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Tracing B cell development in human germinal centres by molecular analysis of single cells picked from histological sections

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Germinal centres are areas of intense B lymphocyte proliferation inside primary B cell follicles in spleen and lymph nodes. Rearranged V genes from single human B cells, isolated from histological sections of two such structures by micromanipulation, were amplified and sequenced. Cells from the follicular mantle were clonally diverse and largely expressed germline V genes. Germinal centres were dominated by a few large B cell clones dispersed throughout these structures and exhibiting intraclonal diversity by ongoing somatic hypermutation. Pronounced counterselection of replacement mutations seen in one of the germinal centres may indicate a late phase of the germinal centre reaction. A polyclonal population of activated B cells expressing unmutated antibodies in the dark zone of the other germinal centre may represent the initial founder cells.

Key words: B cell development/germinal centre/somatic hypermutation/V gene rearrangement/single cell PCR

Introduction

Germinal centres are histologically defined structures in peripheral lymphoid organs. They represent accumulations of predominantly proliferating B lymphocytes and are surrounded by a mantle zone of phenotypically distinct small resting B cells (reviewed in Kroese *et al.*, 1990; Liu *et al.*, 1992). On the basis of histological staining reactions germinal centres can be subdivided into a dark zone with rapidly dividing B cell blasts (centroblasts) and a light zone of non-dividing B cells (centrocytes). Experiments in mice and rats show an oligoclonal development of germinal centres with one to six founder B cells (Kroese *et al.*, 1987; Jacob *et al.*, 1991b; Liu *et al.*, 1991a). Other components of the germinal centres are T helper cells, follicular dendritic cells (FDC) and macrophages (reviewed by Kroese *et al.*, 1990).

Germinal centres arise after antigenic stimulation in T cell dependent immune responses and it has long been suspected that they may be the sites where affinity maturation of antibodies and the generation of memory B cells takes place (see MacLennan and Gray, 1986). Somatic mutation is the hallmark of affinity maturation and the generation of B cell memory, a process by which rearranged antibody variable (V) region genes are modified to give rise to mutant antibodies which are selected for binding of the immunizing antigen with higher affinity (reviewed by Kocks and Rajewsky, 1989). The analysis of splenic germinal centre B cells from immunized mice has indeed shown that in the course of the response these cells not only carry an increasing load of somatic point mutations in rearranged V region genes (Berek *et al.*, 1991), but also that the process of somatic hypermutation is ongoing within the germinal centre (Jacob *et al.*, 1991a). This strongly supports the concept that B cells expressing high affinity mutant antibodies arise in the course of clonal proliferation in the germinal centre microenvironment to become long-lived memory cells. The proliferation kinetics of memory B cells generated upon immunization is consistent with this view (Schittek and Rajewsky, 1990).

The efficiency by which rare high affinity somatic variants are selected at the expense of all other mutants already at early time points of the response (Berek *et al.*, 1991; Weiss *et al.*, 1992) suggests that these B cells are not only generated but also selected within the germinal centre. B cells which through mutations either have lost the ability to produce functional antibodies or whose antibodies have lost the capacity to bind antigen appear to die by apoptosis and to be taken up by macrophages (Liu *et al.*, 1989).

The antigen-mediated signal which rescues germinal centre B cells from apoptosis is not fully understood, but appears to involve surface receptors other than the immunoglobulin receptor complex. Isolated germinal centre B cells undergo rapid apoptosis in tissue culture. However, in the presence of CD23 (the low affinity receptor for IgE) and interleukin 1α or antibodies against the B cell antigen CD40 and surface immunoglobulin, they survive and differentiate into plasmablasts or small, resting (memory?) B cells, respectively (Liu *et al.*, 1989, 1991b). This may indicate that different ligands drive germinal centre B cells into different differentiation pathways, namely either plasma cell or memory cell generation. That germinal centre B cells can indeed differentiate in either direction is supported by evidence from *in vivo* experiments (Coico *et al.*, 1983; Tew *et al.*, 1992).

