Selection of T Lymphocytes Bearing Limited TCR-Vβ Regions in the Lung of Hypersensitivity Pneumonitis and Sarcoidosis

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Hypersensitivity pneumonitis (HP) and sarcoidosis are interstitial lung disorders (ILD) characterized by a lymphocytic alveolitis that, in the active phase of the disease, is sustained by different T-cell subsets, i.e., $CD8^+$ cells in HP and $CD4^+$ lymphocytes in sarcoid patients. To address the question of whether a bias in T-cell selection occurs in the lung of patients with HP and sarcoidosis, we analyzed the T-cell receptor β chain variable region (TCR-Vβ) repertoire by flow cytometry and polymerase chain reaction (PCR) analyses in blood and lung lymphocytes of 14 HP and 25 sarcoid patients. To verify whether these cells can be activated *in vitro* through the TCR, blood and lung lymphocytes were also assessed for their responsiveness to different superantigenic stimuli represented by staphylococcal enterotoxins, including SEA, SEB, SEC1, SEC2, SED, and SEE. Flow cytometry and PCR analyses demonstrated an overexpression of cells bearing Vβ2, Vβ3, Vβ5, Vβ6, and Vβ8 gene segments in the lung of HP patients as compared with the peripheral blood. In sarcoid patients cells bearing Vβ2, Vβ5, and Vβ6 gene segments in the lung of HP patients as compared with the peripheral blood. In sarcoid patients cells bearing Vβ2, Vβ5, and Vβ6 gene segments were overrepresented in the lung rather than in the blood. Both in HP and sarcoid patients almost all T cells bearing the dominant Vβ segment belonged to the T-cell subset that sustains the alveolitis, i.e., CD8 in HP patients and CD4 in sarcoid subjects. Follow-up studies demonstrated that the recovery of the alveolitis was characterized by the disappearance of cells bearing a limited T-cell repertoire. Interestingly, T-lymphocyte response to different superantigens demonstrated that the proliferation elicited by different staphylococcal toxins was more pronounced in the lung than in the blood. Taken together, our findings indicate a compartmentalization of cells bearing discrete Vβ gene products in the pulmonary microenvironment and suggest that the expansion of specific Vβ region subsets occurring in the lung might result from triggering by a specific antigen. In fact, the removal from exposure in HP patients or specific treatment in sarcoidosis resulted in the decrease of the overrepresented cell population accounting for the lymphocytic alveolitis. Trentin L, Zambello R, Facco M, Tassinari C, Sancetta R, Siviero M, Cerutti A, Cipriani A, Marcer G, Majori M, Pesci A, Agostini C, Semenzato G. Selection of T lymphocytes bearing limited TCR-Vβ regions in the lung of hypersensitivity pneumonitis and sarcoidosis.

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Lymphocytic alveolitis represents a common finding in several interstitial lung disorders (ILD), particularly hypersensitivity pneumonitis (HP) and sarcoidosis (1). Although an impairment of T-cell subsets has been observed in the lung of patients suffering from these disorders, mainly represented by $CD8^+$ cells in HP and $CD4^+$ lymphocytes in sarcoidosis, most lung T-cells from these patients showed an activated phenotypic and functional pattern and the α/β T-cell receptor (2–4). Antigens accounting for HP have been characterized, whereas the antigenic stimuli leading to sarcoidosis are still unknown.

T lymphocytes recognize antigens through a receptor structure defined as the T-cell receptor (TCR), which is constructed through rearrangement of variable (V), diversity (D), and joining (J) gene segments. Recent data demonstrated a bias in the

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TABLE 1

PHENOTYPE OF LUNG AND BLOOD T CELLS IN HP AND SARCOID PATIENTS*

Site	Subjects	Cells × 10^3/ml	Lymphocytes (%)	CD4 (%)	CD8 (%)
Lung	HP	345.7 ± 61.9†	47.4 ± 6.6‡	28.0 ± 4.8§	50.4 ± 4.7§
	Sarcoidosis	295.5 ± 31.7†	30.9 ± 2.5‡	66.1 ± 2.4†	20.7 ± 1.9
	Controls	142.2 ± 12.3	7.5 ± 1.3	48.5 ± 1.7	22.5 ± 1.3
Blood	HP	4.26 ± 0.57	20.1 ± 1.78	50.1 ± 2.3	27.3 ± 2.6
	Sarcoidosis	4.56 ± 0.31	24.8 ± 1.95	54.5 ± 2.5	22.4 ± 1.8
	Controls	5.38 ± 0.31	29.7 ± 2.4	52.4 ± 1.6	25.3 ± 2.4

* Data are mean ± SD.
† $p < 0.05$, HP or sarcoid patients versus normal subjects.
‡ $p < 0.005$, HP or sarcoid patients versus normal subjects.
§ $p < 0.01$, HP or sarcoid patients versus normal subjects.

TCR usage in different disease states, including multiple sclerosis, rheumatoid arthritis, primary biliary cirrhosis, and allograft rejection (5–11). In the lung compartment a limited usage of the TCR has been observed in patients with sarcoidosis (12–17). In particular, cells bearing limited numbers of both Vα and Vβ gene segments accumulate within the alveolar structures of sarcoid patients. An increased number of T lymphocytes bearing the Vα2 gene segment was observed, while a consistent variability was detected for the β chain variable region expression (12–17). In HP patients, no data are presently available on the Vβ gene segment usage. In this disorder, Southern blot analysis of the molecular configuration of the T-cell receptor β-gene of lung T cells in HP patients has demonstrated the presence of dominant bands and in some cases an oligoclonal pattern (4). These observations suggested that a T-cell selection with discrete specificities might occur in the lung compartment during exposure to causating antigens.

The mechanisms accounting for the accumulation of lymphocytes with limited specificities in the lung of patients with interstitial lung disorders may be represented by the *in situ* proliferation of Ag-specific T cells and/or redistribution from the blood to the lung of cells with limited specificities. Alternatively, it can be hypothesized that a superantigenic stimulus takes place in the lung of these patients. Superantigens (sAg) are in fact molecules which include endogeneous retroviral gene products and microbial toxins. They can stimulate proliferation and conversely cause anergy or deletion of T cells bearing a particular T-cell receptor β chain variable region (TCR-Vβ) sequence (18, 19).

This study was undertaken to investigate the pattern of accumulation of cells accounting for lymphocytic alveolitis in patients with interstitial lung disorders. We analyzed the TCR-Vβ repertoire in blood and lung by flow cytometry and polymerase chain reaction (PCR) analyses in 14 patients with HP and 25 patients with active sarcoidosis. In six patients the analysis of Vβ segments was performed before and after corticosteroid therapy or removal from exposure in sarcoid and HP patients, respectively. Because T cells bearing defined Vβ regions may be triggered to proliferate by specific superantigenic stimuli, blood and lung lymphocytes were triggered by different staphylococcal enterotoxins (SE), including SEA, SEB, SEC1, SEC2, SED, and SEE.

