

## **Pulmonary alveolar macrophages from patients with active sarcoidosis express type IV collagenolytic proteinase. An enzymatic mechanism for influx of mononuclear phagocytes at sites of disease activity.**

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### Research Article

Alveolar macrophages (AMs) recovered from the bronchoalveolar lavage (BAL) of 44 patients with sarcoidosis were evaluated for their ability to release type IV collagenolytic metalloproteinase (IV-Case). This enzyme, which is produced by peripheral blood monocytes (PBMs) but not by tissue macrophages, degrades type IV collagen, the major structural component of vessel wall basement membranes, and helps to promote the migration of PBMs from the blood compartment to peripheral tissues. Our results demonstrated that AMs from patients with active sarcoidosis released significantly increased levels of IV-Case with respect to patients with inactive disease and control subjects. After in vitro culture, sarcoid AMs secreted IV-Case during the first 24 h of collection; after that time, AMs progressively lost their ability to release IV-Case. Exposition of both sarcoid and normal AMs to recombinant IL 2 or gamma IFN did not influence their property to release IV-Case. The immunoblot analysis of IV-Case demonstrated complete identity between IV-Case released by AMs and the degradative enzyme obtained from PBMs. The increased property to release IV-Case was significantly related to the increase of the absolute number of AMs and, in particular, of AMs bearing two determinants that are usually expressed by most PBMs (CD11b and CD14). Selective depletion of CD11b+/CD14+ AMs from the entire macrophagic population was associated with the recovery of the IV-Case activity to normal [...]

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# Pulmonary Alveolar Macrophages from Patients with Active Sarcoidosis Express Type IV Collagenolytic Proteinase

## An Enzymatic Mechanism for Influx of Mononuclear Phagocytes at Sites of Disease Activity

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### Abstract

Alveolar macrophages (AMs) recovered from the bronchoalveolar lavage (BAL) of 44 patients with sarcoidosis were evaluated for their ability to release type IV collagenolytic metalloproteinase (IV-C<sub>ase</sub>). This enzyme, which is produced by peripheral blood monocytes (PBMs) but not by tissue macrophages, degrades type IV collagen, the major structural component of vessel wall basement membranes, and helps to promote the migration of PBMs from the blood compartment to peripheral tissues. Our results demonstrated that AMs from patients with active sarcoidosis released significantly increased levels of IV-C<sub>ase</sub> with respect to patients with inactive disease and control subjects. After *in vitro* culture, sarcoid AMs secreted IV-C<sub>ase</sub> during the first 24 h of collection; after that time, AMs progressively lost their ability to release IV-C<sub>ase</sub>. Exposure of both sarcoid and normal AMs to recombinant IL 2 or  $\gamma$ IFN did not influence their property to release IV-C<sub>ase</sub>. The immunoblot analysis of IV-C<sub>ase</sub> demonstrated complete identity between IV-C<sub>ase</sub> released by AMs and the degradative enzyme obtained from PBMs. The increased property to release IV-C<sub>ase</sub> was significantly related to the increase of the absolute number of AMs and, in particular, of AMs bearing two determinants that are usually expressed by most PBMs (CD11b and CD14). Selective depletion of CD11b<sup>+</sup>/CD14<sup>+</sup> AMs from the entire macrophagic population was associated with the recovery of the IV-C<sub>ase</sub> activity to normal values. A positive correlation was also found between the increase in the absolute number of lung T cells and the enhanced CD4/CD8 pulmonary ratio. A 6-mo follow-up study indicated a significant association between the positivity for the <sup>67</sup>Gallium scan and the increased property of AMs to release IV-C<sub>ase</sub>. Our data are consistent with the hypothesis that a IV-C<sub>ase</sub> mediated influx of peripheral monocytes takes place in the lung of sarcoid patients. Furthermore, the correlation found between the IV-C<sub>ase</sub> release and disease activity suggests that this assay could represent a useful tool in sarcoidosis disease staging.

### Introduction

Sarcoidosis is a multisystem disease characterized by the accumulation of immunocompetent cells at different sites of dis-

ease activity. T lymphocytes of the helper-related phenotype (CD4) and monocytic-macrophagic cells accumulate within the involved tissues where they aggregate, inducing the formation and the maintenance of the granulomatous process (1, 2). Since bronchoalveolar lavage (BAL)<sup>1</sup> technique has provided access to cell populations accounting for alveolitis in different interstitial lung disorders (3, 4), a variety of information has been generated on the mechanisms promoting the accumulation of mononuclear phagocytes in the lung of sarcoid patients. Studies with incorporation of [<sup>3</sup>H]thymidine and with the cell cycle-related Ki67 MAb have shown that alveolar macrophages (AMs) obtained from patients with sarcoidosis are capable of a limited self-proliferation *in situ*, which could lead to an adaptive increase in the macrophagic population (5, 6). An alternative, but not mutually exclusive hypothesis suggests that macrophagic alveolitis is sustained by an enhanced influx of bone marrow-derived adherent cells via the blood stream. According to this interpretation, phenotypic studies showed that the majority of sarcoid AMs express surface markers related to peripheral blood monocytes (PBMs) (7). In addition, Hunninghake et al. (8) demonstrated that lung T cells from sarcoid patients release a chemotactic factor for PBMs.

Some events accomplishing recruitment of adherent cells from the blood stream to the sites of ongoing inflammation have recently been identified (9). When attracted by relevant chemotactic stimuli, PBMs acquire the property to release type IV collagenase (IV-C<sub>ase</sub>), an enzyme that is capable of binding and degrading type IV collagen, the major structural component of vessel wall basement membranes. By modifying the macromolecular organization of the basement membrane, this proteinase causes discontinuities through which PBMs may enter the inflamed tissues.

