T cells in the lung of patients with hypersensitivity pneumonitis accumulate in a clonal manner

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Abstract: Hypersensitivity pneumonitis (HP) is characterized by an alveolitis sustained by $CD8^+$ T lymphocytes showing a limited expression of the T cell receptor (TCR). We previously demonstrated that a bias in T cell selection occurs in the lower respiratory tract of patients with HP, with a compartmentalization in the lung of $CD8^+$ T cells bearing (TCR)-β variable (TCRBV) #2, 3, 5, 6, 8, and 13 gene segments. We herein characterized the clonal T cell populations present in the lung and in the blood of patients with HP. Heteroduplex analyses, cloning, and sequencing T cells bearing TCR indicate oligoclonal expansions of T cells expressing homologous or identical complementary-determining region 3. Furthermore, T cell clones isolated from the two compartments expressed similar, sometimes identical, junctional regions. Removal from antigenic exposure led to the disappearance of T cell clones. Our findings indicate that expansions of T lymphocytes bearing clonal TCRBV region gene segments take place in the lung of patients with HP during exposure. The evidence that identical T cell clones are present in the lung and the blood of the same patient suggests that the immune reaction occurring at lung level gives rise to a systemic reaction. *J. Leukoc. Biol.* 75: 798–804; 2004.

Key Words: *interstitial lung diseases · T cell receptor · T cell clones · immunopathogenesis*

INTRODUCTION

Hypersensitivity pneumonitis (HP) is an interstitial lung disorder caused by the repeated inhalation of different organic dusts [1, 2]. Dust exposure drives an inflammatory reaction within the lower respiratory tract, which is characterized by a lymphocytic alveolitis and granuloma formation. Repeated exposure determines the progression of inflammation to severe fibrosis [3–5]. A high-intensity lymphocytosis in the bronchoalveolar lavage (BAL) fluid is the main pathological feature of HP and is commonly sustained by $CD8^+$ T cells [6]. Similarly to CD4 cells, CD8 lymphocytes can be defined according to their cytokine secretion pattern: Type 1 CD8 cells (Tc1) are defined by a preferential secretion of interferon-γ (IFN-γ), and Tc2 preferentially synthesize interleukin (IL)-4, IL-5, and IL-10. Recent data indicate that $CD8^+$ T cells obtained from the BAL of patients with HP are Tc1 cells characterized by a predominance of IFN-γ production [7]. The majority of T lymphocytes use the αβ T cell receptor (TCR) to specifically recognize antigens, in association with the class I or class II major histocompatibility complex (MHC). Southern blot analyses of the molecular configuration of the TCR-β genes (TCRB) from $CD8^+$ T cells accumulating in the lung during HP reactions have shown a pattern characterized by the presence of rearranged bands [8]. A bias in the expression of some TCRB variable (TCRBV) regions, as compared with the lung and the peripheral blood (PB) of normal subjects, has been demonstrated. Specifically, the overexpression of the TCRBV families #2, 3, 5, 6, 8, and 13 has been observed in the lung with respect to the blood, suggesting that the exposure to the relevant antigen could induce a bias in TCRBV expression [9]. Furthermore, lung T cells involved in alveolitis expressed CD56 and CD57 molecules and were memory T cells ($CD45R0^+$), providing additional proof that these lymphocytes have already met the antigen [9].

Although the above data indicate the abnormal expression of specific TCRBV regions and the presence of T cells with similar TCR specificities in the lung, no information is yet available concerning the clonality of these cells. In the present study, we investigated, by use of sequencing analyses, the TCRBV genes of expanded $CD8^+$ T cell populations in the lung and in PB of patients with HP. We herein demonstrate the presence of oligoclonal T cell expansions in the lung and in the blood of the patients. Taken together, these data provide evidence of an oligoclonal selection of effector $CD8^+$ T lymphocytes in the BAL and to a lesser extent, in the PB of patients with HP.

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MATERIALS AND METHODS

Study population

Fourteen symptomatic, nonsmoking patients (eight men and six women, aged between 26 and 50 years, mean 34±16) with HP were studied. The diagnosis was based on conventionally reported criteria [2, 10], notably: history of exposure to HP antigens; a symptomatic acute episode with chills, fever, cough, breathlessness 4–8 h after exposure to specific antigens; radiological features (mainly diffuse reticular pattern) and/or a functional pattern of interstitial lung disease; and evidence of antibodies against *Faeni rectivirgula* in all cases. Precipitating antibodies against the etiologic antigens were detected in the BAL and in the serum of the patients under study. All patients were evaluated at least 1 month after the first acute episode as a part of the diagnostic procedures at the time of the onset of the disease.

