Enhanced alveolar macrophage-mediated antigen-induced T-lymphocyte proliferation in sarcoidosis.

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J Clin Invest. 1985;75(1):293-301. https://doi.org/10.1172/JCI111688.

Research Article

Expansion of T-lymphocyte numbers is a characteristic feature of the alveolitis of pulmonary sarcoidosis. One mechanism that may influence the numbers of T-lymphocytes in the lung is the process of antigen presentation in which alveolar macrophages, in the presence of antigen, induce T-lymphocytes to replicate. To evaluate this process in sarcoidosis, alveolar macrophages were obtained by bronchoalveolar lavage, pulsed with tetanus toxoid, and co-cultured with purified autologous T cells. Strikingly, antigen-pulsed alveolar macrophages from sarcoid patients induced more than a twofold increase in autologous T-lymphocyte proliferation compared with the response seen using cells from normal or patients with idiopathic pulmonary fibrosis (P less than 0.001), all comparisons). In contrast, when monocytes were used as the antigen presenting cell, no significant differences were observed in T cell proliferation induced by antigen among the three groups. The enhanced T-lymphocyte proliferation induced by sarcoid alveolar macrophages was not dependent on the compartment from which the T cells were derived, and was independent of the specific antigen used. One possible explanation for augmented antigen presentation seen in sarcoid is that an increased percentage of sarcoid alveolar macrophages express HLA-DR or HLA-DS surface antigens. However, most normal and sarcoid alveolar macrophages express HLA-DR and HLA-DS surface antigens, and the percentage of macrophages expressing these antigens was not significantly different in the two groups. Thus, while […]

Enhanced Alveolar Macrophage-mediated Antigen-induced T Lymphocyte Proliferation in Sarcoidosis

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Abstract

Expansion of T-lymphocyte numbers is a characteristic feature of the alveolitis of pulmonary sarcoidosis. One mechanism that may influence the numbers of T-lymphocytes in the lung is the process of antigen presentation in which alveolar macrophages, in the presence of antigen, induce T-lymphocytes to replicate. To evaluate this process in sarcoidosis, alveolar macrophages were obtained by bronchoalveolar lavage, pulsed with tetanus toxoid, and co-cultured with purified autologous T cells. Strikingly, antigen-pulsed alveolar macrophages from sarcoid patients induced more than a twofold increase in autologous T-lymphocyte proliferation compared with the response seen using cells from normals or patients with idiopathic pulmonary fibrosis ($P < 0.0001$, all comparisons). In contrast, when monocytes were used as the antigen presenting cell, no significant differences were observed in T cell proliferation induced by antigen among the three groups. The enhanced T-lymphocyte proliferation induced by sarcoid alveolar macrophages was not dependent on the compartment from which the T cells were derived, and was independent of the specific antigen used. One possible explanation for augmented antigen presentation seen in sarcoid is that an increased percentage of sarcoid alveolar macrophages express HLA-DR or HLA-DS surface antigens. However, most normal and sarcoid alveolar macrophages express HLA-DR and HLA-DS surface antigens, and the percentage of macrophages expressing these antigens was not significantly different in the two groups. Thus, while the mechanisms of the enhanced antigen presentation in the sarcoid lung are unknown, the process of antigen-driven, alveolar macrophage-modulated lung T cell proliferation may explain, at least in part, the expansion of lung T-lymphocyte numbers that characterizes this disease.

Introduction

Pulmonary sarcoidosis is a chronic disorder characterized by the presence of increased numbers of T-lymphocytes and mononuclear phagocytes in the lower respiratory tract (the "alveolitis" of the disease) and by the presence of noncaseating granuloma within the walls of the alveoli, bronchi, and pulmonary arteries and veins (1, 2). Although the granuloma are the classic hallmark of the disease, it is now recognized that the alveolitis precedes the granuloma and plays a central role in its formation (2–6). In this regard, while the mononuclear phagocyte component of the alveolitis provides the cellular building blocks for granuloma formation, it is the T-lymphocyte component of the alveolitis that attracts monocytes to the lung and likely modulates their differentiation and coalescence into the granuloma (2–6). Thus, central to the understanding of the pathogenesis of pulmonary sarcoidosis is the understanding of the mechanisms underlying the marked expansion of the T-lymphocyte numbers within the lower respiratory tract.

Current evidence suggests that replication of T-lymphocytes within the lung is one important mechanism by which the number of T-lymphocytes is increased in the lungs of patients with sarcoidosis (7–9). First, sarcoid T-lymphocytes are spontaneously replicating at a rate several times higher than lung T-lymphocytes from normals or patients with interstitial lung diseases not associated with lymphocyte accumulation (7, 8). Second, lung T-lymphocytes, but not blood T-lymphocytes from patients with pulmonary sarcoidosis, are spontaneously releasing interleukin-2, a lymphocyte growth factor thought to be required for T-lymphocyte replication (9). It is clear, therefore, that at least some of the increase in lung T cell numbers in sarcoidosis results from mechanisms that induce T cells to proliferate.

One such mechanism is that of antigen presentation, a process by which cells expressing HLA-D region gene products, such as mononuclear phagocytes, interact with antigen and signal T-lymphocytes to proliferate (10–14). While the exact role played by each cell type in antigen presentation is unclear, it is known that enhanced interleukin-2 release is involved (14, 15). Thus, one explanation for why sarcoid lung T-lymphocytes are "spontaneously" releasing interleukin-2 and are replicating at an increased rate is to hypothesize that antigen presentation in the sarcoid lung is enhanced, i.e., the increased numbers of lung T-lymphocytes represent a collective expansion of many different antigen-specific clones of T-lymphocytes that have proliferated more rapidly than normal in response to alveolar macrophages presenting a variety of antigens.

To evaluate this hypothesis, the present study was designed to characterize antigen presentation by alveolar macrophages from the normal lung and to compare this process to antigen presentation by alveolar macrophages in sarcoidosis. As a control, we have studied antigen presentation in idiopathic pulmonary fibrosis, a chronic interstitial disorder not associated with enhanced T-lymphocyte proliferation in the alveolar structures.

Methods

Study population: normals. 14 normals were evaluated. There were 11 men and three women, with a mean age of 30.5±12.2 yr (all data are

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Received for publication 18 October 1983 and in revised form 27 August 1984.

J. Clin. Invest.

0021-9738/85/01/0293/09 $1.00
Volume 75, January 1985, 293–301

presented as mean±standard deviation); 11 were nonsmokers and three were smokers; none was taking medication at the time of the study. The chest roentgenograms and pulmonary function tests were normal in all individuals (Table I).

