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The repertoire of T-lymphocytes recovered by bronchoalveolar lavage from healthy nonsmokers

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ABSTRACT: We reasoned that persistent exposure to a limited set of airborne antigens could drive the preferential expansion of single T-cell clones in the lower respiratory tract of normal individuals. To explore this issue, the normal human α/β T-cell receptor repertoire was studied in lung lymphocytes obtained by bronchoalveolar lavage (BAL) from the lumen of the lower respiratory tract.

BAL T-cells obtained from five healthy volunteers were first analysed using polymerase chain reaction to amplify all known Vα and Vβ genes of the T-cell receptor. T-cells from peripheral blood were used as an internal control. Heteroduplex analysis of the amplified products was then performed, to assess the clonal composition of the repertoire of lung- *versus* blood-derived T-lymphocytes within each amplified variable gene family.

In all subjects, the T-cell repertoire in the lung was largely as heterogeneous as peripheral blood in terms of clonal composition. This indicated lack of preferential expansion of single T-cell clones. A few T-cell clones were simultaneously expanded in blood and lung in all individuals within a limited number of Vβ (mean 2.4; range 2–4) and Vα (mean 1.6; range 1–3) genes. We also found that lung T-lymphocytes expressed all of the V gene families of the T-cell receptor that were expressed by peripheral blood T-cells.

Our results indicate that T-cell clones in the lower respiratory tract of normal individuals are distributed according to a largely polyclonal pattern, which corresponds to that found in peripheral blood.
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The epithelial surface of the lower respiratory tract is constantly exposed to airborne environmental antigens, with a contact area that in humans is above 50 square metres. The T-lymphocytes homing at this site, usually obtained by bronchoalveolar lavage (BAL), have been characterized in normal individuals for a number of distinct phenotypic and functional features that, in general, support the hypothesis of a confined immunological compartment. In fact, lung lymphocytes display the very late activation-1 (VLA-1) surface antigens [1] at a much higher rate than early activation markers, such as the interleukin-2 (IL-2) receptor [2]. They also have low IL-2 messenger ribonucleic acid (mRNA) expression and a "memory" T-cell phenotype, as defined by the CD45 RO membrane molecule [3]. Therefore, it is likely that BAL T-cells represent a compartmentalized lymphoid population with a low capability of proliferation, that is thought to be less efficacious than other more organized structures in driving an optimal immunization against new antigens [4]. Accordingly, the lung epithelial surface may not be the site of a primary immune response [5]. Thus, it may be that immunization *via* the respiratory tract occurs *via* the transport of antigen to lung lymph nodes by macrophages [6] or by dendritic cells, well-characterized as highly efficient antigen presenting cells [7].

The repertoire of antigen specificities of lung T-cells is not known. One speculation is that the local T-cells represent the result of the selection by a persistent antigen challenge by a limited number of airborne antigens. Thus, a restricted repertoire of specificities, *i.e.* a selective expansion of single T-cell clones, would be expected. However, recirculation of T-cells from the lung to peripheral blood and vice versa is an important feature in chronic diseases, such as sarcoidosis and tuberculosis [8], as well as in atopic asthma [9, 10]. This is confirmed in animal models [11], where preferential homing to the lung of lung-derived lymphocytes was demonstrated with adoptive transfer experiments. The combination of these characteristics makes it difficult to predict the degree of diversity generated by locally available antigen specificities in lung T-cells.

In order to explore this issue, five healthy volunteers were involved in the study. Fibreoptic bronchoscopy and BAL were performed in each subject. Polymerase chain reaction (PCR) was used to amplify the genes coding for the variable part of the T-cell receptor in BAL and peripheral blood T-cells. The variable (V) part of the T-cell

receptor (TcR) is the specific element of antigen recognition of T-lymphocytes that gives them the ability to bind antigens in the context of major histocompatibility complex (MHC) molecules of class I or II [12]. Finally, the heteroduplex assay was used to study the clonal composition of the T-cell repertoire. This assay can detect the presence and the relative amount of single T-cell clones within each PCR amplified V gene fragment. With this approach, the pattern of clonality of the T-cells in the lung *versus* periphery was defined.

Materials and methods

Experimental subjects

Five healthy, lifetime nonsmoking volunteers (2 males and 3 females), with no history of pulmonary infection in the previous 6 months, were included in the study. Mean age was 33 (range 21–56 yrs). These subjects had a negative anamnesis for asthma and atopic diseases, a negative skin prick test for a panel of the 12 most common allergens in our country (*Lolium perenne*, Parietaria, Olea, Betula, *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*., Cupressus, cat dandruff, dog dandruff, Alternaria, Cladosporium, Aspergillus), a Tiffenau index and total pulmonary capacity in the normal range [13], and a negative response to methacholine challenge (inhaled cumulative dose 2.4 mg).

