

Human tonsillar germinal center T cells are a diverse and widely disseminated population

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Germinal center (GC) T cells are a poorly investigated population with a unique helper-inducer memory phenotype. Murine GC T cells have been demonstrated to be oligoclonal and antigen specific. The aim of this study was to investigate the diversity of human tonsillar GC T cells. Immunohistochemistry with a panel of different TCRV β family antibodies and sequencing of TCR γ gene rearrangements obtained from individual microdissected GC demonstrated local diversity of human tonsillar GC T cells. No evidence of local clonal dominance or of a local migratory pathway from the T cell zone to adjacent GC was seen. Primers specific to the junctional region of the TCR γ gene of individual GC T cell clones were designed, and used to trace the dissemination of the clones. One was found to be concentrated locally. Both clones studied were present in both of the patient's tonsils, suggesting widespread dissemination. In addition, no evidence of somatic hypermutation was found in the TCR γ gene sequences, or in expressed TCR α and β gene sequences obtained by reverse transcriptase-PCR from isolated tonsillar GC.

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1 Introduction

Germinal centers (GC), which develop in secondary lymphoid tissues during T cell-dependent antibody responses, are the sites of clonal expansion, affinity maturation and isotype switching of B cell blasts, giving rise to plasma and memory B cells. GC contain a unique population of T cells that have a helper-inducer memory phenotype, express the $\alpha\beta$ TCR, CD4 and the memory cell marker CD45RO. Between one and two thirds express NK cell marker CD57 [1]. They also express molecules associated with early T cell activation, such as CD69, but not the late activation markers CD25 or CD71 [1]. In chronically stimulated GC such as those in human tonsil they are non-proliferative [2, 3] and Bcl-2 negative [4].

Using a murine model involving the analysis of the GC T cell response to injected antigens, murine GC T cells have been demonstrated to be specific for the immunizing antigen, and to have proliferated in response to antigen stimulation [5–7]. When GC are induced by injection

with pigeon cytochrome c (PCC) or myelin basic protein, the GC T cell population is significantly enriched in TCRV α families associated with the response to the antigen, in comparison with the surrounding T cell zones (TCZ) and with unimmunized mice. Clonally related antigen-specific T cells have been identified in multiple murine splenic GC and in the surrounding T cell areas [7]. Adoptive transfer of allogeneic antigen-specific cells has demonstrated that the initial site of T-B cell contact is the periphery of the GC and that entry of antigen-specific T cells into the GC occurs subsequently [8].

In contrast to the lymphoid tissues in animal models, human tonsils are exposed to multiple diverse antigens, though individual GC are thought to contain few B cell clones [9]. In microdissected GC an average of three clones were identified [10]. Little is known, however, of the diversity of the associated T cell response. Using antibodies to TCRV β families we have looked for any evidence of local proliferation and clustering of related T cells in GC. We also used microdissection and PCR of rearranged TCR genes to study local T cell diversity, and carried out clone-specific PCR to study T cell dissemination. We have observed that human GC T cells are highly diverse and widely disseminated. The absence of any evidence of local clustering is consistent with a model where the T cells that populate GC divide and disseminate.

[1 19386]

Abbreviations: GC: Germinal Center TCZ: T cell zone
PCC: Pigeon cytochrome c

2 Results

2.1 Immunohistochemical analysis of V β usage in GC and TCZ

Frozen sections from three human tonsil blocks taken from two different patients were stained using a panel of monoclonal antibodies to different TCRV β families (Fig. 1). The number of cells stained brown for each V β and the number stained blue for CD3 were counted in each of four GC and four areas of TCZ. The proportion of total T cells expressing each V β in the different areas was then calculated for each block. These results are shown in Fig. 2. In some cases in patient 1, notably V β 8 in blocks L1 and L2 and V β 6 in block L1, the TCZ contained significantly more cells expressing these TCR compared with the GC ($p < 0.05$). The percentages were consistent between different GC and areas of TCZ within the tissues (data not shown). This observation was confirmed by an independent blind count. Double immunofluorescence staining was performed on sections from patient 1, blocks L1 and L2, using antibodies to TCRV β 8 and CD45RO. The percentage of CD45RO-positive cells also expressing TCRV β 8 was determined in multiple areas of TCZ and found to be approximately 6.9% in block L1 and 7.9% in block L2. This is not significantly different from the proportions of CD3-positive cells expressing TCRV β 8 in the GC (7.3% in block L1 and 8.47% in block L2). When sections were double-stained using Ki67 (immunoperoxidase) followed by CD3, V β 6 or V β 8, proliferating T cells could be observed in the TCZ, but there was no apparent dominance of V β 6 or V β 8 proliferation at these sites (data not shown). The number of cells that were positively stained with each V β antibody was

