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Single-Cell PCR Analysis of T Helper Cells in Human Lymph Node Germinal Centers

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The T helper cell population of human lymph node germinal centers (GCs) was analyzed for clonality and signs of antigen selection. Frozen sections of lymph node biopsies taken from three different individuals were used to micromanipulate single T cells from one particular GC for each of the specimens. T cell receptor (TCR) β gene rearrangements were amplified from these single cells and directly sequenced. Although only unique rearrangements were amplified from T cells of GC2 and GC3, 11 of 28 potentially functional rearrangements amplified from GC1 originated from four different clones. In all three GCs, TCR gene rearrangements neither showed obvious biases in gene segment usage nor similarities in complementarity determining region 3 amino acid sequence. Thus, it appears that T lymphocytes in human GCs usually represent a diverse population of cells. Sequence analysis of V region genes did not provide evidence that in the human the process of somatic hypermutation acts on the TCRβ loci. For one of the GCs (GC3), immunoglobulin heavy chain (IgH) gene rearrangements were amplified and sequenced from single micromanipulated GC B cells. The detection of clonal expansions accounting for more than half of the sampled B cells in addition to ongoing somatic hypermutation of Ig V region genes suggested that GC3 was a fully developed GC. *(Am J Pathol 2000, 156:1067–1071)*

Germinal centers (GCs) are the sites of maturation of T-cell-dependent antibody responses.[1] T helper (T_H) cells account for 5 to 10% of all GC cells and are mainly found in the light zone (LZ) of the GC.[2] GC T cells represent a specialized subset of CD4+ T cells, which seem to be specific for the immunizing antigen.[3] Analysis of the response of mice to nitrophenyl-pigeon cytochrome c (NP-PCC) revealed that although they drive GC B cell development and selection, GC T cells themselves are under selection for antigen recognition.[4] By day 14 after immunization with NP-PCC, the GC T cell population is dominated by cells expressing characteristic T cell receptor (TCR) chains composed of particular gene segments and complementarity determining region 3 (CDR3) sequence motifs known to confer recognition of PCC.[4] It had earlier been shown that in human lymph nodes (LNs), as in hapten-immunized mice, clonal competition within the B cell population of a particular GC results in dominance of few clones that account for the bulk of the population.[5] We have now addressed the question whether T_H cells in GCs of human LNs, like the B cells with which they interact, are an oligoclonal population as a result of selection for peptide/MHC recognition. Single GC T cells were micromanipulated from histological sections of human LNs. TCRβ gene rearrangements were amplified from these single cells and their sequences compared for signs of antigen selection. Because somatic mutations have been observed in TCR genes of murine GC T cells[6] and CD8+ T cells invading GCs in HIV patients,[7] the sequences of TCRβ genes amplified from single micromanipulated GC T cells were also analyzed for mutations which may have been introduced by the process of somatic hypermutation.

Materials and Methods

Patients

Patients 1, 2, and 3 were female. At the time of tissue sampling they were 25, 30, and 17 years of age, respectively.

Immunostaining and Micromanipulation

Frozen sections of LN tissue were stained with the monoclonal antibody Ki67 (Dako, Glostrup, Denmark) or antibodies against $\alpha\beta$ TCR (βF1, T Cell Diagnostics, Woburn, MA), CD8 (C8/144B, Dako) or CD20 (L26, Dako) as described.[8] Single cells were mobilized and transferred into polymerase chain reaction (PCR) tubes using hydraulic micromanipulators as described.[5,8]

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Table 1. Summary of the Single-Cell Analysis of GC T Cells

GC	Micromanipulated cells	Positive for specific PCR product	Rearrangements potentially functional* +	Rearrangements potentially functional* −	Clonally related†	
1	136 GC T cells	37/136 (27%)	28		clone 1	5/28
					clone 2	2/28
					clone 3	2/28
					clone 4	2/28
				8	clone 3	2/8
2	90 GC T cells	20/90 (22%)	16			0/16
				4		0/4
3	129 GC T cells	36/129 (28%)	24			0/24
				8		0/8
Controls	34 GC B cells	0/34				
	34 buffer samples‡	0/34				
	34 water controls	1/34				
	30 single FACS-sorted T cells	23/30 (75%)	18			0/18
				7		0/7

