Release of prostaglandin E₂ and leukotriene B₄ by alveolar macrophages from patients with sarcoidosis

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Abstract

Background – Mediators released by alveolar macrophages, as well as by T cells, play an important part in modulating local immune processes in sarcoidosis. Among alveolar macrophage secretory products, arachidonic acid metabolites are known to regulate inflammatory and immune reactions. It has been suggested that cyclooxygenase and lipoxygenase pathway metabolites of arachidonic acid modulate the evolution of the granulomatous inflammatory response in the lung differently.

Methods – Alveolar macrophages recovered from the bronchoalveolar lavage (BAL) fluid of 32 patients with sarcoidosis in different states of disease activity and 10 normal subjects were evaluated for their ability to release prostaglandin E₂ (PGE₂) and leukotriene B₄ (LTB₄). Alveolar macrophages were cultured in the presence or absence of opsonised zymosan (500 µg/ml), and PGE₂ and LTB₄ levels in the culture supernatants were determined by enzyme immunoassay (EIA).

Results – Stimulated alveolar macrophages from patients with active sarcoidosis released higher LTB₄ levels than those from normal subjects, but no differences in PGE₂ release were observed between the two groups. The time course of LTB₄ release by activated alveolar macrophages showed that normal cells produced similar levels of the hydroxyacid during the early and late times of culture while LTB₄ release by activated cells from patients with sarcoidosis increased markedly after 60 minutes of culture, remaining elevated until 24 hours. Indomethacin (3×10^{-6} M) caused the expected inhibition of PGE₂ formation without affecting LTB₄ release.

Conclusions – These results suggest that alveolar macrophages from the BAL fluid of patients with active sarcoidosis are primed to release LTB₄, which may contribute to the locally heightened immune response.

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Keywords: alveolar macrophages, pulmonary sarcoidosis, arachidonic acid metabolites.

Sarcoidosis is a multisystem chronic granulomatous disorder of unknown aetiology char-

acterised by heightened immune processes at the site of disease activity.¹ In particular, an enhanced recruitment and activation of immunocompetent cells (alveolar macrophages and T cells) occurs within the alveolar structures. Once activated, these cells have a wide spectrum of effector functions including secretion of various mediators that play an important part in the evolution and modulation of local immune processes in sarcoidosis.¹⁻³ Among the alveolar macrophage secretory products, arachidonic acid metabolites are known to regulate inflammatory and immune reactions.³⁻⁵ Although there has been increasing interest in the potential immunomodulatory role of these metabolites, few studies have investigated the characteristics of arachidonic acid metabolism in relation to specific lung diseases.⁶⁻¹²

Arachidonic acid is one of the most represented cell membrane lipids in macrophages and monocytes. Two major pathways account for its metabolism: (1) the cyclo-oxygenase pathway leading to the formation of prostaglandins and thromboxanes, and (2) the metabolism of arachidonic acid through the lipoxygenase pathway leading to the formation of hydroxyeicosatetraenoic acid and leukotrienes (LT).¹³ Several reports suggest that these products have modulatory effects on cellular immune responses. Prostaglandins of the E series (PGE) have been shown to suppress various lymphocyte functions such as T lymphocyte proliferation¹⁴ and lymphokine secretion by antigen-activated lymphocytes,¹⁵ as well as to inhibit Ia antigen expression on macrophages.¹⁶ The role of lipoxygenase products in the regulation of cellular immune responses is more complex and seems to depend on local immune conditions.¹⁷ However, the leukotrienes and other lipoxygenase derivatives are known to possess potent pro-inflammatory activities such as modulation of vascular permeability (LTC₄, LTD₄) and attraction and activation of phagocytes (LTB₄).^{5,18}

Furthermore, several reports suggest that arachidonic acid metabolites released by alveolar macrophages at inflammatory foci might play a part in modulating granulomatous lung inflammation.^{6,19,20} It has been reported that exogenous PGE₁ can suppress schistosome egg-induced lung granuloma formation in mice,¹⁹ and a similar effect has been shown by using lipoxygenase inhibitors which dramatically suppressed the development of pulmonary granulomas in a dose dependent manner.²⁰