Study population: sarcoidosis. The diagnosis of pulmonary sarcoidosis was established in 21 patients using previously described criteria including lung biopsy (6). There were nine men and 12 women with a mean age of 34.2±11.7 yr; 19 were nonsmokers and two were smokers; 14 were receiving no therapy and seven were taking prednisone (22.5±6.6 mg daily). Chest X-rays were grouped as follows: type I (n = 4), type II (n = 7), and type III (n = 10). As a group, these individuals had mildly reduced lung volumes and diffusing capacity, but no limitation to airflow (Table I).

Study population: idiopathic pulmonary fibrosis (IPF).[1] The diagnosis of IPF was established in 12 patients using previously described criteria including open lung biopsy (16). There were nine men and three women with a mean age of 51.8±10.5 yr; seven were nonsmokers and five were smokers; nine patients were receiving therapy at the time of the study (eight were taking prednisone, 16.5±5.5 mg daily; one was taking cyclophosphamide 1.5 mg/kg per d). Chest X-rays demonstrated a diffuse reticulonodular infiltrate in all individuals. As a group, lung function studies showed mildly reduced lung volumes and diffusing capacity, but no limitation to airflow (Table I).

Bronchoalveolar lavage. Bronchoalveolar lavage was carried out with an Olympus BF-B2 bronchoscope using a total of 300 ml saline (100 ml in five 20-ml aliquots in three sites) as previously described (17). The percentage of lavage fluid recovered from patients in each of the groups was: normals, 56±9%, sarcoidosis, 47±11%, and IPF, 48±17% ($P < 0.05$ comparing recovery from normals and patients with sarcoidosis; other comparisons were not significantly different). The cells were immediately centrifuged (600 *g*, 5 min), washed sequentially in phosphate-buffered saline (PBS, Biofluids, Inc., Rockville, MD), and Hanks' balanced salt solution (M.A. Bioproducts, Walkersville, MD), and resuspended in RPMI-1640 (Biofluids Inc.) at 10^7 cells/ml. Total cells recovered averaged $27\pm7 \times 10^6$ in the normal nonsmokers, $88\pm62 \times 10^6$ in the normal smokers, $32\pm31 \times 10^6$ in the sarcoid nonsmokers, $45\pm24 \times 10^6$ in the sarcoid smokers, $44\pm33 \times 10^6$ in the IPF nonsmokers, and $64\pm49 \times 10^6$ in the IPF smokers. Differential cell counts were determined from Diff-Quick (American Scientific Products, McGaw Park, IL) stained cytocentrifuge preparations (Shandon Southern Instruments, Sewickley, PA) by counting a minimum of 500 cells.

Purification of alveolar macrophages. In all cases, the cell suspensions used as a source of alveolar macrophages contained >90% alveolar macrophages with a viability of >95% as assessed by trypan blue exclusion. When alveolar macrophages represented >90% of the effector cells recovered by lavage, the cell suspensions were used as a source of alveolar macrophages without further purification. However, if the percentage of polymorphonuclear leukocytes was >5%, the alveolar macrophages were purified by Ficoll-Hypaque gradient centrifugation (18). If the percentage of lymphocytes was ≥10%, alveolar macrophages were purified by rosetting the T-lymphocytes with neuraminidase-treated sheep red blood cells (N-SRBC) and recovering the nonrosetting population after Ficoll-Hypaque gradient centrifugation (19, 20). In all cases, alveolar macrophages were washed several times in Hank's balanced salt solution and resuspended in RPMI-1640 at 5×10^6 cells/ml before use.

Purification of blood T-lymphocytes. Autologous T-lymphocytes were used for all studies. To isolate blood T cells, mononuclear cells were isolated from blood by Ficoll-Hypaque centrifugation, washed, and resuspended in RPMI-1640 at 10^7 cells/ml. T-lymphocytes were then isolated from the mononuclear cell suspension by allowing the cells to rosette with N-SRBC according to the method of Weiner et al. (19). Equal volumes of blood mononuclear cells (2.5×10^6/ml) and a 0.5% suspension of N-SRBC were mixed and human albumin was added to a final concentration of 8 mg/ml. The mixture was centrifuged (200 *g*, 5 min, 4°C) and incubated for 90 min at 4°C. The resulting rosette forming cells (T-lymphocytes) were separated from nonrosette forming cells by Ficoll-Hypaque centrifugation and the N-SRBC were lysed with ammonium chloride buffer. The recovered rosette forming cells were further purified by rosetting a second time with N-SRBC followed by Ficoll-Hypaque centrifugation. The final T cell-enriched cell suspension contained >98% N-SRBC rosette-forming cells. The purified T cells were then suspended at 10^6 cells/ml in "complete medium" (RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum [Associated Biomedic Systems, Inc., Buffalo, NY], 4 mM glutamine [Sigma Chemical Co., St. Louis, MO], 100 U/ml penicillin [Biofluids Inc.], and 100 μg/ml streptomycin [Biofluids Inc.]).

Purification of blood monocytes. Monocytes were partially purified from blood during the T-lymphocyte purification procedure (19). The nonrosetting blood mononuclear cell population was recovered at the interface of the Ficoll-Hypaque gradient, washed and resuspended at 5×10^6 cells/ml in complete medium. The final preparation contained 60–70% monocytes that were >95% viable; although not completely pure, for convenience, this preparation will be referred to as "monocytes."

Purification of lung T-lymphocytes. Lung T-lymphocytes were purified from bronchoalveolar lavage by double rosetting with N-SRBC as described for blood T cells. The cells were resuspended at 10^6 cells/ml in complete medium. The final preparation contained >95% lung T-lymphocytes that were >95% viable. Because of the limitation of bronchoalveolar lavage in obtaining large numbers of cells, sufficient numbers of T-lymphocytes could only be purified from lung cells obtained from patients with sarcoidosis and not from normals or patients with IPF.

Evaluation of antigen presentation by alveolar macrophages. Evaluation of the ability of alveolar macrophages to present antigen to autologous T-lymphocytes utilized a three-step procedure based on a modification of the method of Todd et al. (13) for studying human monocyte antigen presentation. In this procedure: (*a*) purified alveolar macrophages were "pulsed" with antigen; (*b*) antigen-pulsed macrophages were co-cultured with purified T-lymphocytes; and (*c*) T cell proliferation was quantitated.