Interestingly, a subpopulation of FDC in the light zone of the germinal centre expresses CD23 (Johnson *et al.*, 1989; Liu *et al.*, 1992), and recent experiments suggest that a CD40-mediated signal is involved in the interaction between T helper cells which are mainly found in the light zone (Stein *et al.*, 1980), and B cells in human germinal centres (Lederman *et al.*, 1992). Therefore, the selection processes mediated by interaction of B cells with FDC and T cells may be compartmentalized within the germinal centre.

In collaboration with G.Kelsoe's group we have recently initiated an approach by which B cell differentiation is assessed at the molecular level within the histological structures in which the cells reside. For this purpose we isolated B cell populations from individual germinal centres by micromanipulation from histological sections of mouse spleen, and sequenced rearranged V region genes amplified from those cells by the polymerase chain reaction (PCR)

(Jacob *et al.*, 1991a). This allows the identification of clonally related rearrangements, because each rearrangement is common to and unique for all cell members of a given B cell clone. On the basis of shared and unique point mutations in the sequences, clonal genealogies can then be established (McKean *et al.*, 1984). However, the approach was limited in that a selected set of V genes was amplified and cell populations instead of single cells were analysed. Only the latter would allow the assignment of more than one V gene rearrangement (e.g. both H and L chain gene rearrangements) and thus a given antibody binding site to a given cell. Furthermore, the analysis of single cells is required for the formal demonstration that the clonally related but distinct V region sequences which can be isolated from germinal centres (Jacob *et al.*, 1991a) indeed originate from separate cells and thus reflect somatic hypermutation in the course of clonal expansion. Most importantly, however, the assignment of individual sequences to individual cells picked from the various histologically distinct areas of the germinal centre would allow the determination of the extent and the timing of clonal expansion within this structure and whether clonal expansion, intraclonal diversification and cellular selection are compartmentalized. We have therefore extended our previous approach to the analysis of H and L chain V region gene rearrangements in individual cells picked from various positions in histological sections of human germinal centres. The human system was chosen because human germinal centres are particularly well structured histologically and it also seemed important to demonstrate that somatic hypermutation takes place in germinal centres of the human as it does in the mouse.

Results

PCR analysis of single micromanipulated cells and their histological origin

Frozen sections (8–10 μm thick) derived from human lymph nodes were stained (see below) and single cells were micromanipulated with the help of two hydraulic micromanipulators. Rearranged V_H and V_χ region genes from individual cells were amplified in a semi-nested PCR approach using V gene family specific primers, and the amplification products (defined as bands of ~350 bp in length visible on an ethidium bromide stained agarose gel) were directly sequenced as described in Materials and methods. By direct sequencing of PCR products from both strands somatic mutations can be clearly identified as misincorpration of nucleotides by Taq polymerase is negligible (McHeyzer-Williams *et al.*, 1991).

A double blind control experiment performed with micromanipulated mantle zone cells (>90% of which are B cells) and T cells (see Materials and methods) demonstrated the efficiency and reliability of the method. 25 PCR products representing rearranged V region genes (12 $V_HD_HJ_H$ and 13 $V_\chi J_\chi$ rearrangements) were obtained from 20 B cells. At first glance, this is less than what one might expect. In the case of heavy chains, for example, all cells should harbour an in-frame rearrangement and about one-third should have an additional non-functional $V_HD_HJ_H$ rearrangement (Yamada *et al.*, 1991). However, there are many reasons why the yield of PCR bands must be significantly below 100%, including the fact that many cells in the section lack part of their nucleus (see Discussion). Only two V region genes were amplified from 19 T cells. These two products could be due to cellular or other contamination, or represent true immunoglobulin V gene rearrangements in T cells, although so far only D_HJ_H cross-lineage rearrangements have been described for these cells (Waldmann, 1987). Nonetheless, the PCR approach chosen appears feasible from a technical point of view as it allows the characterization of V gene rearrangements in a large fraction of B cells at a high level of confidence in terms of the assignment of a given rearrangement to a given cell.