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We hypothesised that an imbalance of arachidonic acid metabolites released by alveolar macrophages could occur in sarcoidosis and play a part in modulating the granulomatous response in this disease. To test this hypothesis we have investigated the capacity of alveolar macrophages to release PGE₂ and leukotriene B₄ (LTB₄) in vitro from patients with sarcoidosis in different states of disease activity and compared this with their release by normal alveolar macrophages.

Methods

STUDY POPULATION

Thirty two patients with sarcoidosis (18 men) of mean (SD) age 34.2 (9.18) years were studied. The diagnosis of sarcoidosis was based on consistent clinical features and was confirmed by histopathological findings. Other granulomatous diseases were reliably excluded. Disease activity was evaluated according to the consensus conference on disease activity in sarcoidosis²¹ – that is, on the basis of the following criteria: (1) clinical findings (recently developed or progressing respiratory symptoms such as cough and dyspnoea, or systemic symptoms such as fever, weight loss, severe fatigue, or progressive changes on the chest radiograph or deterioration of lung function within the last three months); (2) percentage of lymphocytes and absolute number of T cells recovered from the bronchoalveolar lavage (BAL) fluid; and (3) 67-gallium citrate (⁶⁷Ga) scan positivity. According to all three 18 patients had active sarcoidosis and 14 patients were in the inactive phase of the disease. In the first group 10 patients were radiographic stage I and eight were stage II, and in the second group six were stage I and eight were stage II. Patients with active disease were newly diagnosed and had a mean disease duration of 3.9 (0.8) months at the time of admission to the study. All had evidence of clinical deterioration defined as worsening of respiratory symptoms or progressive changes on the chest radiograph, or deterioration of lung function. In contrast, patients with inactive disease had a mean disease duration of 2.5 (0.6) years and follow up visits during the year prior to admission to the study showed a clinically stable disease in the absence of steroid treatment – that is, no respiratory symptoms, no progressive changes on the chest radiograph or deterioration of lung function. All patients studied were non-smokers and none had received steroids or non-steroidal anti-inflammatory agents in the previous three months. Ten normal non-smoking volunteers (six men) of mean age 39.2 (9) years acted as controls. All were asymptomatic with normal chest radiographs and pulmonary function. None had recently received any drug.

The study conformed to the Declaration of Helsinki and informed consent was obtained from each subject.

ISOLATION OF ALVEOLAR MACROPHAGES

Alveolar macrophages were obtained by BAL which was performed under local anaesthesia

according to the method previously described.²² Briefly, a fiberoptic bronchoscope was wedged in a segment of the middle lobe or lingula and 150–200 ml of sterile saline solution (warmed to 37°C) was injected in 20 ml aliquots and immediately recovered by gentle suction. BAL fluid was filtered through two layers of surgical gauze to remove mucus and centrifuged at 400 *g* for 10 minutes at 4°C. The resulting pellet was washed twice with phosphate buffered saline (PBS; Sigma Chemical, St Louis, Missouri, USA), resuspended in Hank's balanced salt solution (HBSS), and cells were counted with a haemocytometer. The differential cell count was accomplished according to morphological criteria in cytocentrifuged smears stained with May Grunwald-Giemsa.