Fibreoptic-bronchoscopy and BAL

Bronchoscopy and BAL were performed as described previously [14]. Briefly, after premedication with atropine (0.5 mg *i.m.*) and diazepam (10 mg *i.m.*), local anaesthesia of the nostrils was achieved with lidocaine 2% and adrenaline 0.1/1000 (1 mL each nostril). A fibreoptic bronchoscope (Olympus BF, type P10) was then passed through the nose. The pharynx and the large airways were anaesthetized by instillation of lidocaine 2%, and the tip of the bronchoscope was wedged into a subsegmental branch of the right middle lobe. In patients Nos. 3, 4 and 5 the procedure was repeated in the lingula. Five aliquots of 20 mL each of sterile, warm (37°C) saline were infused through the channel of the bronchoscope and recovered at a negative pressure of 5–120 mmHg.

BAL sample processing

The fluid recovered was filtered through two layers of sterile gauze and centrifuged at 250×g for 5 min to separate the cells from the supernatants. The cell pellet was resuspended in ice cold phosphate-buffered saline (PBS) without Ca^{2+} and Mg^{2+}, at a concentration of 10^6 cells·mL^{-1}. A small sample of the cell suspension was centrifuged by using a cytocentrifuge (Cytospin, Shandon Southern Instruments, Sewickley, PA, USA) spinning approximately 100,000 cells at 500 rpm for 5 min onto a glass slide. BAL cells were air-dried and stained with Diff-Quick staining (Merz & Dade A.G., Dudingen, Switzerland). Cell differentials were determined by counting at least 300 cells per slide, using a light microscope (Carl Zeiss, Oberkochen, Germany). Macrophages, lymphocytes, neutrophils and eosinophils were expressed as percentage of total cells recovered. The remaining cells were immediately lysed in guanidium-isothiocyanate buffer and stored at -20°C until ribonucleic acid (RNA) extraction. We did not *in vitro* amplify BAL cells with any mitogen (IL-2, PHA) before processing them, in order to study the T-cell repertoire without introducing *in vitro* any possible bias.

Isolation of peripheral blood mononuclear cells

In each subject, blood sampling was performed immediately before the beginning of the BAL procedure. Peripheral blood mononuclear cells (PBL) were isolated on a lymphoprep gradient (Sigma Chemical Co.) from heparinized blood, according to standard procedures.

PCR analysis

Total RNA was extracted from unfractionated BAL cells (numbers ranging from 6×10^5 to 3×10^7) and from 2×10^6 PBL using the procedure described by CHOMOCZYNSKI and SACCHI [15]. All of the RNA was used for first-strand complementary deoxyribonucleic acid (cDNA) synthesis using an oligo (dT) primer and murine mammary leukaemia virus (MMLV) reverse transcriptase (BRL, Gaithersburg, MD, USA). Of each cDNA sample, 1/60 was amplified using the Vα-Cα or Vβ-Cβ specific oligonucleotides described previously (table 1) [16, 17]. PCR reactions were performed in a 30 µL final volume using a Thermal Cycler 9600 (Perkin Elmer, Weiterstadt, Germany) under the following conditions: 30 s denaturation at 94°C; 30 s annealing at 60°C for Vβ and at 61°C for Vα; 30 s elongation at 72 °C for 35 cycles preceded by 3 min of denaturation and followed by 5 min for final elongation. One third of the amplified products was run on a 2% agarose, ethidium bromide stained gel; pBr322 MspI digested DNA was used as a molecular weight marker (New England Biolabs, Beverley, MA, USA).

Heteroduplex analysis

The remaining PCR amplified product was subsequently run on a 12% native polyacrylamide gel electrophoresis (PAGE) [18, 19]. In summary, 20 µL of each amplified fragment was heated at 94°C for 5 min to denature DNA, and then reannealed for 1 h at 50°C and kept at 4°C until gel loading. This temperature is permissive for reannealing between strains with minor mismatches (heteroduplex) as well as between homologous strains (homoduplex). Vα and Vβ amplified fragments derived from peripheral blood and BAL of the same subject were

