

Lymphocyte subsets, phytohaemagglutinin responsiveness of blood lymphocytes, and interleukin 2 production in sarcoidosis

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ABSTRACT To test the possibility that T lymphocyte subset imbalance and interleukin 2 (IL₂) play a part in the impairment of cellular immune response in sarcoidosis, the proportion of T lymphocyte subsets in peripheral blood and alveolar lavage fluid from 21 patients with sarcoidosis was studied, monoclonal antibodies OKT3, OKT4, and OKT8 being used. Lectin induced production of IL₂ and phytohaemagglutinin (PHA) responsiveness of peripheral blood lymphocytes were investigated. The percentage of both OKT3⁺ and OKT4⁺ T lymphocytes was significantly lower in peripheral blood from patients with sarcoidosis than in control subjects (control 63% and 46%), more so in patients with chronic sarcoidosis (44% and 23%) than in patients with recent sarcoidosis (56% and 38%). PHA induced IL₂ production from peripheral blood lymphocytes did not differ between patients with sarcoidosis and control subjects. There was a significant positive correlation between PHA responsiveness and the percentage of blood OKT3⁺ and OKT4⁺ cells. Peripheral blood lymphocyte PHA responsiveness was decreased only in patients with an OKT4/OKT8 ratio of less than 1.5. Finally, late addition of exogenous IL₂ to the culture medium on day 5 increased 3(H)Tdr incorporation by PHA stimulated blasts in peripheral blood lymphocytes from normal subjects, but not from those of patients with sarcoidosis. The data suggest that the impairment of cellular immune response in patients with sarcoidosis could in part reflect a decrease in the percentage of blood T helper lymphocytes and impaired IL₂ receptors at the surface of stimulated lymphocytes.

Immunological abnormalities in sarcoidosis arising from cellular and humoral dysfunction include decreased delayed type reactions, a decreased number of circulating T lymphocytes, impaired response of blood mononuclear cells to mitogens, a large number of circulating B lymphocytes, raised serum immunoglobulin concentrations, and the presence of autoantibodies and circulating immune complexes (see ref 1 for review). Alveolar lavage has permitted new insights into these immunological abnormalities, supporting the hypothesis that the immune response is "compartmentalised" in sarcoidosis since the patterns in the lung differ considerably from those in peripheral blood.² Imbalance in

T lymphocyte subsets has been reported in blood^{3,4} and lung.⁵ The lung changes are characterised by an alveolitis with an increased number of in situ activated T lymphocytes,⁶ spontaneously producing lymphokines such as monocyte chemotactic factor⁷ and interleukin 2,⁸ and abnormally high local production of immunoglobulins.⁹ T cell growth factor—TCGF or interleukin 2 (IL₂)—is required for proliferation of activated T lymphocytes. This lymphokine is produced by activated T cells (mostly T4⁺ phenotypes)^{10,11} and its production is controlled by lymphocyte activated monocytes.¹² The aim of this study was to analyse the cellular immune response in 21 patients with sarcoidosis, to determine (1) the proportion of T lymphocyte subsets in peripheral blood and alveolar lavage fluid with the help of monoclonal antibodies OKT3 (identifying mature T cells), OKT4 (identifying the helper subset), and OKT8 (identifying the suppressor-

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Characteristics of patients

Group	Age* (y)	Sex M/F	Smoking history (smokers/no smokers)	VC (% of predicted normal*)	FEV ₁ VC (%*)	Transfer factor (% of predicted normal*)	Radiological stage		ACE (nmol/min/ml*)
							1	2	
Recent sarcoidosis (n = 11)	30.5 ± 8.5	7/4	5/6	108 (17)	83.1 (5.5)	92 (29)	7	4	42.3 (14.8)
Chronic sarcoidosis (n = 10)	44 ± 14	6/4	1/8	90 (15)	74.3 (9.1)	81 (32)	0	10	42.5 (15.3)
	p < 0.02	NS	NS	p < 0.02	p < 0.02	NS	p < 0.01		NS

*Means with standard deviations in parentheses.

(FEV₁—forced expiratory volume in one second; VC—vital capacity; ACE = angiotensin converting enzyme).

cytotoxic subset); (2) the *in vitro* production of interleukin 2 by peripheral blood lymphocytes stimulated by phytohaemagglutinin (PHA); and (3) the effect of exogenous semipurified interleukin 2 on PHA blasts and the effect of interleukin 1 (IL₁) and indomethacin on interleukin 2 production and PHA induced responsiveness.