Five nonsmoking subjects (three men and two women with an average age of 36±4.7 years; 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.

Informed consent was obtained from each HP patient and control subjects.

Recovery and handling BAL and PB cells

BAL was performed under local anesthesia according to the procedure described previously [11]. Briefly, a total of 100–150 ml sterile 0.9% saline solution (warmed to 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 haemocytometer. A differential count of macrophages, lymphocytes, neutrophils, and eosinophils (made from total counts of 300 cells) was performed on cytocentrifuged smears stained with Wright-Giemsa.

For molecular studies, 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 (Flow Laboratories, Irvine, CA). T lymphocytes were 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 [8]. More than 95% of the cell population obtained with this procedure was represented by T lymphocytes, and more than 85% of T cells were constituted by $CD8^+$ lymphocytes, as determined by flow cytometry analysis with CD3, CD4, and CD8 monoclonal antibodies (mAb). The cells were greater than 95% viable, as determined by the trypan blue exclusion test. In selected cases, pulmonary $CD8^+$ T cells were separated from $CD4^+$ T lymphocytes by magnetic separations over columns (Mini MACS, Sunnyvale, CA). Briefly, the cell suspensions obtained as above were incubated at 4°C for 30 min with magnetic beads coated with anti-CD8 mAb (OKT8, Ortho, Raritan, NJ). The $CD8^+$ lymphocytes were then isolated and removed from $CD4^+$ cells by applying a magnetic system to the outer wall of the columns. After this positive selection procedure, more than 97% of the cells were viable, and 95–99% showed $CD8^+$ phenotype.

PB mononuclear cells (PBMC) from patients under study were obtained from freshly heparinized blood following centrifugation on Ficoll-Hypaque gradient and washing with PBS. The cells were then resuspended in RPMI-1640 medium (Gibco, Paisle, Scotland). For molecular analyses, PB lymphocytes were further enriched following rosetting PBMC with SRBC, as reported above [12]. More than 98% of PB lymphocytes obtained with this procedure were represented by T lymphocytes.

In six out of the 14 HP patients with HP, BAL and PB lymphocytes were obtained at diagnosis and 6 months and 12 months after diagnosis, allowing follow-up studies.

Phenotypic evaluation

BAL and blood lymphocytes from patients and normal subjects were characterized using different mAb, including those belonging to CD3, CD4, and CD8. TCRBV segment use was analyzed with the following mAb: BV5a, b, c, BV6, BV8, and BV12 (T Cell Science, Cambridge, MA) and BV2, BV3, BV13, BV17, and BV18 (Immunotech, Marseille, France). Cells were further characterized with mAb anti-IL-4 and anti-IFN-γ (PharMingen, San Diego, CA). Cells were studied for the expression of cell-surface antigens with direct, two-color analysis using fluorescein isothiocyanate-conjugated and phycoerythrin-conjugated mAb using flow cytometry analysis (FACScan, Becton Dickinson, Sunnyvale, CA) as described previously [9]. Expression of the cytoplasmic cytokines was evaluated after permeabilization of the cell membranes with 1:2 diluted PermeaFix (Ortho) for 40 min. After permeabilization procedures, anti-IL-4 and anti-IFN-γ mAb were added. Cells were analyzed using a FACScan analyzer (Becton Dickinson) and using the CellQuest program data, were processed. Ten thousand cells bearing the typical lymphocyte scatter were scored.

RNA extraction, cDNA synthesis, and TCRBV amplification by polymerase chain reaction (PCR)

Total cellular RNA was extracted using the Ultraspec I RNA isolation system (Biotecx Laboratory, Houston, TX) from 5–10 × 10^6 enriched BAL or blood lymphocytes and quantified by measuring absorbance at 260 nm. cDNA was synthesized from 2 μg total RNA at 42°C for 15 min in the presence of avian myloblastosis virus reverse transcriptase (2.5 units), using 2.5 mM oligo-d(T) primer and reaction conditions described by the manufacturer (Promega, Madison, WI).

The TCRBV gene expression in HP patients was evaluated by the method described by Choi et al. [9, 13].