Alveolar macrophages were isolated by adherence procedures on plastic. Briefly, BAL cells were resuspended in RPMI-1640 medium (GIBCO Laboratories, Grand Island, New York, USA) containing 100 U/ml penicillin and 100 µg/ml streptomycin (complete medium) to achieve a concentration of 1×10^6 viable macrophages per ml. An aliquot of 1 ml of the cell suspension was plated into each well of 24 well plastic culture plates (Costar, Cambridge, Massachusetts, USA) and cells were allowed to adhere for one hour at 37°C in 5% CO₂. The cells were then washed three times with warm medium to remove non-adherent cells. The resulting alveolar macrophage population was >95% pure on the basis of non-specific esterase staining and >95% of the cells were viable as judged by the trypan blue exclusion test. Alveolar macrophages were then cultured in complete medium in the presence or absence of opsonised zymosan (final concentration 500 µg/ml) for varying periods of time. Control cultures consisted of medium with or without opsonised zymosan incubated in the absence of alveolar macrophages for the same time periods and processed identically. The viability of adherent macrophages was assessed by the trypan blue exclusion test at the end of each experiment and was found to be at least 90%. The monolayer supernatants were collected, centrifuged at 3000 *g* for five minutes and frozen at –80°C for later analysis of PGE₂ and LTB₄.

To evaluate the kinetics of PGE₂ and LTB₄ release, alveolar macrophage supernatants were harvested at 0, 30, 60, 120 minutes and 24 hours of culture. Because of the high number of cells required, this analysis was carried out in only a limited number of subjects (four controls and five each with non-active and active sarcoidosis).

As the kinetics of LTB₄ release by unstimulated alveolar macrophages showed that higher levels of the hydroxyacid were produced during the early stages of culture with a tendency to decrease over the next 24 hours, in some experiments their ability to metabolise LTB₄ was determined by incubating alveolar macrophage monolayers for 24 hours in the presence of 20 000–30 000 dpm/10⁶ cells of [³H]-LTB₄; control cultures consisted of medium with [³H]-LTB₄ incubated in the ab-

sence of alveolar macrophages for the same time period.

Finally, in some experiments the effect of indomethacin (3×10^{-6} M) on eicosanoid release by cultured cells was evaluated.

PGE₂ AND LTB₄ ASSAYS

PGE₂ and LTB₄ were determined by enzyme immunoassay (EIA) using acetylcholinesterase coupled to different eicosanoids as label.^{23,24} Solid phase EIA was performed by using specialised Titertek microtitration equipment, including an automatic plate washer (Microplate Washer 120), an automatic dispenser (Autodrop), and a spectrophotometer (Multiskan MC) from Flow Laboratories (Helsinki, Finland). Microtitre plates were from Nunc (Denmark). For the quantitative determination mouse monoclonal anti-rabbit IgG antibody was immobilised on 96 well microtitre plates by dispensing 200 µl of a 10 µg/ml anti-IgG solution (phosphate buffer 5×10^{-2} M, pH 7.4, containing 2% glutaraldehyde) automatically into each well using the Autodrop apparatus.

After overnight incubation (at least 18 hours) at room temperature, 100 µl of buffer (NaN₃ 0.03% w/v, NaCl 0.4 M, EDTA 1 mM, BSA 0.3% w/v, in phosphate buffer 0.1 M, pH 7.4) was added and the plates stored at 4°C for 24 hours prior to use. The plates were then washed with 0.01 M phosphate buffer, pH 7.4, containing 0.05% v/v Tween 20. The assay was performed in EIA buffer (0.1 M phosphate buffer, pH 7.4, containing 0.4 M NaCl, 1 mM EDTA, 0.01% BSA and 0.07% NaN₃), total volume 150 µl. Each component was added in a volume of 50 µl: standard or biological sample, enzymatic tracer and specific antiserum (final dilutions of 1:60 000 and 1:40 000, respectively, for LTB₄ and PGE₂). After overnight incubation at 4°C the plates were washed and 200 µl of Ellman's reagent, consisting of enzymatic substrate (acetylthiocholine, 7.5×10^{-4} M) and chromogen (5,5'-dithiobis-(2-nitro-benzoic acid), 5×10^{-4} M) in 0.01 M phosphate buffer, was automatically dispensed into each well with the Autodrop. After three hours the absorbance at 414 nm was measured. In order to evaluate the concentration of LTB₄ and PGE₂ in biological samples a standard curve was used from 7.8 pg/ml to 1 ng/ml. Standard curves and biological samples were analysed with an IBM computer using a linear log-logit transformation. The intra-assay coefficient of variation ranged between 14% and 29% for LTB₄ and between 6% and 15% for PGE₂.

