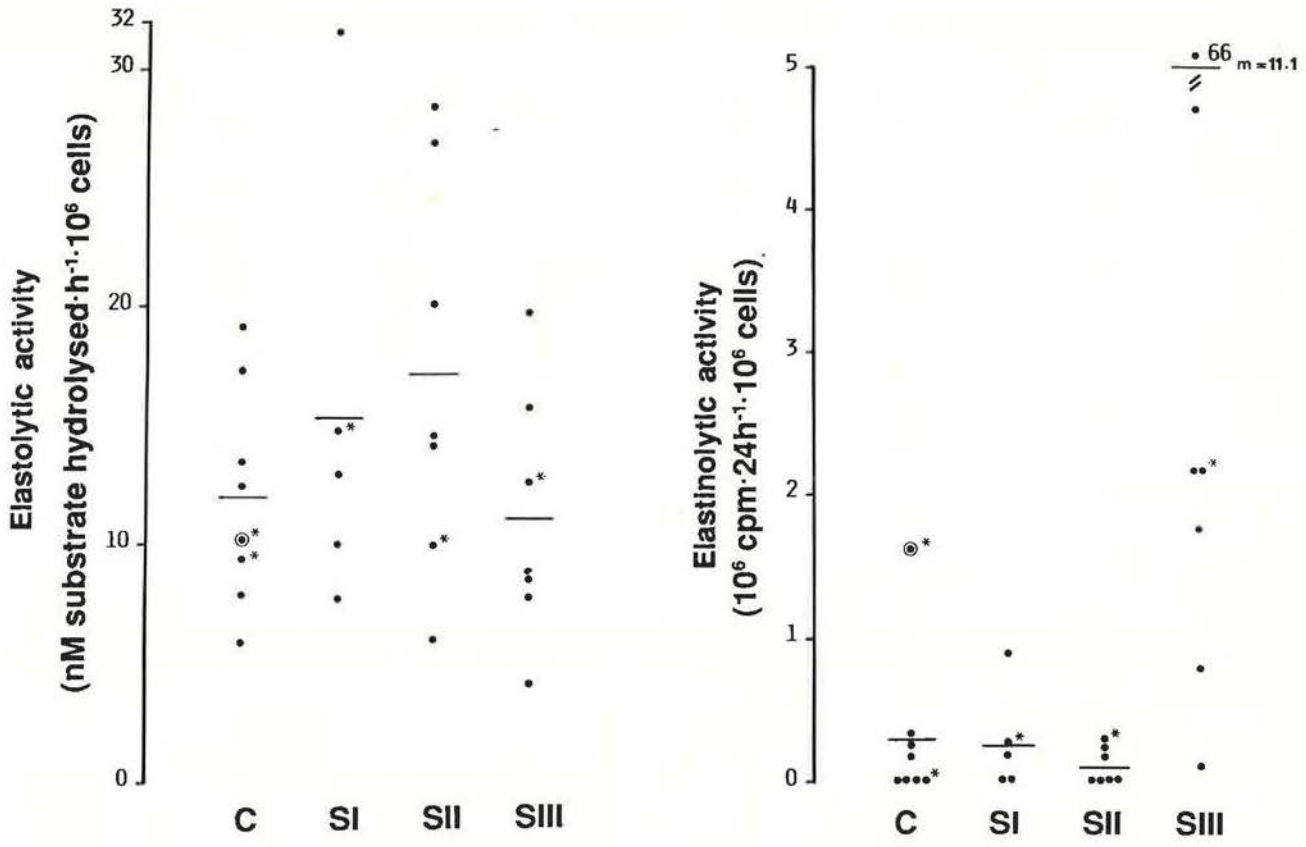

Fig. 1. – BAL cells Elastase-like EA activity (nM SLAPN hydrolysed·h⁻¹·10⁶ cells) and elastinolytic activity ELA (10⁶ cpm·10⁶ cells) in control group (C) and different stages of sarcoidosis (S I, S II, $SIII_A$ $SIII_B$). Points surrounded by circles correspond to the smoking patient. Points accompanied with an asterisk, correspond to the patients whose PAMs were further investigated for EA and ELA.

was obtained in the advanced Stage III of sarcoidosis.