Methods

PATIENTS

The diagnosis of sarcoidosis was established in 21 patients according to clinical and histological criteria, which included a bronchial, lung or sublingual gland biopsy specimen showing non-caseating epithelioid granulomas. Patients were divided into two groups according to disease duration: 11 patients with disease of under three months' duration were considered as having recent sarcoidosis and 10 patients with disease of over 18 months' duration (maximum 65 months') as having chronic sarcoidosis. Details of age, sex, smoking, radiological staging, and functional data in the two groups are shown in the table. Patients with chronic sarcoidosis were older, were all classified as having stage II disease radiographically (versus only four of the "recent" patients), and had greater impairment of respiratory function. Blood angiotensin converting enzyme (ACE) activity was significantly and similarly increased in the two groups. All but one patient with chronic sarcoidosis had received corticosteroid treatment with an initial prednisone dose of 0.5 mg/kg for 6–48 months. Four patients were receiving prednisone at the time of the study (2.5, 5, 10, and 20 mg a day respectively).

LABORATORY METHODS

Isolation and counting of lymphocytes

Bronchoalveolar lavage was performed in all patients by infusion and aspiration of four 50 ml aliquots of sterile 0.9% saline through the channel of a fiberoptic bronchoscope passed transnasally and wedged in a subsegmental bronchus of the right middle lobe or lingula after xylocaine anaesthesia of

the upper respiratory tract and bronchi. The fluid was filtered through two layers of sterile gauze and transported in ice. After evaluation of cell number and differential count, the lavage fluid was centrifuged at 1000 g for 10 minutes. The cell pellet was then washed in Hanks solution and macrophages were eliminated by glass (Petri dishes) adherence. Final non-adherent cell suspensions were adjusted to 10⁶ cells/ml before counting of lymphocyte subsets. Peripheral blood lymphocytes were obtained from heparinised blood by sedimentation on Ficoll-Hypaque and washed twice. Peripheral blood lymphocytes were then frozen in liquid nitrogen until used.

The whole T lymphocyte population was evaluated by the technique of sensitised (EAET—treated by ethylamino isothiuronium) sheep red cell rosettes.¹³ Monoclonal antibodies of the orthodiagnostic series were used to identify mature T cells (OKT3), helper subsets (OKT4), and suppressor-cytotoxic subsets (OKT8). Briefly, after 30 minutes' incubation at 4°C 200 µl of a 5 × 10³ cells/mm³ suspension were mixed with 5 µl of monoclonal antibody and washed three times in isotonic buffer. Fluoresceinated goat antimouse antiglobulin, 100 µl, was then added. After a further 30 minutes' incubation at 4°C the fluorescent cell count was carried out on 200 cells under ultraviolet microscopy. B cells were distinguished by surface membrane immunoglobulins with the aid of fluorescein conjugated F(ab)₂ fragments of goat antihuman immunoglobulin. The number of labelled cells was determined by ultraviolet microscopy. Normal values were established from blood from 35 healthy control subjects.

Assessment of interleukin 2 production from peripheral blood lymphocytes of patients with sarcoidosis

Thawed peripheral blood lymphocytes were routinely checked for viability (>90% required) and adjusted to 1.10⁶/ml in 1% AB human sera supplemented Dulbecco Eagle culture, Medium (DEM) to which fresh glutamine, penicillin (1 U/ml), and streptomycin (100 µg/ml) had been added. Cultures were performed in vertical 25 ml Falcon tissue cul-

ture flasks in 5% carbon dioxide at 37°C for 48 hours in the presence of 1 µg/ml of PHA-P (Difco). Culture supernatants were then harvested and kept at 4°C until assayed for IL₂ activity.

Interleukin 2 assay The amount of IL₂ contained in the supernatant of PHA stimulated blood lymphocytes was assayed on IL₂ dependent concanavallin A (con-A) blasts. Briefly, con-A blasts (10⁴/well) were incubated in 96 wells (0.2 ml) of tissue culture microplates in the presence of log³ dilution of either a reference IL₂ source containing 10 units/ml of IL₂ or the supernatant of control and sarcoidosis blood lymphocytes stimulated by PHA. After 48 hours' culture, 0.25 µCi tritiated thymidine (Amersham, SA = 23 Ci/ml) was added to each well. The cells were harvested 12 hours later with a Skatron multisample harvester and counted for radioactivity. Results are given as units of IL₂/ml and as the average amount of tritiated thymidine incorporated in the presence of the blood lymphocyte supernatants at various dilutions. In this assay con-A blasts gave equally good IL₂ measurement as a CT IL₂ murine cell line when compared in a cross evaluation.¹⁴

Purified interleukin 2 Purified IL₂ (PHA free) was obtained from the supernatant of PHA stimulated lymphocytes precipitated by 80% saturation ammonium sulphate and run on an ultragel (ACA 54) column.¹⁵ The IL₂ that was eluted in the 23–25 000 dalton molecular weight fraction was further concentrated, dialysed against phosphate buffered saline, assayed for IL₂ activity, and used at a final concentration of 5 units/ml.