Frozen sections of two germinal centres derived from human lymph nodes (GC2 and GC3; Figures 1a and 2a) were stained with the Ki-67 antibody which can be used to discriminate between dark and light zones of the germinal centre since it exclusively stains cells in cycle (Gerdes *et al.*, 1984). Therefore, most of the cells in the dark zone are stained by this antibody whereas the majority of the cells in the light zone are Ki-67$^-$. The latter is also true for the mantle zone surrounding the GC. Cells from dark, light and mantle zones were isolated for analysis. A total of 157 cells were analysed by PCR amplification, 90 derived from GC2 and 67 from GC3 (Table I). The efficiency of amplification, defined as the percentage of cells with at least one PCR product, was different for cells from different regions of the GC, ranging from 55% for mantle zone cells to 24% for centroblasts of GC3 (Table I; see Discussion). Sequences were determined for 58 PCR products (Table I). Counting clonally related sequences only once, 15 of 20 $V_HD_HJ_H$ sequences represented in-frame rearrangements whereas only 10 of 20 $V_\chi J_\chi$ rearrangements were in-frame. In the case of the heavy chain genes, the observed frequency of in-frame $V_HD_HJ_H$ rearrangements (75%) is close to values obtained by others for murine B cells and human peripheral blood B cells (Alt *et al.*, 1984; Yamada *et al.*, 1991). The higher fraction of out-of-frame rearrangements among the V_χ sequences is expected considering that 30–40% of human B cells express λ light chains with most of these cells

Table I. Summary of PCR amplification results from two germinal centres

GC	Region	No. of cells analysed	No. of cells positive	% positive	Number of PCR bands	PCR bands sequenced
2	mantle zone	27	15	55	27	8
	dark zone	26	12	46	22	14
	light zone	37	9	24	15	14
3	mantle zone	11	6	54	9	3
	dark zone	25	6	24	7	7
	light zone	31	10	32	12	12

The number of positive cells indicates the number of cells with at least one PCR product for the V_H or V_χ gene amplification.

harbouring one or two non-functional V_xJ_x rearrangements (Graninger *et al.*, 1988).

Nomenclature

Micromanipulated cells were named as follows: a number (2 or 3) indicating the germinal centre from which the cell originates is followed by one or two letters describing the histological origin (M: mantle zone, Ki-67$^-$; MR: mantle zone, Ki-67$^+$; D: dark zone, Ki-67$^+$; L: light zone, Ki-67$^-$; C: light zone, Ki-67$^+$) and a number indicating the numerical order of picking. A V gene rearrangement is described by the designation of the cell from which it originates, followed by 'H' or 'K' to indicate heavy or $\varkappa$ light chain, respectively, and the number of the V gene family.

Mantle zone B cells

V gene rearrangements from a total of 10 mantle zone B cells were analysed by sequencing, seven from GC2 and three from GC3 (Figures 1 and 2 and Table II). Three of