Table 1. – Oligonucleotides used in PCR

Vα		Vβ	
1	5'GGCATTAAGGGTTTTGAGGCTGGA	1	5'GCACAACAGTTCCCTGACTTGCAC
2	5'CAGTGTTCCAGAGGGAGCCATTGT	2	5'TCATCAACCATGCAAGCCTGACCT
3	5'CCCGGGCAGCAGACACTGCTTCTTA	3	5'GTCTCTAGAGAGAAGAAGGAGCGC
4	5'TTGGTATCGACAGCTTCACTCCCA	4	5'ACATATGAGAGTGGATTTGTCATT
5	5'CGGCCACCCTGACCTGCAACTATA	5.1	5'ATACTTCAGTGAGACACAGAGAAAC
6	5'TCCGCCAACCTTGTCATCTCCGCT	5.2-5	5'TTCCCTAACTATAGCTCTGAGCTG
7	5'GCAACATGCTGGCGGAGCACCCAC	5.6	5'TGCTAATGAGTTAAGGAGATCAG
8	5'CATTCGTTCAAATGTGGGCAAAAG	6	5'AGGCCTGAGGGATCCGTCTC
9	5'CCAGTACTCCAGACAACGCCTGCA	7	5'CCTGAATGCCCCAACAGCTCTC
10	5'CACTGCGGCCCAGCCTGGTGATAC	8	5'ATTTACTTTAACAACAACGTTCCG
11	5'CGCTGCTCATCCTCCAGGTGCGGG	9	5'CCTAAATCTCCAGACAAAGCT
12	5'TCGTCGGAACTCTTTTGATGAGCA	10	5'CTCCAAAAACTCATCCTGTACCTT
13	5'TTCATCAAAACCCTTGGGGACAGC	11	5'TCAACAGTCTCCAGAATAAGGACG
14	5'CCCAGCAGGCAGATGATTCTCGTT	12	5'AAAGGAGAAGTCTCAGAT
15	5'TTGCAGACACCGAGACTGGGGACT	13.1	5'CAAGGAGAAGTCCCCAAT
16	5'TCAACGTTGCTGAAGGGAATCCTC	13.2	5'GGTGAGGGTACAACTGCC
17	5'TGGGAAAGGCCGTGCATTATTGAT	14	5'GTCTCTCGAAAAGAGAAGAGGAAT
18	5'CAGCACCAATTTCACCTGCAGCTT	15	5'AGTGTCTCTCGACAGGCACAG
19	5'ACACTGGCTGCAACAGCATCCAGG	16	5'AAAGAGTCTAAACAGGATGAGT
20	5'TCCCTGTTTATCCCTGCCGACAGA	17	5'CAGATAGTAAATGACTTTCAG
21	5'AGCAAAATTCACCATCCCTGAGCG	18	5'GATGAGTCAGGAATGCCAAAGGAA
22	5'CCTGAAAGCCACGAAGGCTGATGA	19	5'CAATGCCCCAAGAACGCACCCTG
23	5'TGCCTCGCTGGATAAATCATCAGG	20	5'AGCTCTGAGGTGCCCCAGAAT
24	5'CTGGATGCAGACACAAAGCAGAGC	21	5'ATTCACAGTTGCCTAAGGATCGA
25	5'TGGCTACGGTACAAGCCGGACCCT	22	5'GGGCAGAAAGTCGAGTTTCTGGTT
26	5'AGCGCAGCCATGCAGGCATGTACC	23	5'TTTATGAAAAGATGCAGAGCGAT
27	5'AAGCCCGTCTCAGCACCCTCCACA	24	5'AAGTCAAGTCAGGCCCCAAAGCT
28	5'TGGTTGTGCACGAGCGAGACACTG		
29	5'GAAGGGTGGAGAACAGATGCGTCG		
Cα		Cβ	
5'CGTATCTGTTTCAAAGCTTTTCTCGACCAG		5'TGCTGACCCCACTGTCGACCTCCTTCCCATT	

PCR: polymerase chain reaction.

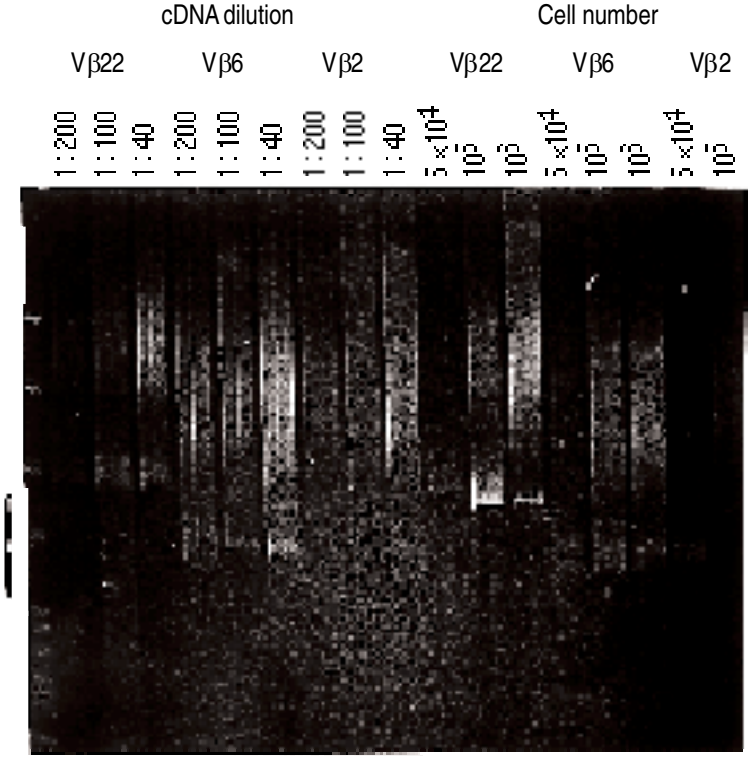

Fig. 1. – Heteroduplex analysis of polyclonal T-cells consistently yielded a smear at increasing dilution of cDNA and at decreasing cell numbers. Three widely represented Vβ families (as indicated) were studied in peripheral blood T-cells from a laboratory worker by using PCR and subsequent heteroduplex analysis of the amplified products. The assay was performed, for each Vβ, at three different dilutions of the cDNA contained in the original 30 μL reaction volume (1:200, 1:100 and 1:40) and at three different cell doses (5×10^4, 5×10^5 and 5×10^6). Bracket indicates where the homoduplex bands migrate (varying between 300 and 400 base pairs). cDNA: complementary deoxyribonucleic acid; PCR: polymerase chain reaction.

run in parallel lanes for comparison of the heteroduplex and homoduplex bands. Gels were run at 10 mA overnight at 4°C, stained with ethidium bromide and photographed. The presence of a single homoduplex band indicated expansion of a single T-cell clone, multiple heteroduplex bands indicated oligoclonal expansion of T-cell clones, and the presence of a smear (both as heteroduplex and/or as homoduplex) reflected a polyclonal T-cell population. At first, the number of emerging bands was determined by three independent observers. The result was then discussed until they reached an agreement.