PHA stimulation Peripheral blood lymphocytes were studied for tritiated thymidine incorporation before and after PHA stimulation in microplates. In these experiments 10⁵ blood lymphocytes were incubated for two or five days in 0.1 ml of RPMI 16-40 supplemented with 10% human AB sera and 1 µg of PHA-P. Purified IL₂ was added to the culture wells, either for the total duration of culture in the case of 48 hour stimulation by PHA or only for the last 24 hours of the culture, to assess the presence of receptor, in the case of five day stimulation.

Interleukin 1 and indomethacin Mitogenic (PHA) responsiveness with or without addition of exogenous purified IL₂ of either sarcoidosis or control blood lymphocytes was studied in the presence or absence of IL₁ (kindly provided by C Damais, Institut Pasteur, Paris). IL₁ was obtained by muranyl dipeptide stimulation of human macrophages.¹⁶ Supernatant containing IL₁ was free of IL₂ in the quantitative IL₂ assay previously described. The supernatant containing IL₁ was added at an optimal final concentration of 10% (v/v) to the various P-PHA stimulated blood lymphocytes. Similarly, IL₂ production by both patient and control blood

lymphocytes was studied in the presence and in the absence of exogenous IL₁ (10% v/v) in the culture. Indomethacin (Sigma) was used at a concentration of 2 µg/ml in the culture medium.

Results

Total lymphocytes, E rosetting lymphocytes, and Sm Ig positive mononuclear cells

Blood Although patients with sarcoidosis had a lower level of E rosetting lymphocytes (68.2 (10%) v (73 (8%)) the difference was not significant. They showed significantly more surface membrane immunoglobulin positive (Sm Ig⁺) cells (24.3 (10%)) than did normal controls (20 (5%)) (p < 0.5). When the data were expressed as absolute values to take account of the number of lymphocytes per mm³, the patients with sarcoidosis showed a significant decrease in the total number of E⁺ lymphocytes (p < 0.01). The total number of Sm Ig⁺ lymphocytes showed no further increase.

Bronchoalveolar lavage Lymphocytes represented (14.4 (10.4%)) and (14.9 (5.8%)) of the mononuclear cells harvested from patients with recent and chronic sarcoidosis respectively and E rosetting cells (75.6 (14.9%)) and (78.8 (9.9%)). When the results were expressed as absolute numbers from the total

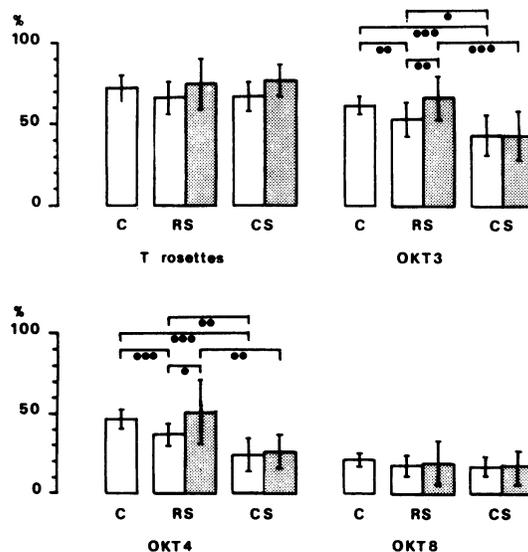


Fig 1 Surface markers of lymphocytes from blood (white columns) and lavage fluid (hatched columns) in control subjects (C), patients with recent sarcoidosis (RS), and patients with chronic sarcoidosis (CS). ●p < 0.05; ●●p < 0.01; ●●●p < 0.001.

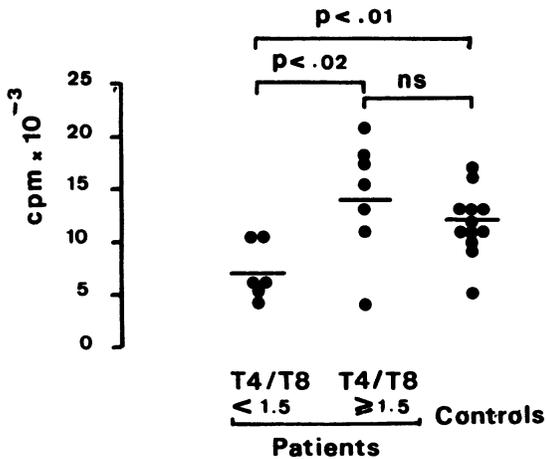


Fig 2 *Phytohaemagglutinin responsiveness of the lymphocytes of patients with sarcoidosis (interleukin 2 added during the last 24 hours of culture).*

number of cells harvested in the lavage fluid, patients with recent and chronic sarcoidosis had a similar number of lymphocytes and E rosetting lymphocytes.