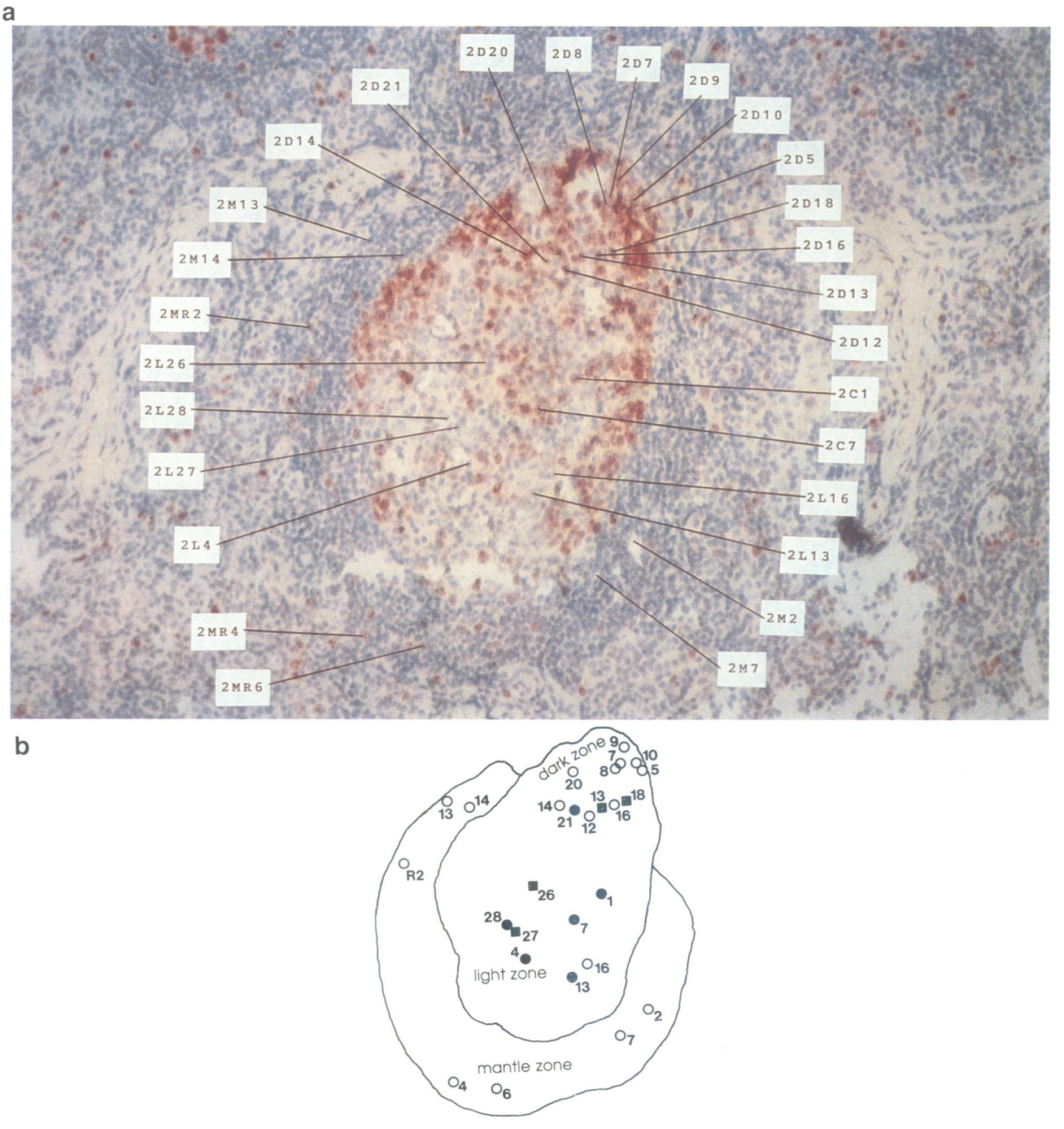

Fig. 1. Histology of germinal centre 2. (**a**) Frozen section of an inguinal lymph node stained with antibody Ki-67 (red) and haematoxylin with a germinal centre (GC2) in the centre. The locations of the cells for which sequence data were obtained are shown. The picture was taken after micromanipulation of cells 2M2 and 2M7. Therefore, for these cells an empty spot is seen. (**b**) Diagrammatic representation of GC2 indicating the distribution of the cells for which sequence information on rearranged V region genes was obtained. Clonally related cells are marked with the same closed symbol (a circle for clone 1 and a square for clone 2, see Table III) and clonally unrelated cells with an open circle. Cell numbers appear next to the cells.