METHODS

Study Population

Fourteen symptomatic, nonsmoking patients (8 men and 6 women; age between 26 and 50 yr, mean 34 ± 16) with HP were studied. All patients had farmer's lung disease and the diagnosis was based on conventionally reported criteria (20, 21), notably: (*1*) history of exposure to HP antigens; (*2*) a symptomatic acute episode with chills, fever, cough, breathlessness 4 to 8 h after exposure to specific antigens; (*3*) radiologic features (mainly diffuse reticular pattern) and/or a functional pattern of interstitial lung disease; and (*4*) evidence of antibodies against *Faeni rectivirgula* in all cases. Precipitating antibodies against the etiologic antigens were detected both in the bronchoalveolar lavage (BAL) and in the serum of the patients under study. All patients were evaluated at least 1 mo after the last acute episode.

Twenty-five patients with active sarcoidosis were analyzed (10 men and 15 women; average age 36.8 ± 5.2 yr). In all cases the diagnosis was made by biopsy obtained either from the lungs or from lymph nodes, and showed noncaseating epithelioid cell granulomas, with no evidence of inorganic material known to cause granulomatous diseases. According to our staging system for sarcoidosis (2), each patient underwent BAL analysis. All sarcoid patients were defined as having an active disease, since they showed the following characteristics: (*1*) lymphocytic alveolitis > 30 × 10^3 lymphocytes/ml; (*2*) positivity to gallium-67 (^{67}Ga) scan; and (*3*) lung CD4/CD8 ratio more than 5.0 (22). Aside from the BAL analysis, the assessment of disease activity included clinical features, chest radiograph, lung function testing, high-resolution computed tomography (HRCT) scan, and routine blood studies. Four patients were defined as having an inactive disease since they had normal lung function tests and BAL cell numbers and the ^{67}Ga scan was negative.

Five normal nonsmoking subjects (3 men and 2 women with an average age of 36 ± 4.7 yr; two nonsmoking healthy persons and three subjects evaluated for complaints of cough without lung disease) were used as controls. They had no clinical symptoms and normal physical examination, chest radiographs, and pulmonary functions.

TABLE 2

COMPARISON OF Vβ REGION VALUES OBTAINED BY PCR AND FLOW CYTOMETRY ANALYSES IN INDIVIDUAL PATIENTS

Patient No.	Vβ2+ Cells		Vβ3+ Cells	
	PCR	Flow Cytometry	PCR	Flow Cytometry
1	7.9	6.0	5.6	2.4
2	16.6	15.2	4.4	3.8
3	5.5	5.8	4.3	4.6
4	15.5	16.6	2.9	4.0
5	7.8	8.2	3.3	4.1
6	7.1	9.0	8.8	8.5
7	2.8	5.2	15.0	15.5
8	7.7	5.9	5.7	6.0
9	4.1	5.0	13.2	10.8
10	8.1	10.0	3.6	4.2
R^2	0.768		0.845	
p	< 0.05		< 0.05	

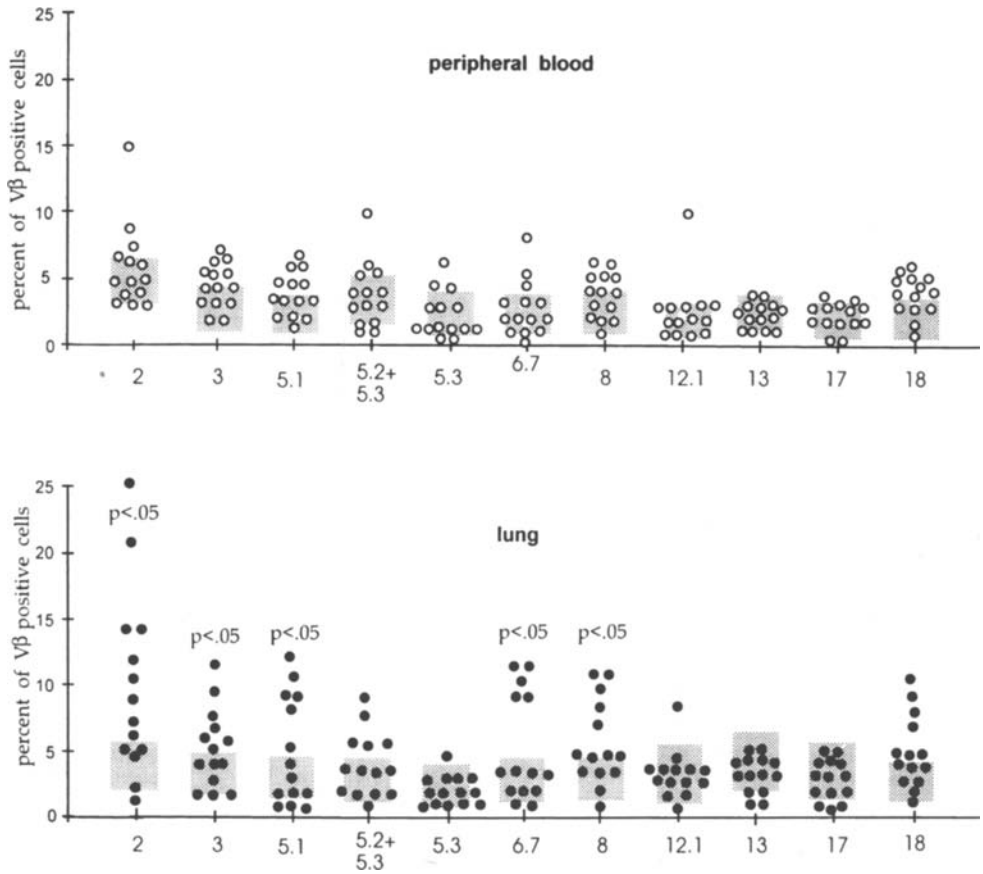

Figure 1. Flow cytometry analysis of TCR-Vβ gene expression in peripheral blood (*top panel*) and lung lymphocytes (*bottom panel*) obtained from patients with HP. The frequency of lymphocytes positive for anti-Vβ region mABS was determined on 10,000 cells bearing typical lymphocyte scatter. Shaded areas represent the range of the mean for controls plus 2 SD for the Vβ percentage in normal peripheral blood and lung. The significances are obtained after comparison of cells expressing of defined Vβ in the lung and blood compartments. The increase of these cells was also statistically significant compared with normal blood.

Recovery and Handling of BAL and Peripheral Blood Cells

BAL was performed under local anaesthesia according to the procedure previously described (23). Briefly, a total of 100 to 150 ml of sterile 0.9% saline solution (warmed at 37° C) was injected in 25-ml aliquots via fiberoptic bronchoscopy. Each aliquot was immediately aspirated and filtered through layers of surgical gauze and the volume was measured. Cells recovered from the BAL were washed with phosphate-buffered saline (PBS), resuspended in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) and counted in a hemocytometer. A differential count of macrophages, lymphocytes, neutrophils, and eosinophils (made from total counts of 300 cells) was carried out on cytocentrifuged smears stained with Wright-Giemsa.