In this study, to verify whether newly recruited mononuclear phagocytes are present in the alveolar space, pulmonary adherent cells freshly recovered from the BAL of 44 patients with sarcoidosis have been evaluated for their capacity to produce IV-C<sub>ase</sub>. Functional analysis has been coupled to a phenotypic study of the expression of monocyte-related markers by AMs. Kinetics of IV-C<sub>ase</sub> production has also been determined by measuring IV-C<sub>ase</sub> activity during AM *in vitro* culture. The metalloproteinase released by pulmonary mononuclear phagocytes has been biochemically characterized and compared to the enzyme produced by PBMs. On clinical grounds, the property of AMs to release IV-C<sub>ase</sub> has been re-

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1. Abbreviations used in this paper: AMs, alveolar macrophages; BAL, bronchoalveolar lavage; IV-C<sub>ase</sub>, type IV collagenolytic proteinase; CD, cluster of differentiation; <sup>67</sup>Ga, <sup>67</sup>Gallium-citrate; PBM, peripheral blood monocyte.

lated to some markers of disease activity and a 6-mo follow-up study of the AM IV-C<sub>ase</sub> activity has been evaluated.

## Methods

**Study population.** 44 sarcoid patients (24 women and 20 men, mean age 33±6 yr) were studied. The diagnosis was based on consistent clinical features and was confirmed with laboratory and histopathologic findings. In all cases, biopsy material obtained from lungs and/or lymph nodes showed the typical noncaseating granulomas with no evidence of inorganic material known to cause other granulomatous diseases. Also, at the time of diagnosis, tissue cultures were negative for fungus and tuberculosis.

All patients had abnormal chest x rays and according to radiologic pulmonary changes, the patients were classified into class I (28 patients) and class II (16 patients) sarcoidosis. No class III patients were included in this study. Disease activity was evaluated according to (a) clinical features (such as fever, cough, dyspnea, weight loss, severe fatigue, progressive changes on chest x ray within the last 3 mo, etc.); (b) percentage of lymphocytes and the absolute number of T cells recovered from the BAL; (c) pulmonary CD4/CD8 ratio; and (d) <sup>67</sup>Gallium-citrate (<sup>67</sup>Ga)-scan positivity as previously described (10). According to these criteria, 25 patients were affected by active sarcoidosis, whereas 19 patients were in the inactive phase of the disease. Both active and inactive sarcoidosis patients were nonsmokers. All subjects with active sarcoidosis were studied at the time of diagnosis, before any therapy. 11 out of the 19 patients with inactive sarcoidosis had previously received corticosteroid therapy, but at the time of the study they had been off therapy for at least 1 mo (specifically, two patients had been off therapy for 1 mo, six patients for 3 mo, and 3 patients for 1 yr).

8 normal nonsmoking volunteers (five men and three women; average age 36±6) were evaluated as controls. They showed normal physical examinations, chest x rays and lung function tests. They were not under therapy at the time of the study.

**Preparation of cell suspensions and purification of adherent cells.** Bronchoalveolar lavage was performed after local anesthesia. A fiberoptic bronchoscope was wedged in a segment of the right lobe or lingula and a total of 100–150 ml of sterile saline solution (warmed to 37°C) was injected in 25-ml aliquots with immediate vacuum aspiration after each aliquot. Immediately after the BAL, the fluid was filtered through layers of surgical gauze and the volume was measured. Cells recovered from BAL were washed three times with PBS, resuspended in HBSS without Ca<sup>2+</sup>, and counted in a hemocytometer. The differential count of macrophages, lymphocytes, neutrophils, and eosinophils (made from a total count of 300 cells) was accomplished according to morphological criteria in cytocentrifuged smears stained with May Grunwald-Giemsa. Cells recovered from the BAL were centrifuged on a Ficoll/Hypaque density gradient and then resuspended in RPMI 1640 culture medium (Gibco Laboratories, Grand Island, NY) supplemented with 2 mM glutamine, 100 µg/ml streptomycin, and 100 U/ml penicillin. PBMCs were obtained from the peripheral blood by a standard Ficoll/Hypaque density gradient technique.

Alveolar macrophages and PBMs were isolated from the entire mononuclear cell population of BAL and from PBMCs by adherence procedures in plastic petri dishes and then removed using a disposable cell scraper as already described (11). The resulting AM and PBM populations were > 94% pure on the basis of nonspecific esterase staining and > 95% of the cells were viable, as judged by the trypan blue exclusion test.

**Evaluation with MAbs.** A panel of commercially available MAbs belonging to the OK (Ortho Pharmaceutical, Raritan, NJ) and Leu (Becton Dickinson & Co., Mountain View, CA) series was used. Monoclonal antibodies used to define the AMs phenotype were OKM1 (CD11b), which recognizes the receptor for C3b on a related structure expressed on more than 90% of PBMs and on < 25% of AMs (7), and LeuM3 (CD14), which defines a differentiation antigen ex-

pressed on > 90% of PBMs, but only to a certain degree by normal AMs (< 10%) (7). Lung T cells were identified by their reactivity with an MAbs (OKT3) belonging to the CD3 cluster, whereas T subpopulations were stained with MAbs belonging to the CD8 (OKT8 and Leu 2) and CD4 (OKT4 and Leu 3) clusters, which include suppressor/cytotoxic and helper-related cells, respectively. The frequency of BAL cells positive for the above reagents was determined by flow cytometry, as previously reported elsewhere (12).