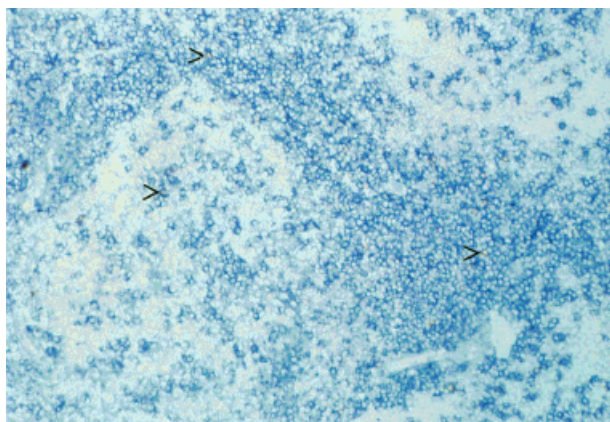


Figure 1. Frozen section of tonsil from patient 2 double stained using V β 8 (immunoperoxidase: brown) and CD3 (alkaline phosphatase: blue). V β 8⁺ cells are indicated.

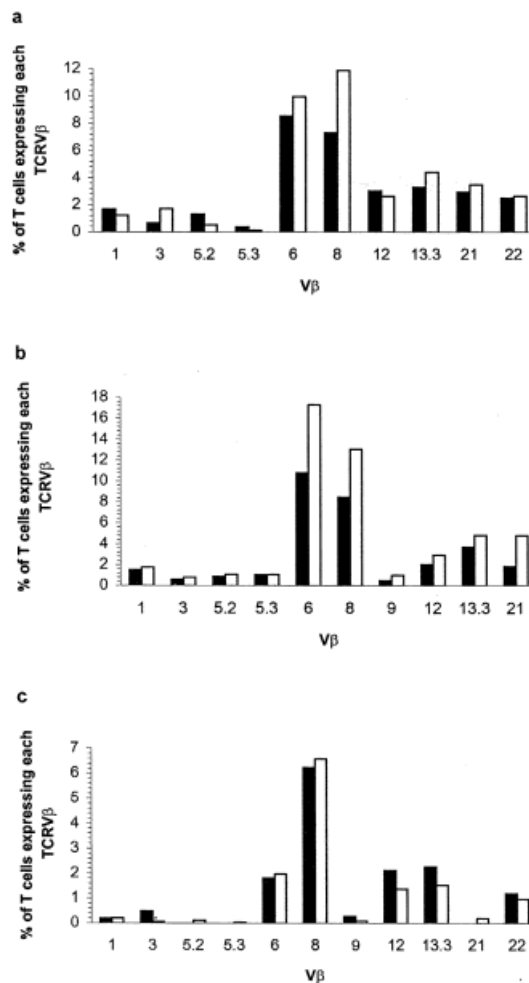


Figure 2. The percentages of T cells expressing each TCRV β were calculated in GC (solid bars) and areas of TCZ (open bars) in tonsils from two patients: (a) patient 1 block L1, (b) patient 1 block L2, (c) patient 2 block L1.

counted in 15 different GC from each section. This was done for all V β antibodies used, in one block from each of the two patients. T cells with rare TCRV β were distributed evenly between GC, with no clustering or local clonal dominance seen (data not shown).