For functional tests, BAL cells from patients under study were initially centrifuged on a Ficoll/Hypaque density gradient, washed and then resuspended in RPMI 1640 culture medium supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% of fetal calf serum (FCS) (Flow Laboratories, Irvine, CA). T lymphocytes were successively enriched from the entire mononuclear cell suspension by rosetting with neuraminidase (Sigma Chemical Co., St. Louis, MO)-treated sheep red blood cells (SRBC) followed by repeated Ficoll/Hypaque gradient separations, as described elsewhere (4). More than 95% of the cell population obtained with this procedure was represented by lymphocytes, as determined by CD3 monoclonal antibody (mAb) and flow cytometry analysis. The cells were greater than 95% viable, as determined by the trypan blue exclusion test.

Peripheral blood mononuclear cells (PBMC) from patients under study were obtained from freshly heparinized blood after centrifugation on Ficoll-Hypaque gradient and washing with PBS. The cells were successively resuspended in RPMI 1640 medium (GIBCO, Paisle, Scotland). For functional *in vitro* assays, peripheral blood lymphocytes (PBL) were further enriched after rosetting of PBMC with SRBC, as reported previously (23). More than 98% of PBL obtained with this procedure were represented by T lymphocytes.

In six patients (3HP and 3 sarcoidosis) BAL and PBL were obtained at diagnosis and 12 mo after removal from exposure in HP or at diagnosis and following 6 mo corticosteroid therapy in sarcoid patients.

Phenotype Evaluation

BAL lymphocytes and PBL from patients and normal subjects were characterized by different mAbs, including those belonging to CD3, CD4, and CD8. TCR Vβ segment usage was analyzed with the following mAbs: Vβ5a,b,c, Vβ6, Vβ8, Vβ12 (T Cell Science, Cambridge, MA), Vβ2, Vβ3, Vβ13, Vβ17, Vβ18 (Immunotech, Marseille, France). Cells were studied for the expression of cell surface antigens with direct two-color analysis using fluorescein isothiocyanate (FITC)-conjugated and phycoerythrin-conjugated mAbs using flow cytometry analysis (FACScan; Becton Dickinson, Sunnyvale, CA) as previously described (4). Cells were analyzed using a FACScan analyzer (Becton Dickinson) and data were processed using the Consort 30 program. Ten thousand cells bearing the typical lymphocyte scatter were scored.

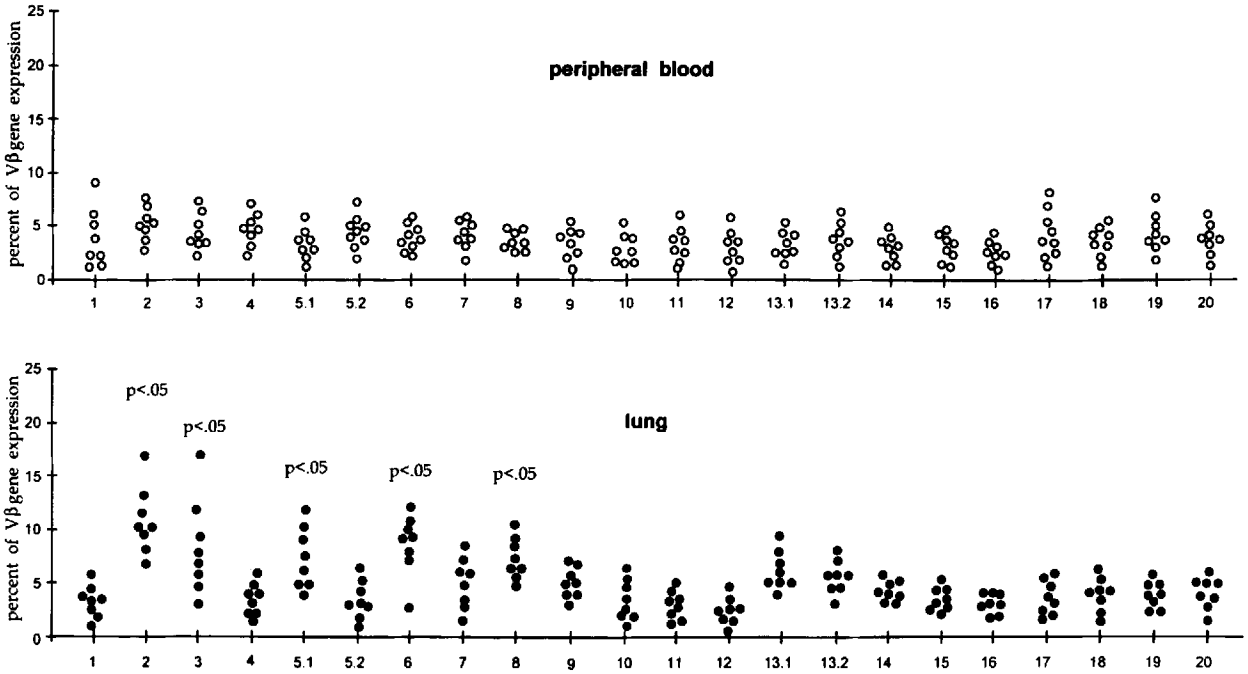

Figure 2. RT-PCR analysis of TCR-Vβ gene expression in peripheral blood and lung lymphocytes obtained from patients with HP. cDNA was obtained from 2 μg of total RNA in a final volume of 20 μl. One microliter of cDNA was amplified in the presence of one of the 22 Vβ primers specific for 20 TCR-Vβ families and a Cβ primer that was labeled with ^{32}P. For quantitation, in each tube the TCRα was amplified by using cold 5' Cα primer and ^{32}P-3'Cα primer. The samples were electrophoresed on 2% TBE agarose gels, the amplified bands were cut from the gels and the incorporated radioactivity was determined using a β-scintillation counter. The lower recovery of BAL T cells recovered from the lung of healthy subjects prevents PCR analysis. The significances are obtained after comparison of cells expressing a defined Vβ in the lung and blood districts.

Extraction, cDNA Synthesis, Amplification, and Quantitation by PCR

Total cellular RNA was extracted from 5 to 10 × 10^6 purified BAL lymphocytes after lysis with 4 M guanidine isothiocyanate and by centrifugation through a 5.7 M cesium chloride gradient, according to the method previously described (23) and quantified by measuring absorbance at 260 nm. Due to the low number of lymphocytes recovered from normal lungs (Table 1), not enough RNA was obtained in normal subjects for PCR analysis since the T-cell enrichment technique performed on BAL after performing flow cytometry and morphologic analyses did not allow for recovery of a discrete amount of cells for PCR studies. Complementary DNA (cDNA) was synthesized from 2 μg of total RNA at 42° C for 1 h in the presence of Moloney murine leukemia virus reverse transcriptase (2.5 units), using 2.5 μM oligo-d(T) primer and reaction conditions described by the manufacturer (Invitrogen Corp., San Diego, CA). The reaction was stopped by heating the sample of 99° C for 5 min.