Elastase activity of BAL cells (EA and ELA)

There was a wide variation in EA of BAL cells, in all groups of patients (fig. 1). No difference in EA could be observed in any of the sarcoidosis groups as compared to the healthy control group. When BAL cell EA obtained from each patient was tested for inhibition by EDTA, there was a mean of 80±5% inhibition, indicating that the majority of this enzyme activity could be attributed to a metalloprotease. When BAL cell EA was tested for inhibition by bestatin, an inhibitor of aminopeptidase, the extent of inhibition was found to be approximately 50%.

In contrast, ELA was only increased in the BAL cells obtained from Stage III sarcoidosis patients, and from the one healthy smoking patient examined. There was a correlation between the ELA and the PMN number and/or their percentage in BAL (n=27; $p<0.001$), but no correlation was found between EA and ELA when expressed on a total cell basis. When BAL cell ELA from the smoking patient was tested with EDTA and PMSF as inhibitors, 80% of inhibition of enzyme activity was found to be metallodependant. ELA associated with BAL cells from Stage III sarcoidosis, was partly inhibited by metal chelating agents (67% inhibition by EDTA) and partly by serine active site titrants (80% inhibition by PMSF).

Elastase activity of cultured PAMs

The results related to the quantitative variations of EA and ELA in cultured PAMs are summarized in table 2. PAMs were obtained from one healthy patient, one healthy smoker, and one patient at each stage of sarcoidosis. The three sarcoidosis patients exhibited a detectable level of ELA in BAL cells. For each patient, EA and ELA were investigated simultaneously in adherent PAMs, non-adherent PAMs and culture conditioned medium from total PAMs.

Secretion of EA was not detectable at any time in the culture media, whatever the stage of sarcoidosis. In contrast, release of EA was observed in the culture medium conditioned by PAMs from the healthy smoker: this enzyme activity remained constant at all times of the culture period and represented about 30% of total EA distributed between the culture medium, the adherent PAMs and the non-adherent PAMs. In contrast, ELA was never detectable in the culture medium at any time of culture which ever the patient studied.

When expressed in enzyme activity per 10^6 cells,

Table 1. – Percentage of fluid recovery and variation of BAL cells (number and percentage) in the control group and at different stages of sarcoidosis

Groups	% recovery Fluid	Macrophages 10^6	Macrophages %	Lymphocytes 10^6	Lymphocytes %	Neutrophils 10^6	Neutrophils %
Control n=8	62.6 ±3.8	47.1 ±10	87.7 ±3.7	4.3 ±1.1	9.9 ±2.6	0.8 ±0.3	2.1 ±0.8
Stage I n=8	62.4 ±3.4	43.9 ±13.4	76.6 ±7.1	19.5* ±11.9	22.7* ±7.2	0.5 ±0.2	0.7 ±0.2
Stage II n=7	67.6 ±2.3	31.2 ±2.1	64.0** ±8.3	20.9** ±6.5	34.8** ±8.3	0.6 ±0.2	1.2 ±0.3
Stage III n=7	64.3 ±1.7	44.9 ±10.9	63.4** ±7.4	25.2** ±8.1	31.1** ±7.6	5.2** ±2.8	5.4 ±2.1

*:$p<0.02$; **:$p<0.01$.

