Clin Exp Immunol 1996; **104**:144–153

Analysis of the T cell receptor Vγ region gene repertoire in bronchoalveolar lavage (BAL) and peripheral blood of atopic asthmatics and healthy subjects

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(Accepted for publication 28 November 1995)

SUMMARY

We analysed the T cell receptor (TCR) Vγ repertoire in BAL and peripheral blood (PBL) of three mild stable atopic asthmatics and two non-asthmatic controls. We used the polymerase chain reaction (PCR) to establish the expression of the four Vγ families, and to detect oligo or monoclonal expansion of $\gamma\delta^+$ T cells, we resolved the PCR products on denaturing and non-denaturing gels to find the extent of junctional diversity arising from differences in the lengths of the V(D)J junctions. We also subcloned and sequenced the PCR products to characterize fully the sequence diversity. BAL T lymphocytes from two asthmatic patients (treated with inhaled steroids) expressed only VγII and, in one of them, VγIIJγ usage was restricted to JγP and JγP1 gene segments, contrasting with the VγJγ repertoire found in his respective PBL. Analyses in denaturing and non-denaturing gels showed that the BAL VγIIJγP and VγIIJγP1 PCR products resolved into few bands, suggesting deletions at the junctions due to oligoclonal expansion. BAL T lymphocytes from the third asthmatic (not receiving inhaled steroids) expressed VγI, II and III, and the sequences of the in-frame TCR transcripts from this asthmatic and one healthy volunteer who expressed a similar BAL Vγ TCR repertoire showed clonal expansion of T cells expressing all three Vγ families. Our analyses showed that much of the $\gamma\delta$ T cell population found in BAL fluid of humans derives from clonally expanded T cells.

Keywords asthma inflammation T cells hsp lung

INTRODUCTION

Asthma is characterized by tracheobronchial hyperresponsiveness, variable airflow obstruction and airway inflammation [1]. Although the cellular and molecular mechanisms perpetuating airway inflammation in asthma are not entirely known, there is evidence that T cells could contribute to establish and/or maintain the airway inflammation seen in asthmatic subjects [2]. In atopic asthma, activated T cells found in BAL are of the Th2 type which can elicit inflammatory changes through the production of interleukins [3,4]. This pattern of lymphokines increases bronchial hyperresponsiveness in animals [5,6], and provokes chronic inflammation of the airways [7–10]. Drugs such as inhaled steroids that reduce lymphocyte activation [11] and reduce the number of lymphocytes infiltrating the airways [12] improve clinical asthma and bronchial hyperresponsiveness [11,12].

T cells become activated upon recognizing an antigen through the T cell receptor (TCR) a CD3-associated heterodimeric surface complex that defines the specificity of the T lymphocyte [13]. In healthy subjects, about 90% of blood T lymphocytes express a TCR composed of α- and β-chains that collectively recognize a broad range of antigens [14]. The remaining T cells have TCRs composed of γ- and δ-chains. The function and specificity of $\gamma\delta$ T cells are unclear. In a number of different systems, $\gamma\delta$ T lymphocytes, that generally exhibit the $CD4^-\,CD8^-$ or $CD4^-\,CD8^+$ phenotype, provide a regulatory role over the $\alpha\beta^+$ T cell effector subset [15–22]. However, the role of $\gamma\delta^+$ T lymphocytes in the human lung or airways [23] is not yet known. It is plausible to think that they could play a significant role in inflammatory airway disease; several authors have noted increased numbers of $\gamma\delta^+$ T cells in a variety of pathological conditions, and have proposed that $\gamma\delta$ T cells provide a first line of defence [24] on epithelial surfaces. For example, it has been shown that heat shock proteins (hsp) are ligands for $\gamma\delta$ T cells [25,26], suggesting that the appearance of hsp on the surface of stressed cells may begin a system of immune surveillance [27]. This reactivity to hsp may carry with it an inherent risk of autoimmunity [27–29]. Yet an immune reaction against conserved epitopes would provide an immediate response to diverse pathogens and cellular changes. Activation of the regional $\gamma\delta$ T cells to produce lymphokines would then promote non-specific inflammation and macrophage activation, providing a degree of non-specific resistance [30].

Since the factors involved in the maintenance of chronic airway inflammation and hyperresponsiveness in asthma are poorly

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understood, in the present study we examined the TCR Vγ repertoire in asthmatic subjects. The human TCR γ repertoire is *per se* limited, with only eight functional Vγ gene segments and five Jγ gene segments [24,31]. The Vγ gene segments group into four families according to sequence similarities. The VγI family has five functional gene segments: Vγ2, 3, 4, 5, and 8. The VγII, III and IV families have one member each, called Vγ9, 10 and 11, respectively.

We focused our attention on the γδ repertoire based on the observations that: (i) effectors $CD4^+$ and $CD8^+$ αβ T lymphocytes play a role in asthma [32]; (ii) γδ T cells seem to regulate such αβ T cells in a number of typically T cell-mediated conditions [15–22]; (iii) γδ T cell subsets home in the epithelial surface [23,33], of several species; (iv) one of the most important ligands γδ T cells respond to are hsp [34]; (v) hsp have been shown to be up-regulated in the stable asthmatic airway epithelium [35] or following allergen challenge [36]; and (vi) genetic linkage has been reported between the α/δ loci and atopy [37].