Results are expressed in terms of percentage B/B₀ where B and B₀ represent the absorbance measured on the bound fraction in the presence or absence of eicosanoid competitors, respectively.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

The identity of both LTB₄ and PGE₂ was checked in representative samples (three patients with sarcoidosis and three control subjects) by combined HPLC-EIA coupled with UV detection.

HPLC purification and analysis of LTB₄

Supernatants from alveolar macrophages (1 ml) were extracted using a solid phase cartridge (Supelclean C18). Ninety percent aqueous methanol eluates were evaporated to dryness using a SpeedVac evaporating centrifuge (Savant Instruments, Farmingdale, New York, USA), reconstituted in HPLC solvent A (methanol/acetonitrile/water/acetic acid, 10:10:80:0.02 v/v/v/v, pH 5.5, with ammonium hydroxide) and analysed using a gradient pump system (Beckman mod.168). UV absorbance was monitored at 235 nm and 280 nm, and full UV spectra (210–340 nm) were acquired at a rate of 0.5 Hz. A multilinear gradient from solvent A to solvent B (50% methanol, 50% acetonitrile) was used. A semi-micro column (Superspher 15 × 0.2 cm, 4 µm particle size, Merck, Darmstadt, Germany) was used at a flow rate of 0.25 ml/min to increase the sensitivity of the analysis. This method allowed easy separation of LTB₄ from both 5S,12S-dihydroxyeicosatetraenoic acid (5S,12S-diHETE) and Δ6 trans LTB₄ epimers, with a lower detection limit for positive identification through UV spectral analysis of chromatographic peaks of 3 pmol LTB₄. Synthetic standards were used to determine daily retention time of arachidonic acid metabolites (Cascade Biochem., Reading, UK).

COX activity in the supernatant of alveolar macrophages

Alveolar macrophages were cultured at a density of 1×10^6 /ml in RPMI containing 100 U/ml penicillin and 100 mg/ml streptomycin, in the presence or absence of opsonised zymosan, for varying periods of time. One hour before the end of the incubation period ¹⁴C-arachidonic acid (0.5 µCi) was added for evaluation of its conversion rate to COX products. The supernatant, approximately 1 ml, was collected, 1 ml ethanol was added, and the mixture was kept at -20°C until HPLC analysis. Samples were thawed, acidified by adding 10 µl formic acid (18.5 N), and centrifuged at 2500 g for 15 minutes at room temperature. The supernatant (approximately 1.8 ml) was diluted with 4 ml ultrapure water (Millipore MilliQ, fed with bi-distilled water) and loaded onto BondElut cartridge (Varian, Milan, Italy) which had been previously activated with 3 ml methanol and 3 ml ultrapure water. Samples were then washed with 4 ml water followed by 6 ml petroleum ether to get rid of any residue of water; elution was performed with 5 ml ethyl acetate. The organic phase was taken to dryness and redissolved using 0.6 ml of a 1:2 mixture of methanol:water. The samples were then injected into a Beckman Gold HPLC equipped with a reverse phase column (LiChroCart RP-18, 250 × 4 mm, 5 µm particles, Merck-Bracco, Milan, Italy) connected to a radioactivity detector (Ramona-Raytest, Straubenhardt, Germany; liquid scintillator FloScint-V Packard, flow 3 ml/min, liquid scintillator cell 0.8 ml). Elution was carried out at a flow rate of 1 ml/min using a multilinear gradient from solvent

Table 1 Bronchoalveolar lavage data from normal subjects and patients with active and inactive pulmonary sarcoidosis