Table 2. – Elastase activities associated with cultured adherent pulmonary alveolar macrophages (Ad PAMs), non-adherent (NAd PAMs) and corresponding culture media from the control group, the smoker and the different stages of sarcoidosis

Patients	cells	EA (SLAPN) Total activity $nM \cdot h^{-1}$	EA (SLAPN) %	EA (SLAPN) $nM \cdot h^{-1} \cdot 10^6$ cells $ng \cdot h^{-1}$	ELA (^{3}H Elastin) Total activity	ELA (^{3}H Elastin) %	ELA (^{3}H Elastin) $ng \cdot h^{-1} \cdot 10^6$ cells
Control	Ad PAM	30	91	27	0	0	0
	NAd PAM	3	9	8	0.5	100	1
	MEM	0	0	0	0	0	0
Smoker	Ad PAM	23	26	17	37	60	27
	NAd PAM	31	37	70	25	40	45
	MEM	31	37	31	0	0	0
Stage I	Ad PAM	64	91	22	3	16	3
	NAd PAM	6.6	9	44	16	84	26.6
	MEM	0	0	0	0	0	0
Stage II	Ad PAM	17.6	100	23	24	84	40
	NAd PAM	0	0	0	4.5	16	4
	MEM	0	0	0	0	0	0
Stage III	Ad PAM	10.2	28	12	0	0	0
	NAd PAM	26.6	72	148	14	100	140
	MEM	0	0	0	0	0	0

EA appeared comparable in cultured adherent PAMs obtained from healthy or sarcoidosis patients, and was unchanged whatever the culture time (about 20 $ng \cdot h^{-1} \cdot 10^6$ adherent PAMs). Nevertheless, EA decreased in adherent PAMs obtained from the smoker and Stage III sarcoidosis patient, when expressed as percentage of total EA distributed between adherent PAMs, non-adherent PAMs and culture medium. This finding could be explained by the increase of EA in non-adherent PAMs from the smoker and Stage III sarcoidosis patient, whatever the time of culture (from 1–24 h). When adherent PAMs Triton X-100 extracts (obtained from Stage II sarcoidosis patient) were incubated with EDTA and several protease inhibitors, the inhibition profile of EA (table 3) indicated that this enzyme activity was due partly to the action of a metalloprotease (85% inhibition by EDTA) and partly to a serine protease (35, 47 and 65% inhibition, respectively, obtained with Ol-$(Ala)_2$-Pro-Val-CH_2Cl, Eglin C and PMSF).

Only traces of ELA were detectable in PAMs from the healthy non-smoker investigated. In contrast, ELA was present in PAMs from the three sarcoidosis patients and the smoker (table 2); it appeared associated

Table 3. – Inhibition profile of elastase activity of adherent PAMs obtained from a Stage II sarcoidosis patient

	Inhibitors	Final Concentration	Substrate	
			SLAPN % inhibition	Insoluble ^{3}H Elastin % inhibition
Adherent PAMs	0	0	0	0
	PMSF	4 mM	65	75
	EDTA	2 mM	85	18
	N-Ethyl Maleimide	4 mM	0	nd
	Leupeptin	40 µM	33	nd
	Pepstatin	20 µg·ml^{-1}	0	nd
	Eglin C	5 µM	47	95
	Oleoyl-(Ala)$_2$-Pro-Val-CH_2Cl	10 µM	35	100
BAL cell	Bestatin	58 µM	40	0
Rat leucocyte elastase*	Bestatin	58 µM	0	0
Human fibroblast metalloelastase**	Bestatin	58 µM	100	0

*: obtained from rat pleural exudates; **: obtained from human skin fibroblasts in culture

mostly with the non-adherent culture PAMs. The inhibition profile of ELA from adherent PAMs (from Stage II sarcoidosis patients) was due mainly to a serine protease (100, 100 and 75% inhibition by Ol-(Ala)$_2$-Pro-Val-CH_2Cl, Eglin C and PMSF, respectively).

Discussion

The purpose of our study was to determine whether elastase activity associated with BAL cells and with PAMs, was correlated with the evolutive stage of sarcoidosis, and so might yield prognostic information concerning the progression of the disease.