**Negative selection of CD11b<sup>+</sup> and CD14<sup>+</sup> phagocytes from the entire AM population.** Pulmonary macrophages bearing the CD11b and CD14 monocyte-related markers were removed from the entire AM population using magnetic microspheres coated with goat anti-mouse IgG (Dynabeads; Dynal Oslo, Norway), according to the method of Lea et al. (13). After incubation of AMs with CD11b and CD14 MAbs (45 min at 4°C) in two separate tubes and washing, 40 × 10<sup>6</sup> beads/ml were incubated with 10 × 10<sup>5</sup> AMs/ml for 30 min at 4°C under continuous slow rotation. The CD11b<sup>+</sup> and CD14<sup>+</sup> AMs rosetting with antibody coated beads were isolated and removed by applying a magnetic system on the outer wall of the test tubes for 2 min. The incubation with the beads and the magnetic separations were repeated twice. Less than 2% of the remaining cells were CD11 and CD14 positive. More than 98% of the cells obtained with this procedure was viable when tested by trypan blue exclusion.

**Evaluation of the type IV collagenolytic activity.** Type IV collagen was biosynthetically labeled with [<sup>14</sup>C]proline from organ cultures of Engelbreth-Holmes-Schwarm sarcoma, as already reported (14). Each substrate preparation was checked for purity by SDS-PAGE and stored frozen in 0.5 M acetic acid until use. The quantitation of in vitro type IV-collagen degradation by monocyte-macrophages was performed according to the method previously described (9). Briefly, 24 [<sup>14</sup>C]type IV collagen-coated flat-bottom wells (Becton Dickinson & Co., Oxnard, CA) were sterilized by exposure to UV light for 20 min. 3 × 10<sup>5</sup> effector cells (entire AM population, CD11b- and CD14-depleted AMs or PBMs freshly recovered, as above reported from BAL and peripheral blood, respectively) were plated with 1.0 ml HB 101 medium in 16-mm wells coated with 2 × 10<sup>3</sup> cpm of <sup>14</sup>C-labeled type IV collagen. Control wells contained 1.0 ml of HB 101 medium with or without bacterial collagenase (10 µg/ml, Advanced Biofactures Co., USA). After 18 h of incubation, 600 µl of supernatants were mixed with 5 ml of Insta-Gel (Packard Instruments, Downers Grove, IL) and counted for <sup>14</sup>C radioactivity in a scintillation counter (Wallace 1210 Ultrabeta; LKB Instruments, Gaithersburg, MD). The mean value of triplicate assays was used to calculate the percentage of IV-C<sub>ase</sub> activity according to the following formula: (counts per minute bacterial collagenase – counts per minute control)/counts per minute test – counts per minute control) × 100.

To study the production kinetics of the enzyme, the differential secretion of IV-C<sub>ase</sub> by AMs and PBMs from eight patients with active sarcoidosis and AMs from four control subjects was verified by conditioned media dilution and digestion time studies. Progressive dilutions (done twice) of the conditioned media from 3 × 10<sup>5</sup> cells showed a linear decrease of IV-C<sub>ase</sub> activity. In particular, IV-C<sub>ase</sub> released by as little as 3 × 10<sup>4</sup> PBMs was still detectable, whereas IV-C<sub>ase</sub> released by 3 × 10<sup>4</sup> AMs was not significant even after a 16-h incubation period (data not shown). Accordingly, the time course determinations have been performed using undiluted conditioned media. Alveolar macrophages and PBMs isolated as described above were plated in 25-ml culture flasks (Becton-Dickinson & Co.) and cultured (a) in Iscove's medium supplemented with transferrin, albumin, soybean lecithin, glutamine, and Hepes (Flow Laboratories, Inc., Rockville, MD); and (b) in culture medium supplemented with recombinant (r) IL 2 (500 IU/ml; Glaxo Institute for Molecular Biology S.A., Geneva, Switzerland). Determinations of type IV collagenolytic activity were performed after 6, 24, 48, and 96 h, as reported above. The viability of cultured monocyte-macrophages was always > 80% during the entire culture period.

**PAGE and immunoblotting.** Conditioned media (0.2 ml) from 24-well plates, obtained as reported above, were precipitated by 90% cold

ethanol, centrifuged at 27,000 g (for 30 min), and solubilized in sample buffer. SDS-PAGE was performed using a Tris-glycine buffer system as described by Laemmli (15) with 10% separating and 5% stacking gels. Transfer of proteins from polyacrylamide gels to Hybond-C Extra (Amersham International, Amersham, UK) was performed using 12 mM Tris, 100 mM glycine buffer containing 10% methanol with a Trans-blot cell (Bio-Rad Laboratories) for 2 h at 0.2 A. The transfer efficiency was verified by staining the gels after transfer with Coomassie blue. The Hybond-C Extra was then saturated with 3% gelatin solution, and treated with rabbit anti-human-type IV collagenolytic proteinase polyclonal antibodies (Garbisa, S., M. Onisto, G. Fastelli, and L. A. Liotta, manuscript submitted for publication) (1:300) and a second antibody as in the Immuno-Blot goat-anti-rabbit-horseradish peroxidase protocol (Bio-Rad Laboratories).

**Follow-up study of the type IV-C<sub>ase</sub> activity.** A 6-mo follow-up study of the AM IV-C<sub>ase</sub> activity was performed in nine patients (seven patients with active sarcoidosis and two patients with inactive disease). During this period patients with active sarcoidosis received corticosteroid therapy (40 mg prednisone/d), whereas the two patients with inactive disease were followed without any therapy.