In order to quantify Vβ gene expression in HP and sarcoid patients, the method described by Choi and coworkers was used (24). Briefly, PCR was performed in 100-μl reactions for 30 cycles with 30 s melting at 94° C, 60 s annealing at 55° C, and 90 s extension at 72° C in a Cetus/Perkin Elmer thermal cycler using 1 μl of cDNA and 2.5 U of Taq polymerase (Perkin Elmer, Norwalk, CT) in the buffer recommended by the manufacturer. The primers used were represented by 22 Vβ primers specific for 20 TCR-Vβ families, as well as one Cβ primer for Vβ amplification and 3' and 5' Cα primers for use as standards for quantitation. For the PCR reaction, a solution consisting of 1.3 mM $MgSO_4$, 50 mM KCl, 10 mM TRIS-HCl, 0.2 mM concentrations of each deoxynucleotide triphosphate, 0.4 mM concentration of 3'Cα and 3'Cb 32P-end labeled primers, about 10^6 cpm per tube, cDNA from 2 μg of total RNA, and 2.5 units of ampliTaq polymerase (Perkin-Elmer-Cetus) was added into each of the 22 tubes containing 20 pmol of one of the Vβ primers. To avoid contamination, reagent controls were performed. Ten microliters of PCR products were electrophoresed on 2% tris borate edta (TBE) agarose gels. The amplified Cα and Vβ bands were cut out from the gel and the amount of ^{32}P radioactivity incorporated was determined by liquid scintillation counting. Under these conditions, the amount of radioactivity incorporated into the Cα and Vβ bands is proportional to the amount of input RNA. To correct for differences due to pipetting errors and efficiency of amplification in each well, the number of counts in each Vβ band was normalized to the number of counts in the Cα band for each Vβ region and expressed as a percentage of the total counts incorporated in all Vβ bands. The cycle number was chosen to ensure that the amount of product synthesized was proportional to the amount of specific messenger RNA (mRNA) in the original preparation. This was performed by using different concentrations of cDNA. To further validate quantitative PCR analysis of TCR-Vβ expression, in some patients in which both flow cytometry analysis and PCR analysis were performed the values obtained with the two different methods were compared. In particular, the percentage of cells expressing a defined Vβ region as detected by flow cytometry was compared with the percentage of cells positive for the same Vβ obtained by reverse transcription–polymerase chain reaction (RT-PCR). In Table 2, the percentage values of Vβ2+ and Vβ3+ T cells obtained by flow cytometry analysis in 10 patients showed a correlation (R2 = 0.768 and 0.845, respectively) when compared with the data obtained by PCR. Furthermore, these correlations were stronger when the values were greater than 4%. In some subjects the analysis was performed 2 or 3 times for each Vβ; the data obtained differed less than 3% of the total Vβ gene expression. For this reason, a skewing of the TCR repertoire in the lung in individual patients was considered significant when the difference was greater than twice that of peripheral blood or at least at a level higher than 10%.

Cell Proliferation Assay

BAL T cells and PBL from HP and sarcoid subjects were resuspended

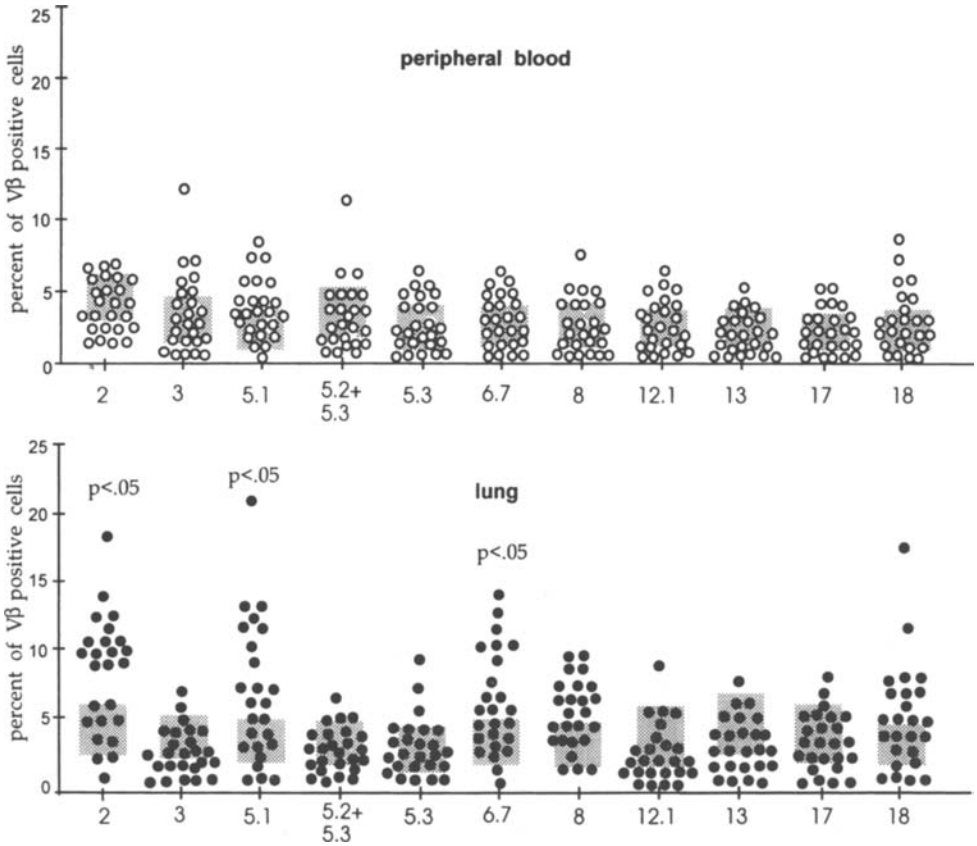

Figure 3. Flow cytometry analysis of TCR-Vβ gene expression in peripheral blood (*top panel*) and lung lymphocytes (*bottom panel*) obtained from patients with sarcoidosis. The frequency of lymphocytes positive for anti-Vβ region mAbs was determined on 10,000 cells bearing typical lymphocyte scatter. Shaded areas represent the range of the mean for controls plus 2 SD for the Vβ percentage in normal peripheral blood and lung. The significances are obtained after comparison of cells expressing a defined Vβ in the lung and blood compartments. The increase of these cells was also statistically significant compared with normal blood.

at a concentration of 1×10^6/ml in RPMI 1640 medium supplemented with 10% FCS. Cultures were carried out in triplicate with each well containing a total volume of 0.2 ml of culture medium and 1×10^5 cells/well. Cells were incubated for 3 d at 37° C in a humidified atmosphere of 5% CO_2 and 95% air. Different concentrations of staphylococcal enterotoxins (0.01, 0.1, 1 ng/ml) were used. The proliferation was determined by pulsing plates with 1 μCi/well of ^{3}H-thymidine (^{3}H-TdR; DuPont De Nemours, Belgium) for the last 12 h of culture; cells were then harvested and ^{3}H-TdR incorporation measured in a β-scintillation counter.