Sampling error could occur because of the low level of T-cells in the BAL once their T-cell receptors are further amplified by PCR. A single clonotype could emerge and then over-represent the *in vivo* distribution. To assess this possibility, experiments were performed in which cDNA was exhaustively diluted and others where the cell number from which RNA was extracted and the cDNA prepared were progressively lowered. Vβ2, Vβ6 and Vβ22 specific probes were used as representative of widely used V gene families.

In order to establish the limit of detection of single clones in our system, we performed an experiment where a single T-cell clone (TCC A) was mixed at different cell doses with a constant amount (8×10^6) of a polyclonal population of peripheral blood T-cells from a normal donor (not included in the BAL study). Heteroduplex analysis was then performed on the PCR amplified product of the Vβ gene family expressed by that clone. Thus, detection of one single emerging band was expected. Moreover, in order to mimic the situation where two T-cell clones in a polyclonal population are expanded within a given V gene family, two T-cell clones were used which expressed the Vβ17 gene (denominated TCC A and TCC B) both CD4+, expressing two different Jβ and Dβ genes. They were raised from a seronegative donor against the gp120 envelope protein of the HIV-1 virus (curtesy of L. Furci). These two T-cell clones were mixed (TCC A: 2×10^6 cells; TCC B: 10^5 cells) with a polyclonal population of T-lymphocytes obtained from peripheral blood of the same normal donor (8×10^6 cells), and the heteroduplex assay was then performed. Two heteroduplex and two homoduplex bands were expected in this case.

Results

Heteroduplex assay on T-cell receptor gene segments at low cell number and high cDNA dilution in peripheral blood lymphocytes

This assay did not introduce signs of oligoclonality in a polyclonal T-cell sample in forms of emerging bands, either at high cDNA dilution or at low cell number (fig. 1). In both cases, a weakening of the smear was observed at cDNA dilution (1:200) or at number of cells (10^5) lower than those usually handled when analysing BAL T-lymphocytes.

Sensitivity of the heteroduplex assay in detecting single T-cell clone in a polyclonal population and pattern of bands expected in the case of the expansion of two T-cell clones within a given Vβ family

The sensitivity of the heteroduplex assay in detecting a single T-cell clone mixed in a polyclonal population of T-lymphocytes was around 1 cell out of 3,200 (fig. 2). When two clones (expressing the same Vβ) were mixed in a polyclonal population of T-lymphocytes, the following pattern was found: two homoduplex bands and two heteroduplex bands, superimposed to a smear corresponding to the polyclonal population of peripheral T-cells (fig. 2).

BAL cellularity

Subjects characteristics, total number of cells recovered by BAL and proportions of the different cell types are shown in table 2. Lack of erythrocytes and low numbers of polymorphonuclear leucocytes indicated that BAL was not contaminated by peripheral blood.

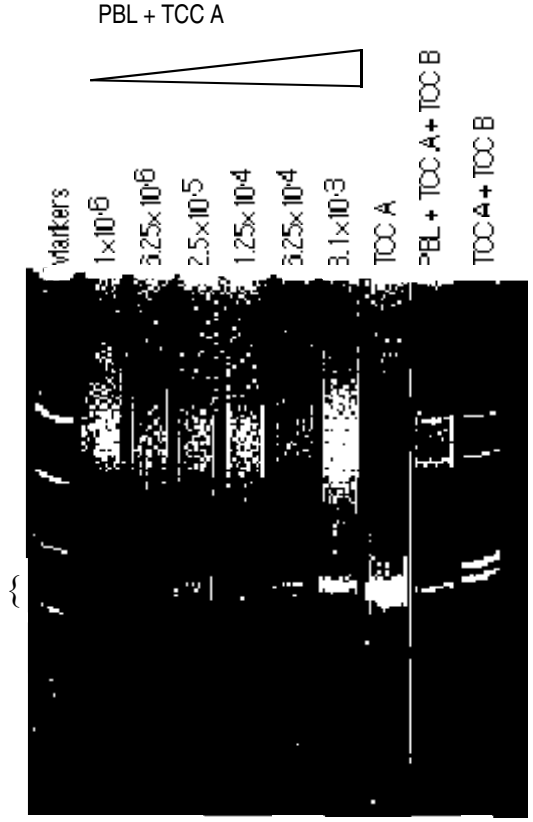

Fig. 2. – The sensitivity of the heteroduplex assay was assessed in the detection of a single T-cell clone (TCC A, Vβ17) mixed with a constant number (8×10^6) of peripheral blood mononuclear cells (PBL) to emulate the physiological condition where a single T-cell clone is expanded in a polyclonal population of T-lymphocytes. The ratio between the number of T-cells from TCC A and the number of PBL is indicated. Heteroduplex analysis of TCC A (2×10^6 cells) + a second T-cell clone expressing the same Vβ (TCC B, 10^5 cells) + peripheral blood T-cells (8×10^6 cells) are shown as an example of the result expected when a biclonal expansion is taking place in a polyclonal population of PBL (TCC A + TCC B + PBL).