PATIENTS AND METHODS

Subjects

Three atopic asthmatics with mild, stable disease and two healthy non-atopic non-asthmatic volunteers gave written consent to participate in the study, which had the University of Toronto Human Ethics Committee approval. All subjects underwent spirometric testing and skin prick tests to 12 common aeroallergens. On the following day we repeated spirometry, and if the FEV_1 was $>80\%$ of the predicted value, we obtained BAL specimens and simultaneous peripheral blood samples from an antecubital vein as previously described [38]. Table 1 describes the demographic data, pulmonary function test and prick test results, and medications received by the patients.

Method of analysis

Four discrete yet interdependent analyses were used to study TCR diversity. First, using polymerase chain reaction (PCR), the level of expression from each Vγ family was investigated. Second, the PCR products were resolved on denaturing gels to find the junctional diversity arising from differences in the lengths of the V(D)J junctions. Third, we resolved the PCR products on non-denaturing gels to find the diversity arising from conformational differences. Fourth, we subcloned and sequenced the PCR products to characterize fully the sequence diversity.

Amplification of TCR Vγ transcripts

We transcribed cDNA from total RNA, extracted from unfractionated BAL cells and peripheral blood cells (post Ficoll), and amplified the cDNA as previously described [39]. For the analysis of the TCR Vγ repertoire, we amplified the cDNA using the PCR with a constant region oligonucleotide and one of the four Vγ oligonucleotides [40]. To transcribe RNA into cDNA and subsequently to amplify the cDNA, we used two constant region oligonucleotides. We used the first (reverse transcriptase primer) to prepare cDNA from the patients' mRNA. We labelled the 5′ end of the second constant region primer (nested 5′ to the first) with ^{32}P-ATP and T_4 polynucleotide kinase. We used the labelled primer with an unlabelled V region primer to amplify the DNA over 25 cycles of the PCR. Each cycle of amplification involved denaturation for 30 s at 94°C, annealing for 30 s at 63°C, and extension for 1 min at 72°C. In our analysis of patient A4, we also amplified cDNA using a labelled VγII oligonucleotide with each of four Jγ primers. Three of the Jγ primers are homologous to one of JγP, JγP1 and JγP2. The fourth Jγ primer is homologous to both Jγ1 and Jγ2. The annealing temperature for these amplification reactions was 53°C. We used a Perkin Elmer 9600 Biocycler for all PCR reactions.

Oligonucleotide sequences [41]

The two Cγ primers were as follows: reverse-transcriptase primer, CATGGTGTTCCCCTCCTGGGA; amplification primer, CGTGTTGCTCTTCTTTTCTTGC. The Vγ oligonucleotide sequences were: VγI, GAGGGGAAGGCCCCACAGCGT; VγII GGAATCTGGCATTCCGTCAGGC; VγIII CCGCAGCTCGACGCAGCATGG and VγIV, ATGTCTTCTTGACAATCTCTGCTC. Each Vγ oligonucleotide contains at least 10 nucleotide differences compared with any sequence from a different family. All members of the Vγ1 family share the VγI sequence. The four Jγ primers were as follows: Jγ1/2 ACTGCCAAAGAGTTTCTTATAATA; JγP TACCTTGATTTTTTTGCCCAACTC; JγP1 CAGCAAATATCTTGAACCAAC and JγP2 TTGCAAACGTCTTGATCCAAT.

Internal standards

As positive control for the expression of each Vγ and Jγ region, we used internal standards ranging in size from approximately 800 to 1000 base pairs. One of the standards contained the sequences corresponding to the primer for reverse transcriptase and each of two primers required for the PCR. The synthesis of each polynucleotide internal standard required the PCR and 8–12 oligonucleotides ranging in size from 80 to 120 nucleotides. For the first step in the synthesis two oligonucleotides of opposite polarity

Table 1. Characteristics of the study subjects

Subjects	Age (years)/sex	Years of asthma	FEV_1 (*l*/%)	Positive skin tests	Medications
A4	67/M	22	3·0 (80)	hdm, cat, dog	Becl, Salb
A10	31/F	30	3·0 (96)	hdm, cat, rag	Becl, Salb
A13	20/F	10	3·3 (89)	hdm, cat	Salb, Nedoc
C5	38/M	N/A	3·8 (100)	Neg.	Nil
C8	25/F	N/A	3·9 (113)	Neg.	Nil

FEV_1, forced expiratory volume in 1 s; %, per cent predicted; hdm, house dust mite; rag, ragweed; Becl, beclomethasone; Salb, salbutamol; Nedoc, nedocromil; nil, no medication.

derived from sequences ultimately at the centre of the construction and hybridizing over a stretch of 20–25 nucleotides were designed. Taq polymerase adds dNTP to the 3′ ends of the annealed oligonucleotides and by that generates double-stranded DNA. At each later step, each strand of DNA anneals to one of two new oligonucleotides. Through repeated rounds of amplification the two oligonucleotide primers generate single-stranded and overlapping DNAs. In the later cycles of amplification (25 in total), the major product results from the annealing and subsequent filling in of these two strands of DNA. The internal standard also acted as control for possible nucleotide misincorporation that might have occurred during PCR (see below).