2.2 Microdissection and sequencing of TCR γ gene rearrangements

Microdissection of GC was carried out on sections double-stained for Bcl-2 to define the limits of the GC and for CD3 to identify the T cells. Three microdissections from each of four GC (2 from patient 1 and 2 from patient 2) were performed on serial sections (Fig. 3). PCR to amplify TCR γ genes was carried out and the products cloned. In total 12 TCR from each of the 4 GC

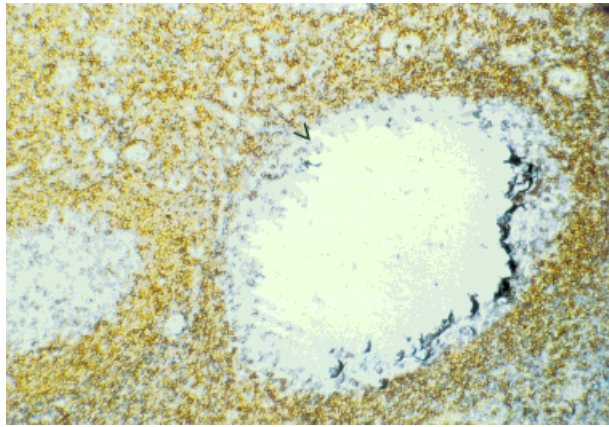


Figure 3. Frozen sections of tonsil double stained using Bcl-2 (immunoperoxidase: brown) and CD3 (alkaline phosphatase: blue). GC are Bcl-2 negative. A microdissected GC is indicated.

were sequenced. Analysis of the sequences showed that from patient 1, 9 different sequences were seen in GC A and 11 in GC B. From patient 2, 11 different sequences were seen in GC A and 8 in GC B (Fig. 4). Duplicate sequences came from the same PCR reaction. The number of different sequences seen demonstrates that the GC T cell populations are locally polyclonal. The mean number of N nucleotides added between the V and J regions in patient 1 was 4.65, with a range of between 1 and 10. For patient 2 the mean number was 8, with a range of 2 to 17. In most cases, due to the short length of the junctional region, or the number of repeated nucleotides, it was not possible to generate a clone-specific primer.

2.3 Wide dissemination of T cell clonotypes

Primers were designed to bind to the junctional regions of the TCR γ gene rearrangements obtained from the clones designated SK10 (containing 11 N nucleotides) and SK55 (containing 8 N nucleotides), both originally found in the left tonsil of patient 2. The primers and their binding sites are illustrated in Fig. 5. For clone SK10, semi-nested PCR with the same V region primer used in the original PCR reaction and the same cycle conditions was performed. The presence of a band of 330 bp when visualized on a gel was taken to be a positive result, indicating the presence of the clone. This was confirmed in one case by sequencing. The specificity of the primers was tested using 40 samples of DNA prepared from whole sections from two other patients. No PCR products of the correct size (compared to the original sample used as a positive control) were seen. For clone SK55,

a)

GC	V region used	V	N	J
A	1S2	GGACG	ACGCAGGG	TATAAGAACTCTTTGG
	1S3	ACAGG	CATG	AGAAACTCTTTGG
	1S3	ACAGG	CTAGAG	GAAACTCTTTGG
	1S4	GGGAT	TCGGAA	TTATTATAAGAACTCTTTGG
	1S4	GGATG	TAAC	TATAAGAACTCTTTGG
	1S4	TGGGA	G	GAAACTCTTTGG
	1S5	CTGGG	CCCTATGGG	CTTTGG
	1S8	ATAGG	CCGTAA	TTATTATAAGAACTCTTTGG
	1S8	ATAGG	GG	AGAAACTCTTTGG
B	1S3	ACAGG	CCCCAG	TAAGAACTCTTTGG
	1S4	TGGGC	CGCCG	GG
	1S4	ACCTG	TT	TTATTATAAGAACTCTTTGG
	1S4	TGGGC	CCCG	TAAGAACTCTTTGG
	1S4	ATGGG	GC	TAAGAACTCTTTGG
	1S4	TGGGA	TTCCCT	TTATTATAAGAACTCTTTGG
	1S4	CCTGG	C	TATTATAAGAACTCTTTGG
	1S4	TGGGC	CGGGAC	TTATTATAAGAACTCTTTGG
	1S4	GATGG	CCTTAGCAC	TATAAGAACTCTTTGG
	1S5	CTGGG	GTCGG	AAGAACTCTTTGG
	1S8	ACCTG	CCAGTC	ATTATAAGAACTCTTTGG

b)