OKT (T3, T4, T8) cell surface markers (fig 1)

Blood The percentages of OKT3⁺ lymphocytes from patients with sarcoidosis (55.7 (10)) for recent, (44 (12.4) for chronic) were significantly lower than those from normal controls (63 (6); $p < 0.01$). In addition, OKT3⁺ lymphocytes were significantly lower in patients with chronic than in

those with recent sarcoidosis ($p < 0.05$).

The percentage of OKT4⁺ cells in blood was decreased in patients with both recent (37.6 (6.9); $p < 0.001$) and chronic sarcoidosis (23.2 (10.4); $p < 0.001$), but more pronounced in the chronic form ($p < 0.01$).

The percentage of OKT8⁺ cells in blood was slightly but significantly lower in the patients with sarcoidosis than in normal controls ($p < 0.05$), with no difference between patients with chronic and recent disease. Only patients with chronic sarcoidosis had a significantly low OKT4⁺/OKT8⁺ ratio (1.3 (0.4); $p < 0.001$). The findings were similar when the data were expressed as absolute values to take the lymphopenia already noted into account (details not given).

Bronchoalveolar lavage fluid The percentage of OKT3⁺ cells was higher in lavage fluid than in blood (67.3 (12.6) v 55.7 (10); $p < 0.01$, paired *t* test) (fig 1) in patients with recent sarcoidosis, but not in those with chronic sarcoidosis. OKT4⁺ cells were also more frequent in lavage fluid than in blood from patients with recent sarcoidosis (50.4 (19) v 37.6 (6.9); $p < 0.05$), but the two counts were similar in chronic sarcoidosis. The percentage of OKT8⁺ cells was similar in lavage fluid and in blood in patients with recent and chronic sarcoidosis. Thus a high OKT4⁺/OKT8⁺ ratio was found in lavage fluid from patients with recent sarcoidosis (6.5 (6.4)), but not in that from patients with chronic sarcoidosis (1.7 (1.2)). Again, expressing the data as absolute number of cells gave similar results. In addition, the OKT3⁺/EAET⁺ ratio was significantly lower ($p <$

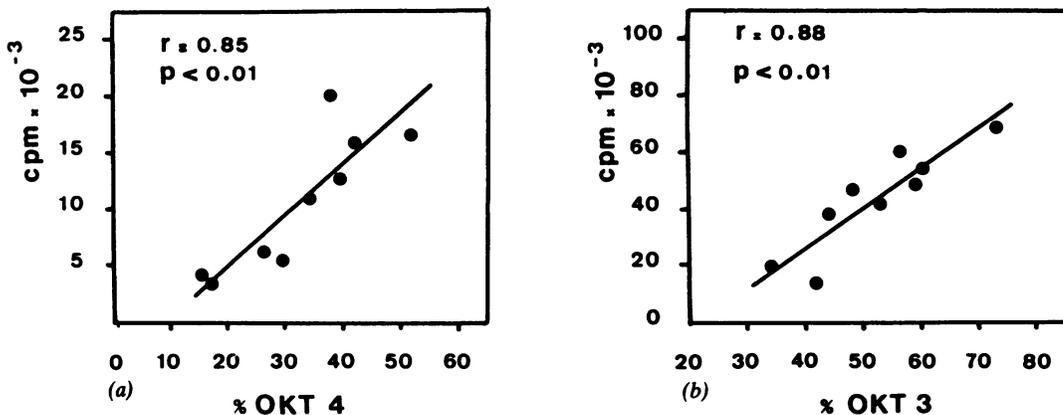


Fig 3 *Correlation between phytohaemagglutinin (PHA) responsiveness and OKT3⁺-OKT4⁺ cell surface markers in peripheral blood lymphocytes of sarcoidosis patients: (a) two days' culture and (b) five days' culture with PHA.*

0.05) in both blood and lavage fluid in patients with chronic sarcoidosis.

Interleukin 2

Interleukin 2 production from lectin stimulated peripheral blood lymphocytes was similar in 14 patients with sarcoidosis and normal controls (4.9 (2.3) v 3.7 (2) U/ml). Addition of interleukin 1 or indomethacin did not alter the yield of interleukin 2 in either group. Patients receiving corticosteroids had significantly lower interleukin 2 production (0.2 (0.45) v 4.9 (3.4) U/ml; $p < 0.05$).