Table 2. – Cellular components of bronchoalveolar lavage

	Subject No.				
	1	2	3	4	5
Age yrs	33	21	56	24	29
Sex	F	M	F	M	M
Right middle lobe					
Total cell number $\times 10^6$	5.0	8.7	4.5	2.4	2.9
Macrophages %	88	94	91	89	93
Lymphocytes %	10	5	7	6	5
Neutrophils %	1	1	1	4	1
Eosinophils %	1	0	1	1	1
Lingula					
Total cell number $\times 10^6$	ND	ND	4.9	3.1	3.1
Macrophages %			89	95	92
Lymphocytes %			10	5	6
Neutrophils %			1	0	1
Eosinophils %			0	0	1

M: male; F: female; ND: not determined.

V gene usage by α/β T-cell receptor positive lymphocytes in the lung versus *peripheral blood*

Gene segments belonging to virtually all the single Vβ genes of the panel tested could be amplified by PCR from cDNA obtained by T-lymphocytes from peripheral blood as well as from BAL in the five individuals studied (not shown). A representative result (subject No. 1) is shown in figure 3. In figure 4, results of PCR on T-cells from two segments of the same lung (subject No. 3) are shown. Although no quantitative information can be obtained concerning the relative usage of single variable genes coding for the α/β T-cell receptor, there was not any single Vβ gene amplifiable from peripheral blood which was excluded from lung T-lymphocytes in all individuals studied. Analysis of Vα genes gave similar results (not shown).

Clonal composition of the T-cell receptor repertoire in the lung versus *peripheral blood*

Heteroduplex analysis of the amplified PCR products obtained from peripheral blood and from BAL cells yielded a smear type of pattern in the vast majority of V genes analysed. This reflected the polyclonality of T-cells within each V gene family (figure 5 shows representative results of heteroduplex analysis of Vβ1, 2, 5.1, 6, 7 and 22 gene segments amplified from peripheral blood and from BAL T-lymphocytes in subject No. 1). Polyclonality of BAL T-cells was confirmed by the analysis of the Vα genes (not shown). In a limited number of V gene products, co-migrating bands were simultaneously detected both in the peripheral blood- and in the lung-derived sample. Overall, the mean number of emerging bands was 2.4 among Vβ genes (range 2–4) and 1.6 among Vα genes (range 1–3). Single V gene products where an expanded band was visible are listed in table 3. Representative examples of these bands are shown in figure 5 (Vβ5.1) and figure 6 (fig. 6a from subject No. 2; fig. 6b from subject No. 3). In subjects where BAL samples were available from two different lung segments, co-migrating bands were present in both (fig. 6b). In subjects Nos. 3 and 5 a V gene product was found (Vβ7 and Vβ13.1, respectively) which showed an expanded band in the peripheral blood-derived sample not accompanied by a corresponding expanded band in the BAL-derived sample (table 3).