GC	V region used	V	N	J	
A	1S2	ACCTG	TCTTGTAGGGG	AGAAACTCTTTGG	
	1S2	TGGGA	TTTTCTTTGGTCCGAA	TTATTATAAGAACTCTTTGG	
	1S2	GGGAC	TCAATTCAGGG	AGAAACTCTTTGG	
	1S4	ATGGG	AGAA	TTATTATAAGAACTCTTTGG	
	1S4	TGGGC	TAGGAGATTGG	TATTATAAGAACTCTTTGG	
	1S4	TGGGA	GAGTGAA	TTATTATAAGAACTCTTTGG	
	1S4	TGGGC	ATACTA	TTATTATAAGAACTCTTTGG	
	1S4	CTGGG	TCTG	TTATTATAAGAACTCTTTGG	
	1S4	CCTGG	CCCTTACCGGGG	TATAAGAACTCTTTGG	
	1S8	ATAGG	GGTAGC	TATAAGAACTCTTTGG	
	1S8	TGGGA	CCC	ATAAGAACTCTTTGG	
	B	1S2	CTGGG	GTATTTC	CTCTTTGG
		1S2	TGGGA	AGGGG	GAAACTCTTTGG
		1S4	GATGG	AACCCCTAA	TTATTATAAGAACTCTTTGG
1S4		TGGGA	GTTAT	TTATTATAAGAACTCTTTGG	
1S5		ACAGG	TTGGGT	TTATAAGAACTCTTTGG	
1S8		CTGGG	TCCCAACTTTA	TTATTATAAGAACTCTTTGG	
1S8		CTGGG	GC	TTATTATAAGAACTCTTTGG	
1S8		GATAG	TTCACAATTCGG	TTATAAGAACTCTTTGG	

Figure 4. Diverse TCR γ gene rearrangements were sequenced from microdissected GC (a) Patient 1. (b) Patient 2.

nested PCR was performed using an internal 5' primer VG2, which was designed to bind only to TCRGV1S2 and 1S4 genes. The same cycle conditions were used except for the annealing temperature, which was slightly increased in order to improve the specificity of the reaction. The presence of a band of 250 bp on a gel was taken to be a positive result. The specificity of the primers was tested on 69 DNA samples from two other patients, and no PCR products of the correct size were seen.

The primers were then used to investigate the dissemination of the clones through both tonsils. For SK10, a total of 90 DNA samples prepared from whole sections were taken from 5 different blocks of the left tonsil and 90 DNA samples from 6 different blocks were prepared from the right tonsil. All samples were approximately the same size; 43 out of 90 samples from the left tonsil and

SK10 5' -TGGGATGGGCT**AGGAGATTGGT**TATTATAAGAAAC-3'
 10A 3' -GATCCTCTAACCATAATATTCT-5'
 10B 3' -TACCCGATCCTCTAACC-5'
 SK55 5' -TGTGCCACCTGGGG**TATTTC**ACTCTTTGGCAGTGG-3'
 55A 3' -CATAAAGTGAGAAACCGTC-5'
 55B 3' -GGTGGACCC**CATAAAGT**-5'

Figure 5. Primers 10A and 10B were used in a semi-nested PCR reaction with a 5' V region consensus primer to amplify the SK10 TCR. Primers 55A and 55B were used in a nested PCR reaction with a 5' V region consensus primer and an internal TCRGV1S2 and 1S4 specific primer to amplify the SK55 TCR. The primers bind the junctional regions of the clones (shown in bold).