Resolution of amplification products into component bands

The subsequent analysis provided a detailed view of the extent of junctional diversity. Diversification of the junctions occurs by a largely random process that deletes variable, diversity and joining region nucleotides by a double-stranded DNA exonuclease, and adds nucleotides as P elements or non-germ-line elements (NGE). We resolved Vγ-Jγ amplification products into component bands using a 5% denaturing polyacrylamide gel. This provides single base resolution. The population of PCR products separates into components according to the different lengths at the junctions. The labelled PCR products were diluted 2 : 10 with loading dye (98% formamide, 20 mmol/*l* ethylenediaminetetraacetate, 0·05% bromphenol blue and 0·05% xylene cyanol), heated at 80°C for 3–5 min and promptly loaded onto a 5% polyacrylamide gel containing 40% urea ($20 \times 40 \times 0{\cdot}2$ cm, acrylamide: N,N′ bisacrylamide = 49 : 1; LiquidGel; Novex, San Diego, CA) and subjected to electrophoresis in 1·0 times Tris-borate-ethylenediaminetetraacetate). After electrophoresis for 5 h at a constant power of 50 W, the gel was fixed in 10% acetic acid and 12% methanol for 15 min and then dried on Whatman 3MM paper, and the bands were visualized by autoradiography at −70°C with intensifying screens after 12 h exposure.

A junction that maintains the reading frame will have no loss of nucleotides at the junction compared with the germ-line sequence or will have a loss or addition of multiples of three nucleotides. An analysis of previously reported VγII sequences [42] showed that 44% of polyclonal peripheral blood T cells and 52% of lung T cells expressed VγIIJγ sequences with identical lengths resulting from a net loss at the junctions of three nucleotides compared with the germ-line length. The junctional sizes distributed normally about this mean value [42]. On a sequencing gel we would observe a pattern of one dominant central band flanked by bands of progressively decreasing intensity. The presence of a T cell clone would produce some departure from the normal distribution of intensities [41]. The internal standards also controlled for diversity due to misincorporation of nucleotides during the PCR by generating always a single band. The addition or deletion of nucleotides during the process would produce the appearance of diversity.

In addition, we also resolved the Vγ-Jγ amplification products using a non-denaturing 5% polyacrylamide gel. This is an adaptation of the technique that detects single-strand conformation polymorphisms [43–46]. PCR products were diluted as described above and heated for 10 min at >80°C. These gels were prepared with MDE (Hydrolink, San Diego, CA), did not contain urea, had 5% glycerol and were run at room temperature at a constant power of 40 W for 8 h. The rate of migration through a non-denaturing gel will vary according to the nature of the backfolding of single-stranded DNA [43–46]. This technique detects differences in sequence, even single-base differences, that alter the conformation of single-stranded DNA [43–46]. The analysis of a polyclonal population would give a smearing based on differences in the junctional sequences. The emergence of a clone or group of clones produces discrete bands within the smear.

Subcloning and sequencing

We examined those families clearly implicated by PCR to be markedly expanded in use or showing evidence of clonal or oligoclonal expansion after resolution of the amplified fragments on polyacrylamide gels. This analysis established the degree of clonal expansion. Subcloning and sequencing of 15–20 independent clones showed the proportion of repeated sequences. To subclone the fragments derived from each family we appended the sequence CUACUACUACUA to the 5′ end of each oligonucleotide. We used these modified oligonucleotides with the CloneAmp subcloning system (GIBCO-BRL, Gaithersburg, MD) to subclone the PCR products. We screened individual colonies for plasmids containing VγI, II, III or IV inserts using ^{32}P-labelled random primed PCR products derived from the amplification of mixed populations of patient derived cDNA. We sequenced using the Sanger technique and the Cγ oligonucleotide primer GAAGAAAAATAGTGGGCTTGGGGGAA.

RESULTS

The Vγ TCR repertoire was restricted in BAL but not in PBL of asthmatics that were treated with inhaled steroids. Further, in subjects with no restrictions of the Vγ TCR repertoire, clonal expansion to T cells was found in BAL fluid.

Table 2 gives a summary of the TCR Vγ expression by BAL and peripheral blood T cells of asthmatics and healthy volunteers. BAL T cells from the two asthmatics who used inhaled steroids (A4 and A10), expressed only VγII (Fig. 1); and one of them (A4) showed limited Jγ region usage by BAL T cells. VγII rearranged with JγP and JγP1 only (Fig. 2) while the peripheral blood T lymphocytes showed expression of VγI, II and III in combination with all J segments (Fig. 3). Moreover, the VγIIJγP PCR products yielded a single dominant band on a denaturing polyacrylamide gel and resolved into two fractions on a non-denaturing polyacrylamide gel (Fig. 4); VγJγP1 products resolved into two fractions with either approach (Fig. 4). The data indicate an oligoclonal population of VγII$^+$ T lymphocytes in the lungs of both these patients.

The BAL T cells of the third asthmatic not receiving inhaled steroids (A13), expressed VγI, II and III (Fig. 5). Subcloning and sequencing of the BAL Vγ transcripts showed that all three expressed Vγ families at least in part derived from clonally expanded T cells (Table 3). One sequence constituted 36% of in frame VγI sequences. Two sequences constituted 55% of in frame VγII sequences. We found a higher percentage of repeated sequences with our analysis of the VγIII family, but distributed among more sequences. Six repeated sequences constituted 81% of the in frame VγIII repertoire. The BAL from one control (C5) expressed VγI, VγII and VγIII (Fig. 6). Subcloning and sequencing of BAL Vγ transcripts showed that the bulk of them also came from clonally expanded T cells (Table 4).

In summary, a stable asthmatic and a healthy volunteer exhibited oligoclonally expanded $\gamma\delta^+$ T cells in the lung compartment.