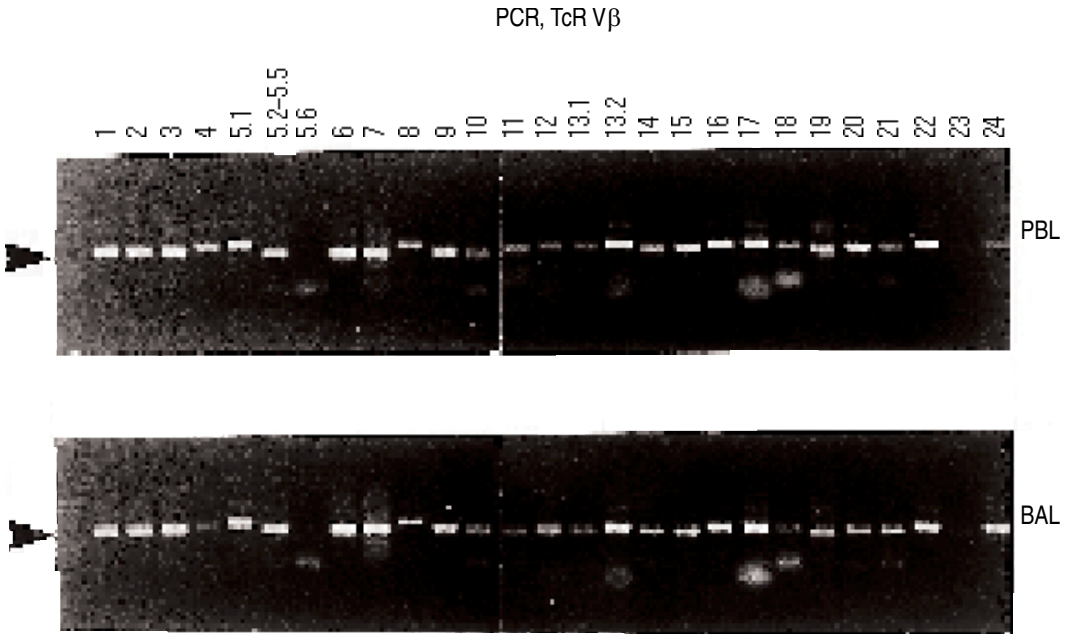

Fig. 3. – Polymerase chain reaction (PCR) analysis of T-cell receptor (TcR) Vβ genes in bronchoalveolar lavage (BAL) and peripheral blood from subject No. 1. PCR products obtained using the panel of Vβ gene specific oligonucleotides described in table 1 on the cDNA derived from peripheral blood lymphocytes (PBL) and BAL T-cells. Arrow heads indicate the 307 base pair fragment.

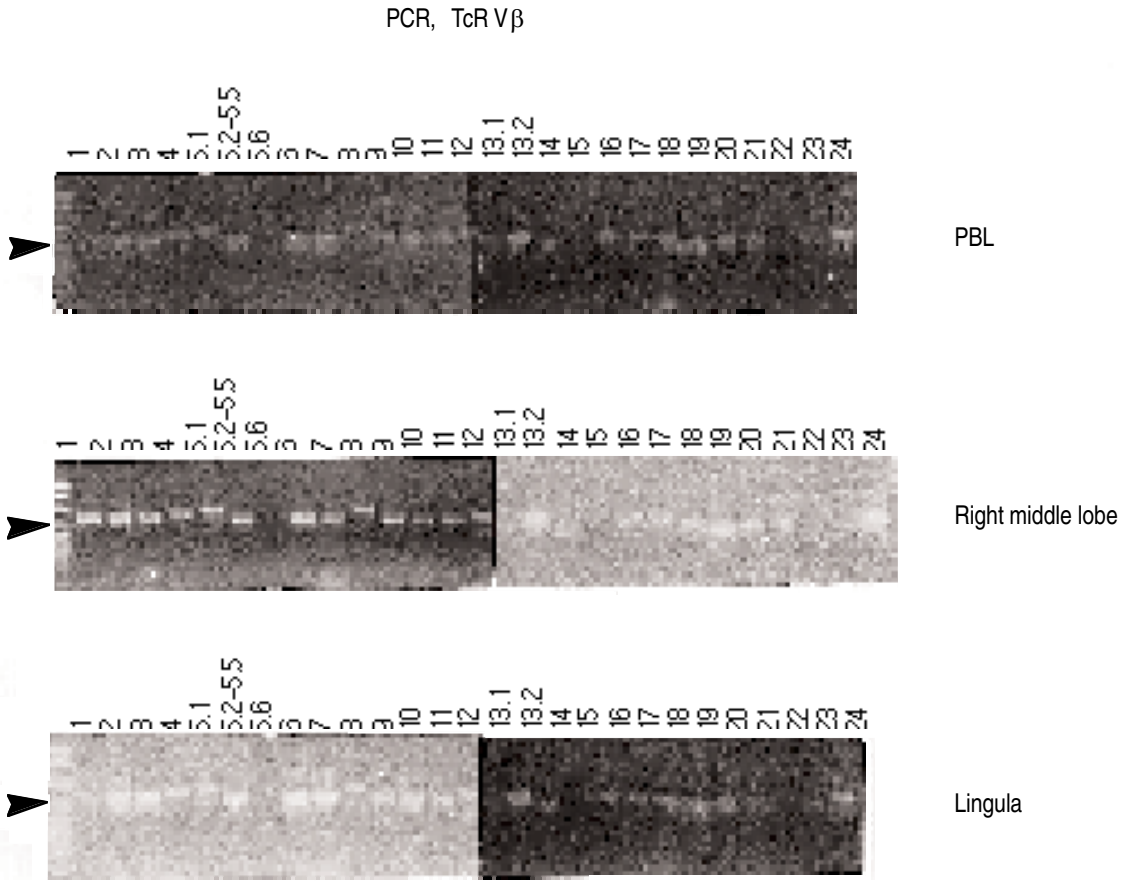

Fig. 4. – Polymerase (PCR) analysis of T-cell receptor (TcR) Vβ genes in bronchoalveolar lavage (BAL) performed in two different segments and peripheral blood from subject No. 3. For explanation see legend to figure 3. Cells from symmetrical sites of the two lungs were analysed, as indicated. PBL: peripheral blood lymphocytes.