13 out of 90 from the right tonsil contained clone SK10 (Fig. 6). This is a significant difference ($p = 1.4 \times 10^{-6}$) using the χ^2 test. All of the different blocks tested from the left tonsil contained clone SK10, with a range of 15–75 % positive samples. This was not the case for the right tonsil, where one block gave no positive samples, and the maximum seen was 30 % (Fig. 6). Twenty-four microdissected GC and eight microdissected areas of TCZ (all from the left tonsil) were also tested with the clone-specific primers. Two samples of TCZ, but no GC, contained clone SK10.

For clone SK55, 80 DNA samples were prepared from 5 blocks of the left tonsil and 88 samples from 6 blocks of the right tonsil; 35 out of 80 samples from the left tonsil and 47 out of 88 from the right tonsil contained clone SK55. This is not a significant difference using the χ^2 test. All of the different blocks from both tonsils contained the clone, with a range of 10–55 % positive samples from the left tonsil and 20–78 % from the right tonsil (Fig. 6).

2.4 No evidence of hypermutation of TCR genes

In addition to the 29 TCR γ gene sequences obtained from microdissected GC, 11 different TCR α and 4 different TCR β gene sequences were obtained by reverse transcriptase (RT)-PCR from GC isolated by sedimentation from fresh tonsil. The sequences were analyzed for mutations by comparison to published germ-line sequences taken from the GenBank database. Forty-three base changes from germ line were found in a total of 18956 bp of sequence (Table 1). This gives an overall mutation frequency of 1 in 440.1 bp, compared to an expected PCR error rate of approximately 1 in 500 bp.

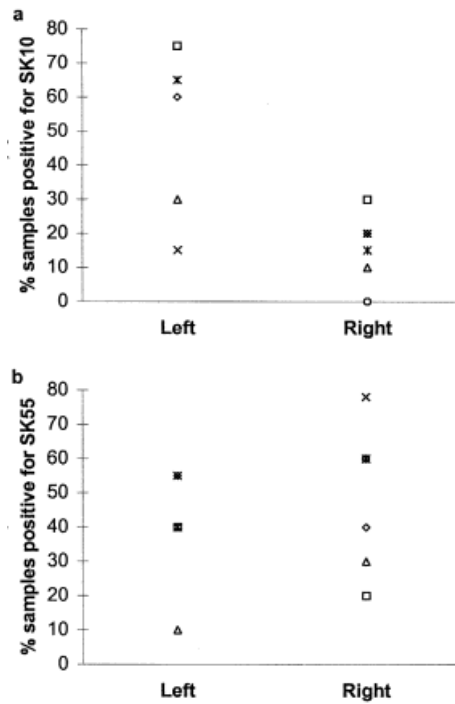


Figure 6. The percentage of sections from each block from the left and right tonsils that contained each clone are shown. For each tonsil, the different blocks are indicated by different symbols. (a) Clone SK10. (b) Clone SK55.

No significant differences in mutation frequencies were seen between the TCR α , β , and γ genes using the χ^2 test.

3 Discussion

The number of different TCR γ gene rearrangements found within individual GC strongly suggests that GC T cells are a locally polyclonal population. Twenty out of 24 clones sequenced from patient 1 and 18 out of 21 clones from patient 2 gave different sequences and in each

Table 1. Summary of base changes from germ line observed in TCR genes

	Number of sequences analyzed	Total number of bases analyzed	Changes from published germ-line sequences	Frequency of base changes from germ line
TCR α	11	3815	8	1 in 476.8
TCR β	4	1667	3	1 in 555.7
TCR γ	29	13360	32	1 in 417.5

case where duplicates were found, they came from the same PCR reaction. It is likely that this reflects cloning of identical PCR products, rather than sampling of related cells. This is a common problem when using small microdissected samples. As the TCR γ gene is the first of the TCR genes to rearrange during T cell development, it can be used as a clonality marker of both $\gamma\delta$ and $\alpha\beta$ T cells. Less than 1 % of the T cells in the GC of the tonsils studied were $\gamma\delta$ T cells (data not shown) and therefore the diversity observed is within the population of GC $\alpha\beta$ T cells. It might be argued that the diverse TCR γ sequences observed may come from B cells which have rearranged their TCR genes. However, this is highly unlikely because of the larger number of sequences obtained, compared with the small number which could be expected from an oligoclonal B cell population. The range of TCR sequences observed therefore undoubtedly represents a high degree of diversity within the GC T cell population. This is supported by the lack of variation in the composition of individual GC and the fact that no clusters of cells expressing rare V β segments were seen either in the GC or in the TCZ.