Table 2. Vγ region gene usage in the study subjects

	Bronchoalveolar lavage repertoire				Peripheral blood repertoire			
Subject	VI	VII	VIII	VIV	VI	VII	VIII	VIV
A4	−	+	−	−	+	+	+	−
A10	−	+	−	−	+	+	+	−
A13	+	+	+	−	+	+	+	−
C5	+	+	+	−	+	+	+	−
C8	+	+	−	−	+	+	+	−

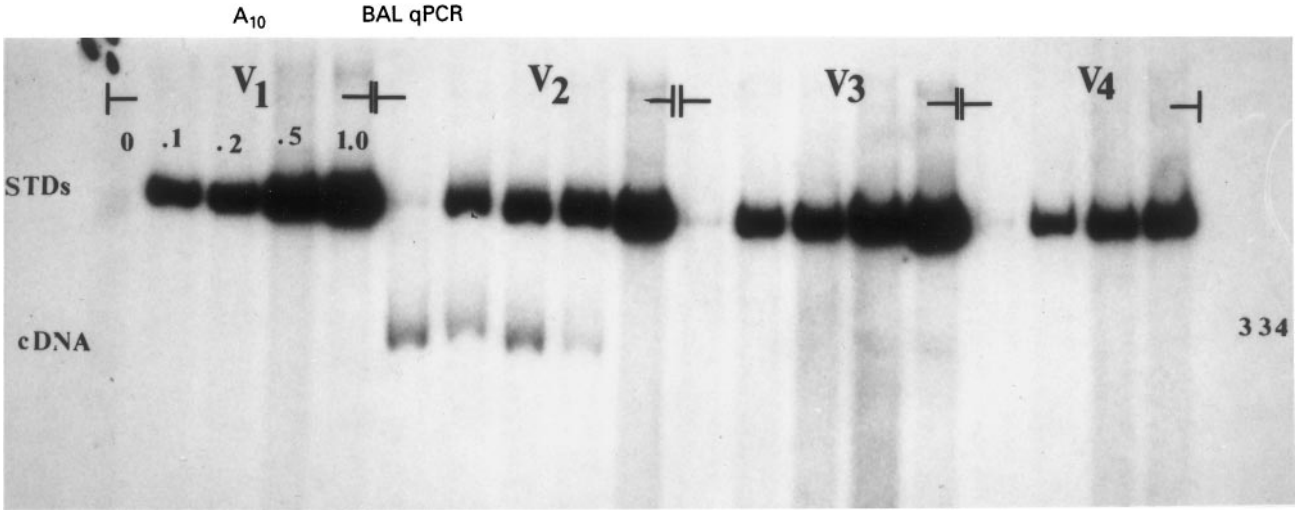

Fig. 1. The expression of each Vγ family was analysed in quadruplicate with a constant amount of cDNA and various amounts of internal standard (0 (negative control), 0·1, 0·2, 0·5 and 1·0 pg) with appropriate negative controls [66]. These results show cDNA encoding for VγII (about 334 base pairs). STDs denotes DNA internal standards and were confirmed by repeating polymerase chain reaction (PCR) separately using VγI, VγIII and VγIV 5′ primers.

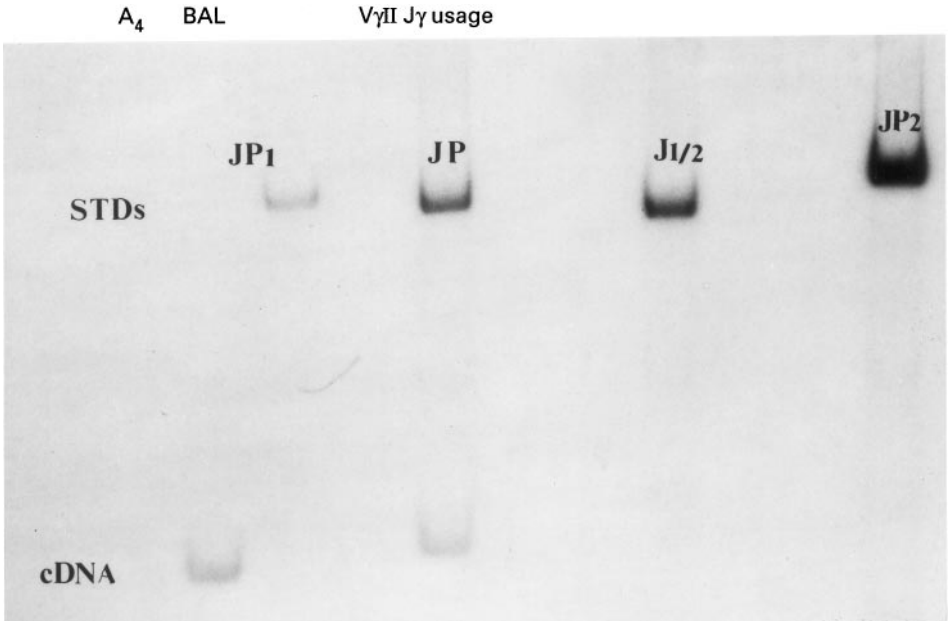

Fig. 2. A4 cDNA amplified using a labelled VγII oligonucleotide with each of the four Jγ primers. VγII rearranged with JγP and JγP1 only. Internal standards acted as controls. To test for competition between templates, JγP1 cDNA was amplified in a separate tube from the internal standard, while JγP cDNA and internal standard were amplified in the same tube. No inhibition or competition were apparent.