To "pulse" the alveolar macrophages with antigen, purified alveolar macrophages recovered by bronchoalveolar lavage were resuspended at 5×10^6 cells/ml in complete medium. To prevent subsequent incorporation of ^{3}H-thymidine by the macrophages (or any minor cell types contaminating the macrophage preparation), mitomycin-C (Sigma Chemical Co.) was added to a final concentration of 30 μg/ml and the cells were incubated for 1 h at 37°C in 95% air/5% CO_2. The macrophages were then washed four times and resuspended at the same concentration in complete medium. Tetanus toxoid (Wyeth Laboratories, Marietta, PA) was dialyzed extensively against RPMI-1640 and then added to a final concentration of 12 U/ml and the cells incubated at 37°C in 95% air/5% CO_2. After 24 h, the cells were washed extensively to remove unbound antigen and were resuspended at 10^5 cells/ml in complete medium.

Co-culture of antigen-pulsed macrophages with purified T-lymphocytes was carried out in triplicate in round bottom wells of microtiter plates (Flow Laboratories, McLean, VA). Unless otherwise specified, each well contained T-lymphocytes (10^5 cells/well), alveolar macrophages (10^4 cells/well), and complete medium (200 μl). The cultures were then incubated for 5 d at 37°C in 95% air/5% CO_2.

To quantify T cell proliferation, the co-cultures were pulsed with ^{3}H-thymidine for the last 18 h of incubation by addition of 4 μCi ^{3}H-thymidine (23 Ci/mM, Amersham Corp., Arlington Heights, IL) in 20 μl of complete medium. The plates were then harvested using a

1. *Abbreviations used in this paper:* FITC, fluorescein isothiocyanate; IPF, idiopathic pulmonary fibrosis; IFN_γ, gamma interferon; N-SRBC, neuraminidase-treated sheep red blood cells; SKSD, streptokinase-streptodornase.

multiple sample harvester (Mash II, M.A. Bioproducts) and the incorporation of ^{3}H-thymidine assayed as previously described (13).

As controls, parallel-triplicate cultures (final volume 200 μl) were prepared which contained: (*a*) T-lymphocytes (10^5 cells/well) in complete medium; (*b*) T-lymphocytes (10^5 cells/well) plus tetanus toxoid (12 U/ml final concentration); (*c*) alveolar macrophages (not pulsed, not treated with mitomycin-C, 10^5 cells/well); (*d*) alveolar macrophages (not pulsed, not treated with mitomycin-C, 10^5 cells/well) plus tetanus toxoid (12 U/ml final concentration); and (*e*) T-lymphocytes (10^5 cells/well) plus alveolar macrophages (not pulsed, but treated with mitomycin-C, 10^4 cells/well).

To evaluate the dependence of the assay on the concentration of the antigen, the pulsing of alveolar macrophages was performed using a variety of concentrations of tetanus toxoid (3–60 U/ml). To evaluate the time dependence of the assay, co-cultures of T cells and antigen-pulsed macrophages were incubated for 3–7 d and ^{3}H-thymidine was added for the last 18 h of co-culture. To evaluate the importance of the ratio of alveolar macrophages to T cells in the efficiency of antigen presentation, macrophage to T cell ratios ranging from 1:100 to 1:2 were evaluated with the other aspects of the assay performed as described above.

To compare the ability of blood monocytes versus alveolar macrophages to present antigen to autologous T-lymphocytes, antigen presentation by blood monocytes was investigated in each study population as described for alveolar macrophages. The monocytes were pulsed with tetanus toxoid at a concentration of 12 U/ml and co-cultured with autologous T-lymphocytes at a monocyte to lymphocyte ratio of 1:10 for 5 d.

Evaluation of antigen presentation in patients with sarcoidosis and IPF. To evaluate the ability of alveolar macrophages from patients with pulmonary sarcoidosis and IPF to present antigen to autologous T-lymphocytes, the assay was carried out as described for normal individuals. For comparison, similar studies were performed with blood monocytes.

Evaluation of antigen presentation of other antigens. To evaluate whether the observations concerning antigen presentation by macrophages from normals, sarcoid patients, and IPF patients were unique to tetanus toxoid or whether it was similar using a variety of other antigens, antigen presentation by alveolar macrophages to autologous T cells was carried out in each of the study populations with tetanus toxoid, streptokinase-streptodornase (SKSD; Eli-Lilly & Co., Indianapolis, IN), and mumps antigen (Lederle, Pearl River, NY). All antigens were dialyzed against RPMI-1640 before use. The assays were identical to that described above except that SKSD (100 U/ml final concentration) or mumps antigen (0.1 complement-fixing units per milliliter final concentration) were substituted for tetanus toxoid.

Quantification of alveolar macrophages expressing HLA-D region surface antigens. To evaluate whether differences observed in antigen presentation among the study populations were associated with differences in the proportion of antigen-presenting cells expressing gene products of the HLA-D locus on their cell surface, the proportion of monocytes and alveolar macrophages from normals and patients with sarcoidosis and IPF expressing HLA-DR and HLA-DS antigens was quantitated.

Monoclonal antibodies used were: L243 (HLA-DR, Becton-Dickinson Inc., Sunnyvale, CA), which reacts with a nonpolymorphic HLA-DR epitope (21) and SK10 (Leu 10, Becton-Dickinson Inc.), which reacts with a common polymorphic HLA-DS epitope (22). As controls, mouse monoclonal antibodies of heavy chain subclass IgG_1 (MOPC-21, Bionetics, Kensington, MD) and IgG_{2a} (RPC-5, Bionetics), which do not react with human cells, were used. Fluorescein isothiocyanate (FITC) conjugated rabbit anti-mouse IgG $F(ab')_2$ was obtained from Cappel Laboratories (Cochranville, PA). This fluorescent antibody was further purified by chromatography on Protein A Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) and human IgG-Sepharose to remove residual antibody which could cross-react with Fc receptors or human IgG present on the surface of the macrophages (23). The FITC conjugated antibody was then titered using fluorescence-activated flow cytometry (24) to give maximum fluorescence without staining cells nonspecifically.

Peripheral blood mononuclear cells or cells obtained by bronchoalveolar lavage (5×10^5 cells in each case) were preincubated in V-shaped microtiter wells in 50 μl of 3% rabbit serum in PBS to block Fc receptors. Cells were washed, and suspended in 20 μl of 1% bovine serum albumin and 0.02% sodium azide in PBS (staining buffer) containing 0.5 μg purified monoclonal antibody for 30 min at 4°C and washed three times with 200 μl of staining buffer. Cells were resuspended in 20 μl of staining buffer containing 5 μg FITC-conjugated $F(ab')_2$ rabbit anti-mouse IgG for 30 min at 4°C. Cells were then washed three times in 200 μl of staining buffer, suspended in 10% formalin in PBS for 15 min, washed two times in staining buffer, and stored in 50% glycerol in PBS.