We classified sarcoidosis patients as a function of radiological pulmonary changes. This classification paralleled the intensity of alveolitis evaluated according to the percentage of T-lymphocytes. Only Stage II and Stage III sarcoidosis patients presented a high intensity of alveolitis with greater than 30% of T-lymphocytes, probably due to active T-cell proliferation and to more pronounced attraction of lymphocytes from peripheral blood to the parenchyma and to the alveolar space [29]. The percentage of PMNs was very small, except in most Stage III sarcoidosis patients with clinical signs of evolutive pulmonary fibrosis. This simultaneous increase of both PMNs and lymphocytes in the BAL fluids from patients with advanced sarcoidosis, may indicate an evolution of the granulomatous process toward pulmonary fibrosis. Because PMNs are well known to elaborate proteolytic enzymes such as elastase as well as arachidonate and O_2 derived products, they can exert a potentially aggravating role by enhancing lung injury and local inflammatory reaction [30].

The classification of sarcoidosis patients as a function of radiological changes also appears to closely parallel the variations in SACE activity. This enzyme is concentrated in endothelial lining cells of the blood vessels especially those lining the pulmonary circulation. As pulmonary endothelial metabolism may well be affected by lung injury, modification of SACE may reflect an alteration of the pulmonary endothelial system. High values of SACE were found in serum of most patients with granulomatous diseases including sarcoidosis [31]. Moreover, in these diseases, PAMs appear transformed into either ACE storage or secretory cells, respectively, and are known to be enriched in ACE [32]. Our own results were in agreement with those of other authors [33], who demonstrated a good correlation between the granulomatous process and SACE in sarcoidosis.

We used SLAPN as a convenient synthetic substrate which fits the active site of proteases such as pancreatic or leucocyte elastase, bearing in mind that sometimes the activity may not represent that of a true elastase (*i.e.* an enzyme capable of hydrolysing insoluble elastin), and results from the combined action of an endo- and an exopeptidase [33]. On the contrary, ELA is due to the proteolytic action of a true elastase. EA was demonstrated in all the BAL cells obtained either from healthy smokers or non-smoking patients, and from sarcoidosis patients, but no difference in the total amount of SLAPN enzyme activity could be shown between the different groups. On the basis of inhibitory studies, we showed that EA was due mainly to a metalloprotease, whatever

the stage of the disease. This may be combined with the finding of FERA *et al.* [17] who demonstrated that approximately two thirds of elastase-like activity in BAL from smokers was due to a metalloprotease. Moreover, this enzyme activity was largely inhibited by bestatin, an inhibitor of leucine aminopeptidase. This finding suggests that for complete processing SLAPN requires the combined action of a metalloendopeptidase cleaving SLAPN between the P_1 and P_2 Ala-Ala peptide bond, and an exopeptidase hydrolysing the residual Ala-NA. Such a combined action of two proteases in SLAPN hydrolysis was observed in cultured skin fibroblasts [34] and in human plasma [33, 35].

In contrast, even by using a sensitive assay capable of detecting subnanograms of elastase, we only detected ELA in BAL cells obtained from the smoker and Stage III sarcoidosis patient. This finding allowed us to differentiate the more advanced stage of sarcoidosis from the others, by quantitating ELA in BAL cells. In particular, patients with a fibrotic or bullous roentgenographic pattern (Stage III_B), exhibited the highest ELA values. In this case, ELA could be attributed to the action of both a serine protease and a metalloprotease, whereas ELA of BAL cells from the smoker was primarily due to a metalloprotease. Metalloenzyme activity against ^{14}C elastin was also detected by NIEDERMAN *et al.* [36] in BAL of smokers and may be derived from PAMs [5]. Indeed, neutral metallo-ELA was found to be secreted by PAMs of mouse, bovine, baboon and human lungs [8, 10, 12, 13] and appears to reflect the stage of maturation, or an activation of PAMs in response to tobacco smoke inhalation or exposure to various noxious agents. NIEDERMAN *et al.* [36] have recently proposed that human BAL PAMs were the source of metalloelastase activity against both radiolabelled elastin and SLAPN in lavage fluids. Our own studies on BAL cell extracts allowed us to confirm this proposition, and also suggested that this elastase activity would be associated with two distinct enzymes, one elastase-like metalloendopeptidase active against SLAPN only, acting together with an aminopeptidase and one true elastase active against insoluble elastin.