These results contrast with studies in mice, which find that the GC T cell population is clonally restricted, compared to the polyclonal T cell area. Fuller et al. [5] observed a sevenfold higher prevalence of V α 11⁺ T cells in lymph node GC (an average of 50 %) compared to the surrounding paracortex when the mice were immunized with PCC, and a similar difference in V β 8⁺ cells when immunized with MBP. These ratios were maintained at least until the GC began to diminish (by day 24). Others have reported similar findings [6, 7] although Gulbranson-Judge and MacLennan [6] noted that a substantial proportion of T cells migrating into follicles did not appear to be PCC specific. They suggested this contrast with the results of Fuller et al. [5] and Zheng et al. [7] might be due to differences in immunization conditions. Zheng et al. [7] also employed microdissection and PCR techniques on murine spleen and found that more than 90 % of the TCR rearrangements recovered from the GC could also be found in both nearby T cell areas and in distant GC, suggesting a specific migration pathway from a site of proliferation in the T cell area into GC.

The observations described here and the contrast to murine studies could be explained by the recently proposed theory of T cell cross-reactivity [11] which argues that because there are far more potential foreign peptides than there are T cells produced by a mouse, each individual cell must be able to recognize multiple peptides. Mason calculated that between 10² and 10⁵ different T cell clonotypes (out of about 10⁹ naive T cells found in the mouse) could react (with varying affinities) with a single peptide-MHC complex. This figure is comparable

with that calculated in a recent study by Butz and Bevan [12]. Given that the total number of T cell clonotypes in man is significantly greater, the number of different potentially reactive T cells must be correspondingly greater. Since so many different T cells can react with the same peptide, the GC T cell population could be polyclonal but still antigen specific. This does leave the question of why GC T cells in mice appear to be clonally restricted. In the case of PCC, the 23 amino acid peptide recognized by the V α 11V β 3 T cells differs from its murine homologue by only one amino acid. It is possible that the majority of the T cells recognizing this peptide were also self reactive and were therefore deleted in the thymus before they could ever reach the circulation and that the V α 11V β 3 subset were the only population to consistently avoid negative selection. It could also be possible that the “non-specific” T cells observed in the follicles of PCC-immunized mice by Gulbranson-Judge and MacLennan [6] were in fact part of a diverse population responding to the same antigen. It should, however, be remembered that human tonsils are exposed to multiple diverse antigens which could generate a diverse local effector T cell population. This would contrast with the locally oligoclonal B cell response generated in GC by the same antigenic load.

Clusters of proliferating T cells were observed in the TCZ of the tonsil, consistent with previous studies on the T cell areas in mice [13, 14] and in human Peyer's patches [15]. The failure to observe local clusters of cells expressing any given V β suggests that no accumulation of dividing T cells occurs in the microenvironments of the human tonsil and no evidence of a local migration pathway from the TCZ to the adjacent GC can be seen. Two T cell clones have been shown to be widely disseminated, occurring in virtually all of the different blocks studied from both the left and right tonsils. However, local concentration of clone SK10 was observed, since it was seen significantly more frequently in the left tonsil, from which it was originally isolated. This suggests that a precursor cell proliferated in the TCZ or the early GC, the progeny migrated throughout this tonsil and some of them returned to the blood and localized in the other tonsil. The chemokine DC-CK1, which may be produced by T cell-stimulating dendritic cells [16], is proposed to attract naive T cells into the GC [17], whilst ABCD-1 attracts activated T cells [18]. It is difficult to see how they could attract only highly antigen-specific cells.

Hypermutation of TCR α genes in mice [19] and TCR β genes in HIV-positive humans [20] have been reported. In this study, changes from germ line in the TCR genes were only seen at a rate equivalent to that expected to occur by PCR error. It can be concluded that no evidence of hypermutation in TCR genes could be found.