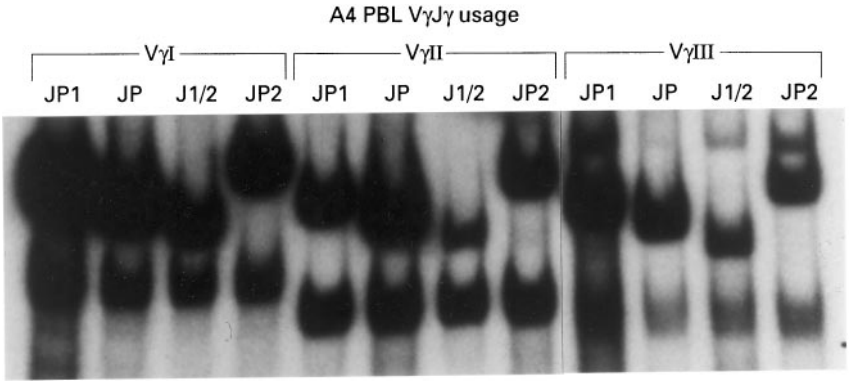

Fig. 3. A4 PBL cDNA amplified using a labelled VγII oligonucleotide with each of four Jγ primers. Internal standards acted as controls. So far the results suggested that BAL T cell receptor (TCR) Vγ repertoire was narrow in asthmatics but not in PBL.

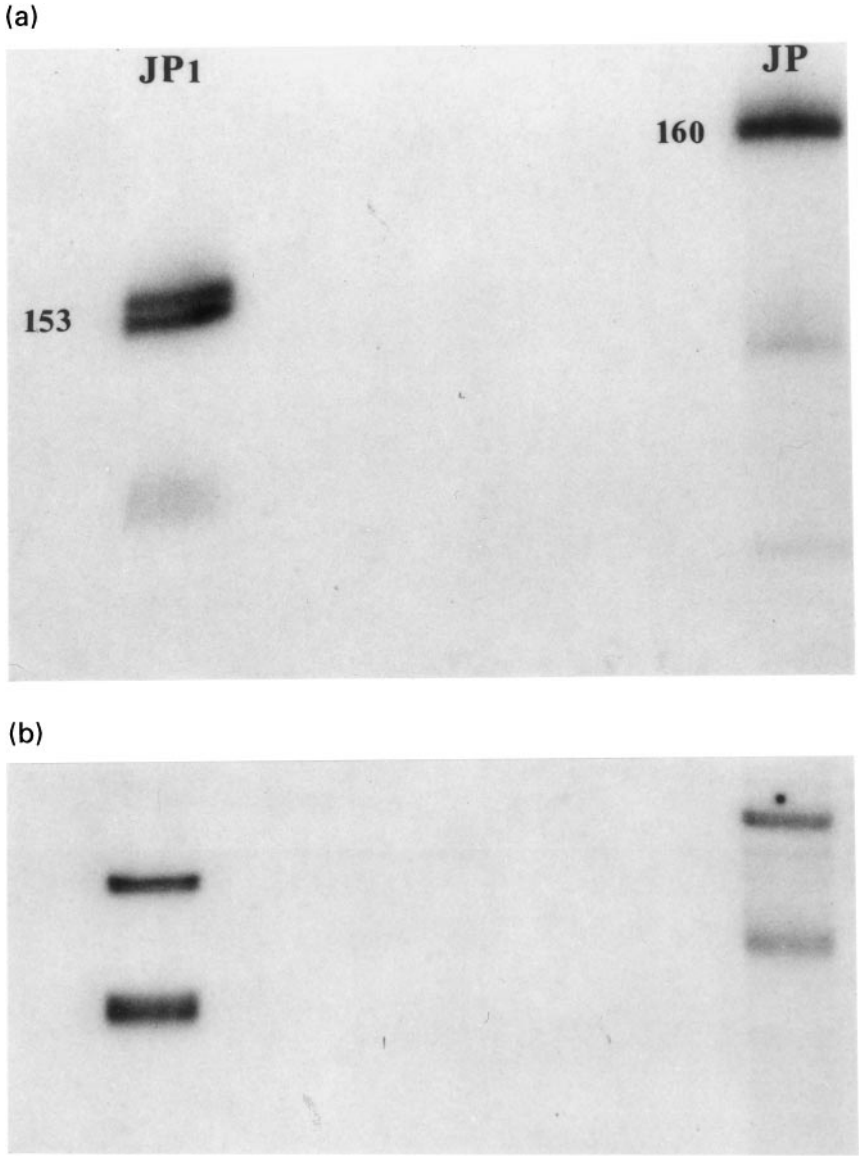

Fig. 4. Data showing the bulk of VγIIJγP1 and VγIIJγP in the A4 BAL appears to originate from an oligoclonal population of $V\gamma II^+$ T lymphocytes. (a) Bands separated in a denaturing (urea) gel. (b) Non-denaturing (SSCP) gel (see text). JP1 resolves into two major bands, JP remains as one major band. The sizes of the major bands were determined by a known sequence; JP1 runs at 153 and JP at about 160; the germ-line sequences would run at 174 nucleotides. There appears to be substantial deletion at the junctions.