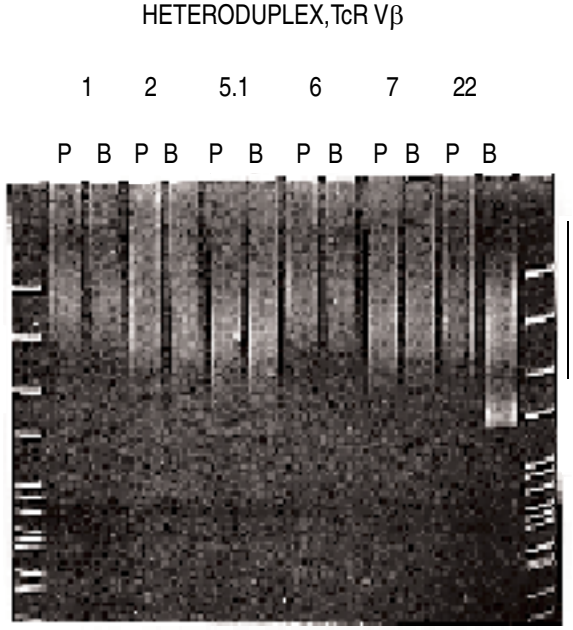

Fig. 5. – Heteroduplex assay of BAL and peripheral blood T-cells from subject No. 1. Heteroduplex analysis of PCR products of five representative Vβ genes (listed on top), comparing peripheral blood (P) and BAL (B) derived T-cells. The vertical line indicates the region where the smear is distributed.

Table 3. – V gene products which yielded a single expanded band at heteroduplex analysis

Subject No.	TcR V gene	PBL	BAL 1	BAL 2
1	Vβ5.1	+	+	ND
	Vβ10	+	+	
	Vα4	+	+	
2	Vβ2	+	+	ND
	Vβ14	+	+	
	Vβ21	+	+	
	Vβ22	+	+	
	Vα2	+	+	
	Vα4	+	+	
	Vα20	+	+	
3	Vβ7	+	-	-
	Vβ8	+	+	+
	Vα21	+	+	+
4	Vβ1	+	+	+
	Vβ5	+	+	+
	Vα6	+	+	+
	Vα17	+	+	+
5	Vβ13.1	+	-	-
	Vβ7	+	+	+
	Vα7	+	+	+

TcR: T-cell receptor; V: variable part; PBL: peripheral blood mononuclear cells (lymphocytes); BAL: bronchoalveolar lavage.

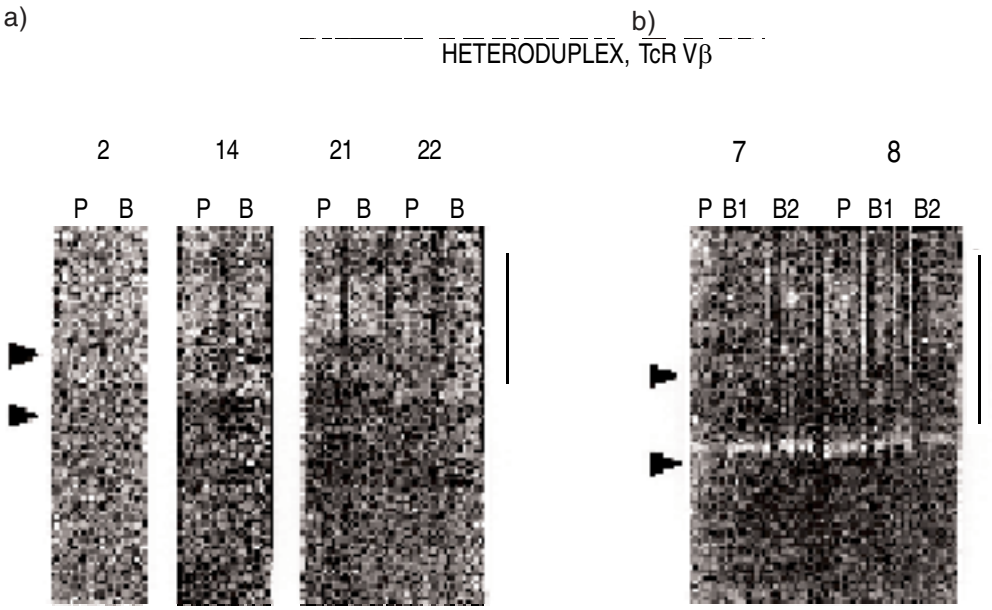

Fig. 6. – V genes from BAL and peripheral blood T-cells from subject No. 2 and No. 3 (a) and b), respectively) where expanded bands were observed. Heteroduplex analysis of PCR products of the Vβ genes (listed on top), comparing peripheral blood (P) and BAL (B) derived T-cells. B1 and B2 indicate two different lung segments of subject No. 3 from which BAL T-cells were recovered. The vertical lines indicate the region where the smear is distributed. Arrows indicate the positions of 527 and 307 molecular weight markers.

Discussion

The results of this study indicate that in the normal lower respiratory tract the T-cell repertoire is qualitatively similar to the peripheral blood, not only in terms of V gene usage (all V gene families of the T-cell receptor are represented) but also in terms of degree of diversity (prevalent polyclonality). A limited number of expanded clones is present in the BAL T-cell repertoire, which are detectable in the periphery as well. We conclude that a large repertoire of T-cells with different antigen specificities is maintained in the lumen of the normal lower respiratory tract. The heteroduplex assay represents a technological novelty, compared to other methods for studying the relative usage of the different V gene families (quantitative PCR, Vβ specific monoclonal antibodies). In fact, this assay reveals the clonal composition of T-cells within each population of T-lymphocytes expressing a given V gene family. This approach can discriminate situations where predominant clones emerge (*e.g.* for the effect of an antigen drive) from others where the population of single T-cells is distributed in a homogeneous (polyclonal) fashion.