Moreover, on the basis of correlation studies between ELA and the number of PMNs in BAL, the detection of serine ELA in BAL cells from Stage III sarcoidosis patients appears to be mostly related to the increased number of PMNs. This protease activity may also originate from activation of PMNs and/or from internalized leucocyte elastase into PAMs [14, 37, 38]. EA was not, however, increased in Stage III sarcoidosis; this apparant discrepancy could be explained by the small number of BAL PMNs as compared to those of PAMs. Finally, the absence of correlation between EA and ELA, suggested the presence of at least two different elastase activities associated with BAL cells.

The expression of EA and ELA by macrophages obtained from BAL was qualitatively studied; these investigations were primarily undertaken to give further insight into the type of elastase activity synthesized by human macrophages in culture. ELA was not demonstrated in PAM serum-free culture medium at any time, whatever the stage of sarcoidosis. The possible presence of a proenzyme such as prostromelysin in these culture media cannot be excluded [39, 40]. Nevertheless, EA was only detected in the culture medium conditioned by PAMs from one smoker investigated. These findings must be viewed in the context of many contradictory reports in the literature, concerning elastase activities released by macrophages in culture. HINMAN *et al.* [41], showed that cultured human PAMs secreted a metallo-EA, but not ELA. EA was detected by MCLEOD *et al.* [42] in the culture medium of PAMs obtained from smokers but not in those from healthy patients. BANDA and WERB [8] detected a metalloelastase secreted into the culture medium conditioned by peritoneal murine macrophages capable of hydrolysing ^3H elastin but devoid of activity against SLAPN. VALENTINE and FISHER [12] showed that bovine PAMs released into serum-free media, a calcium dependant protease active against radio-labelled elastin substrate. It appears, therefore, that the presence of an elastolytic activity secreted into the culture media conditioned by macrophages, might depend on several parameters: origin, activation state of macrophages, direct contact of macrophages with ^3H elastin substrate [11] or with other extracellular matrix components [43, 44] or overall, on the level of secreted macrophage inhibitors of elastases [45–47]. The absence of elastolytic proteases released into the culture medium conditioned by PAMs does not exclude the presence of exogenous or endogenous membrane bound elastase activities which might be secreted *in vivo*. We found that total EA was comparable in cultured PAMs issued from healthy or sarcoidosis patients. This result is in keeping with findings obtained in BAL cells. Nevertheless, whether expressed per 10^6 cells or as a percentage, EA was higher in non-adherent than in adherent PAMs obtained from the smoker or Stage III sarcoidosis patient. This finding suggests that a minor population of PAMs might express EA in response to inflammatory lung injury. These differences in enzyme activity could not be explained by the presence of PMNs contaminating PAM cultures, since no PMN could be morphologically detected in these cell cultures.

ELA was detectable in non-adherent PAMs (but not in adherent PAMs) obtained from the smoker and Stage III sarcoidosis patients. This result confirms the above suggestion concerning the presence of a minor population of non-adherent PAMs capable of expressing a particular elastinolytic activity in response to stimuli associated with smoking and sarcoidosis. Recent data suggest that alveolar macrophages are functionally and biochemically heterogeneous [48, 49]. Our present study tends to confirm the functional heterogeneity of PAMs with respect to their elastase activity and adherence capacity.