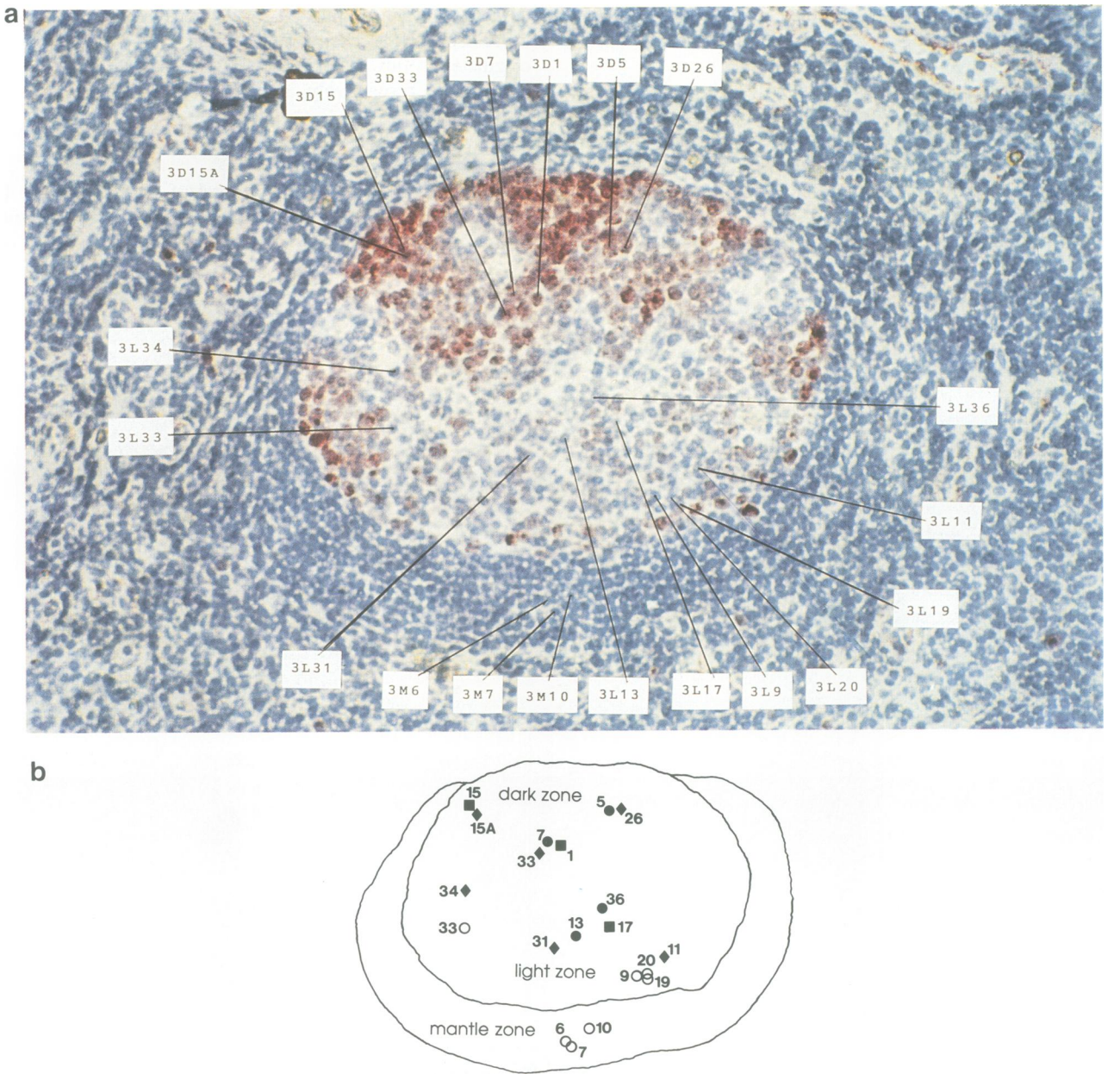

Fig. 2. Histology of germinal centre 3. (**a**) Frozen section of a cervical lymph node stained with antibody Ki-67 (red) and haematoxylin, with a germinal centre in the centre. The locations of the cells for which sequence data were obtained are shown. The picture was taken before micromanipulation. (**b**) Diagrammatic representation of GC3 indicating the distribution of the cells for which sequence information on rearranged V region genes was obtained. Clonally related cells are marked with the same closed symbol (a square for clone 1, a circle for clone 2 and a diamond for clone 3; see Table IV) and cells for which no clonal relationship is evident (although one or the other of these cells—with the exception of 3L19—might belong to clone 1 or 2 for which only a functional V_H rearrangement was obtained) with an open circle. Cell numbers appear next to the cells.

these cells (2MR2, 2MR4 and 2MR6) were Ki-67$^+$ and therefore belong to the very small fraction of proliferating cells in the mantle zone. V genes from different V_H and V_x families were found to be rearranged in the mantle zone cells. Of the 11 V genes sequenced, nine are unmutated by comparison with published germline genes and—in one case—with an expressed V_H gene (VH4-Wil). 2MR6K4 shows one nucleotide difference from the published V_x4 germline gene whereas 3M10K3 has five nucleotide differences from the V_x3 gene A27 (Table II). These differences could be due to polymorphism, somatic mutation or to the expression of a previously undescribed V_x3 gene in the case of 3M10K3. Clonally related cells could not be identified in the mantle zone of either germinal centre.