Group	Lavage fluid recovery (%)	Total cells (10 ³ /ml)	Lymphocytes		Macrophages		Neutrophils	
			%	(10 ³ /ml)	%	(10 ³ /ml)	%	(10 ³ /ml)
Normal control subjects (n=10)	56.8 (2.3)	99.9 (2.05)	6 (0.45)	6.02 (0.51)	93.8 (0.39)	93.67 (1.7)	0.2 (0.13)	0.21 (0.14)
Active sarcoidosis (n=18)	56.4 (1.4)	305.37 (25.4)*	46.2 (3.4)*	139 (2)*	53.61 (3.43)*	165.5 (21.6)†‡	0.11 (0.08)	0.62 (0.43)
Inactive sarcoidosis (n=14)	55.4 (1.71)	106 (3.44)	12.9 (1.4)	22.02 (2.8)	86.83 (1.49)	92.03 (2.1)	0.14 (0.1)	0.15 (0.26)

All values are expressed as mean (SE).

* p<0.001 compared with both controls and inactive sarcoidosis; † p<0.05 compared with controls; ‡ p<0.01 compared with inactive sarcoidosis.

A (ultrapure water with 0.1% acetic acid, v/v) to solvent B (acetonitrile with 0.1% acetic acid, v/v).

STATISTICAL ANALYSIS

Statistical analysis was performed using the Dunn test²⁵ for the comparison of the three groups and a paired Student's *t* test for within group comparisons. Data are expressed as mean (SE).

Results

BAL FLUID DATA

The total cell recovery, percentage of lymphocytes, and the absolute number of lymphocytes were found to be significantly increased in the BAL fluid of patients with active sarcoidosis compared with the corresponding values obtained from both patients with inactive disease and control subjects (in both cases p<0.001; table 1). The absolute number of alveolar macrophages was also significantly greater in the BAL fluid of patients with active sarcoidosis compared with that of both patients with inactive disease (p<0.01) and control subjects (p<0.05).

EICOSANOID RELEASE

Both PGE₂ and LTB₄ were undetectable in supernatants from cell free incubations or from cells at time zero. The time course of PGE₂ release by unstimulated alveolar macrophages showed detectable levels only after 24 hours of culture and a similar profile was observed in stimulated alveolar macrophages (data not shown).

PGE₂ levels produced after 24 hours of culture by both unstimulated and zymosan stimulated alveolar macrophages from the overall population of patients with sarcoidosis and normal subjects are shown in table 2. Under resting conditions low levels of the eicosanoid were produced by cells from both normal subjects

and those with sarcoidosis. After zymosan stimulation a significant increase in PGE₂ release was observed in both normal cells and those from patients with sarcoidosis (p<0.05 and p<0.001, respectively, compared with unstimulated cells). No significant differences in PGE₂ release by either unstimulated or stimulated alveolar macrophages were seen between the groups.

The kinetics of LTB₄ release by unstimulated alveolar macrophages showed that higher levels of the hydroxyacid were produced during the early stages of culture (30–120 minutes) in both normal cells and those from patients with sarcoidosis, with a tendency to decrease over the next 24 hours; this decrease, however, was statistically significant only for normal alveolar macrophages (fig 1A). The analysis of some

Figure 1 Time course of release of leukotriene B₄ (LTB₄) by (A) unstimulated and (B) zymosan-stimulated alveolar macrophages from healthy controls (n=4) and patients with inactive (n=5) and active sarcoidosis (n=5). Alveolar macrophages were cultured in RPMI 1640 in the absence (A) or presence (B) of opsonised zymosan (500 µg/ml) for the indicated time and LTB₄ release was evaluated. Data are expressed as mean (SE). * p<0.05 compared with values at 0.5, 1 and 2 hours; ** p<0.05, *** p<0.02 compared with values at 0.5 hours.