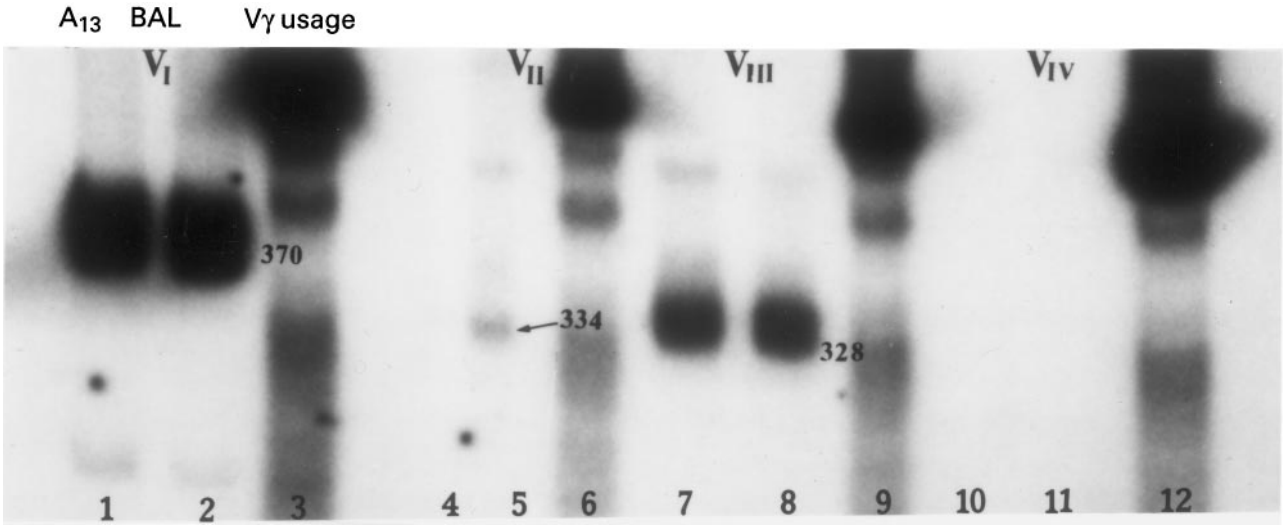

Fig. 5. Polymerase chain reaction (PCR) results from BAL of an asthmatic on no steroid therapy (A13). Each Vγ family if shown in three lanes. Lanes 1, 2 and 3 show VγI (cDNA duplicates and internal standard respectively); lanes 5 and 6 show VγII; lanes 7, 8 and 9 show VγIII, while lanes 10, 11 and 12 show VγIV.

Another two asthmatics (on inhaled steroids) expressed only VγII in BAL, one of whom exhibited rearrangement of VγII with JP and JP1. These PCR products resolved into few (four) bands in denaturing and non-denaturing gels, suggesting oligoclonality and deletions at the junctions. Moreover, subcloning and sequencing the TCR repertoire of one asthmatic and one non-asthmatic exhibited clonal expansion of cells expressing VγI, II and III.

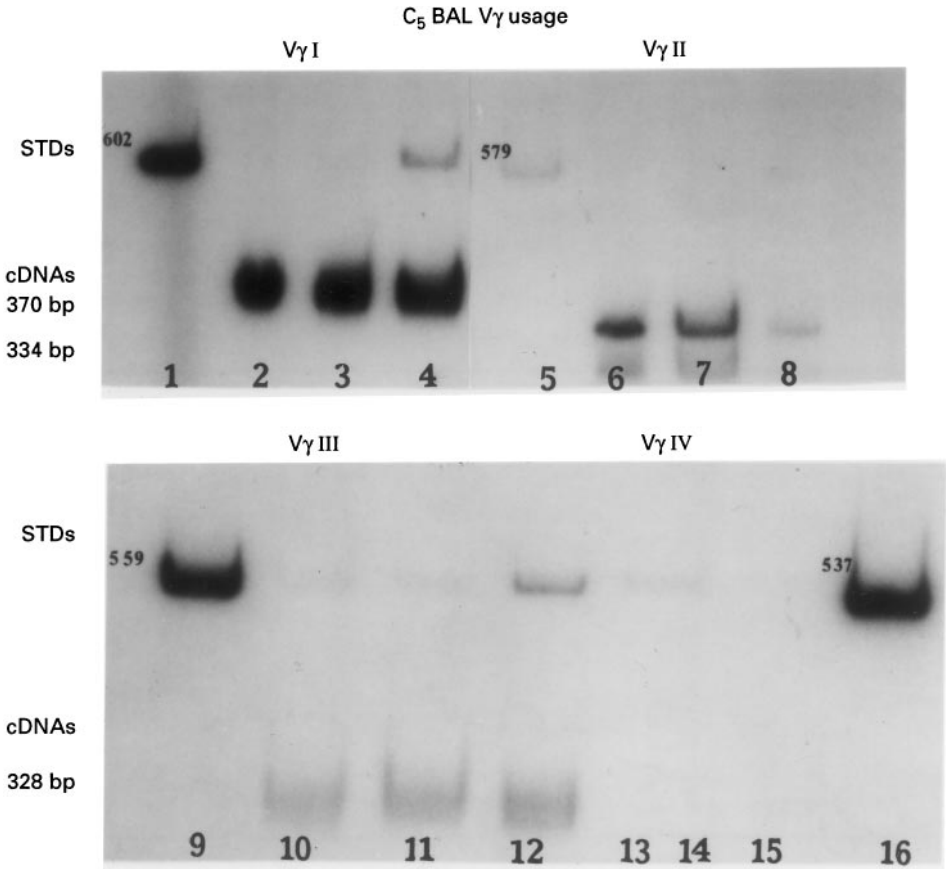

Fig. 6. Polymerase chain reaction (PCR) results from BAL of a healthy normal volunteer (C5). Each family is shown in four lanes. Lanes, 1, 5, 9 and 13 show internal standards only running between 537 and 602 base pairs. Lanes, 2, 3, 6, 7, 10, 11, 14, and 15 show cDNA only (duplicates), and lanes 4, 8, 12 and 16 show both internal standard and cDNA in the same PCR reaction.