Monocytes and macrophages were identified in suspension using a phase contrast microscope at 1,000× magnification (Carl Zeiss, Inc., New York, NY) and fluorescence determined by examining the cells under epifluorescent illumination using an FITC filter pack (Carl Zeiss Inc., 487716-9901). Only monocytes and macrophages having a distinct rim of green fluorescence were scored as positive. Using these criteria, <1% of cells reacted in a positive fashion with MOPC-21 or RPC-5 control antibodies.

Evaluation of the surface phenotype of T-lymphocytes in sarcoidosis. To evaluate the surface phenotype of lung and blood T-lymphocytes from sarcoid patients, the proportions of lymphocytes expressing surface antigens detected by monoclonal antibodies Leu 3 (25, 26) ("helper/inducer" T-lymphocyte subset, Becton Dickinson Inc.) and Leu 2 (25, 26) ("suppressor/cytotoxic" T-lymphocyte subset, Becton Dickinson) were determined by indirect immunofluorescence as described above.

To determine the proportion of lung T-lymphocytes which were expressing surface antigens associated with T-lymphocyte activation, two-color immunofluorescence was performed on peripheral blood mononuclear cells and cells recovered by bronchoalveolar lavage. 5×10^5 cells were incubated with 1 μg FITC-conjugated Leu 4 (26) (Becton-Dickinson Inc.) in 20 μl of staining buffer for 30 min, washed three times, reacted with 0.5 μg biotin-conjugated monoclonal antibody 4F2 (27, 28) or L243 (HLA-DR, Becton Dickinson Inc.) for 30 min, washed three times, and reacted with 0.125 μg rhodamine-conjugated avidin (Vector Laboratories, Burlingame, CA) in 25 μl staining buffer for 20 min. Cells were then washed, fixed, and stored as described above.

Leu 4^+ cells (T-lymphocytes) were identified by examining the cells under epifluorescent illumination using an FITC filter set, and the proportion of T-lymphocytes expressing HLA-DR and 4F2 surface antigens determined by examining these cells under epifluorescent illumination using a rhodamine filter set (Carl Zeiss, Inc., 487715-9902).

Measurement of gamma interferon production by T-lymphocytes. To determine if lung T-lymphocytes from sarcoidosis patients could be contributing to enhanced antigen presentation by spontaneously releasing gamma interferon (IFN_γ), lung and blood T-lymphocytes (purified as described above) from these patients were incubated at 5×10^6 cells/ml in complete medium for 24 h at 37°C, and the IFN_γ production quantified by comparing the ability of serial dilutions of supernatant fluid and partially purified human IFN_γ (10^6 U/ml, Meloy Laboratories, Springfield, VA) to inhibit vesicular stomatitis virus-induced lysis of human WISH cells (29). The interferon was classified as gamma on the basis of pH sensitivity, species specificity, and inhibition of activity by preincubation with mouse anti-human IFN_γ monoclonal antibody (Meloy Laboratories) (30).

Analysis of data. All data are presented as mean±SD. Statistical comparisons of the effect of addition of antigen-presenting cells or antigen on T-lymphocyte proliferation were made using the signed rank test of Wilcoxon (31). All other statistical comparisons were made using the Mann-Whitney test (31). For all statistical tests, if the

calculated significance probability was >0.05, the result is reported as "not significant."

Results

Characterization of cell populations recovered by lavage. Bronchoalveolar lavage demonstrated that the cell populations present in the lung were as previously reported for normals, patients with sarcoidosis, and patients with IPF (Table I). In the normal individuals, macrophages comprised >90% of the effector cells, with the remainder being lymphocytes. In contrast, patients with sarcoidosis had a marked expansion of lung lymphocyte numbers, such that lymphocytes were proportionally increased more than threefold compared with both normals and patients with IPF ($P < 0.05$). In comparison, patients with IPF had an accumulation of polymorphonuclear leukocytes (neutrophils greater than eosinophils), but the proportion of lymphocytes was not significantly different from that of normals.

Ability of normal human alveolar macrophages to present antigen to autologous T-lymphocytes. Alveolar macrophages from normal individuals were capable of presenting antigen to autologous T-lymphocytes, which resulted in a marked stimulation of T cell proliferation (Fig. 1). When purified blood T-lymphocytes were cultured for 5 d, either alone or in the presence of tetanus toxoid, there was minimal incorporation of tritiated thymidine (difference not significant between T cells alone vs. T cells plus antigen). Similarly, alveolar macrophages (that had not been treated with mitomycin-C) showed no significant proliferation when cultured alone or in the presence of tetanus toxoid. As has been observed when human blood monocytes and autologous T cells are co-cultured (13), co-culture of alveolar macrophages and autologous blood T-lymphocytes induced a small increase in T cell proliferation. This likely represents an example of the "autologous mixed leukocyte reaction," i.e., when mitomycin-C treated alveolar macrophages were co-cultured with autologous T cells, there was some stimulation of T cell replication even in the absence of added antigen ($P < 0.01$ compared with T cells cultured alone). However, when autologous T cells were co-cultured with alveolar macrophages pulsed with antigen, there was a marked increase of T-lymphocyte proliferation ($P < 0.01$ compared with T cells co-cultured with macrophages that were not exposed to antigen; $P < 0.001$ compared with T cells cultured alone).

Characterization of the culture parameters for antigen presentation by normal alveolar macrophages to autologous T-lymphocytes demonstrated a significant dependence on the concentration of antigen used (Fig. 2 *A*). Evaluation of the time dependence of the assay showed, as expected, an increase in T cell replication as a function of time in culture (Fig. 2 *B*). Furthermore, there was clearly a dependence on the relative numbers of T cells and alveolar macrophages used in the assay, but this dependence exhibited a broad range of T cell to alveolar macrophage ratios at which T cell proliferation was optimal (Fig. 2 *C*).