Table 2 Mean (SE) PGE₂ release by alveolar macrophages from normal subjects and patients with inactive and active pulmonary sarcoidosis

Group	PGE ₂ (ng/10 ⁶ cells)	
	Resting	Opsonised zymosan
Controls (n=10)	0.8 (0.27)	1.88 (0.57)*
Inactive sarcoidosis (n=14)	1.35 (0.73)	2.28 (0.82)**
Active sarcoidosis (n=18)	0.75 (0.22)	2.44 (0.77)**

* p<0.05; ** p<0.001 compared with unstimulated cells.

Figure 2 Release of leukotriene B₄ (LTB₄) by alveolar macrophages from normal subjects ($n=10$) and patients with inactive ($n=14$) and active ($n=18$) sarcoidosis after 30 minutes of culture. Alveolar macrophages were cultured for 30 minutes in RPMI 1640 in the absence or presence of opsonised zymosan (500 μ g/ml). Data are expressed as mean (SE). Compared with normal alveolar macrophages, stimulated alveolar macrophages from patients with active sarcoidosis released significantly higher amounts of LTB₄ ($p<0.05$).

alveolar macrophage supernatants with added [³H]LTB₄ by HPLC (see methods for details) did not reveal omega hydroxy or omega carboxy-LTB₄, suggesting that the decrease of LTB₄ release observed after 24 hours of culture was not attributable to omega oxidation. No evidence for conversion of LTB₄ to 10, 11-dihydro-LTB₄ was found (data not shown). Furthermore, as >95% of the counts were recovered in the supernatants, we can safely exclude the possibility that the decrease in LTB₄ levels at 24 hours was due to adherence of the product to the surfaces of the culture well. After zymosan stimulation the kinetics of LTB₄ release showed a different behaviour in normal cells and those from patients with sarcoidosis, with normal cells producing similar levels of the hydroxyacid during the early and late times of culture while LTB₄ release markedly increased after 60 minutes of culture in cells from patients with active sarcoidosis, remaining at a high level for 24 hours (fig 1B).

In fig 2 LTB₄ levels released after 30 minutes of culture by both unstimulated and zymosan stimulated alveolar macrophages from the overall population studied are shown. Only minute amounts of the hydroxyacid were produced during resting conditions by both normal cells and those from patients with sarcoidosis. Zymosan stimulation induced a significant increase in LTB₄ release in alveolar macrophages from both normal subjects and patients with sarcoidosis ($p<0.05$ and $p<0.01$, respectively, compared with unstimulated cells).

No differences were observed during resting conditions in the levels of LTB₄ released by cells from the three groups, but after zymosan stimulation alveolar macrophages from patients with active sarcoidosis released significantly higher amounts of LTB₄ than those from control subjects ($p<0.05$). The same behaviour was observed after 24 hours of culture when, in fact, basal LTB₄ levels produced by alveolar macrophages from all three groups were low (controls, 0.50 (0.13) ng/10⁶ cells; non-active

Figure 3 Effect of indomethacin on release of (A) LTB₄ and (B) PGE₂ by stimulated alveolar macrophages from patients with inactive ($n=5$) and active ($n=5$) sarcoidosis. Alveolar macrophages were cultured for 24 hours in the presence of opsonised zymosan (500 μ g/ml) or opsonised zymosan + indomethacin (3×10^{-6} M). Data are expressed as mean (SE). In the presence of indomethacin, the addition of zymosan resulted in no detectable release of PGE₂ but no significant effect was observed on LTB₄ release.

sarcoidosis, 0.61 (0.17) ng/10⁶ cells; active sarcoidosis, 0.56 (0.19) ng/10⁶ cells) and no differences were observed between the groups. Stimulated alveolar macrophages from patients with active sarcoidosis were still found to produce significantly higher levels of LTB₄ than those from normal subjects (active sarcoidosis, 8.82 (1.92) ng/10⁶ cells; controls, 2.81 (0.51) ng/10⁶ cells, $p<0.05$), whereas no significant differences were observed between LTB₄ levels released by alveolar macrophages from patients with inactive disease (6.01 (2.44) ng/10⁶ cells) and normal subjects.