Heteroduplex analysis

Heteroduplex analysis was performed on the complete TCR repertoire of the lung and blood lymphocytes of the patients with HP. As the limited number of lung T cells recovered from the BAL of normal subjects prevented the heteroduplex analysis on the complete TCR repertoire expressed by BAL T cells, normal PB T cells were used to control the efficiency of the technique. The TCRBV PCR products (20 μl) were denatured at 95°C for 5 min and then kept at 50°C for 1 h. This temperature is permissive for the annealing between homologous (homoduplex) and heterologous DNA strands, which belong to the same TCRBV family but differ for the N and the TCRBJ regions (heteroduplex). The annealed PCR samples were separated on a 12% acrylamide gel (29:1 acrylamide/bisacrylamide) in 1× Tris-boric acid-EDTA (TBE) buffer for 6 h at 200 V. Gels were then stained with ethidium bromide (0.75 μg/ml) for 30 min in 1× TBE and were photographed under UV light. Unrelated by the TCRBV expression, polyclonal PCR products smear in the gel, and monoclonal PCR products give a definite single band. TCRBV oligoclonal pattern is characterized by the presence of more distinct bands on a smeared background.

Cloning and sequencing PCR products

TCRBV repertoires of four patients were cloned and sequenced. The PCR products were purified by cutting the band with the expected size from a 2.5% NuSieve GTG low-melting agarose gel (FMC BioProducts, Rockland, ME) and eluting the melted gel through an ion-exchange resin column (Qiagen tip5, Qiagen, Chatsworth, CA). Purified DNA fragments were ligated to pCRII vector. Plasmids were grown in INVα-F′-modified, competent *Escherichia coli* cells in Luria-Bertani agar plates, and several plaques for each TCRBV, which showed an oligoclonal pattern at the heteroduplex analysis, were picked up and expanded according to the manufacturer's instructions (TA cloning kit, Invitrogen, San Diego, CA) for subsequent sequencing. To verify the presence of the correct insert, recombinant plaques were tested by PCR with the above-described TCRBV-specific family primers and with two primers specific for the TCRBC segments (the antisense primers βAI, 5′ CCCACTGTGCACCTCCTTCC 3′, and the sense oligonucleotide Cβ1, 5′ GTCGCTGTGTTTGAGCCATCAGAA 3′). Plasmid DNA from TCRBV-positive plaques was sequenced with automated laser fluorescent A.L.F. DNA sequencer (Pharmacia LKB, Uppsala, Sweden) using the AutoRead sequencing kit (Pharmacia LKB) [14]. Sequences were compared with published data of TCRBV, TCRBD, and TCRBC segments [15, 16].

Statistical analysis

Data are expressed as mean ± SD, and comparison between values was made using the *t*-test. A P value <0.05 was considered significant.

TABLE 1. Phenotype of Lung and Blood Lymphocytes in HP Patients and Control Subjects

Study population	Site	Cells $\times 10^3$/ml	Lymphocytes %	CD4 %	CD4 $\times 10^3$/ml	CD8 %	CD8 $\times 10^3$/ml
HP (n: 14)	Lung	362.9* ± 65.8	49.8† ± 7.6	26.0‡ ± 6.0	47.0 ± 10.8	53.2‡ ± 5.3	96.1 ± 9.6
Controls (n: 5)		139.7 ± 13.4	7.9 ± 1.7	46.2 ± 1.5	5.1 ± 0.2	23.6 ± 1.9	2.6 ± 0.2
HP (n: 14)	Blood	5.7 ± 0.7	18.7 ± 2.3	48.4 ± 2.6	0.5 ± 0.0	28.6 ± 2.8	0.3 ± 0.0
Controls (n: 5)		5.9 ± 0.5	26.9 ± 3.7	52.8 ± 2.1	0.8 ± 0.0	26.8 ± 2.2	0.4 ± 0.0

Comparison between HP patients and normal subjects as follows: * $P < .05$; † $P < .005$; ‡ $P < .01$.

RESULTS

Morphological and phenotypical analyses

Morphological and phenotypical features of cells obtained from the BAL and the blood of 14 patients with HP are reported in **Table 1**. All the subjects showed a high-intensity $CD8^+$ lymphocytic alveolitis sustained by Tc1 cells: in fact, these cells bore IFN-γ but not IL-4 (data not shown). $CD4^+$ and $CD8^+$ subsets detected in the PB were superimposable to those observed in the controls.