**Statistical analysis.** All data were presented by mean±SEM and comparisons between mean values were made using the Cockran-Cox test. Because the data were not normally distributed, statistical analysis was made using a nonparametric test (i.e., Spearman's rank correlation test) to test correlation coefficients between the distribution of disease activity markers, the frequency of CD11b and CD14 AMs and the AM production of IV-C<sub>ase</sub>.

## Results

As shown in Table I, the percentage of pulmonary lymphocytes, the absolute number of pulmonary lymphocytes, the absolute number of AMs, and the CD4/CD8 ratio were found to be increased in the BAL of patients with active sarcoidosis when compared with the corresponding values obtained from both patients with inactive disease and normal subjects (in both cases  $P < 0.001$ ).

Data concerning IV-C<sub>ase</sub> activity by PBMs and AMs from 34 sarcoid patients are shown in Fig. 1. After 18 h of incubation, AMs recovered from controls degraded only small amounts of type IV-collagen (4.3±1.2%). By contrast, IV-C<sub>ase</sub> expressed by AMs recovered from patients with active sarcoidosis was significantly increased compared with control subjects (26.1±2.9%;  $P < 0.001$ ). AMs from patients with inactive disease displayed intermediate levels of IV-C<sub>ase</sub> (11.9±1.2%). The difference was found to be statistically significant with respect to both patients with active disease ( $P < 0.001$ ) and controls ( $P < 0.05$ ). Despite the interruption of

the steroid therapies at different times, there were not differences among randomly chosen inactive patients for IV-C<sub>ase</sub> production.

As far as the production of IV-C<sub>ase</sub> by PBMs is concerned (Fig. 1, top), we demonstrated that PBMs recovered from both sarcoid patients and controls degraded high amounts of type IV-collagen (87.1±4.7%, 82.5±5.1% and 86.2±6.2% in patients with active and inactive disease and controls, respectively). No statistically significant differences were observed in the values obtained between the different groups of patients.

Type IV-C<sub>ase</sub> activity by sarcoid and normal monocyte-macrophages was also tested after collection periods ranging between 6 and 96 h at resting conditions and after exposure to  $\gamma$ IFN and rIL 2 (Fig. 2). AMs cultured in medium alone from patients with sarcoidosis degraded significant amounts of type IV-collagen during the first 24 h of culture (the maximum IV-C<sub>ase</sub> activity was found between the third and ninth hours; data not shown). When the time of culture was prolonged, IV-C<sub>ase</sub> activity gradually decreased and became undetectable after 4 d. Kinetics production of IV-C<sub>ase</sub> by PBMs was similar to that of sarcoid AMs. In fact, after 4 d of culture normal and sarcoid PBMs failed to degrade type IV collagen (Fig. 2). Exposure of AMs from sarcoid individuals with rIL 2 and  $\gamma$ IFN failed to stimulate the release of the enzyme. Normal AMs expressed very low levels of type IV-C<sub>ase</sub> during the entire period of culture. When we tested the effect of  $\gamma$ IFN and IL 2 on normal AMs, we demonstrated that both lymphokines were unable to influence the enzyme release (3.9±1.9, 1.6±0.9, and 1.9±0.8%, by unstimulated AMs and after stimulation with  $\gamma$ IFN and IL 2, respectively).

As shown in Fig. 3, immunoblot analysis of the neutral proteinase revealed no differences between IV-C<sub>ase</sub> produced by PBMs and the degradative enzyme of AM origin. Antibodies against the type IV collagenolytic metalloproteinase purified from peritoneal inflammatory macrophages recognized an identical antigenic band of ~ 70,000 D in SDS-PAGE.

Fig. 4 shows the results of the relationship between the property of AMs to express IV-C<sub>ase</sub> activity and some markers of disease activity in 34 sarcoid patients. The property of sarcoid AMs to express IV-C<sub>ase</sub> significantly correlated with the increase of the CD4/CD8 pulmonary ratio, the enhanced absolute number of lung T lymphocytes and the enhanced absolute number of AMs from the BAL of sarcoid patients ( $r = 0.61$ ,  $P < 0.01$ ;  $r = 0.43$ ,  $P < 0.05$ ;  $r = 0.48$ ,  $P < 0.05$ , respectively).

In 10 additional patients (6 with active sarcoidosis and 4 with inactive sarcoidosis) we evaluated the relationship be-

Table I. Cell Types Recovered from Bronchoalveolar Lavage Fluid of Patients with Sarcoidosis

Group	Lavage fluid recovery	Total cells × 10 <sup>-3</sup>	Differential cell count								
			Lymphocytes × 10 <sup>-3</sup>		Macrophages × 10 <sup>-3</sup>		Neutrophils × 10 <sup>-3</sup>		Eosinophils × 10 <sup>-3</sup>		CD4/CD8 ratio
			%	ml	%	ml	%	ml	%	ml	
Active sarcoidosis (n = 25)	57.1±6.9	351.6±18.1**	43.6±3.1**	133.6±17.1**	54.6±4.1**	220.4±18.2**	1.0±0.4	2.9±1.1	0.2±0.2	0.8±0.3	7.5±1.5**
Inactive sarcoidosis (n = 19)	55.2±5.9	101.2±16.4	10.8±2.1	8.2±1.3	87.5±6.3	97.3±10.6	1.1±0.6	0.1±0.1	0.1±0.1	0.1±0.1	1.7±0.4
Controls (n = 8)	57.8±5.2	106.9±15.7	7.9±1.5	7.1±1.5	90.8±2.1	97.1±15.1	0.8±0.2	0.8±0.2	0.5±0.2	0.4±0.2	1.9±0.3

Active sarcoidosis vs. inactive sarcoidosis, \*  $P < 0.001$ ; active sarcoidosis vs. controls, †  $P < 0.001$ ; inactive sarcoidosis vs. controls, NS.