The possibility that it occurred at lower and hence undetectable rates cannot be excluded, but it must be considered whether such a low rate would have any real biological significance, particularly in the light of the theory of T cell cross-reactivity.

4 Materials and methods

4.1 Tissues

Fresh human tonsils were received on the day of surgery. Tissue was either placed in culture medium for isolation of GC, or cut into blocks, snap frozen and stored in liquid nitrogen until use. Tonsils from two female patients aged 5 and 12 years were investigated. Blocks from the left and right tonsils were designated L1, R1, L2, R2 etc. Tonsil tissue from several other patients was used as controls for clone-specific PCR.

4.2 Isolation of GC

Cell suspensions were produced by gently teasing blocks of fresh tonsil in RPMI medium (Imperial Laboratories, Andover, GB) containing 10 % FCS. The solution was left for 5 min for the GC to sediment and the supernatant removed. The GC were resuspended and the sedimentation repeated four times. Immunohistochemistry using Ki67 and R4/23 was carried out using cytocentrifuge preparations to confirm GC origin of the vast majority of cells. It was not possible to quantify the T cells sampled since the GC remained in three-dimensional clusters.

4.3 Immunohistochemistry

Acetone-fixed 8- μ m frozen sections and 100 μ l cytocentrifuge preparations were stained using an indirect immunoperoxidase method with a horseradish peroxidase-conjugated rabbit antiserum to mouse Ig (Dako Ltd, High Wycombe, GB) and diaminobenzidine substrate. The sources and specificities of primary antibodies used are shown in Table 2. For double staining an anti-CD3 or an anti-TCRV β antibody was added and the sections incubated overnight at 2–4 °C. These antibodies were detected using biotin-conjugated rabbit anti-mouse monoclonal antibody (Dako Ltd), followed by an avidin-alkaline phosphatase conjugate. Bound alkaline phosphatase was visualized using fast blue substrate. It was not necessary to use isotype-specific antibodies for primary and secondary stainings, as identification of double-stained cell surfaces was not required. Sections intended for microdissection were left unmounted and air-dried.

For immunofluorescence, a biotin-conjugated anti-TCRV β 8 antibody (Pharmingen, Oxford, GB) and a FITC-conjugated anti-CD45RO antibody (Pharmingen) were added to fixed sections, and incubated at room temperature for 1 h. This

Table 2. Primary antibodies used in enzyme-linked immunohistochemistry

Antibody specificity	Dilution	Clone	Isotype	Source
V β 1	1:400	AHUT7	IgG1	Pharmingen
V β 3	1:100	LE89	IgG2a	Immunotech
V β 5.2	1:200	36213	IgG1	Immunotech
V β 5.3	1:100	3D11	IgG1	Immunotech
V β 6	1:400	OT145	IgG1	T Cell Sciences
V β 8	1:400	56C5	IgG2a	Immunotech
V β 9	1:200	AMKB-1	IgG1	Pharmingen
V β 12	1:400	S511	IgG2a	T Cell Sciences
V β 13.3	1:200	JU-74	IgG1	Immunotech
V β 21	1:200	IG125	IgG2a	Immunotech
V β 22	1:200	IMMU546	IgG1	Immunotech
CD3	1: 50	UCHT1	IgG1	Dako Ltd
Bcl-2	1: 50	124	IgG1	Dako Ltd
Ki67	1: 50	Ki67	IgG1	Dako Ltd
Dendritic reticulum cell	1: 20	R4/23	IgM	Dako Ltd

was followed by an avidin-tetramethylrhodamine (TRITC) conjugate for a further 30 min. Sections were mounted in fluorescent mounting medium (Dako Ltd) and stored at 2–4 °C in the dark until viewing.

4.4 Counting the proportion of T cells expressing each TCRV β

The number of cells stained with each V β antibody and the number stained with CD3 were counted separately in a minimum of four GC and four areas of TCZ. The proportions of T cells expressing each V β were calculated. An independent assessor confirmed the results by blind counts.