Antigen presentation by alveolar macrophages of patients with sarcoidosis and IPF. Compared to normal macrophages, antigen presentation by alveolar macrophages from patients with sarcoidosis to their autologous blood T cells was markedly enhanced (Fig. 3). Purified T cells from patients with sarcoidosis showed a minimal proliferation when cultured alone, although thymidine incorporation was somewhat greater than for normal blood T cells (Table II; $P < 0.02$ compared with normals). As with normal blood T cells, the proliferation of sarcoid blood T cells alone was not modified by the addition of tetanus toxoid (difference not significant; data not shown). Co-culture of sarcoid blood T cells with autologous alveolar macrophages that had not been exposed to antigen resulted in a small increase in proliferation compared with that of sarcoid blood T cells cultured alone ($P < 0.01$), but this increase was similar to that observed under similar conditions in normals (Table II; difference not significant). As with normal macrophages, antigen-pulsed sarcoid alveolar macrophages added to T cells significantly increased T cell proliferation compared with T cell proliferation produced by sarcoid alveolar macrophages not exposed to antigen ($P < 0.01$). Strikingly, however, the ability of antigen-pulsed sarcoid alveolar macrophages to present antigen to autologous blood T-lymphocytes was markedly enhanced compared with the ability of antigen-pulsed normal alveolar macrophages to present antigen to autologous blood T cells (Fig. 3; $P < 0.001$). This was true, even though the antigen concentration and time of co-culture producing maximal proliferation was similar for sarcoid patients and normals (data not shown). Furthermore, the increased ability of antigen-

Table I. Physiologic and Bronchoalveolar Lavage Data from Normals, Patients with Sarcoidosis, and Patients with Idiopathic Pulmonary Fibrosis

		Physiologic data*				Bronchoalveolar lavage data‡				
		Vital capacity	Total lung capacity	FEV_1/FVC	Diffusing capacity	Total cells	Differential cell count			
Study group	*n*	(% predicted)	(% predicted)	(% predicted)	(% predicted)	($\times 10^{-6}$)	Macrophages	Lymphocytes	Neutrophils	Eosinophils
							%	%	%	%
Normals	14	99.7±11.5	96.2±11.9	111.1±5.7	112.3±19.9	40±36	94.5±4.1	4.3±4.4	0.5±1.3	0.7±1.1
Sarcoidosis	21	61.7±14.7	65.1±13.4	99.4±13.2	66.0±26.1	34±27	78.1±15.7	17.9±15.8	2.3±4.0	1.7±2.2
IPF§	12	65.4±24.8	62.6±19.9	108.7±11.2	47.5±20.5	54±44	82.7±7.8	3.0±4.7	9.5±7.5	4.8±4.0

* Physiologic methods and predicted values were carried out as previously described (6, 16); diffusing capacity based on alveolar volume and corrected for hemoglobin. FEV_1/FVC = forced expiratory volume in 1 s/forced vital capacity. ‡ Total cell counts are presented as total number of cells recovered $\times 10^{-6}$; differential cell counts are presented as percentage of all inflammatory and immune effector cells recovered. § IPF, idiopathic pulmonary fibrosis.

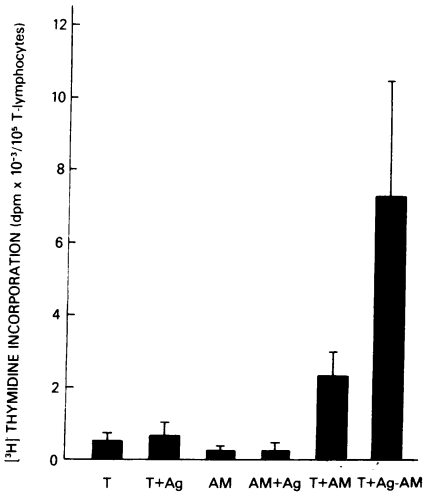

Figure 1. Evaluation of antigen presentation by normal alveolar macrophages to autologous T-lymphocytes. Triplicate cultures containing T-lymphocytes cultured alone ("T," n = 14), T-lymphocytes with tetanus toxoid ("T + Ag," n = 14), alveolar macrophages alone ("AM," n = 4), alveolar macrophages with tetanus toxoid ("AM + Ag," n = 4), T-lymphocytes with alveolar macrophages ("T + AM," n = 14), or T-lymphocytes with tetanus toxoid-pulsed alveolar macrophages ("T + Ag-AM," n = 14), were cultured for 5 d at 37°C. During the last 18 h of cultivation, ^{3}H-thymidine was added, and proliferation was assessed by determining the amount of ^{3}H-thymidine incorporated by the cells.

pulsed sarcoid alveolar macrophages to induce T-lymphocyte proliferation was not restricted to cultures performed at an alveolar macrophage/lymphocyte ratio of 1:10. When an alveolar macrophage/lymphocyte ratio of 1:5 was used, antigen presentation by sarcoid alveolar macrophages was also increased (normals, 7,161±2,725 dpm/10^5 lymphocytes; sarcoid patients, 14,086±4,039 dpm/10^5 lymphocytes; n = 4; $P < 0.01$).

In contrast to macrophages from patients with sarcoidosis, the ability of alveolar macrophages from patients with IPF to present antigen to autologous blood T cells demonstrated a pattern similar to macrophages from normals (Fig. 3). As in normals, blood T cells from patients with IPF showed minimal incorporation when cultured alone and thymidine incorporation was slightly enhanced when alveolar macrophages which had not been exposed to antigen were added; these observations were not significantly different from those made in normals (Table II). As with normals, antigen-pulsed alveolar macrophages significantly stimulated autologous T cell proliferation ($P < 0.01$ compared with T cells cultured alone or with alveolar macrophages not exposed to antigen). However, the increase in T cell proliferation induced by the antigen-pulsed macrophages was not significantly different from that observed in normals, but twofold less than observed in sarcoidosis ($P < 0.001$).

Interestingly, alveolar macrophages from untreated sarcoid patients with an increased percentage of lymphocytes recovered by lavage were more efficient antigen-presenting cells than alveolar macrophages from untreated patients with a normal percentage of lymphocytes recovered by lavage (>10% lymphocytes, n = 6: 19,947±3,168 dpm/10^5 lymphocytes; <10% lymphocytes, n = 8: 13,727±2,085 dpm/10^5 lymphocytes; $P < 0.01$). In some cases, alveolar macrophages from sarcoid patients receiving corticosteroid therapy induced less T-lymphocyte proliferation than that produced by alveolar macrophages from untreated sarcoid patients (Fig. 3). However, in other cases, alveolar macrophages from treated patients continued to show enhanced antigen presentation, and when the two groups were compared, there was no difference in antigen presentation between treated and untreated patients (treated, n = 7; 12,525±7,217 dpm/10^5 lymphocytes; untreated, n = 14; 16,393±4,050 dpm/10^5 lymphocytes; difference not significant). Similarly, there was no significant difference in antigen presentation by alveolar macrophages among sarcoid patients grouped on the basis of age (<35 yr, n = 11: 12,612±10,246 dpm/10^5 lymphocytes; >35 yr, n = 10: 14,566±7,188 dpm/10^5 lymphocytes; $P > 0.2$), sex (males, n = 9; 14,374±5,792 dpm/10^5 lymphocytes; females, n = 12: 15,657±5,383 dpm/10^5 lymphocytes; $P > 0.2$), or chest X-ray type (type I, n = 4: 16,031±3,584 dpm/10^5 lymphocytes; type II, n = 7: 14,523±6,075 dpm/10^5 lymphocytes; type III, n