Statistical Analysis

Data are expressed as mean ± SD and comparisons between values were made using the *t* test. A p value < 0.05 was considered as significant.

RESULTS

Relevant morphologic and flow cytometry analyses of cells obtained from the lung and blood HP and sarcoid patients are reported in Table 1. Both groups of patients showed a lymphocytic alveolitis characterized by $CD8^+$ cells in HP patients and by $CD4^+$ lymphocytes in sarcoid patients. The frequency of $CD4^+$ and $CD8^+$ subpopulations in the peripheral blood of the patients was more or less superimposable to that of control subjects (Table 1).

Flow cytometry analysis of TCR-Vβ gene usage by lung and peripheral blood of patients with HP is reported in Figure 1. We observed a trend in the overexpression of some Vβ region products, particularly Vβ2, Vβ3, Vβ5, Vβ6, and Vβ8, in the lung with respect to the blood of the same subjects. Cells bearing the aforementioned Vβ gene products were significantly increased in the lung of patients with HP as compared with normal subjects ($p < 0.05$). Vβ18+ cells also seem to be increased in the lung of these patients, but the difference was not significant when compared with blood of patients and blood of normal subjects. PCR analysis confirmed the presence of an increased number of lymphocytes expressing Vβ2, Vβ3, Vβ5, Vβ6, and Vβ8 segments in HP (Figure 2) and extended the observation to other Vβ families, which cannot be explored with monoclonal antibodies. In fact, using PCR analysis we also observed an overexpansion of lymphocytes bearing Vβ13 segment in the lung with respect to the blood ($p < 0.05$).

Figure 3 shows the data obtained from sarcoid patients. Flow cytometry analysis demonstrated an increased expression of cells expressing Vβ2, Vβ5, and Vβ6 in the lung with respect to peripheral blood ($p < 0.05$). The difference between sarcoid patients and normal subjects was statistically significant at lung level but not in the peripheral blood. Data from PCR analysis in sarcoid patients (Figure 4) confirmed the flow cytometry results

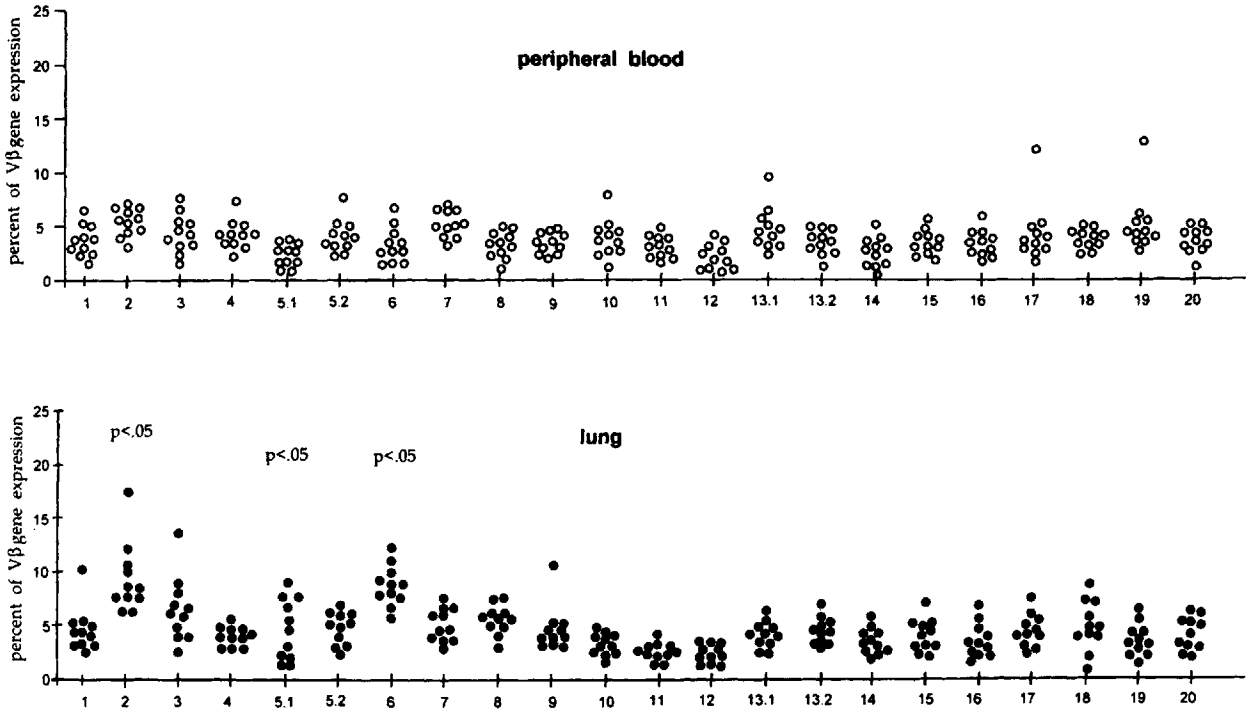

Figure 4. RT-PCR analysis of TCR-Vβ gene expression in peripheral blood and lung lymphocytes obtained from patients with sarcoidosis. cDNA was obtained from 2 μg of total RNA in a final volume of 20 μl. One microliter of cDNA was amplified in the presence of one of the 22 Vβ primers specific for 20 TCR-Vβ families and a Cβ primer that was labeled with ^{32}P. For quantitation, in each tube the TCRα was amplified by using cold 5' Cα primer and ^{32}P-3'Cα primer. The samples were electrophoresed on 2% TBE agarose gels, the amplified bands were cut from the gels, and the incorporated radioactivity was determined using a β-scintillation counter. The low recovery of BAL T cells recovered from the lung of healthy subjects prevents PCR analysis. The significances are obtained after comparison of cells expressing a defined Vβ in the lung and blood districts.

and did not demonstrate the accumulation of cells expressing additional Vβ regions.

To examine the pattern of distribution of different Vβ specificities in individual patients, the data related to PCR analysis in six representatives subjects (3 with HP and 3 with sarcoidosis) are given in Figure 5. The panels show a different TCR repertoire usage by lung and blood lymphocytes of six individual patients. In particular, Patients A and D showed an increase in lung T cells bearing the Vβ2 region with respect to blood lymphocytes; Patients C and E showed an overexpression of Vβ3+ cells in the lung as compared with the blood. On the contrary, in Patient B an overexpression of Vβ2+ lymphocytes was observed in the blood rather than in the lung compartment.

To investigate whether this skewing of the T-cell repertoire is associated with the T-cell subset that usually sustains the alveolitis in the lung of these patients ($CD8^+$ lymphocytes in HP and $CD4^+$ cells in sarcoidosis), double-staining analysis of lung T cells with PE-CD8 or PE-CD4 and FITC-Vβ mAbs was performed in patients with increased number of cells bearing a discrete Vβ region. Flow cytometry analysis in four representative patients (2 HP and 2 sarcoidosis) characterized by an overexpression of one Vβ segment is reported in Figure 6. It can be seen that lung lymphocytes obtained from patients with HP and expressing a particular Vβ region belong to the CD8 subset, while lung lymphocytes from sarcoid patients expressing a prominent Vβ coexpress the CD4 antigen.