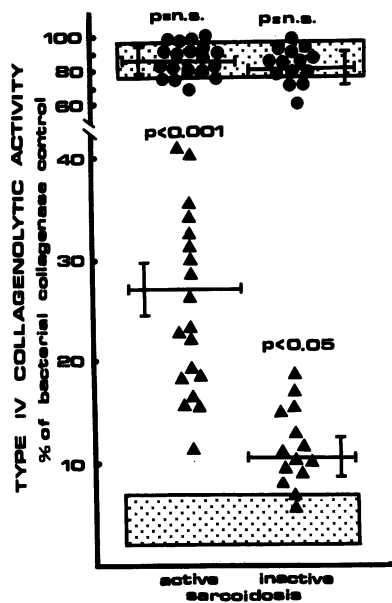


Figure 1. Type IV collagenolytic activity by alveolar macrophages ( $\Delta$ ) and PBMs ( $\bullet$ ) of patients with active and inactive sarcoidosis. Each data point represents the mean of three determinations. Shaded areas represent the control ranges of normal alveolar macrophages (bottom) and monocytes (top).

tween type IV- $C_{ase}$  activity and the expression of the monocyte-related CD11b and CD14 markers by AMs. A significant correlation was demonstrated between type IV-collagen degradation and the absolute number of CD11b- and CD14-positive AMs ( $r = 0.77$ ,  $P < 0.01$ ; and  $r = 0.71$ ,  $P < 0.01$ , respectively) (Fig. 5).

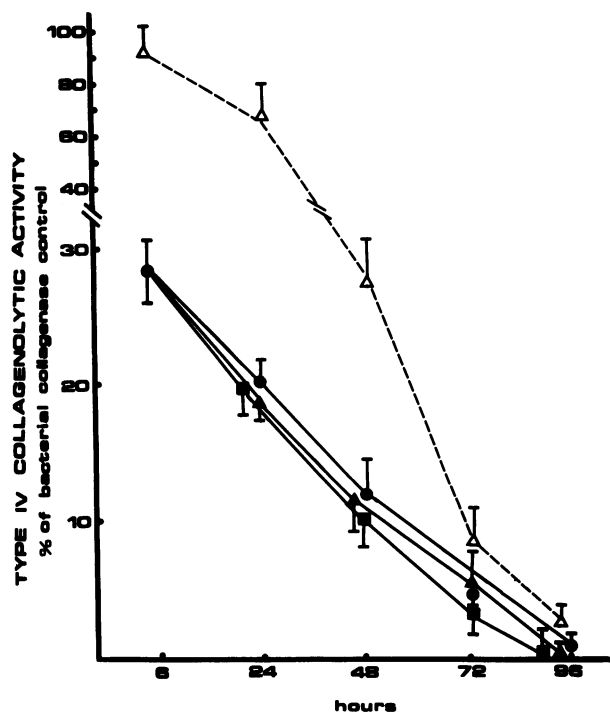


Figure 2. Type IV collagenolytic activity by alveolar macrophages recovered from BAL of eight patients with active sarcoidosis after different culture periods ( $\bullet$ , unstimulated AMs;  $\Delta$ , AMs cultured in presence of rIL 2;  $\blacksquare$ , AMs cultured in presence of  $\gamma$ IFN;  $\triangle$ , unstimulated PBMs). Controls with Iscove's medium alone have been subtracted.

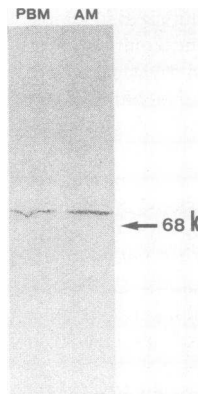


Figure 3. Immunoblot analysis of culture media conditioned by PBMs and AMs from a representative patient with active sarcoidosis. After SDS-PAGE, proteins have been transferred to Hybond-C Extra and identical antigenic bands are recognized by anti-type IV collagenase polyclonal antibodies.