*The reading frame status could be determined unequivocally in 113 of the 125 Vβ gene rearrangements amplified from micromanipulated and control T cells. Among these 113 rearrangements, 27 (24%) were nonfunctional (*ie*, rearrangements of a pseudogene, rearranged out-of-frame, or nonfunctional due to a stop-codon in CDR3).

†Identical rearrangements amplified from different cells are listed as clonally related.

‡Samples of buffer covering the sections during the micromanipulation procedure.

Amplification of TCR and Ig Gene Rearrangements from Single Cells

TCR Vβ gene rearrangements were amplified in a seminested PCR as described.[8] Briefly, a first round of amplification was performed using a mix of 25 Vβ gene family-specific and seven Jβ-specific primers. In a second round of PCR, the same Vβ primers were used together with a mix of seven internal Jβ-specific primers.

Ig heavy chain gene rearrangements were amplified from samples containing two cells each as previously described.[9,10] Forty samples were analyzed using V_H FRI family-specific primers,[10] and 46 samples were analyzed using V_H leader region family-specific primers[9] together with a collection of J_H segment primers in a seminested PCR using internal J_H primers in the second round of amplification.

Sequence Analysis

PCR products were gel-purified and directly sequenced using the Ready Reaction DyeDeoxyTerminator cycle sequencing kit (Perkin Elmer, Foster City, CA) and an ABI 377 automatic sequencer. DNASIS software (Pharmacia, Freiburg, Germany) and the GenBank data library (release 93.0) were used to analyze the V gene sequences. Sequences were deposited in the EMBL database under accession numbers AJ389943-AJ390023 (TCR sequences) and AJ389914-AJ389942 (Ig sequences).

Results

Micromanipulation and PCR Analysis of GC T Cells

Biopsies of cervical LNs from three patients were analyzed. All patients had suffered from lymphadenopathy, and LNs were excised to rule out a neoplastic process. The lymph node swelling was clinically manifest several weeks and several months before biopsy in patient 1 and 2, respectively. (This information was not available for patient 3.) Histopathologically, a normal LN architecture with numerous GCs was found. An immune response against an unknown pathogen was considered the most likely explanation for the clinical symptoms.

Frozen sections were stained for the $\alpha\beta$TCR, CD20, and Ki67 to identify T cells, B cells, and proliferating cells, respectively. For each LN specimen, one GC was selected (GC1, GC2, and GC3 from tissue samples of patients 1, 2, and 3, respectively) that showed clear polarization into a dark zone (DZ) with predominantly proliferating Ki67+ cells and few T cells and a LZ mainly composed of Ki67− cells and numerous T cells.

Sections stained for the $\alpha\beta$TCR were used to isolate single GC T cells. Most T cells were micromanipulated from the LZ of the GCs. For GC1, a minor fraction (about 10%) and about one-third of the T cells in GC2 and 3 were micromanipulated from the junction of the LZ and the follicular mantle. The cells were analyzed for TCR Vβ gene rearrangements on the level of genomic DNA by single cell PCR using mixtures of Vβ family-specific and Jβ-specific primers.[8] $\alpha\beta$TCR− cells were micromanipulated from the same GC as negative controls. Aliquots of the buffer covering the sections during the micromanipulation procedure were aspirated as additional negative control (buffer samples, Table 1). Single T cells from blood of healthy donors were sorted into PCR tubes as described earlier[8] and served as positive controls. All control samples were analyzed in parallel with the GC T cells. Of the sorted single T cells, 75% were positive for at least one specific product. The sequences obtained from these cells were all unique and unrelated to those obtained from GC T cells. Of 355 T cells micromanipulated in total from the three GCs, 93 (26%) yielded a specific product. One of 34 tubes containing PCR buffer but no

cell (water controls, Table 1) yielded a unique Vβ gene rearrangement, which likely represents a rare incidence of PCR product contamination. The results of the control amplifications are in accordance with our earlier data, which suggest that the single-cell amplification of TCRβ gene rearrangements is reliable with respect to unbiased amplification of the majority of all possible rearrangements and the assignment of sequences to individual cells.[8]