Table 3. Junctional sequences of Vγ1–8$^+$ (VγI) mRNA transcripts from BAL of an asthmatic patient A13

Sample	Total in frame	No. of clones	V	N	J	Jγ	+/−
BAL	14	1	ACCTGGGACAGG	CCCAG	ATTATAAGAAA	V3J2	0
		1	ACCTGGGATG	AACCCAATCAATT	TTATTATAAGAAA	V4J2	+9
		1	ACCTGG	AAAAGGCGAAGT	AGTAGTGATTGGATC	V8JP2	+6
		1	(G)	GTGG	TTATTATAAGAAA	V4J2	−12
		1	ACCTGGGACAG	TTGG	TATAAGAAA	V3J2	−3
		5	ACCTGGGACGGG	CCCGT	A	V2J2	−9
		1	ACCTGGGACGGG	CCCCGAA	ATTGGATC	V2JP2	0
		1	A	TCCAG	TATAAGAAA	V3J2	−12
		1	ACCTGGG	CC	TATTATAAGAAA	V3J2	−3
		1	ACCTGGGA	CCTCG	(tc)	V4J2	

Junctional sequences of Vγ9$^+$ (VγII) mRNA transcripts

Sample	Total in frame	No. of clones	V	N	J	Jγ	+/−
BAL	9	2	TTG	CTGAAG	–	J2	−21
		3	TTGTGGGAGGTG	CG	TTATAAGAAA	J2	−3
		1	TTGTGGGAGGTG	CTGTTG	AAGAAA	J2	−3
		1	TTGTGGGAGGTG	CTG	GAGTTGGGC	JP	−3
		1	TTGTGGGAGG	GGGGTCAGG	ATTATAAGAAA	J2	+3
		1	TTGTGGGA	TAG	TTATAAGAAA	J2	−6

Junctional sequences of Vγ10$^+$ (VγIII) mRNA transcripts

Sample	Total in frame	No. of clones	V	N	J	Jγ	+/−
BAL	16	1	TGTGCTGCGTGGGAT	TATTTG	ACTGGTTGGTTC	JP1	+3
		2	TGTGCTGCGTGGGA	CCGGGACCTCA	(tt)	JP1	−9
		2	TGTGCTGCGTGGGA	ATG	TAAGAAA	J2	−6
		2	TGTGCT	CCACTACCTGAGAAG	–	J2	−9
		2	TGTGCTGCGTGGGAT	TATCCACCG	AATTATTATAAGAAA	J2	+9
		2	TGTGCTGCGTGGG	GTCCCCTGG	ATTATAAGAAA	J1	+3
		1	TGTGCTGCGTGGGAT	TATAG	TAAGAAA	J1	−3
		3	(tac)	GGTTGGTTTAGCGGGAATGGGG	ATTATTATAAGAAA	J2	+6
		1	TGTGCTGCGTGGGA	GACACGG	AATTATTATAAGAAA	J2	+6

Nucleotides in parentheses denote the first or last coding sequence of a longer sequence that has been spliced out. Positive and negative figures denote the number of nucleotides added or spliced from the germinal sequence.

DISCUSSION

Using a number of approaches, we have detected restricted Vγ and Jγ expression and oligoclonal expansion of $\gamma\delta^+$ T cells recovered from BAL of asthmatic and healthy volunteers. We have studied the Vγ repertoire of BAL and PBL T cells of both asthmatics and non-asthmatics using PCR (Figs 1–3). We have resolved the amplification products of VγJγ combinations by size and conformation to reveal limited diversity (Fig. 4). We have subcloned and sequenced the VγI, II and III amplification products to establish the extent of clonal or oligoclonal expansion (Tables 3 and 4).

Our PCR results show a restricted Vγ repertoire in two of the asthmatics' BAL, while the sequences of the complete Vγ repertoire in another asthmatic (not receiving inhaled steroids) and in one healthy volunteer were clearly oligoclonal. Taken together, these data support the view that the human lung may have clonally expanded $\gamma\delta^+$ T lymphocytes in the absence of drug therapy and disease. In this regard, our findings lend support to and extend those of Tamura *et al.* [42], who analysed only the VγII (Vγ9) transcripts in BAL from four healthy individuals, demonstrating that the VγII$^+$ population of T cells were monoclonally expanded. However, since corticosteroids can induce T cell apoptosis [47–49] and inhaled steroids can reduce both the total number of airway lymphocytes [12] and activation markers on the surface of T cells [11,12], the restricted expression of VγII by BAL T cells from two of our patients may be the result of the inhaled steroid therapy. The persistence of the most abundant VγII$^+$ T cell subset despite an overall decrease of $\gamma\delta$ T cells in BAL of our subjects is a plausible explanation for our findings in the two subjects using inhaled steroids [42,50]. Nevertheless, we cannot exclude with our data that the differences in the BAL Vγ repertoire among our asthmatics were related to differences in the clinical behaviour of their asthma, the patients expressing only VγII being the ones requiring inhaled