On the basis of elastase inhibitory studies performed on extracts from adherent PAMs from Stage II sarcoidosis patients, we found that EA was due primarily to a metalloproteinase and partly to a serine protease; in contrast, we demonstrated that elastase activity against ^{3}H elastin was completely inactivated by potent inhibitors of serine protease and also partially by EDTA. These findings, in addition to those obtained in PAMs from smokers or in BAL cell extracts, suggest that three distinct types of elastase activity are liable to be present in PAMs. The first is a neutral metalloelastase-like activity, capable of hydrolysing SLAPN between but P_1 and P_2 Ala-Ala peptide bond but exhibiting no solubilizing elastin potential; it might be analogous to the endopeptidase found largely in cells such as lung cells and skin fibroblasts [50, 51], bone marrow cells [52] or neutrophils [53] and in body fluids such as serum [33, 54–57) rheumatoid synovial fluid [58]; the complete processing of SLAPN cleavage would require the combined action of this endopeptidase and an aminopeptidase. The second type of PAM elastase is a metalloelastase capable of hydrolysing ^{3}H elastin; it shares several similarities with proteoglycanase also designated as stromelysin [28, 39]. The third type of PAM elastase is a serine elastase possibly of neutrophilic or monocyte origin; it has been proposed that PAMs may have a protective function by binding, internalizing and degrading leucocyte elastase released during neutrophil degranulation [37, 38], but they may also release endocytosed neutrophil elastase and cause tissue injury at sites of mononuclear phagocyte accumulation. Moreover, the serine elastase of PAMs may also originate from monocytes or young macrophages [59].

In conclusion, the elastase-like activity present in BAL cell extracts and PAMs does not appear to reflect the evolutive stage of sarcoidosis. Nevertheless, elastase activity against ^{3}H elastin is particularly increased in sarcoidosis patients with advanced sarcoidosis associated with fibrotic or bullous roentgenographic patterns. Moreover, our results suggest strongly that three distinct types of elastase activity could be associated with PAMs and that their relative distribution depends upon the activation state of PAMs and the evolutive stage of sarcoidosis.

Acknowledgements: We wish to thank D. Guillemot and N. Rennard for their excellent typewriting assistance and Dr H.P. Schnebli (Ciba-Geigy, Basel) for providing us with the Eglin C sample.

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RÉSUMÉ: Les activités élastasiques ont été étudiées dans les cellules du lavage alvéolaire provenant de 8 sujets contrôles (C) et de patients atteints de sarcoïdose médiastino-pulmonaire non traitée à différents stades: I (n=5), II (n=7), III_A (n=3) : micronodules pulmonaires sans adénopathie, et III_B (n=4) : lésions fibrotiques ou bulleuses. Les activités de type élastase ont été mesurées vis-à-vis du substrat synthétique le succinyl-trialanine-paranitroanilide (SLAPN) pour EA, et vis-à-vis de l'élastine insoluble radiomarquée du ligament nucal bovin (ELA 3.7 MBq H/mg de radioactivité spécifique). (EA=activité élastase; ELA=activité élastolytique). On a observé une augmentation significative de ELA dans les cellules de lavage broncho-alvéolaire dans la sarcoïdose au stade III_B, particulièrement chez les patients présentant des lésions fibrotiques et un nombre élevé de poly-morphonucléaires. Au contraire, on n'a observé aucune modification de EA dans les cellules du lavage broncho-alvéolaire dans les différents stades de sarcoïdose. ELA n'a jamais été détectée dans le milieu de culture des macrophages, et est principalement associée aux macrophages non adhérents; EA est recouvre au niveau des macrophages adhérents. Les profils inhibiteurs de ELA et EA ont été déterminés à l'aisle d'inhibiteurs généraux des protéinases, ainsi que de composés agissant spécifiquement sur l'élastase leucocytaire. ELA est due principalement à une serine protease qui a une sensibilité à l'égard des inhibiteurs similaire à celle de l'élastase leucocytaire, et est augmentée aux stades avancés de la sarcoïdose. EA n'est pas, ou seulement légèrement modifié, dans la sarcoïdose, et est probablement en relation avec l'action à la fois d'une enzyme sérique et d'une métallo-enzyme. En conclusion, seul ELA fournit des informations d'utilité diagnostique concernant le stade II de sarcoïdose associée à des anomalies radiologiques fibrotiques ou bulleuses.