In order to evaluate whether cyclo-oxygenase pathway inhibition could affect LTB₄ release by alveolar macrophages from patients with sarcoidosis, in some experiments indomethacin (3×10^{-6} M) was added to cultured cells 30 minutes before the addition of opsonised zymosan. As expected, in the presence of indomethacin no detectable synthesis of PGE₂ was observed, even after zymosan stimulation; moreover, no significant effect was observed on LTB₄ release (fig 3). These results suggest that, in the presence of indomethacin, stimulation with zymosan of alveolar macrophages from patients with sarcoidosis resulted in almost complete inhibition of the release of cyclo-oxygenase products and no shunting through the lipoxygenase pathway.

Discussion

This study shows that stimulated alveolar macrophages from patients with active sarcoidosis release significantly higher levels of LTB₄ than normal alveolar macrophages, suggesting that alveolar macrophages in the BAL fluid of these patients are primed to release LTB₄. In contrast, no differences were observed in PGE₂ release between normal cells and those from patients with sarcoidosis. Our observations are consistent with previous results showing that stimulated alveolar macrophages from rabbits immunised with BCG released larger amounts of LTB₄ than cells from non-immunised rabbits while the release of prostaglandin was decreased,²⁶ but differ from those of Bachwich *et al*⁶ who failed to detect an increase in LTB₄ production by alveolar macrophages from patients with sarcoidosis and observed a reduced capacity of these cells to produce arachidonic acid metabolites compared with normal alveolar macrophages.

Many explanations can account for these discrepancies. Bachwich *et al*⁶ evaluated lipoxygenase metabolism in a smaller number of patients, only two of whom had active disease, different culture conditions and a different stimulus were employed, and lipoxygenase metabolism was evaluated following the addition of exogenous arachidonate. Wide differences in the metabolism of exogenous versus endogenous fatty acid in both resting and agonist stimulated cells have been described by Peters-Golden *et al*²⁷ showing that, whereas leukotrienes (especially LTB₄) were major products of endogenous arachidonic acid released by agonist stimulation, exogenous arachidonate was metabolised predominantly to cyclo-oxygenase products and mono-HETE but only minimally to leukotrienes.

In the present study only the monolayer supernatants were analysed for LTB₄ and PGE₂ so the results only reflect the amounts of eicosanoid released by the cells and not the total alveolar macrophage production of these metabolites. To the best of our knowledge, no intracellular storage site for eicosanoids has been described and found to be of physiological relevance. Eicosanoids are synthesised "de novo" by the interaction of different stimuli with the cell membrane and diffuse immediately into the surrounding environment. However, Damon *et al*²⁸ recently observed no significant differences in total LTB₄ levels produced by alveolar macrophages from healthy subjects and from asthmatic patients, whereas intracellular LTB₄ levels were slightly higher in the asthmatic patients. This suggests that differences in eicosanoid production between cells from different subjects are not always the same when the products are analysed in supernatants, after alcohol destruction of the cells, or a combination of the two. On the basis of the results of the present study, we cannot therefore exclude the possibility that the increased LTB₄ levels released by alveolar macrophages from patients with sarcoidosis do not reflect an increase in the total production of the eicosanoid, but rather an increased transport across the cell membrane. In any case, the

increased levels of LTB₄ occurring in the inflammatory environment in patients with active sarcoidosis might contribute to the locally heightened immune response.

Our study also showed different kinetics of release of cyclo-oxygenase and lipoxygenase metabolites by resting alveolar macrophages. Whereas PGE₂ levels were undetectable in the early stages of culture, increasing only at 24 hours, LTB₄ levels were higher during the first hours of culture with a trend to decrease over the next 24 hours. This pattern is consistent with previous reports of arachidonic acid metabolism by animal and human alveolar macrophages.²⁹⁻³¹

The decrease in release of LTB₄ did not seem to be attributable to omega oxidation since analysis of some alveolar macrophage supernatants with [³H]-LTB₄ by HPLC did not reveal omega hydroxy or omega carboxy-LTB₄; these findings are also consistent with previous observations.^{29 32-35}