Study of TCR diversity by heteroduplex analysis

We previously demonstrated that T cells isolated from the lung of patients with HP overexpress TCRBV #2, 3, 5, 6, 8, and 13 gene segments [9]. In this paper, the analysis with heteroduplex techniques was focused on each single TCRBV PCR product obtained from BAL and PB lymphocytes of patients with HP. **Figure 1** shows heteroduplex analysis of one representative patient (A) and a control subject (B). Generally, amplified products of control subjects were smeared with some weak homoduplex bands, and TCRBV PCR products obtained from patients with HP migrate as homoduplex bands in a context of a more-or-less evident background of heteroduplex bands or smears. This pattern of migration strongly suggests the presence of oligoclonal T cell subsets in the context of polyclonal T cell populations in the lung or in the blood. Noteworthy, the oligoclonal pattern was shown by the entire TCRBV repertoire, and it was not restricted to the single TCRBV overexpressed by the T cell populations that sustained the alveolitis.

Furthermore, some TCRBV PCR products obtained from the lung and the blood of the same patient showed different migratory patterns, suggesting that the lung and the blood have dominant T cell populations, but the clones are different (**Fig. 2**).

Sequencing TCRBV clones

A further step was the demonstration of the presence of oligoclonal T cell populations in the lung and the blood of patients with HP by cloning and sequencing the TCRBV PCR products. The sequences confirmed the existence of dominant T cell clones in the lymphocyte populations obtained from the lung and the blood of the patients with HP (**Table 2**). As predicted by heteroduplex analysis, the presence of T cell clones was not

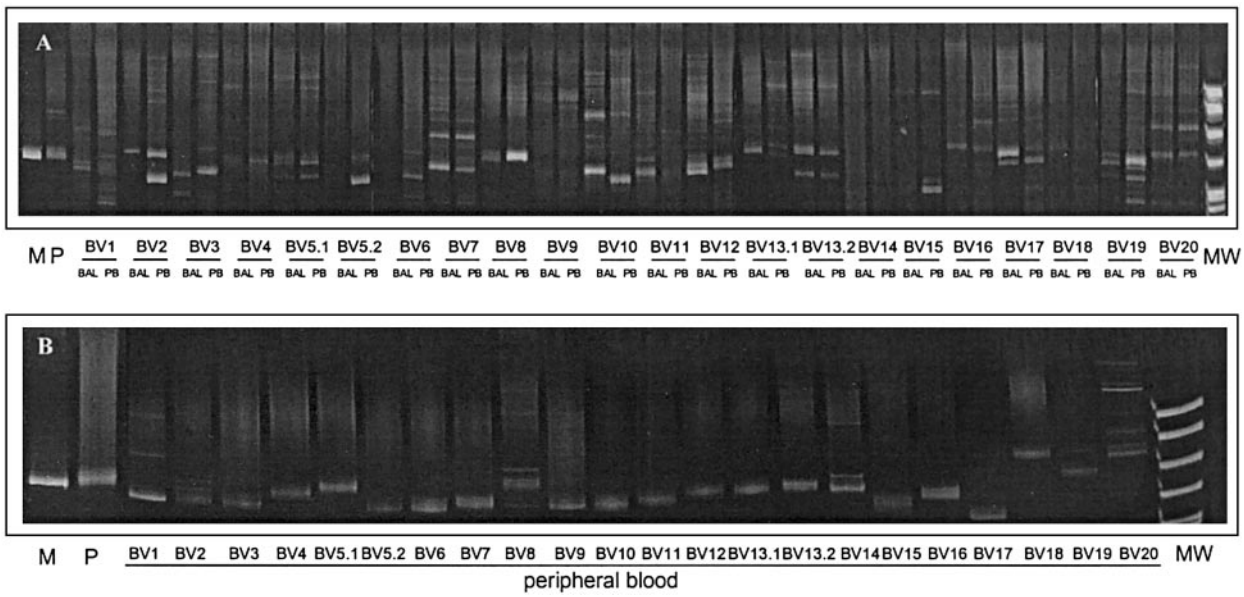

Fig. 1. Heteroduplex analysis of TCRBV gene PCR products obtained from BAL and PB lymphocytes from one representative HP patient (A) and one control subject (B). TCRBV8-amplified products, obtained from the amplification of J77 and C1-632 cell RNA, were used as mono (M)- and polyclonal (P) controls and loaded, respectively, in the first two lanes of both gels. MW, Molecular weight marker pBR322.