In six patients (3 sarcoid and 3 HP), phenotypic analysis of the expression of variable regions of the TCR was performed in two consecutive BAL and PBL samples, which were obtained after the removal from exposure in HP and after corticosteroid therapy in sarcoidosis (Figure 7). Two patients, who displayed an overexpanded cell population with a limited T-cell repertoire at the first BAL determination (i.e., Vβ5.1 in the sarcoid patient and Vβ2 in the HP patient), experienced a considerable decrease in this cell subset following corticosteroid therapy in sarcoidosis and removal from exposure in HP. This trend was associated with the resolution of the lymphocytic alveolitis as well as the normalization of the number of CD4 and CD8 subsets in the lung. No differences were observed at blood level in terms of cells bearing the same Vβ region segment at different times of evaluation.

To investigate whether lung T cells from HP and sarcoid patients proliferate in response to superantigens, cell proliferation assays were performed in the presence of different staphylococcal enterotoxins, including SEA, SEB, SEC1, SEC2, SED, and SEE. The data related to the proliferation assay in two representative patients (1 HP and 1 sarcoidosis) are reported in Figure 8. A different pattern of cell responsiveness between lung and blood compartments was demonstrated in these patients. In the majority of subjects studied, a discrete or high proliferative rate was determined by several toxins on BAL lymphocytes. Furthermore, superantigenic stimuli elicited a different T-cell responsiveness in the lung with respect to the blood in individual patients. In particular, ranges of proliferation in cpm of lung lymphocytes to increasing concentrations of toxins in the HP patient were 27,531 to 44,382; 19,472 to 24,826; 4,982 to 13,497; 7,390 to 16,589; 4,934 to 11,846; and 14,396 to 46,321 for SEA, SEB, SEC1, SEC2, SED, and SEE, respectively. Ranges of proliferation in cpm of blood lymphocytes in this HP patient

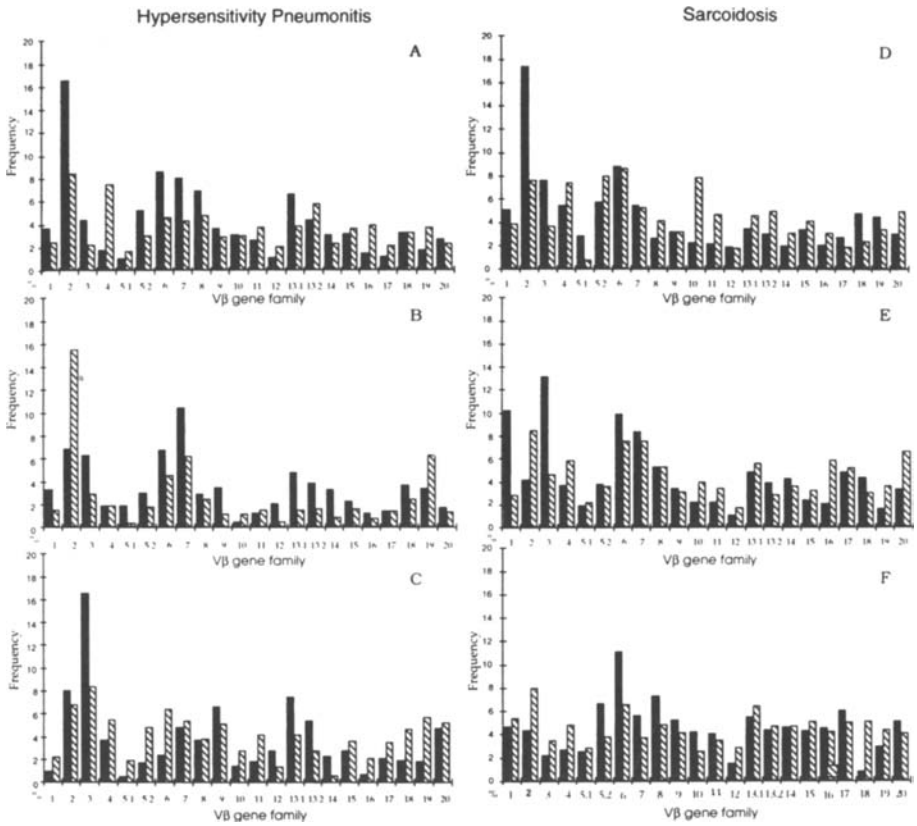

Figure 5. RT-PCR analysis of TCR–Vβ gene expression in peripheral blood (*shaded columns*) and lung lymphocytes (*dark columns*) obtained from three individual patients with HP and three with sarcoidosis. cDNA was obtained from 2 μg of total RNA in a final volume of 20 ml. One microliter of cDNA was amplified in the presence of one of the 22 Vβ primers specific for 20 TCR-Vβ families and a Cβ primer that was labeled with ^{32}P. For quantitation, in each tube the TCRα was amplified by using cold 5' Cα primer and ^{32}P-3'Cα primer. The samples were electrophoresed on 2% TBE agarose gels, the amplified bands were cut from the gels and the incorporated radioactivity was determined using a β-scintillation counter.