Interestingly, after zymosan stimulation different kinetic behaviour was observed between normal cells and cells from patients with active sarcoidosis; whereas normal cells released similar levels of the hydroxyacid during the early and late times of culture, LTB₄ release by cells from patients with active sarcoidosis increased significantly after 60 minutes of culture, remaining increased until 24 hours. These results further support the hypothesis that alveolar macrophages from patients with active sarcoidosis appear to be primed to release LTB₄. The use of cyclo-oxygenase inhibitors resulted in almost complete inhibition of the release of cyclo-oxygenase products and no shunting through the lipoxygenase pathway in cells from patients with sarcoidosis.

Pulmonary sarcoidosis is a disorder characterised by a variety of enhanced immune and inflammatory processes localised in the lung. Activated T lymphocytes and alveolar macrophages are present in increased numbers in the alveoli of patients affected by this disease. These cells interact by spontaneously releasing large amounts of mediators, and these interactions appear to be crucial for the development of the granuloma. In this context, the enhancement of LTB₄ release by the alveolar macrophages from patients with active disease in response to a phagocytic stimulus is potentially relevant. LTB₄ is a 5-lipoxygenase product of arachidonic acid metabolism that has potent chemotactic activity for polymorphonuclear and mononuclear leucocytes^{5 36-39} and its release by alveolar macrophages in patients with sarcoidosis may play a part in the amplification of the granulomatous response by recruiting mononuclear phagocytes from the blood into the lung. Furthermore, even if the contribution of LTB₄ to the regulation of cellular immunity is complex,¹⁷ some observations suggest that it might play an amplificatory part in the immune response. LTB₄ has been shown to enhance interleukin 1 (IL-1) production by human monocytes⁴⁰ and to replace IL-2 mediated helper cell requirement for interferon-γ (IFN-γ) production in the mouse spleen cell system.⁴¹ In addition, Rola-

Pleszczynski *et al*⁴² showed that IL-2 and IFN- γ production by human lymphocytes was significantly enhanced in the presence of LTB₄ and indomethacin and that depletion of the T8⁺ suppressor cell subset resulted in enhanced lymphokine production and further augmentation by LTB₄. It thus appears that LTB₄ can generate a number of "positive" signals when "negative" signals leading to enhanced suppressor cell function are blocked. Such a condition could occur in the alveoli of patients with active sarcoidosis in whom a significant increase in T helper lymphocytes, which spontaneously release increased levels of IL-2 and IFN- γ , has been described.⁴³⁻⁴⁶ In this context, an enhanced release of LTB₄ in the inflammatory environment of sarcoidosis by alveolar macrophages might contribute to the amplification of the immune response which characterises this disease. Even if the enhanced LTB₄ release by stimulated alveolar macrophages might be considered an additional marker of functional activation of these cells in sarcoidosis, it does not seem to be suitable as a new marker of disease activity. In fact, although patients with active disease release higher levels of LTB₄ compared with patients with inactive disease, the difference is not statistically significant, probably due to the large interpatient variation. Additional studies including a larger number of patients and serial assessments of LTB₄ release by alveolar macrophages are needed in order to define the clinical relevance of these findings and, in particular, the relationship between LTB₄ levels released by alveolar macrophages and the outcome of the disease. This is particularly interesting in the context of the recent pharmacological advances in the field of leukotriene antagonists that might represent a new potential therapeutic strategy for inhibiting the enhanced inflammatory/immune processes in sarcoidosis.

In conclusion, the results of this study show that stimulated alveolar macrophages from patients with active sarcoidosis release higher LTB₄ levels than those from healthy controls during both the early and late stages of culture and appear to be primed to release LTB₄. The enhanced release of LTB₄ probably reflects in vivo cellular activation which occurs in the lung in active sarcoidosis, and it might contribute to the locally heightened immune response.

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