4.5 Microdissection and preparation of DNA samples for PCR

Tonsil sections to be used for microdissection were double-stained with Bcl-2, to outline the GC and CD3 to identify the T cells. GC cells (approximately 30–100 T cells sampled per GC) and cells from small areas of TCZ (approximately 500–1000 T cells) were microdissected from sections under

50 % ethanol using a Leitz micromanipulator. DNA was released from microdissected fragments using 10 μ l proteinase K as previously described [21].

4.6 Amplification and sequencing of TCR γ gene rearrangements recovered from GC

All PCR reactions were performed on a Hybaid Omnigene thermal cycler using 1.5 mM MgCl₂, 200 μ M each dNTP, 1 \times reaction buffer and 0.5 U Taq DNA polymerase (Promega, Southampton, GB) in a total volume of 50 μ l. Of the extracted DNA 5 μ l was amplified using 100 ng of a V γ consensus primer 5'-TCTTCCAACCTTGAAGGGAGA-3' and 250 ng of a J region primer 5'-GTTACTATGAGC(T/C)TAGTCC-3' [22, 23]. After a hot start of 7 min at 95 °C, 40 cycles of 93 °C for 1 min, 55 °C for 1 min and 73 °C for 1 min were performed, followed by a final extension time of 7 min at 73 °C. PCR products were cloned and sequenced as previously described [24]. Some sequencing was also performed by Qiagen Sequencing Services.

4.7 RT-PCR and sequencing of expressed TCRV α and V β 8 genes

RNA was extracted from the isolated GC and reverse transcription performed as previously described [24]. Semi-nested PCR to detect expressed TCR α genes was carried out as previously described [25] on 5 μ l of cDNA using 100 ng each of the following primers: 5'-TGAGTGTCCC(G/A)GA(G/A)GGG(T/G/A)G-3' (V α consensus), 5'-GAGGG-AGCACAGGCTGTCTT-3' (C α 1 – first round only), 5'-GGCAGACAGACTTGTCACTGGAT-3' (C α 2 – second round only). Semi-nested PCR to detect expressed TCRV β 8 genes was performed on an additional 5 μ l of cDNA using 100 ng each of the following primers: 5'-TTGCATCTTGGTAGC-AAAGCACAC-3' (V β 8), 5'-GCCTTTTCCCTGTGGGAGAT-3' (C β Q – first round only) and 5'-GGCTCAAACACAGCGACCT-3' (C β N – second round only) [26]. For the first round, after a hot start of 96 °C for 2 min, 5 cycles of 96 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min 30 s were performed, followed by 35 cycles of 96 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min 30 s and a final extension time of 72 °C for 10 min. For the second round: after a hot start of 94 °C for 2 min, 40 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min 30 s were performed, followed by a final extension time of 72 °C for 10 min. PCR products were cloned and sequenced as described above.

4.8 Clone-specific PCR

Nested PCR primers were designed to bind specifically to the junctional regions of a number of TCR γ gene sequences. They were tested using the original sample from which the sequence was obtained as a positive control, and 40 samples in total taken from two other patients as negative con-

trols. In the initial set of experiments, the V region consensus primer, reaction mix and PCR conditions were as described above for TCR γ gene PCR. Primers appearing exclusive to the clone SK10 were identified and their specificity confirmed by the sequencing of a PCR product. These primers were used in further reactions performed on samples of DNA prepared from whole sections and from microdissected areas of GC and areas of TCZ. The presence of a 330-bp band in the PCR products indicated the presence of at least one cell of clonotype SK10 within a sample. To identify further clone-specific primers, fully nested PCR was performed using primers to the junctional regions as above, the V region consensus primer and an internal 5' primer designed to bind only on the TRGV1S2 and 1S4 genes: 5'-GCTGAAGGAAGTAACGG-3'. The annealing temperature was increased from 55 °C to 58 °C. Primers exclusive to clone SK55 were identified and used to amplify DNA samples prepared from whole sections. A 250-bp band indicated the presence of at least one cell of clonotype SK55 within a sample.

4.9 Statistical analysis

The χ^2 test was used to test the significance of all comparisons.

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