Expanded GC T Cell Clones Are Detectable in Only One of Three GCs

Twenty-eight potentially functional Vβ gene rearrangements were obtained from T cells of GC1 (Table 1). Among these, the rearrangements from five different cells were identical to each other, indicating that these cells belonged to the same T cell clone (clone 1). Three additional rearrangements were amplified from two different cells each (clones 2–4). Clone 3 also harbored an out-of-frame rearrangement. The remaining 17 potentially functional and seven nonfunctional rearrangements were unique, ie, they were each amplified from one cell only. Members of the four expanded clones in GC1 were in three instances located in proximity to a clonally related cell compatible with recent division of a common progenitor, whereas other clonally related cells were found at locations distant from each other (data not shown).

All 16 and 24 potentially functional rearrangements (and all four and eight non-functional rearrangements) amplified from T cells of GC2 and GC3, respectively, were unique.

Rearrangements that potentially encode a functional TCRβ V region may have been subject to selection by antigen. Therefore, the potentially functional rearrangements obtained from each GC were compared with each other and with a collection of 60 potentially functional Vβ gene rearrangements that were amplified from blood of four healthy donors[8] in an attempt to find signs of antigen selection. However, the usage of gene segments from the various Vβ gene families and of Jβ gene segments as well as the distribution of CDR3 lengths in TCRβ gene rearrangements from GC T cells did not show obvious biases in comparison to the control collection[8] (clonally related sequences from GC1 were counted only once; data not shown).

The Vβ gene rearrangements were also compared with respect to deduced CDR3 amino acid sequences. Sequences were aligned according to the conserved CASS amino acid motif encoded by Vβ and the conserved Jβ-encoded FG motif. In GC1, the TCRβ gene rearrangement of clone 4 showed some similarity to a TCR V region sequence, which was obtained from one cell only. The rearrangements used Vβ gene segments from closely related Vβ families (BV5 and BV23), used the same Jβ gene segment, and had an identical CDR3 length. The two amino acids encoded by the hypervariable VDJ junction, however, were dissimilar (CASS*TR*SYEQYFG (clone 4) and CASS*LD*SYEQYFG).

TCRβ Gene Rearrangements of Human GC T Helper Cells Are Not Subject to Somatic Hypermutation

Because products of single target amplification were directly sequenced without prior subcloning, the sequences are essentially devoid of errors introduced by the thermostable polymerases. Therefore, the sequences amplified from GC T cells allow investigation at a high level of resolution of whether somatic mutations are introduced into TCRβ V region genes of these cells in the course of the GC reaction. An unambiguously readable Vβ gene segment sequence (1.7×10^4 bp) from GC T cells contained only 1 bp that could not be matched with published human Vβ gene segment sequences and may thus represent either a rare mutation event or a polymorphism.

B Cells of GC3 Are Characterized by Clonal Expansions and Ongoing Hypermutation of Ig V Region Genes

The composition of the GC T cell population is likely dependent on the maturity of the GC reaction. All three GCs analyzed showed the clear polarization into DZ and LZ that usually develops several days after the onset of the GC reaction. Clonal expansion of GC B cells and the load of mutations introduced into Ig V region genes are additional indicators of GC maturity. Therefore, for one of the GCs, GC3, single B cells were micromanipulated from Ki67-stained sections and analyzed for rearrangements of the IgH locus by single cell PCR. Single Ki67+ and Ki67− cells were isolated from DZ and LZ. Because B cells account for about 90% of all GC cells, most of the cells isolated in this manner are B cells.[5] A total of 172 cells yielded 29 V_H gene rearrangements. As positive control for the V_H gene PCR, single sorted B cells were used. Twenty-eight V_H gene rearrangements (2 V_H1, 15 V_H3, 9 V_H4, and 2 V_H5) were amplified from 45 B cells, demonstrating that V_H region genes of various families were efficiently amplified.