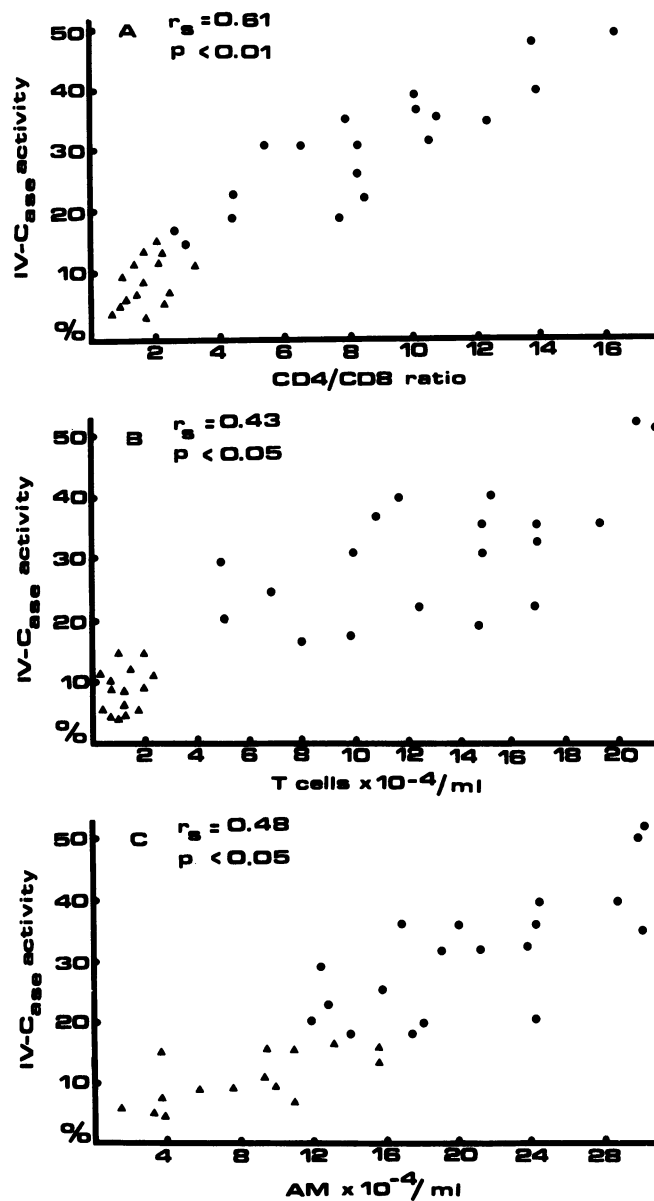


Figure 4. Relationship between the property of alveolar macrophages to release type IV collagenase and the pulmonary CD4/CD8 ratio (A), the absolute number of T lymphocytes (B), and the absolute number of AMs (C) in patients with active ( $\bullet$ ) and inactive ( $\Delta$ ) sarcoidosis. Coefficient of correlation ( $r$ ) has been calculated using Spearman's rank correlation test.

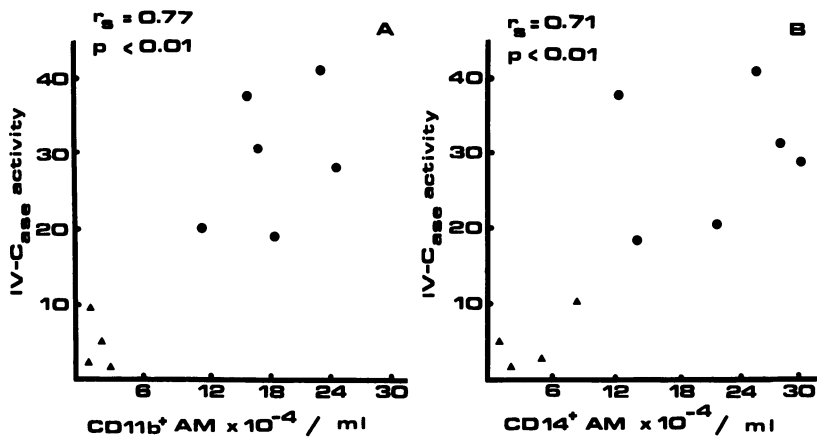


Figure 5. Relationship between the property of alveolar macrophages to release type IV collagenase and the absolute number of AMs bearing CD11b (A) and CD14 (B) monocyte-related determinants in patients with active (●) and inactive (▲) sarcoidosis. Coefficient of correlation ( $r$ ) has been calculated using Spearman's rank correlation test.

In four patients with active sarcoidosis, the CD11b and CD14 AM fractions were removed from the entire AM population and different fractions were evaluated for their ability to release IV-C<sub>ase</sub>. As shown in Fig. 6, selective depletion of AMs bearing CD11b and CD14 determinants was associated with a recovery of IV-C<sub>ase</sub> expression to the normal range.

Concerning the relationship between the <sup>67</sup>Ga-scan positivity and the AM capacity to produce IV-C<sub>ase</sub>, 11 out of the 19 patients with positive <sup>67</sup>Ga scans showed a level of IV-C<sub>ase</sub> activity > 3 SD of the mean value obtained in subjects with inactive disease and negative <sup>67</sup>Ga scans. A 6-mo follow-up study of AM IV-C<sub>ase</sub> activity was performed in nine patients: in four out of seven patients with positive <sup>67</sup>Ga scans, the persistent <sup>67</sup>Ga positivity was still associated with a consistently increased expression of the enzyme at the end of the study (Fig. 7 A); by contrast, in three <sup>67</sup>Ga<sup>+</sup> patients, the recovery of the

<sup>67</sup>Ga-positivity after 6 mo of steroid therapy was followed by a parallel decrease of type IV collagenolytic activity (Fig. 7 B). In two additional cases, low levels of IV-C<sub>ase</sub> activity were associated with a persistent negativity for <sup>67</sup>Ga scan during the entire follow-up period (Fig. 7 C).

## Discussion

In this study, we demonstrated that (a) mononuclear phagocytes recovered from the BAL of patients with active sarcoidosis released a type IV-collagenolytic proteinase that degrades the major structural component of vessel basement membranes; (b) similar to PBMs, AMs lost this property after in vitro culture; (c) on the basis of immunoblot analysis, the enzyme released by pulmonary macrophages and the degradative enzyme from blood monocyte origin were virtually identical; (d) the property of pulmonary phagocytes to release IV-C<sub>ase</sub> was significantly related to the absolute number of AMs bearing monocyte-related markers and to the main indices of disease activity of sarcoidosis; (e) selective depletion of AMs expressing monocyte-related determinants was associated with a recovery of IV-C<sub>ase</sub> production to normal values; and (f) a 6-mo follow-up study of IV-C<sub>ase</sub> activity indicated a positive association between <sup>67</sup>Ga scan positivity and the secretory capacity of AMs. These data support the hypothesis that an increased IV-C<sub>ase</sub>-mediated influx of PBMs occurs in the lung of patients with active sarcoidosis. Moreover, the correlation we demonstrated between the property of AMs to produce IV-C<sub>ase</sub> and the disease activity underlines the potential use of this assay in the staging of sarcoidosis.