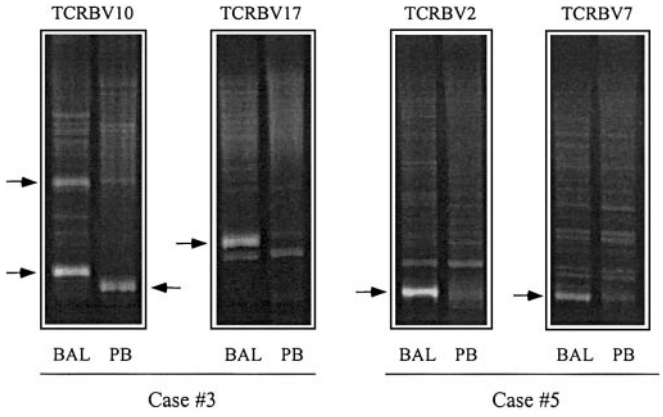

Fig. 2. Heteroduplex analysis of TCRBV gene PCR products obtained from BAL and PB lymphocytes from two representative HP patients. The migration patterns of TCRBV2 and TCRBV7 PCR products, obtained from lung T lymphocytes of case #5, and the migration of TCRBV17 PCR product, obtained from lung T lymphocytes of case #3, showed the presence of one homoduplex band that was absent in the homologous TCRBVs of PB. This would suggest the existence of dominant T cell clones in the lung that are completely absent in the T cell populations expressing the same TCRBVs in the blood. Furthermore, the migration of TCRBV10 PCR product obtained from T cells of lung and blood of patient #3 showed the presence of homoduplex bands with different positions, suggesting that the lung and the blood have dominant T cell populations but that the clones are different.

limited to the TCRBV overexpressed by T population, but it was extended to other β-variable regions. Noteworthy, we found the presence of clones with different TCRBV regions but similar CDR3 in the same patient (Table 2, case #13). These data suggest the presence of an antigenic stimulus that was recognized by different TCRBV segments but by the same or similar CDR3.

The sequenced cases were all characterized by a CD4/CD8 ratio <0.1. Nevertheless, in two selected cases, we separated $CD8^+$ BAL T cells from $CD4^+$ BAL lymphocytes with magnetic beads coated with anti-CD8 mAb. Cloning and sequencing the purified CD8 and CD4 populations showed the presence of dominant T cell clones only in the $CD8^+$ cells. On the contrary, $CD4^+$ pulmonary lymphocytes of HP patients were polyclonal, and there was no CDR3 identity between the two populations (**Table 3**).

Follow-up studies

In six patients with HP, phenotypic analysis and molecular studies of the expression and the sequence of TCRBV regions were repeated on BAL and PB samples obtained at 6 months and 12 months after diagnosis. Four patients were removed from antigenic exposure, and two patients continued to be exposed to the relevant antigen. Patients who displayed overexpanded T cell populations with dominant clones at diagnosis and who continued to be exposed to the relevant antigen maintained the overexpression of the specific TCRBV region. On the contrary, the patients removed from antigenic exposure were characterized by a discrete decrease in the T cell subset previously overexpressed (**Table 4**). Furthermore, with heteroduplex analyses performed on TCRBV PCR products obtained from BAL and PB lymphocytes of patients removed from antigenic exposure, we did not detect the homoduplex bands previously identified from lung and PB T lymphocytes (**Fig. 3**).

TABLE 2. Junctional Amino Acid Sequences of the Indicated TCRBV Segments, Obtained from BAL and PB Lymphocytes Prepared from Three Patients with HP

				Location	
	TCRBV	CDR3	TCRBJ	BAL	PB
HP case #1					
TCRBV4 clones	YLCSV	EGPSSGGG	ETQYFG 2S5	11/11	0/9
TCRBV12 clones	*YFCAISE*	*SEEA*	*YEQYFGP 2S7*	*10/16*	*6/14*
	YFCAISE	SEGA	YEQYFGP 2S7	2/16	0/14
	YFCAIS	VSQGDL	TEAFFG 1S1	2/16	0/14
HP case #11					
TCRBV7 clones	*YLCASS*	*DGPPPP*	*YEQYFGP 2S7*	*11/12*	*7/10*
	YLCASS	QVNRV	YEQYFGP 2S7	0/12	2/10
HP case #13					
TCRBV8 clones	SAVYFCASS	RLAGD	NEQFFG 2S1	4/10	0/8
	SAVYFCAS	RLAGVG	YNEQFFG 2S1	3/10	0/8
	SAVYFCASS	R*GSL*	*N*TEAFFG 1S1	1/10	0/8
	SAVYFCASS	RW*GSLN*	TDTQYFG 2S3	0/10	7/8
	SAVYFCASS	F*GLAGG*	EQYFG 2S7	0/10	1/8
TCRBV13.1 clones	*SVYFCAS*	*NSPPGTGG*	*DTQYFG 2S3*	*11/15*	*4/8*
	SVYFCASS	FLPDV	NTEAFFG 1S1	2/15	0/8
	SVYFCA	TS*GLAG*	TGELFFG 2S2	1/15	0/8