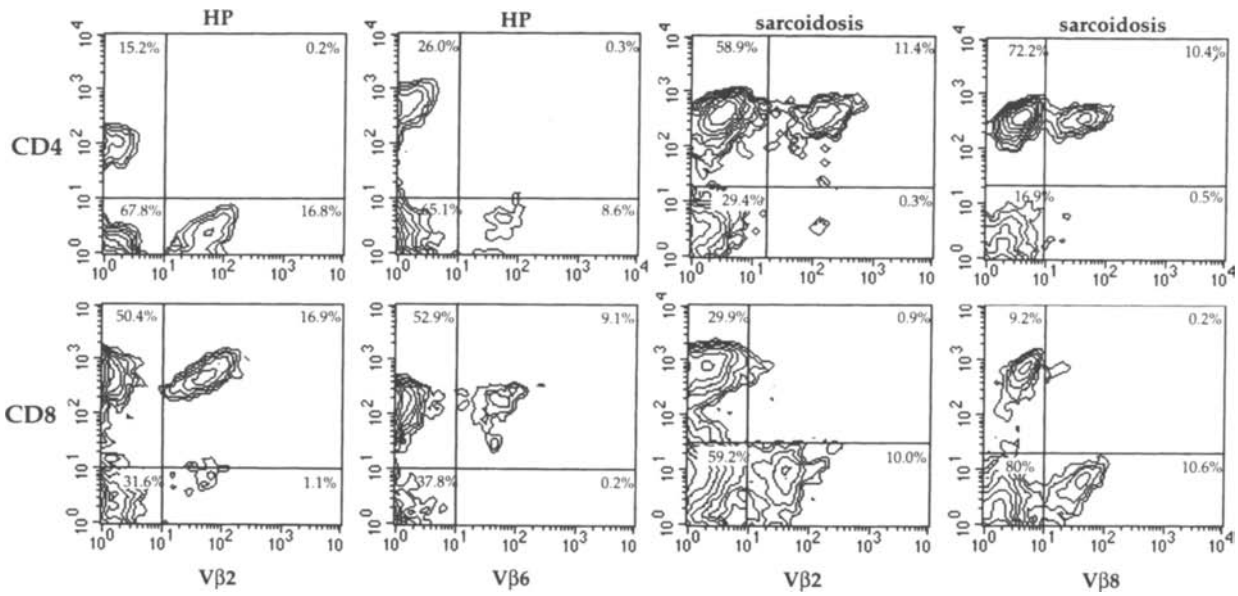

Figure 6. Flow cytometry analysis of CD8 and TCR-Vβ gene segment coexpression in lung lymphocytes obtained from four different patients (2 HP and 2 sarcoid). Lung lymphocytes were stained with FITC anti-Vβ mAb and PE-CD8 mAb. The frequency of lymphocytes positive for the above mAbs was determined on 10,000 cells bearing typical lymphocyte scatter. All T-cell subsets bearing a defined Vβ gene region belong to the CD8 subset.

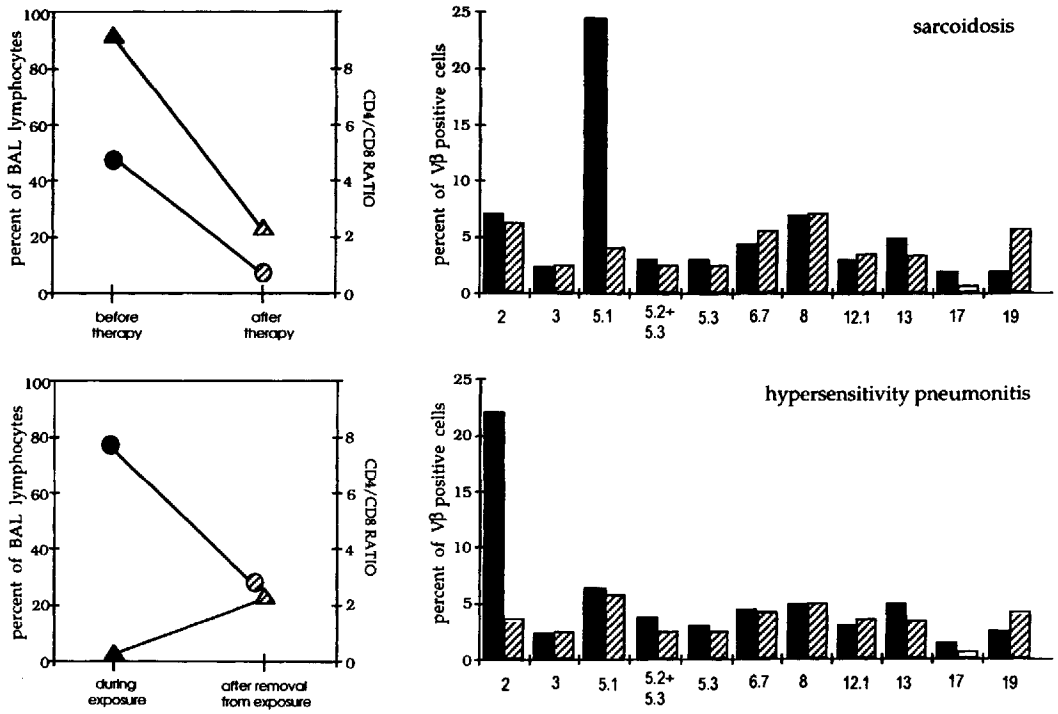

Figure 7. Follow-up analysis of TCR-Vβ gene expression in lung lymphocytes obtained from one patient with sarcoidosis and one with HP in two different bronchoalveolar lavages. The analysis was performed using anti-Vβ region mAbs. The frequency of lymphocytes positive for anti-Vβ region mAbs was determined on 10,000 cells bearing typical lymphocyte scatter. Panels on the left indicate data regarding the percentage of lung lymphocytes (*circles*) and the CD4/CD8 ratio (*triangles*) before (*closed symbols*) and after therapy (*shaded symbols*) in the sarcoid patient or after removal from exposure in the HP patient.

were 2,072 to 6,919; 1,481 to 3,127; 834 to 1,034; 607 to 1,575; 352 to 1,239; and 672 to 5,424 for SEA SEB, SEC1, SEC2, SED, and SEE, respectively. In the patient with sarcoidosis, ranges of proliferation in cpm of lung lymphocytes were 22,134 to 23,136; 25,512 to 45,950; 17,515 to 40,513; 23,958 to 46,700; 20,153 to 28,590; and 436 to 4,131 for SEA, SEB, SEC1, SEC2, SED, and SEE, respectively. Ranges of proliferation in cpm of blood lymphocytes in this sarcoid patient were 16,151 to 17,174; 11,424 to 17,150; 2,172 to 11,440; 4,331 to 12,336; 12,323 to 15,515; and 1,165 to 1,930 for SEA, SEB, SEC1, SEC2, SED, and SEE, respectively. Because of the low number of BAL lymphocytes recovered in normal subjects, lung T-cell proliferation assays were not performed on this group. Furthermore, staphylococcal toxins elicited similar proliferation rates in blood lymphocytes in both patients and control subjects.

DISCUSSION

To investigate the pattern of T-cell accumulation in the lungs of patients with HP and sarcoidosis, TCR-Vβ gene usage has been analyzed both on lung and blood lymphocytes. We demonstrated a bias in the expression of some Vβ region products in the lung of these patients as compared with the blood and lung of normal subjects. Cells overexpressing a particular Vβ segment belong to the lymphocyte subset which accounts for the alveolitis in these patients, i.e., $CD8^+$ cells in HP and $CD4^+$ lymphocytes in sarcoidosis. These cells decreased significantly in the lung of patients after removal from exposure in HP or corticosteroid therapy in sarcoidosis. Furthermore, lung lymphocytes recovered from the lung of these patients proliferate to different superantigens.

Several mechanisms may account for the alveolitis in the pulmonary microenvironment of patients with HP and sarcoidosis. Lymphocytes with limited specificities observed in lung structures may be the consequence of a migration process of a homogeneous cell subset from the blood to this site of involvement and/or, alternatively, of the proliferation in the lung of a selected cell subpopulation in response to foreign antigens. Whether this latter process occurs as a consequence of the effect of the putative antigen on resident lung T cells or following a preliminary recruitment of selected lymphocytes from the blood remains to be clarified. However, a bias toward use of specific Vβ regions might be viewed as the consequence of the triggering of lung T cells by a limited number of antigens, which can be found in different environments (25).