During chronic inflammatory processes of the lung, multiple strategies can account for sustaining the increased demand of AMs: enhanced proliferative activity, release of preexisting cells from reservoirs within the lung, or increased recruitment of PBMs (16, 17). Providing new evidences for an enhanced traffic of monocytes, our study supports the concept that the last mechanism predominates in sarcoid patients. Because PBMs, but not mature macrophages, are the source of IV-C<sub>ase</sub> (9), the demonstration that BAL mononuclear phagocytes release high amounts of type IV-C<sub>ase</sub> per se indicates the presence of freshly recruited PBMs. Kinetic studies of enzyme production further confirm this interpretation. It is well known that in vitro culture induces the terminal differentiation of mononuclear phagocytes. Thus, the lack of IV-C<sub>ase</sub> production by

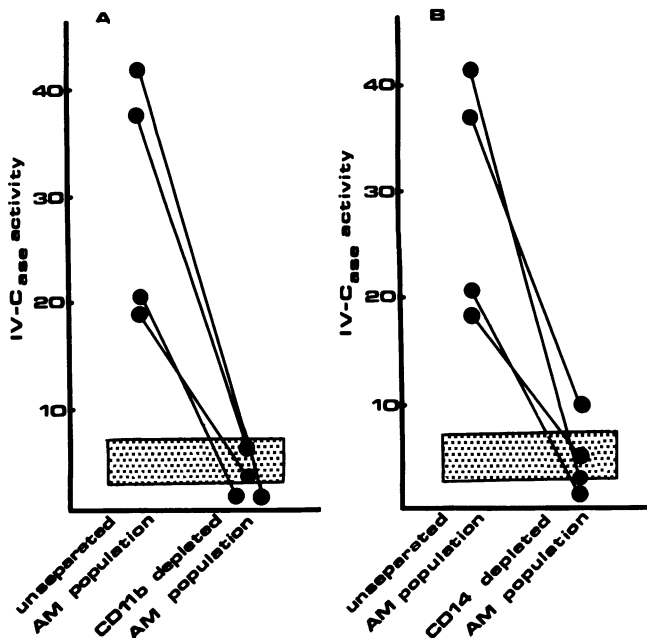


Figure 6. Release of type IV collagenase by sarcoid AMs before and after the selective depletion of CD11b- (A) and CD14-positive (B) cells from the entire macrophagic population. Shaded areas represent the control ranges of type IV collagenase release by normal AMs.

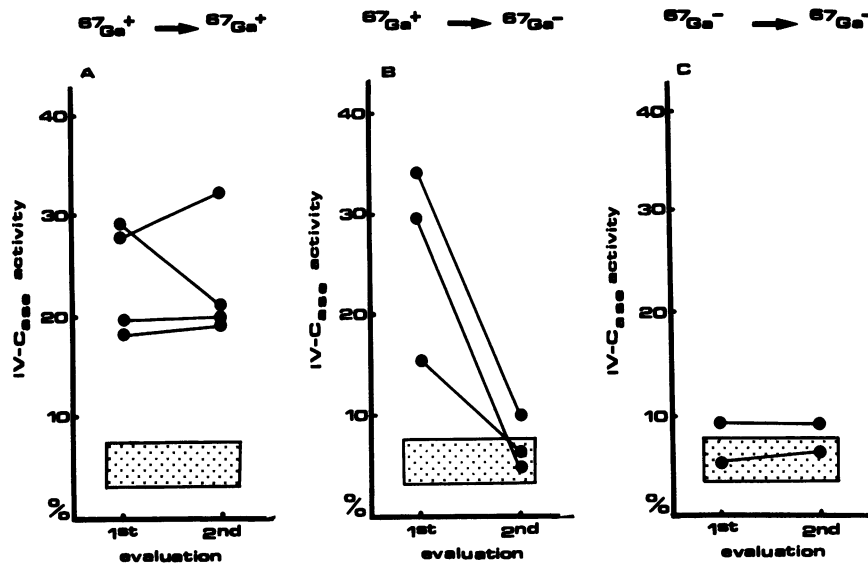


Figure 7. Relationship between the property of alveolar macrophages in releasing type IV collagenase and the positivity or negativity of the  $^{67}\text{Ga}$  scan in nine patients with sarcoidosis during a 6-mo follow-up. Shaded areas represent the control ranges of type IV collagenase release by normal AMs.

cultured AMs is probably related to the differentiation of the young, recruited adherent cells into mature, unproductive macrophages. Furthermore, (a) the immunoblot demonstration of a complete identity between the proteinase of AM source and the monocytic IV-C<sub>ase</sub>; (b) the finding of a significant relationship between the IV-C<sub>ase</sub> activity and the enhanced expression of monocyte-related markers, and (c) the demonstration that the removal of CD11b and CD14 cells abolishes IV-C<sub>ase</sub> expression are in line with the interpretation that this proteinase is a product of young macrophages.