The patients with HP presented identical T cell clones in the lung and the blood. Noteworthy, in case #13, 11/15 TCRBV13.1 clones obtained from T cells isolated from BAL presented the same complementary-determining region 3 (CDR3) region (NSPPGTGG) and the same J region (DTQYFG 2S3). The same clone was found in the blood and was the dominant form (4/8 TCRBV13.1 clones). In the same patient, 7/8 TCRBV8 clones obtained from T cells isolated from blood showed the same CDR3 region (RWGSLN); this CDR3 is very similar to the same region sequenced in one TCRBV8 clone of the BAL (RGSLN). Finally, the same GLAG sequence was present in one TCRBV8 clone of PB and in one TCRBV13.1 clone of BAL. The data were deduced from nucleotide sequences and are displayed in a standard one-letter code. Only the last 3′ amino acids of the TCRBV segments and the first 5′ amino acids of TCRBJ chains are shown. The same CDR3 amino acid sequences were encoded by identical TCRB nucleotide sequences. These sequence data are available from GenBank under accession numbers AY292261–AY292274.

TABLE 3. Junctional Amino Acid Sequences of the TCRBV4 Segments, Obtained from BAL $CD8^+$ and $CD4^+$ Lymphocytes Prepared from One Representative Patient with HP (Case #8)

BAL $CD8^+$ T cells				BAL $CD4^+$ T cells			
TCRBV4 clones	CDR3	TCRBJ	Frequency	TCRBV4 clones	CDR3	TCRBJ	Frequency
YLCSVE	*KAA*	*NTGELFF 2S2*	*14/22*	YLCSVE	LAV	NTGELFF 2S2	2/20
YLCSVE	LARN	SYEQYFG 2S7	4/22	YLCS	ALRGM	NTEAFFG 1S1	2/20
YLCSVE	GSS	NTGELFF 2S2	2/22	YLCS	ATVGIQ	QPQHFGD 1S5	2/20
YLCSVE	RAT	NTGELFF 2S2	2/22	YLCSVE	DVGY	TGELFF 2S2	1/20
				YLCSVE	APA	NTGELFF 2S2	1/20
				YLCSVE	QEMGS	SYEQYFG 2S7	1/20
				YLCSV	ASREEGG	TEAFFG 1S1	1/20
				YLCSV	GQGLD	QPQHFGD 1S5	1/20
				YLCSV	VYLQTSGKN	NEQFFG 2S1	1/20
				YLCSV	GQGSLA	GELFF 2S2	1/20
				YLCSV	FVRESP	GELFF 2S2	1/20
				YLCSV	RLRLAGGD	EQYFG 2S7	1/20
				YLCSV	RMSFRGKPD	EQYFG 2S7	1/20
				YLCS	AVDRAPG	NTEAFFG 1S1	1/20
				YLCS	AGLRGVG	YNEQFFG 2S1	1/20
				YLCS	DIRDSVN	NEQFFG 2S1	1/20
				YLCS	GRQN	GELFF 2S2	1/20

The data were deduced from nucleotide sequences and are displayed in a standard one-letter code. The same CDR3 amino acid sequences were encoded by identical TCRB nucleotide sequences. These sequence data are available from GenBank under accession numbers AY424671–AY424691. Frequency, frequency of cDNA clones with the same sequences within the total number of clones sequenced.

This behavior was associated with the resolution of the lymphocytic alveolitis and the recovery of the number of CD4 and CD8 T cell populations in the lung (**Table 5**).

DISCUSSION

In this paper, we analyzed the TCR configuration of T lymphocytes accumulating in the lung of patients with HP. Heteroduplex and sequencing analyses of TCRBV regions provided evidence for the presence of dominant T cell clones in the lung and the blood of HP patients. Furthermore, clones of some TCRBV products of lung and blood T cells showed similar or identical CDR3 sequences.