Spontaneous and mitogenic blastogenesis

Peripheral blood lymphocyte incorporation of tritiated thymidine measured after two or five days of "spontaneous" or lectin (PHA) stimulated culture did not differ between patients with sarcoidosis ($n = 14$) and control subjects ($n = 11$). Addition of IL₁ or indomethacin to the culture did not modify the growth of blood lymphocytes from either patients or normal subjects. When IL₂ was added to the medium on day 5 of the PHA stimulated culture, there was a borderline increase in incorporation of tritiated thymidine in lymphocytes from normal subjects ($p < 0.05$), but no effect on those from patients (fig 2).

Correlation between blastogenesis and percentages of lymphocyte subsets

There was a significant correlation between PHA responsiveness and the level of T4⁺ or T3⁺ lymphocyte subpopulations when corticosteroid treated patients were excluded: tritiated thymidine incorporation correlated after two days of culture with the percentage of OKT4⁺ cells ($r = 0.85$, $p < 0.01$), and after five days with the percentage of OKT3⁺ cells ($r = 0.88$, $p < 0.001$) (fig 3).

When patients were classified according to the T4/T8 blood ratio decrease in PHA responsiveness was significantly lower in patients with a low ratio (< 1.5) than patients with a T4/T8 ratio closer to normal values (> 1.5).

Discussion

The most striking feature of OKT subpopulation studies in sarcoidosis is the increase in the proportion of OKT4⁺ cells and OKT4/OKT8 ratios in bronchoalveolar lavage fluid in patients with acute sarcoidosis.^{5,17-19} Our results in patients with recent sarcoidosis are in agreement with these reports. Data concerning the proportions of lymphocyte subsets in blood are more contradictory: the percentage of OKT3⁺ cells and OKT4⁺ cells has usually been found to be decreased,^{17,20} whereas the proportion

of OKT8⁺ cells has been reported as both high⁵ and low,^{17,20} with the OKT4/OKT8 ratio either low or normal. Our results for patients with chronic sarcoidosis show a decreased percentage of OKT3⁺ and OKT4⁺ cells in both blood and lavage fluid. Corticosteroids may decrease the percentage of OKT4⁺ cells,²¹ though in our study there was no difference in the percentage of OKT3⁺ and OKT4⁺ cells between patients receiving and not receiving corticosteroids.

A depressed peripheral blood lymphocyte proliferative response to polyclonal mitogens such as PHA in sarcoidosis has been described by previous authors.¹ The decreased proliferative response might be due to an intrinsic defect in sarcoid lymphocytes,²² to factors inhibiting mitogen response,²³ or to antilymphocyte antibodies.²⁴ Alterations in the distribution of helper and suppressor lymphocyte subpopulations have also been described,^{3,4} and an interaction between immune complexes and T suppressor cells has been suggested.²⁵ High suppressor activity in sarcoidosis could derive from prostaglandin secretion by monocytes, since for some investigators²⁶ removal of the adherent cell population from peripheral blood mononuclear cells as well as addition of indomethacin to culture increased the lymphocyte response to PHA. Our results did not show a significant decrease in PHA responsiveness in the patients with sarcoidosis as a whole. When patients were classified according to their T4/T8 ratio, however, those with a low ratio (< 1.5) had less PHA responsiveness than those with a T4/T8 ratio closer to normal values (> 1.5). This discrepancy in PHA responsiveness could be related to differences in disease activity since, on the basis of lavage lymphocyte counts, our patients had mainly low intensity alveolitis.²

Pinkston *et al* have shown that lung T lymphocytes of patients with sarcoidosis and high intensity alveolitis spontaneously release interleukin 2, which could have a crucial role in increasing the number of lung T cells in active pulmonary sarcoidosis.⁸ Our study, restricted to interleukin 2 release by lectin stimulated blood lymphocytes, showed that after PHA stimulation lymphocytes from patients and controls produced similar amounts of interleukin 2. This suggests that defect in endogenous interleukin 2 production is not a major factor in the impaired mitogenic response in sarcoidosis since interleukin 2 has been shown to be necessary for lectin induced proliferation.¹⁰ Moreover, late addition of exogenous interleukin 2 to the culture medium of PHA stimulated blasts on day 5 increased incorporation of tritiated thymidine in normal individuals but not in patients with sarcoidosis, suggesting that interleukin 2 receptors at the surface of lectin stimulated

blast cells might be impaired in sarcoidosis. This hypothesis requires further study, with direct binding of labelled interleukin 2 on PHA blasts from patients and normal subjects.

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