Several reasons might account for the relatively low efficiency of the V_H gene PCR analysis of GC cells. First, about 10% of the micromanipulated cells may have been non-B cells, since the sections were not stained with a B cell-specific antibody (see above). Second, somatic mutations in a V_H region gene can prevent amplification. If such mutations are present in a V_H region gene of a large B cell clone, many cells will remain negative in the PCR analysis. Third, because centroblasts are large cells, often a fraction of the nucleus will not be present in the section used for single cell isolation (the thickness of the section was in the same range as the diameter of small lymphocytes, ie, about 10 μm).

Among 18 potentially functional rearrangements amplified from GC B cells of GC3, 8 belonged to one of three clones (Table 2). In addition, 8 of 11 non-productive rearrangements could be assigned to two clones (Table 2). Thus, besides several unique sequences, three to five expanded B cell clones were detected in GC3. Twenty-eight of the 29 V_H region genes were somatically mu-

Table 2. Summary of V_H Gene Analysis of B Cells Isolated from GC3

Micromanipulated cells	Specific PCR products	Rearrangements potentially functional +	Rearrangements potentially functional −	Clonally related*		Mutation frequency (%)
88 Ki67$^+$ centroblasts†	17	12		clone 2	3/12	2.7–3.8
				clone 3	1/12	3.4
				clone 4	1/12	1.3
			5	clone 1	3/5	3.8–5.1
				clone 5	2/5	3.5, 3.8
84 Ki67$^-$ centrocytes†	12	6		clone 2	1/6	3.3
				clone 3	1/6	5.4
				clone 4	1/6	2.2
			6	clone 1	2/6	3.9, 4.5
				clone 5	1/6	3.0
Controls						
9 buffer samples‡	0					
45 single FACS-sorted B cells	28			0		

*Identical rearrangements amplified from different cells are listed as clonally related. Because each PCR tube contained two single GC cells, it was not possible to determine whether one or both of the two groups of clonally related nonproductive Ig gene rearrangements belonged to one of the clones defined by a productive rearrangement. For each of the clones, members were found among centroblasts as well as centrocytes.

†A total of 86 PCR tubes (44 centroblast samples and 42 centrocyte samples) containing two cells each was analyzed.

‡Samples of buffer covering the sections during the micromanipulation procedure.

tated, with an average mutation frequency of 3%. Intraclonal diversity of V gene rearrangements was observed in each of the five groups of clonally related sequences. Clonally related sequences differed from each other by 4 to 24 sequence differences (single nucleotide exchanges, deletions, and/or duplications; not shown), demonstrating ongoing somatic hypermutation during clonal expansion of the respective GC B cells.

Discussion

In the present study, clonal composition and TCRβ repertoire of the T helper cell population in human LN GCs were analyzed. For each of three patients investigated, T cells were micromanipulated from one particular GC, and TCRβ gene rearrangements were amplified from these single cells. This technique[8] allows comprehensive, quantitative analysis of the TCRβ repertoire expressed by small T cell populations.

In all three GCs analyzed, the GC reaction had reached a degree of maturity characterized by clear polarization into DZ and LZ as revealed by immunohistochemical staining for a proliferation marker. Furthermore, sequence analysis of IgH V region genes from B cells of GC3 demonstrated the presence of expanded B cell clones. Eight of 18 potentially functional and 8 of 11 nonfunctional IgH gene rearrangements amplified from B cells could be assigned to one of few clones. The finding of these expanded B cell clones, which were all characterized by somatically mutated Ig genes showing considerable intraclonal diversity, suggests that in GC3 the GC reaction must have commenced several days before the biopsy.