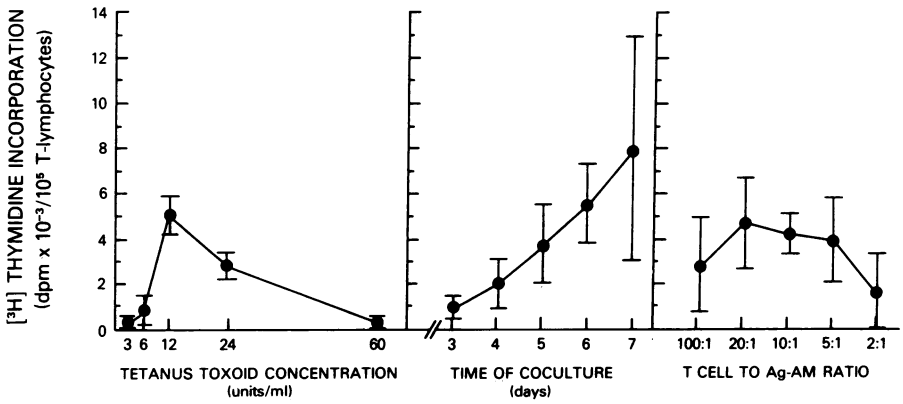

Figure 2. Evaluation of antigen concentration, time of co-culture, and T cell to macrophage ratio on the efficiency of antigen presentation by normal alveolar macrophages to autologous T-lymphocytes. (*A*) Antigen concentration dependence of the assay. Macrophages were pulsed with various concentrations of tetanus toxoid, but otherwise the assay was carried out as described in Methods. The assay was performed using cells from two individuals, each in triplicate. (*B*) Time dependence of the assay. Antigen-pulsed macrophages were co-cultured with T cells for various times; otherwise the assay was as in *A*. The assay was performed using cells from three individuals, each in triplicate. (*C*) Dependency of the assay on the T cell to macrophage ratio. Various ratios of T cells to antigen-pulsed macrophages (Ag-AM) were used, but the assay was otherwise as in *A*. The assay was performed using cells from four individuals, each in triplicate. Data is presented on the ordinate as ^{3}H-thymidine incorporation of: (co-cultures of T cells plus antigen-pulsed macrophages) − (co-cultures of T cells plus macrophages that were not pulsed with antigen).

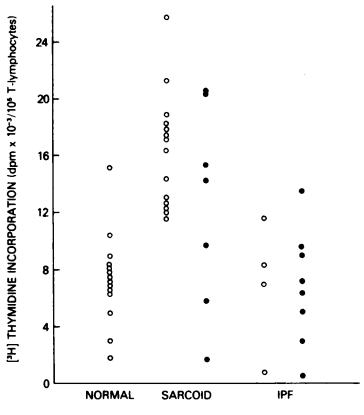

Figure 3. Evaluation of antigen presentation by alveolar macrophages to autologous T-lymphocytes from normals, patients with sarcoidosis, and patients with idiopathic pulmonary fibrosis. The assays were carried out as described for antigen presentation by normal alveolar macrophages. All studies were performed using 12 U/ml tetanus toxoid, 5 d of co-culture, and a T cell to alveolar macrophage ratio of 10:1. Open symbols indicate individuals not receiving corticosteroids; closed symbols indicate patients receiving corticosteroid therapy.

= 10: 14,958±6,109 dpm/10^5 lymphocytes; $P > 0.2$ for each comparison).

Evaluation of alveolar macrophage presentation of antigens other than tetanus toxoid. Importantly, the enhanced antigen presentation by alveolar macrophages observed in patients with sarcoidosis was not unique to tetanus toxoid. When two other common antigens were evaluated (SKSD and mumps antigen), the T-lymphocyte responses for both antigens were similar to that observed with tetanus toxoid (Table II). With both antigens, antigen presentation by sarcoid macrophages was increased compared with antigen presentation by normal macrophages or macrophages from patients with IPF (Table II).

Presence of HLA-D region antigens on the surface of alveolar macrophages. Since the expression of HLA-D region gene products on the cell surface of antigen-presenting cells is a necessary requirement for the ability to present antigen (10–14, 32), the increased T cell proliferation triggered by antigen presentation by sarcoid macrophages might be explained by differences in the study groups in the proportion of macrophages that had HLA-D locus gene products on their cell surface. However, the majority of macrophages from normals and from patients with sarcoidosis express both HLA-DR and HLA-DS antigens. The proportion of HLA-DR bearing alveolar macrophages in patients with sarcoidosis was similar to that of normals (Table III, difference not significant). The percentage of macrophages from patients with IPF which expressed HLA-DR antigens (97.5±0.5) was also similar to that of normals and patients with sarcoidosis (no significant difference for both comparisons). Similarly, the proportions of alveolar macrophages from normals and patients with sarcoidosis bearing HLA-DS surface antigens were also not significantly different (Table III).

The percentage of normal and sarcoid macrophages expressing sufficient HLA-DR and HLA-DS surface antigen to be detectable by indirect immunofluorescence was significantly higher than the percentage of blood monocytes expressing detectable levels of these surface antigens (Table III). In addition, the density of HLA-DR and HLA-DS antigens, as assessed by the intensity of fluorescence, was considerably higher on pulmonary macrophages than on blood monocytes from both normals and sarcoidosis patients.

The percentages of blood monocytes from normals and sarcoid patients expressing sufficient HLA-DR to be detectable by indirect immunofluorescence were similar. However, significantly more blood monocytes from patients with sarcoidosis expressed detectable HLA-DS surface antigens ($P < 0.05$, Table III).

Comparison of monocyte antigen presentation to autologous T-lymphocytes in the study populations. In each of the groups evaluated, antigen-pulsed blood monocytes induced significant amounts of proliferation of autologous blood T-lymphocytes. However, the amount of T cell proliferation induced by antigen-pulsed monocytes was similar for normals, sarcoidosis patients, and IPF patients. (Normals, n = 8: 41,868±13,179 dpm/10^5 T-lymphocytes; sarcoidosis, n = 6: 37,495±12,513 dpm/10^5 T-lymphocytes; IPF, n = 2: 35,260±9,236 dpm/10^5 T-lymphocytes; difference not significant for all comparisons).