Table 4. Junctional sequences of mRNA transcripts from control C5 BAL

Sample	Total in frame	No. of clones	V	N	J	Jγ	+/−
Junctional sequences of Vγ1–8$^+$ (VγI)							
BAL	5	3	ACCTGGGAC	CTCG	(tc)	V4J2	−15
		1	ACCTGGG	CG	AGTAGTGATTGGATC	V4JP2	−3
		1	AC	GACC	TATAAGAAA	V8J2	−12
Junctional sequences of Vγ9$^+$ (VγII)							
BAL	10	3	TTGTGGGAGGT	CCACAACCCA	TATTATAAGAAA	J2	+6
		1	TTGTGGGAG	ATA	CAAGAGTTGGGC	JP	−3
		1	TTGTGGGA	TGTT	CAAGAGTTGGGC	JP-3	
		1	TTGTGGGAGGTG	–	CAAGAGTTGGGC	JP	−3
		2	TTGTGGGA	A	CAAGAGTTGGGC	JP-6	
		1	TTGTGGGAGGT	CTAT	GAGTTGGGC	JP	−3
		1	TTGTGGGAGGTG	CAGGGT	GAGTTGGGC	JP	0
Junctional sequences of Vγ10$^+$ (VγIII)							
BAL	2	1	TGTGCTGCGTGGGA	ACAT	ACCACTGGTTGGTTC	JP1	+3
		1	TGTGCTGCGTGGGA	CCGGGACCTCA	(tt)	J2	−9

Subcloning and sequencing of BAL Vγ transcripts form a control BAL showed that the bulk of them also came from clonally expanded T cells. Nucleotides in parentheses denote the first or last coding sequence of a longer sequence that has been spliced out. Positive and negative figures denote the number of nucleotides added or spliced from the germinal sequence.

steroid therapy rather than such treatment producing the restricted repertoire.

Studies aimed at determining clonal expansion of the γδ T cell population in the human lung are scarce. Groh *et al.* [31] did not detect γδ$^+$ T cells in fetal and adult lung using MoAbs, while Fajac *et al.* [51], using immunohistochemical techniques and specific MoAb, reported that T lymphocytes present in normal bronchi and alveolar parenchyma were predominantly of the αβ TCR phenotype, whereas γδ$^+$ T lymphocytes represented about 1% of the total CD3$^+$ cells. Agostini *et al.* recently reported [50] that Vδ2$^+$ T cells (which usually pair with VγII) comprise approximately 70% of the γδ$^+$ T cells in the normal lung. More recently it has been reported that the number of γδ$^+$ T cells in bronchial biopsies of asthmatics was not different from controls [52]. Yet in none of these studies was the functional status of such cells investigated.

The major limitations of our study are related to the number of subjects involved and the number of γδ$^+$ T cells that might have been present in the BAL fluids of these individuals. Because of these limitations, it is difficult to extrapolate our results to the whole lung compartment and/or to the broader population of atopic asthmatics. However, our study is one of the first that has analysed the Vγ TCR repertoire in humans, and our approach may serve the basis for future larger studies in Vγ or Vβ repertoires in asthma [53], particularly in light of the genetic linkage found between atopy and the α/δ locus in certain populations [37]. Because we studied γδ T cells recovered from BAL we cannot draw conclusions regarding the status of the airway mucosal γδ$^+$ T cell population. This population has been better studied in the intestine, where it comprises about 10% of the total epithelial cell number, and about 50% are thymus-independent.

γδ T lymphocytes are considered to be pre-activated T cells that are functionally activated whenever foreign antigenic material is encountered, and this rapid response system may help to keep an antigen in check during the first few days following exposure. Several lines of evidence suggest that γδ$^+$ T cells are involved in some early, non-specific resistance mechanisms [54–56], particularly the localization of different γδ subsets to different epithelia that are in contact with the external environment [24, 57–60] where they appear to function as activated cytotoxic T lymphocytes [61,62]. There is also evidence from both mice and humans [63,64] that γδ T cells originating from the recirculating pool are reactive to self 60–70-kD heat shock proteins. In this regard, Vignola *et al.* [35] have demonstrated up-regulation of hsp in the asthmatic airway epithelium by the technique of *in situ* hybridization. In addition, Hastie *et al.* reported an increase of hsp following allergen segmental challenge in atopic asthmatics [36]. Moreover, studies in animal models of viral pneumonia have demonstrated recruitment of non-cytotoxic γδ T cells with transcripts of IL-2, interferon-gamma (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF) or IL-10 long after virus are cleared in response to hsp65. These findings suggest that the cells might be clearing the inflammatory process by secreting the appropriate spectrum of interleukins [65].

In conclusion, our results show that γδ$^+$ T cells are expanded in the lung compartment of healthy and atopic asthmatic subjects. It

remains to be determined whether inhaled steroids restrict the Vγ region gene expression or the number of $\gamma\delta^+$ T lymphocytes, or if patients with a more restricted repertoire are the ones with more severe asthma and likely to receive inhaled steroids. Knowledge of the TCR repertoire, state of activation, function and specificity of $\gamma\delta^+$ T cells in asthma is relevant, since T cell-mediated immunity appears to be an important component of asthma.

ACKNOWLEDGMENTS

We thank Dr J. Jongstra and Dr A. Cohen for helpful comments, and The Toronto Hospital Asthma Centre personnel for help with the patients. N.A.M. was funded by the Medical Research Council of Canada.

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