When a redistribution of cells with limited specificities from the blood to the lung is considered, a decrease in lymphocytes with a limited TCR would be predicted in the blood of the same subject. Our data, however, did not demonstrate any depletion of lymphocyte subsets in the blood of these patients, nor in patients with a very high number of cells expressing a peculiar Vβ in the lung, arguing against a T-cell redistribution from the blood to the lung.

Selection and accumulation of T lymphocytes bearing a predominant Vβ region family may be due to at least two types of antigenic stimuli. One is represented by peptides, derived from antigen processing, which form complexes with major histocompatibility complex (MHC) molecules. These complexes are recognized by the Ag-specific TCR. The formation of Ag-MHC-TCR complex triggers a cascade of events resulting in T-cell activation and proliferation. A second stimulus may be represented by superantigens, molecules which include endogeneous retroviral gene products and microbial toxins. They can stimulate proliferation and conversely cause anergy or depletion of T cells bear-

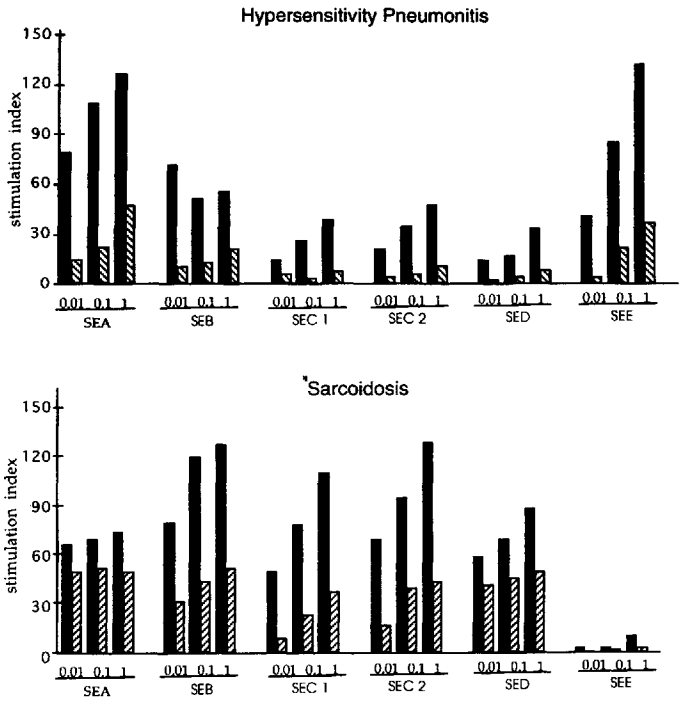

Figure 8. Proliferation of lung (*dark bars*) and peripheral blood (*shaded bars*) lymphocytes elicited by different concentrations (0.01, 0.1, and 1 ng/ml) of staphylococcal enterotoxins (SEA, SEB, SEC1, SEC2, SED, SEE) in two patients (1 HP and 1 sarcoid). Data are reported as stimulation index.

ing a particular TCR-Vβ sequence. The first type of antigen induces a lymphocyte accumulation characterized by a limited number of specific T cells which would lead to a clonal proliferation. By contrast, superantigens commonly determine the accumulation of cells expressing the same V region but maintain a polyclonal pattern of response. Data herein reported demonstrated that T cells in HP lung are commonly characterized by a bias in TCR usage. Coupled to the evidence that alveolitis may sometimes end in the accumulation of oligoclonally expanded T lymphocytes (4), our data suggest that the pattern of accumulation we observed is better related to the expansion of cells with a limited number of specificities and, of course, of restricted TCRs. It can be hypothesized that the antigen itself, i.e., *Faeni rectivirgula* in our patients, might be involved in determining this T-cell selection. In this context, this suggestion might be validated by the analysis of TCR-β region usage after stimulation of lung lymphocytes by the putative specific antigen, i.e., *F. rectivirgula*. The lack of purified antigen and/or of synthetic peptides for the time being prevents this analysis. Nevertheless, the majority of lung CD8 cells express cytotoxic related antigens, including CD56 and CD57 molecules (4), and almost all BAL lymphocytes belong to memory T-cell subsets (CD45R0) (data not shown). These observations provide an additional piece of information supporting the concept that cells under study have just seen the antigen. Since our study was performed in ill patients exposed to *F. rectivirgula*, it will be also interesting to evaluate the pattern of TCR-Vβ gene usage in the lung of asymptomatic but similarly exposed individuals who commonly have BAL findings similar to those observed in symptomatic HP patients (3). With regard to sarcoidosis, our results are consistent with data reported in literature in terms of preferential usage of Vβ regions by lung T cells (4, 12, 14–17), i.e., the presence of oligoclonal Vβ-specific T cells in lung of sarcoid patients. These data favor the idea of an involvement of putative specific antigens in the development of T-cell alveolitis in sarcoid patients.

Phenotypic data demonstrated that lung T cells bearing the overrepresented Vβ gene segment belong to the CD8 subset in HP and to the CD4 population in sarcoidosis thus providing an additional proof that these cells play a key role in lung microenvironment. Our data also showed that discrete T-cell subsets bearing the same Vβ gene segments can be identified in these two different disorders. In fact, the common use of some Vβ regions (Vβ2, Vβ5, and Vβ6) both in HP and sarcoid patients indicates that the same variable region may be used in response to different antigens. Differences in the TCR-Vβ region usage detected between normal and HP/sarcoid lung suggest that the preferential usage of defined V regions in patients with these interstitial lung disorders is by no means related to homing mechanisms.

Functional data in terms of staphylococcal enterotoxin-induced proliferation of lung lymphocytes indicated that a different pattern of growth exists in the lung with respect to the blood. In fact, lung lymphocytes from our patients proliferate in a more pronounced manner than blood lymphocytes, thus confirming a difference in the TCR usage between lung and blood T cells. Furthermore, the different degree of responsiveness to each enterotoxin by lung lymphocytes belonging to the same patient represents a further proof of the presence of cell subsets with limited specificities. These suggestions are supported by the demonstration that staphylococcal enterotoxins selectively stimulate T cells bearing specific Vβ elements (19). On the other hand, it cannot be excluded that the different behavior of response between these two districts may be related to characteristics of residual antigen presenting cells.

The clinical relevance of the expansion of a discrete cell subset with a limited usage of the TCR in the lung of patients with HP and sarcoidosis deserves a final comment. Follow-up analysis performed after removal from exposure in HP and after corticosteroid therapy in sarcoidosis shows a close correlation between the presence in the lung of discrete populations of lymphocytes with a limited T-cell repertoire and the resolution of the alveolitis with a normalization of T-cell subsets. This observation strengthened the role of putative causative antigens in determining and maintaining the lymphocytic alveolitis in patients with HP and sarcoidosis. In this regard, it can be hypothesized that relevant antigens might exhibit an antigenic pressure that ultimately triggers proliferation of T cells with a selected TCR. Sequencing studies of the TCR of T lymphocytes in lung and peripheral blood of these patients, and the analysis of the effect of the involved antigens on the regulation of *in vitro* selection of cells with limited T-cell repertoire could support this suggestion and further clarify this issue.

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