Although the blood monocyte preparations were not as pure as the alveolar macrophage preparations, it was apparent that under identical conditions (numbers of mononuclear phagocytes, numbers of autologous blood T-lymphocytes, antigen concentration, time, and mononuclear phagocyte to T cell ratio), that in the presence of antigen, the blood monocyte

Table II. Ability of Alveolar Macrophages from Normals, Patients with Sarcoidosis, and Patients with IPF to Present a Variety of Antigens to Autologous T-Lymphocytes

	Patient group					
	Normal		Sarcoidosis		IPF	
Culture conditions*	n	^{3}H-thymidine incorporation	n	^{3}H-thymidine incorporation	n	^{3}H-thymidine incorporation
T-cells alone	14	558±212	21	802±298	12	615±230
+AM	14	2,335±684	21	2,784±1,011	12	2,445±950
+TT-AM	14	7,325±3,212‡	21	15,103±5,457‡§	12	6,763±4,041‡
+SKSD-AM	7	7,195±1,421‡	5	13,835±3,330‡§	6	5,912±2,296‡
+Mumps-AM	7	7,755±1,527‡	6	10,855±3,485‡	6	6,058±2,032‡

^{3}H-Thymidine incorporation equals dpm/10^5 T-lymphocytes. * AM, alveolar macrophages; TT-AM, alveolar macrophages pulsed with tetanus toxoid; SKSD-AM, alveolar macrophages pulsed with streptokinase-streptodornase; Mumps-AM, alveolar macrophages pulsed with mumps antigen; n, number of individuals in each group. ‡ Values significantly different ($P < 0.05$, all comparisons) than those of T cells co-cultured with autologous alveolar macrophages not pulsed with antigen. § Values significantly different ($P < 0.05$, all comparisons) than similar co-cultures of normals or patients with IPF.

Table III. Presence of HLA-DR and HLA-DS Antigens on the Surface of Alveolar Macrophages and Blood Monocytes of Normals and Patients with Pulmonary Sarcoidosis‡*

Study group	n	HLA-DR (%)		HLA-DS (%)	
		Lung	Blood	Lung	Blood
Normal	10	94.2±2.4	68.6±6.7	83.0±4.4	4.0±1.5
Sarcoidosis	15	97.5±1.5	63.5±4.1	90.8±3.4	10.6±2.5

* Alveolar macrophages were incubated for 30 min at 4°C with murine monoclonal antibody anti-human HLA-DR or HLA-DS, then washed and incubated 30 min with a fluorescein-conjugated $F(ab')_2$ rabbit anti-mouse IgG. Data is presented as percentage of blood monocytes or alveolar macrophages expressing HLA-DR or HLA-DS antigens as evaluated visually by fluorescence microscopy.

‡ Significant differences were noted between alveolar macrophages and blood monocytes for normals and sarcoid patients ($P < 0.05$, all comparisons), but not between alveolar macrophages of normals versus sarcoidosis patients for either DR or DS.

populations induced far more T cell proliferation than did the autologous alveolar macrophage populations ($P < 0.05$, all comparisons of blood monocytes to alveolar macrophages). However, the difference between alveolar macrophage antigen presentation and monocyte antigen presentation was most dramatic in the normals (alveolar macrophage preparations were 18±2% as efficient as the blood monocyte preparations in inducing T cell proliferation) and patients with IPF (15±4%). In contrast, the difference between alveolar macrophage antigen presentation and monocyte antigen presentation in the patients with sarcoidosis was far less (41±8%, $P < 0.05$ compared with normals and IPF). Thus, while alveolar macrophages were generally less efficient as antigen-presenting cells compared with blood monocytes (or possibly nonmonocyte blood cells contained in the monocyte preparation), this was less so for the alveolar macrophage population in sarcoidosis.

Characterization of lung and blood T-lymphocytes in sarcoidosis. Evaluation of the lung and blood T-lymphocytes from the sarcoid patients suggested that more of the lung T-lymphocytes were activated than were those in the blood. The ratio of Leu 3^+ to Leu 2^+ T-lymphocytes in the lung was 6.6±1.3, compared with 2.0±0.33 in the blood ($P < 0.01$). Furthermore, a greater percentage of the Leu 4^+ lymphocytes (T-lymphocytes) in the lung were expressing surface antigens characteristic of activated cells than was present in the blood. Among the total population of Leu 4^+ cells in the lung, 31±10% were HLA-DR^+, compared with 9±4% of blood T-lymphocytes ($P < 0.05$), and 14±4% of lung T-lymphocytes were $4F2^+$, compared with 2±0.5% of blood T-lymphocytes ($P < 0.05$). In addition, lung T-lymphocytes from sarcoidosis patients spontaneously released 96±40 U $IFN_\gamma/10^6$ cells/24 h. In contrast, blood T-lymphocytes from these patients released no or negligible amounts of IFN_γ (<10 U/10^6 cells/24 h, $P < 0.02$ compared with lung), although blood T-lymphocytes could be readily induced to produce IFN_γ by the addition of phytohaemagglutinin.

Comparison of alveolar macrophage antigen presentation to lung versus blood T-lymphocytes. There were not sufficient numbers of lung T-lymphocytes recovered by lavage from normals or patients with IPF to permit purification of sufficient numbers of T-lymphocytes to determine whether the lung T cells and blood T cells from these groups responded in a similar or dissimilar manner to antigen presentation by autologous alveolar macrophages. However, in five sarcoid patients, sufficient numbers of highly purified T-lymphocytes could be obtained to allow such comparisons. Lung T-lymphocyte proliferation in response to presentation of tetanus toxoid by autologous alveolar macrophages was not significantly different from that observed for blood T-lymphocytes (lung T-lymphocytes: 15,753±10,666 dpm/10^5 T-lymphocytes; blood T-lymphocytes: 12,671±5,365 dpm/10^5 T-lymphocytes; $n = 5$; difference not significant).

Discussion

A central feature of pulmonary sarcoidosis is the presence of large numbers of spontaneously replicating T-lymphocytes in the lower respiratory tract (7–9). One mechanism that may contribute to the increased replication of T-lymphocytes in this disorder is enhanced alveolar macrophage-mediated antigen-induced lymphocyte proliferation. According to this hypothesis, the population of alveolar macrophages and/or T-lymphocytes present in the lungs of patients with pulmonary sarcoidosis are different from those normally present such that, for the same antigen burden, the sarcoid macrophages stimulate increased T-lymphocyte proliferation in the local milieu. In this context, we have shown that alveolar macrophages from patients with sarcoidosis present antigen to autologous T cells in a manner such that there is more than a twofold increase in the T-lymphocyte proliferative response compared with that observed in normals. In contrast, the T-lymphocyte proliferative response induced by antigen-pulsed blood monocytes is similar in the two groups. Furthermore, the increased T-lymphocyte proliferation induced by antigen-exposed sarcoid alveolar macrophages is not dependent on the compartment from which the T-lymphocytes are obtained, and is independent of the specific antigen used. However, the difference between antigen presentation by sarcoid and normal alveolar macrophages does not appear to be related to the proportion of macrophages that express HLA-DR and HLA-DS surface antigens.