TABLE 4. TCRBV Region Expression Values Obtained by Flow Cytometry from BAL T Cells of Patients with HP in Follow-Up Studies. The Frequency of Lymphocytes Positive for Anti-BV Region mAb Was Determined on 10,000 Cells Bearing Typical Lymphocyte Scatter. TCRBV Expression of Control Subjects Range from 1.0 to 10.0%

			TCRBV values after removal from antigenic exposure	
Patients	TCRBV region overexpressed	TCRBV values during exposure	6 months	12 months
case #2	BV2	26.4	13.2	6.2
case #4	BV6	18.7	11.2	3.1
case #10	BV8	16.3	10.6	4.6
case #12	BV8	17.9	8.3	2.0
			TCRBV values without removal from antigenic exposure	
			6 months	12 months
case #6	BV5	19.6	18.7	19.0
case #14	BV13	16.8	17.1	16.3

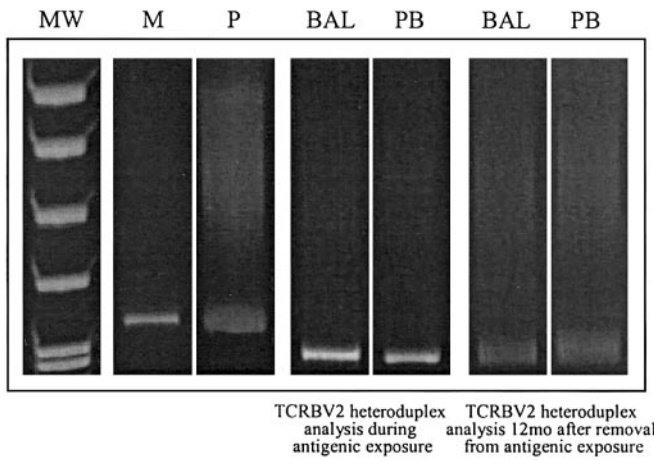

Fig. 3. Heteroduplex analysis of TCRBV2 gene PCR products obtained from BAL and PB lymphocytes from a representative HP patient (case #2) during exposure and 12 months after removal from antigenic exposure. The migration patterns of TCRBV2 PCR products, obtained from lung and blood T lymphocytes during the patient's antigenic exposure, showed the presence of the same homoduplex bands. On the contrary, the migration patterns of TCRBV2 PCR products, obtained from lung and blood T lymphocytes of the same patient 12 months after removal from *F. rectivirgula* exposure, showed a polyclonal pattern of TCRBV2 PCR products, and the homoduplex bands corresponding to T cell clones were no longer detected in the lung nor in the blood. TCRBV8-amplified products, obtained from the amplification of J77 and C1-632 cell RNA, were used as mono (M)- and polyclonal (P) controls and were loaded, respectively, in the first two lanes of gels. MW, Molecular weight marker pBR322.

TABLE 5. Phenotype of Lung Cells of Patients with HP in Follow-Up Studies

	During antigenic exposure				12 Mo after removal from antigenic exposure			
	Cells × 10^3/ml				Cells × 10^3/ml			
Patients	Cells	Lymphocytes	CD4	CD8	Cells	Lymphocytes	CD4	CD8
case #2	343.2	185.2	37.3	129.1	137.4	8.2	3.7	1.9
case #4	410.6	229.6	57.8	148.1	152.0	11.6	5.3	2.4
case #10	305.9	195.5	56.1	119.2	145.1	10.6	4.5	2.3
case #12	297.0	162.5	30.9	115.8	133.2	9.3	4.1	2.2

Our data provide the formal proof of the presence of $CD8^+$ T cell clones in the pulmonary microenvironment of HP patients [8, 17]. The persistence of T lymphocytic alveolitis and the presence of clonal T populations are likely to represent the result of an antigen-driven process, triggered by epitopes derived from *F. rectivirgula,* i.e., the agent accounting for HP in our patients [18]. On the contrary, the presence of clonality in T cell expansions strongly argues against the hypothesis that a superantigen accounts for the expansions in these patients. In fact, superantigens selectively stimulate T lymphocytes to express the appropriate TCRBV segment but generate a polyclonal T cell proliferation [19]. Noteworthy, some T cell clones expressing different BV segments, identified in the lung and in the blood, have similar or identical CDR3 regions. As the CDR3 region is directly involved in antigen recognition, these results support the idea that T cell clones have interacted with a common epitope derived from *F. rectivirgula*.