The lung is a model of a normal tissue chronically exposed to antigens. Inhaled antigens reach the bronchial mucosa at the upper and/or lower respiratory tract with a pattern dependent on the size of the particles that carry them [20]. Lung lymphocytes represent a population of mature differentiated T-cells that developed in lung lymph nodes as antigen specific effector cells. This probably occurred during acute exposure to invaders or otherwise immunologically relevant substances; subsequently, these cells have migrated to the lung mucosa [21]. Alveolar macrophages, physiologically endowed with inhibitory functions over T-lymphocytes [4], in certain circumstances allow antigen presentation and local T-cell activation (*e.g.* in a fraction of patients with asthma) [22]. Thus, they could significantly influence the progression of the immune response in atopic mucosas. Indeed, we recently demonstrated oligoclonal T-cell expansion upon antigen provocation in BAL T-cells from atopic asthmatics [23]. T-cells obtained by BAL represent the epithelium-associated lymphocytes. How much of this cell population is segregated and how much is actively recirculating is a matter of debate [21]. In fact, the pattern of adhesion molecules expressed by lung lymphocytes is suggestive of the defined homing properties of a resident cell population [1, 2]; on the other hand, recirculation of disease-associated lymphocytes has been found in the course of certain diseases characterized by chronic inflammation (tuberculosis, sarcoidosis) [8], or in individuals with atopic asthma [9, 10].

The investigation of the clonal composition of antigen specific T-cells is a novel approach to the study of the immune response in the lung. Although antigen specific T-cells have been isolated and cloned from BAL samples obtained from atopic individuals [24], to our knowledge, there is still no information available on the overall distribution of the T-cell repertoire in the lower respiratory tract in healthy individuals. The α/β T-cell receptor is the element of antigen recognition of MHC class I and II restricted (CD3+) T-lymphocytes [12].

Amplification with PCR of the genes coding for the T-cell receptor is a valuable procedure in ascertaining the presence of T-lymphocytes which express a given V gene family. In this work, PCR was used as a first step in screening, to assess whether qualitative differences (absence or presence of clones using each single known V gene) could be seen in the lung *versus* the periphery. This analysis was further extended with the heteroduplex assay [18, 19], to assess the clonal composition within each V gene family. This assay is based on the principle that a mixture containing highly homologous DNA can renature after boiling not only by reannealing

of homologous strains (homoduplex), but also of partly heterologous ones (heteroduplex). The latter migrate in a polyacrylamide nondenaturing gel with a higher apparent molecular weight. Moreover, heteroduplexes composed by the same but complementary strains are endowed with different migration properties (see figure 2, TCC A + TCC B). By applying this method to the analysis of the T-cell receptor in peripheral blood T-cells [18, 19], a typical smear-type distribution of bands representing the multiplicity of the T-cell clones is obtained. This result depicts the extreme polyclonality and heterogeneity of the peripheral repertoire.

A theoretical limit of the heteroduplex assay is the small sample size, either in terms of low cell number or of high cDNA dilution. This fact could over-represent the frequency of single T-cell clones, since PCR is made in a nonquantitative fashion. In preliminary tests, working with cell numbers in the range of those contained in BAL samples, we demonstrated that neither a limiting number of T-cells nor a high cDNA dilution caused the artificial appearance of emerging bands (fig. 1). In addition, we tested the sensitivity of our assay by titrating the number of T-cells in a polyclonal T-cell population; frequencies of single T-cell clones in the range of 1/3,200 could be detected as emerging bands by our method (fig. 2).

In agreement with a previous report by other investigators [25] we found that all known Vβ T-cell receptor genes that are expressed in peripheral blood T-cells can also be detected in T-cells recovered from the lower respiratory tract (figs. 3 and 4). The novel finding of this study is that we found a T-cell receptor repertoire in the lung that was as heterogeneous and polyclonal as that found in peripheral blood (fig. 5). Moreover, when single emerging bands were visible in the periphery, they were present in the lung-derived sample as well (fig. 6 and table 3). These may represent populations of chronically expanded clones, that have been described in normal individuals and whose function is still debated [18]. In this case, one has to hypothesize that they possess homing receptor for the lung tissue. Alternatively, they may be T-cells specific for respiratory antigens, which expanded in the lymph nodes and recirculated back to the site of antigen encounter. However, the negative anamnesis for respiratory diseases in the last 6 months discourages this hypothesis.

We want to make the following points about the results of this study: 1) our method was sensitive to modifications of the lung T-cell repertoire, which induce the appearance of emerging bands; and 2) the lung T-cell repertoire in healthy subjects did not show organ-specific signs of clonal expansion (as one could expect in a segregated immune compartment). In individuals who underwent BAL in two different lung segments, these single expanded bands were present in samples derived from both (fig. 6 and table 3), indicating a homogeneous distribution of the lung T-cell repertoire in different areas of this organ. In conclusion, in physiological conditions the persistent exposure to airborne antigens does not imply the local expansion of single T-cell clones and/or the unbalanced homing to the lumen of the lower respiratory tract of single T-cell clones with selected antigen specificities.

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