Antigen presentation by normal alveolar macrophages. These studies demonstrate that normal human alveolar macrophages are capable of stimulating autologous T-lymphocyte proliferation in response to soluble antigens. The extent of T-lymphocyte proliferation is dependent on the concentration of antigen to which the alveolar macrophages are exposed and to the time for which the alveolar macrophages and T-lymphocytes are co-cultivated. In addition, this process is significantly modified by the relative numbers of antigen presenting alveolar macrophages and the responding T-lymphocytes present. In this regard, highly purified T-lymphocytes proliferated maximally in response to antigen when small numbers of antigen-presenting cells were present (lymphocyte to macrophage ratio of 10:1); when proportionally more alveolar macrophages were present (lymphocyte to macrophage ratio of 2:1), T cell replication was partially suppressed. However, even when the proportion of antigen presenting and responding cells are optimized, the extent of T-lymphocyte proliferation induced by normal alveolar macrophages is far less than that induced by autologous blood monocytes. These observations are consistent with previous studies evaluating the ability of antigen-

exposed alveolar macrophages from humans (33–37) and other species (38–41) to induce T-lymphocyte proliferation.

Antigen presentation in pulmonary sarcoidosis. These studies also demonstrate that the ability of antigen-exposed alveolar macrophages to induce T-lymphocyte proliferation is increased in sarcoidosis. In these experiments, autologous alveolar macrophages and T-lymphocytes were used. Thus, changes in the alveolar macrophage population and/or changes in the T-lymphocyte populations could explain the increased alveolar macrophage-mediated antigen-induced T-lymphocyte proliferation observed in these sarcoid patients. However, several lines of evidence suggest that this augmented antigen-induced proliferation did not result from activation of the responder T-lymphocyte population. First, maximal T-lymphocyte proliferation (i.e., that induced by autologous antigen-pulsed blood monocytes) was of similar magnitude whether the responder T-lymphocytes were obtained from normals, patients with sarcoidosis, or patients with IPF. Second, T-lymphocytes purified from the lungs of patients with sarcoidosis, which were demonstrated by several criteria to be more "activated" than blood T-lymphocytes from these patients, did not show significantly increased proliferation in response to antigen-primed autologous alveolar macrophages when compared with the response of the blood T-lymphocytes. Third, the proliferation of sarcoid blood T-lymphocytes induced by the autologous mixed leukocyte reaction (i.e., T-lymphocyte proliferation induced by autologous alveolar macrophages or blood monocytes in the absence of antigen) was no different than that seen in normals. Finally, other studies have shown that peripheral blood T-lymphocytes from patients with sarcoidosis do not show enhanced proliferation in response to mitogens (7, 42). Thus, it is likely that changes in the population of antigen-presenting cells, and not the responding T cells, explain the increased antigen-induced T-lymphocyte proliferation observed in these studies.

Although our results suggest that the increased antigen-induced T-lymphocyte proliferation results from the fact that sarcoid alveolar macrophages are better antigen-presenting cells than normal alveolar macrophages, it is still possible that the T-lymphocytes play a primary role in this phenomenon. In this context, these studies also demonstrate that lung T-lymphocytes, but not blood T-lymphocytes, from sarcoid patients are spontaneously releasing IFN_γ. IFN_γ has been shown to enhance antigen presentation by accessory cells (43, 44), and therefore sarcoid alveolar macrophages may be better antigen-presenting cells because they have been stimulated by the activated T-lymphocytes. Since IFN_γ is also known to induce the expression of HLA-D locus surface antigens by accessory cells, it has been suggested that this is one mechanism by which IFN_γ enhances the ability of accessory cells to present antigen (44). However, our results demonstrate that most macrophages from normals and sarcoid patients express both HLA-DR and HLA-DS antigens, and as a group, the percentage of alveolar macrophages from normals and sarcoid patients expressing these antigens is not different. We were not able to quantify the density of these surface antigens on the alveolar macrophages in the two groups, and therefore cannot exclude the possibility that differences in the density of HLA-D locus surface antigens on alveolar macrophages explain the observed differences in antigen presentation. Further studies are required to determine the mechanisms accounting for the enhanced antigen presentation seen in sarcoidosis, and to clarify the role of the T-lymphocyte in this process.

It should be emphasized that the magnitude of the difference observed in these studies between antigen-induced T-lymphocyte replication in normals and patients with sarcoidosis cannot be extrapolated to the in vivo situation. When T-lymphocytes and antigen-exposed alveolar macrophages were co-cultivated in vitro at a 10:1 ratio (i.e., under conditions producing maximal T-lymphocyte proliferation), sarcoid alveolar macrophages induced more than twofold more T-lymphocyte replication than that produced by normal alveolar macrophages. When a 5:1 ratio was used, a similar difference was observed. However, if such comparisons were made at other T-lymphocyte to alveolar macrophage ratios, the difference observed between antigen presentation by normal and sarcoid alveolar macrophages may differ. Furthermore, the relative proportions of lymphocytes and alveolar macrophages present on the epithelial surface of the lower respiratory tract of normals and patients with sarcoidosis are different. Since the lymphocyte to macrophage ratios encountered on the epithelial surface of patients with active sarcoidosis (1:1–1:2) are more conducive to antigen presentation than those present in normals (1:5–1:10) (35, 37), it is likely that antigen presentation in sarcoidosis is enhanced by more than the twofold difference demonstrated in these studies.

Although augmented antigen presentation could account for the increased T-lymphocyte proliferation characteristic of pulmonary sarcoidosis, it is also possible that the T-lymphocytes are activated by an independent mechanism, and these activated T-lymphocytes secondarily stimulate the ability of sarcoid alveolar macrophages to present antigen. These studies do not distinguish whether or not increased antigen presentation is a primary event in the pathogenesis of sarcoidosis or represents an effect of this chronic inflammatory process. However, antigen-induced T-lymphocyte proliferation is augmented in sarcoidosis, which is an observation consistent with the possibility that enhanced antigen presentation of a variety of antigens by alveolar macrophages promotes T-lymphocyte replication and therefore may play an important role in initiating or perpetuating the T-lymphocyte alveolitis of sarcoidosis.

Acknowledgments

Dr. Saltini was supported by USL16 and by University of Modena, Italy.

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