The presence of oligoclonal $CD8^+$ T cells was already demonstrated in the PB of healthy individuals [20, 21]. For this reason, finding homoduplex bands in the blood of our control subjects was not unexpected. Our hypothesis that oligoclonality demonstrated in our HP patients is not a simple reflection of the presence of $CD8^+$ T cell clones in the blood but that it is strictly correlated to the exposure to *F. rectivirgula* has been confirmed by the follow-up studies. In fact, the removal from antigenic exposure is associated with the resolution of the alveolitis and the normalization of pulmonary T cell populations and of the CD4/CD8 ratio (Table 5). Furthermore, the heteroduplex analyses performed during the follow-up on BAL and blood T lymphocytes of HP patients removed from antigenic exposure showed a polyclonal pattern of TCRBV PCR products, and homoduplex bands previously corresponding to T cell clones were no longer detected (Fig. 3). On the contrary, no relevant differences were shown in TCRBV expressions and TCRBV heteroduplex patterns on BAL and blood T lymphocytes of HP patients who maintained exposure to the relevant antigen and a persistent disease (without acute episodes). Thus, our data are consistent with the fact that *F. rectivirgula* causes an antigenic pressure, triggering the proliferation of T cells bearing a restricted TCR repertoire characterized by different TCRBV regions and sharing the same hypervariable region. Noteworthy, we demonstrated the same T cell clones in the lung and blood of the same patient, but we did not find the presence of identical CDR3 regions among the patients under study. Probably, this is a result of the antigenic complexity of *F. rectivirgula* and to the MHC molecules of each patient. It would be interesting to analyze the TCRBV regions in these patients following in vitro stimulation of lung and blood lymphocytes by *F. rectivirgula*. At present, this study is hindered by the lack of the purified antigen and/or of synthetic peptides.

A large number of antigens may cause HP; most of them belong to thermophilic actinomycetes, including *F. rectivirgula, Thermoactinomyces vulgaris, Tetraselmis viridis, Thermoactinomyces saccharii*, and *Thermoactinomyces candidus*, and are implicated in farmer's lung, bagassosis, Mushroom Worker's disease, and ventilation pneumonitis [22]. Other antigens are related to fungi, animal proteins (avian proteins accounting for Pigeon Breeder's disease), and more, but currently, only few data are available about the preferential use of TCRBV regions in HP caused by antigens other than *F. rectivirgula*. In particular, studies have been performed on summer-type HP, the most prevalent form of HP in Japan, induced preferentially by *Trichosporon cutaneum*, which demonstrated a preferential TCRV gene use but no common T cell expansions [23]. Bird Fancier's disease, induced by avian serum proteins [24], has also been studied; however, these studies have not provided any formal evidence of T cell clonality (e.g., heteroduplex and sequence analyses were not assessed).

The presence of T cell clones in the BAL of patients with farmer's lung is not specific for this illness. In fact, a skewing of the TCR repertoire has been detected in other interstitial lung diseases. Chronic Beryllium disease is characterized by TCRBV repertoire alterations in the BAL compared with the blood of patients and by oligoclonal expansions of the BAL $CD4^+$ TCRBV3 subset [25]. A discrete use of some TCRBV segments and the TCRAV 2.3 region in the lung T lymphocytes has been demonstrated in sarcoidosis patients [9, 26]. Some viruses, such as human immunodeficiency virus (HIV), determine a pulmonary involvement characterized by an alveolitis sustained by the proliferation of $CD8^+$ T cells that overexpress specific TCRBV regions [27].

Even if the lung is the predominant organ involved in interstitial lung diseases, other organs may be affected. Chronic Beryllium disease and sarcoidosis are multisystem disorders involving the lymphatic system, skin, liver, and spleen. The evidence that T cell populations obtained from the lung and blood of HP patients express the same or similar T cell clones suggests that HP itself is not exclusively restricted to the lung but is likely to represent a systemic disease. The pathogenic relevance of this phenomenon is still unknown, and further studies are needed to assess the TCR repertoire in other organs of patients with HP and the molecular and functional mechanisms possibly involved in the distribution of T cell clones. In fact, recent data in other interstitial lung disorders,

such as sarcoidosis and the T-lymphocytic alveolitis detected in patients with HIV, pointed out the central role of the cytokine/chemokine networks in the recruitment and accumulation of T cells in the lung [28, 29]. Analysis of the functional and molecular interactions between chemokines and T cells is needed to comprehend the contribution of these molecules in triggering an antigen-specific TCR oligoclonal response in the lung and the possible distribution of clonal T cells in different organs of patients with HP.

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