The TCRβ V region sequences from GC T cells were compared for clonal identity, Vβ and Jβ gene segment usage and CDR3 amino acid sequence. In GC1, 11 of 28 potentially functional rearrangements originated from four different clones while the remaining 17 rearrangements were unique. In GC2 and GC3, only unique TCRβ gene rearrangements were obtained. Thus, the clonal composition of the B and T cell populations of GC3 differed significantly from each other, since about half of the IgH V region sequences originated from three to five expanded B cell clones, showing that the GC T cell population can be much more diverse than the GC B cells with which they are interacting. It is possible that the immune response was less mature in GC2 and GC3 as compared to GC1. At late stages of the GC response, effects of T cell selection, including expansion of clones with optimal specificities, may be more evident. This interpretation is not supported by the fact that the duration of the lymphadenopathy before biopsy was longer in patient 2 (GC2) as compared to patient 1 (GC1). However, this time interval may not be a reliable measure of the duration of a GC reaction, as the reaction may go on for long periods of time without clinically manifest lymph node swelling. On the other hand, lymphadenopathy may be sustained by different subsequent immune responses. An alternative explanation for the difference observed between GC1 and the GCs 2 and 3 is that GC1 was involved in a response against an antigen that selects a more restricted repertoire of T cell specificities.

Except for the clonal expansions detected in GC1, comparison of TCR V region sequences provided little evidence that the micromanipulated T cells were sampled from a population of cells selected by one or few antigenic peptides. The gene rearrangements did not exhibit apparent biases in V and J gene segment usage or CDR3 length in any of the three GCs analyzed. Similarities in CDR3 amino acid sequence, which might reveal antigen selection, were rare. Taken together, it appears that the human lymph node GC T cell population is usually not dominated by a few expanded clones. One may speculate that the selection of GC T cells allows survival of a broad spectrum of different clones. The diversity of the GC T cells may also reflect a high number of T cell

epitopes linked to one or few B cell epitopes that are recognized by the oligoclonal population of GC B cells.

There is evidence that the process of somatic hypermutation, which introduces point mutations into Ig V region genes of GC B cells, is not entirely specific for cells of the B cell lineage. Somatically mutated TCRα, but not TCRβ V region, genes were described in murine GC T cells,[6] and ongoing mutation of a TCRα gene rearrangement was observed *in vitro* in a murine T cell hybridoma.[11] Recently, somatically mutated TCRβ V region genes were detected in CD8 T cells invading GCs of HIV patients,[7] implying that the human TCRβ locus may be accessible for hypermutation.

Among 68 potentially functional as well as 21 nonproductive TCRβ gene rearrangements amplified in total from GC T cells of three individuals in the present study, only a single sequence contained one mismatch with published TCR Vβ gene segment sequences. This mismatch may be due to a so far undescribed polymorphism of the gene segment. We conclude that TCRβ V region genes of human GC T helper cells are in general not subject to somatic hypermutation. Mutation of TCRβ V region genes in CD8 T cells recovered from GCs of HIV+ individuals may therefore be a phenomenon unique to HIV infection.

In several autoimmune diseases like rheumatoid arthritis, Sjögren's syndrome, Hashimoto's disease, and myasthenia gravis, GC-like structures develop in extranodal sites.[12] Little is known about T cell differentiation processes taking place in these ectopic GCs in comparison to normal GCs. Moreover, GC T cells are found in association with malignant B cells in follicular lymphoma.[13] It will be interesting to investigate whether the T cell populations found in these diseases have a composition distinct from GC T cells in normal GCs. For example, it may well be that protracted interaction of T cells with B cells in autoimmune diseases and B cell lymphomas results in the selective outgrowth of few T cell clones.

Note Added in Proof

Evidence that T cells in human GCs usually are a diverse population of cells was recently also provided by Golby et al (Eur J Immunol 1999, 29:3729–3736).

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