An increased production of IV-C<sub>ase</sub> by cells belonging to the monocytic lineage has been demonstrated in other diseases that are characterized by a quick recruitment of PBMs to sites of ongoing inflammation. Monocytes recruited into the peritoneal cavity of subjects with acute peritonitis release high levels of IV-C<sub>ase</sub> (9). Furthermore, in a patient with systemic lupus erythematosus who developed pleurisy sustained by an accumulation of monocyte-like cells, we evidenced the presence of great amounts of IV-C<sub>ase</sub> in the pleural fluid (Garbisa, S., M. Onisto, G. Fastelli, C. Caenazzo, C. Agostini, R. Zambello, L. Trentin, and G. Semenzato, manuscript submitted for publication). These data suggest that the release of IV-C<sub>ase</sub> can be considered as a recruitment mechanism that is common to different pathological conditions and contributes to the expansion of the macrophagic population of PBMs when an increased demand of mononuclear phagocytes occurs. Unfortunately, molecular pathways that regulate this migration mechanism are still unknown in both normal subjects and in different disease states. To approach this problem, as rIL 2 and  $\gamma$ IFN are actively released in the lung of sarcoid patients (18, 19), we verified whether these biological response modifiers were capable of influencing IV-C<sub>ase</sub> production. Regarding the experimental system we assessed, our finding indicates that these lymphokines are not involved in the modulation of the IV-C<sub>ase</sub> release. However, it should be mentioned that we observed a strict correlation between the increase in the absolute number of T cells, the enhanced CD4/CD8 ratio, and the capacity of AMs to release IV-C<sub>ase</sub>. These data suggest that helper T cells are probably involved in the regulation of the

enzyme-mediated influx of PBMs (perhaps through the release of lymphokines other than IL 2 and  $\gamma$ IFN). Because this is a crucial point in the comprehension of the mechanisms that initiate the sarcoid inflammatory process, further studies are needed to determine whether or not and which soluble factors modulate the release of IV-C<sub>ase</sub>. Another noteworthy piece of evidence emerging from our kinetic studies is the lack of any IV-C<sub>ase</sub> activity after the exposure of normal AMs to  $\gamma$ IFN and IL 2. These data, contradicting the hypothesis of a potential role of these lymphokines in the induction of IV-C<sub>ase</sub> on pulmonary phagocytes, indirectly support our interpretation of the monocyte recruitment.

Several authors provided evidence that AMs are activated in patients with sarcoidosis. This statement is supported by the fact that AMs (a) secrete biological mediators of the immune response (19–23); (b) display enhanced antigen presenting capacity (10, 24, 25) and (c) express determinants related to the activation state of the monocytic-macrophagic lineage (7, 11). As we demonstrated that alveolar macrophages recovered from patients with active sarcoidosis represent a heterogeneous population encompassing both monocytes and mature tissue macrophages, a major factor at this point is establishing whether the cells that account for the increased state of activation of the macrophagic component of alveolitis are the newly recruited phagocytes and/or resident macrophages that became “activated.” Studies in animal models have demonstrated that during a chronic inflammatory process of the lung, the cells that acquire the functional properties of activated macrophages are newly recruited PBMs, rather than preexisting AMs (17). Conceivably, the hypothesis could be formulated that in the lung of sarcoid patients, monocytes newly recruited from peripheral blood could be responsible for the above reported activation of the macrophage population.

Evidence herein presented that the ability of AMs to produce type IV collagenolytic proteinase is related to the main laboratory indices of disease activity suggests that the release of IV-C<sub>ase</sub> could serve as a parameter of activity of the sarcoid process. A further confirmation of this hypothesis comes from the fact that increased levels of IV-C<sub>ase</sub> were always signifi-

cantly associated to positivity for the  $^{67}\text{Ga}$  scan (Fig. 7). This technique, taking advantage of the high affinity of  $^{67}\text{Ga}$  for monocytic-macrophagic cells (26), gives a measurement of the degree of the macrophagic alveolitis. Although the concept must be substantiated by more extensive follow-up studies, we believe that the determination of IV-C<sub>ase</sub> production might be helpful in defining the intensity of the macrophagic component of alveolitis. Because alternative and accurate in vitro assays for monitoring the degree of the macrophagic alveolitis are lacking, this test could be a useful clinical tool. In this regard, the determination of IV-C<sub>ase</sub> activity, a simple and easy test to perform in every laboratory, could be applied to the study of other interstitial lung diseases characterized by a macrophagic alveolitis (such as histiocytosis X, berylliosis, silicosis, etc.). However, we are standardizing a sandwich enzyme immunoassay (EIA) for the quantitative determination of IV-C<sub>ase</sub> levels in serum, other extracellular body fluids, or cell culture supernatants. This assay will allow the detection of this protease in any form: proenzyme or either active or inactive (inhibited) enzyme. Additional studies will determine whether EIA determination of IV-C<sub>ase</sub> will be advantageous in monitoring the clinical course of disorders which are characterized by a rapid recruitment of immunocompetent cells, including sarcoidosis.

A further observation suggested from our follow-up study deals with the possible interference of corticosteroid therapy with the property to express IV-C<sub>ase</sub> by adherent cells. Interestingly, after six months of steroid treatment three out of seven patients with active sarcoidosis showed a marked reduction of the IV-C<sub>ase</sub> expression that paralleled the regression of the  $^{67}\text{Ga}$  scan positivity. Because it has been reported that corticosteroids are capable of inhibiting the secretion of neutral protease by macrophages (27), the hypothesis can be formulated that steroid therapy might inhibit the migrative capacity of PBMs by interfering with their property to release IV-C<sub>ase</sub> and/or other proteases. Currently, in vitro studies are in progress in our laboratory to investigate whether glucocorticoids influence the production of IV-C<sub>ase</sub> by monocyte-macrophages.

In conclusion, our results indicate the presence of an increased release of type IV collagenolytic protease by AMs of patients with active sarcoidosis that is significantly associated with an increase in the AM population and with the expression of monocyte-related markers. Together, these data suggest that the influx of monocytes in the blood into the alveolar space could represent an important mechanism in the development of a macrophage accumulation in sarcoid lung. Because the enzymatic activity of AMs seems to be related to disease activity, more extensive follow-up studies are needed to verify the usefulness of this test in monitoring the macrophagic component of alveolitis in interstitial lung disorders.

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