Germinal centre 2, derived from an inguinal lymph node of a 6 year old child

Sequence data were obtained for 28 V gene rearrangements from a total of 20 cells (Figure 1 and Table III). 14 sequences originated from 12 dark zone B cells. Six V region genes of those cells use unmutated gene segments and four further sequences have only two nucleotide differences relative to published germline genes (Table III). The remaining four V_H4 rearrangements (2D9H4, 2D10H4,

Table II. V region sequence analysis of mantle zone B cells derived from GC2 and GC3

Cell	V gene family	Germline gene	Base pairs difference	In-frame?	Ref.
2M2	VH4	VH4-Wil[a]	0	+	1
2M7	VK4	VK4	0	−	2
2M13	VK2	A3	0	+	3
2M14	VH4	VH4-Wil[a]	0	+	1
2MR2	VK1	O18	0	−	4
2MR4	VK3	A27	0	+	5
2MR6	VH3	VH26	0	−	6
	VK4	VK4	1	+	2
3M6	VK1	L4	0	−	7
3M7	VK3	A27	0	−	5
3M10	VK3	A27	5	+	5

The sequences are compared with those of the most homologous known germline gene. In-frame rearrangements are marked by '+', non-functional (out-of-frame) ones by '−'. References for germline genes: 1: Pratt *et al.* (1991); 2: Klobeck *et al.* (1985); 3: Straubinger *et al.* (1988); 4: Scott *et al.* (1991); 5 (VK325): Radoux *et al.* (1986); 6: Chen (1990); 7 (Va): Pech *et al.* (1984).
[a]In this case the V_H sequence is compared with that of a rearranged V_H4 gene.

Table III. V region sequence analysis of germinal centres cells derived from GC2

Cell	V gene family	Germline gene	Base pairs difference	Clone	In-frame?	Ref.
Dark zone						
2D5	VH4	VH4-Wil[a]	0		+	1
2D7	VH3	DP58	2		+	2
2D8	VK2	A17	0		−	3
2D9	VH4	VH416	5		+	4
	VK3	A27	2		+	5
2D10	VH4	VH421	17		+	4
2D12	VH4	VH421	13		+	4
2D13	VH4	VH411	18	2	+	4
2D14	VK4	VK4	0		−	6
2D16	VK4	VK4	0		−	6
2D18	VK4	VK4	0	2	−	6
2D20	VH4	VH421	2		−	4
	VK2	A3	0		−	7
2D21	VK3	L6	2	1	+	8
Light zone						
2C1	VH4	VH411	14	1	−	4
	VH3	VH26	8		+	9
	VK2	A3	2		+	7
2C7	VH4	VH411	14	1	−	4
	VK3	L6	2		+	8
2L4	VH3	VH26	11	1	+	9
2L13	VH3	VH26	11	1	+	9
2L16	VH4	VH4-Wil[a]	4		+	1
2L26	VH4	VH411	17	2	+	4
	VK1	L9	18		+	10
2L27	VH4	VH411	16	2	+	4
	VK1	L9	15		+	10
	VK4	VK4	0		−	6
2L28	VK3	L6	2	1	+	8

Cells belonging to one of the two clones are indicated. The sequences are compared with those of the most homologous germline genes. '+' means in-frame, '−' a non-functional (out-of-frame) rearrangement. References for germline genes: 1: Pratt *et al.* (1991); 2: Tomlinson *et al.* (1992); 3: Lautner-Rieske *et al.* (1992); 4: Sanz *et al.* (1989); 5 (VK325): Radoux *et al.* (1986); 6: Klobeck *et al.* (1985); 7: Straubinger *et al.* (1988); 8 (Vg): Pech and Zachau (1984); 9: Chen *et al.* (1990); 10 (Ve): Pech *et al.* (1984).
[a]In this case the V_H sequence is compared with a rearranged V_H4 gene.

2D12H4 and 2D13H4) have 5−18 base pair differences from their closest germline homologue. Whether any or all of these four V_H4 sequences are somatically mutated cannot be determined as there is considerable polymorphism in the V_H4 family, and it is not known whether all members of this family have yet been cloned (Weng *et al.*, 1992). Within

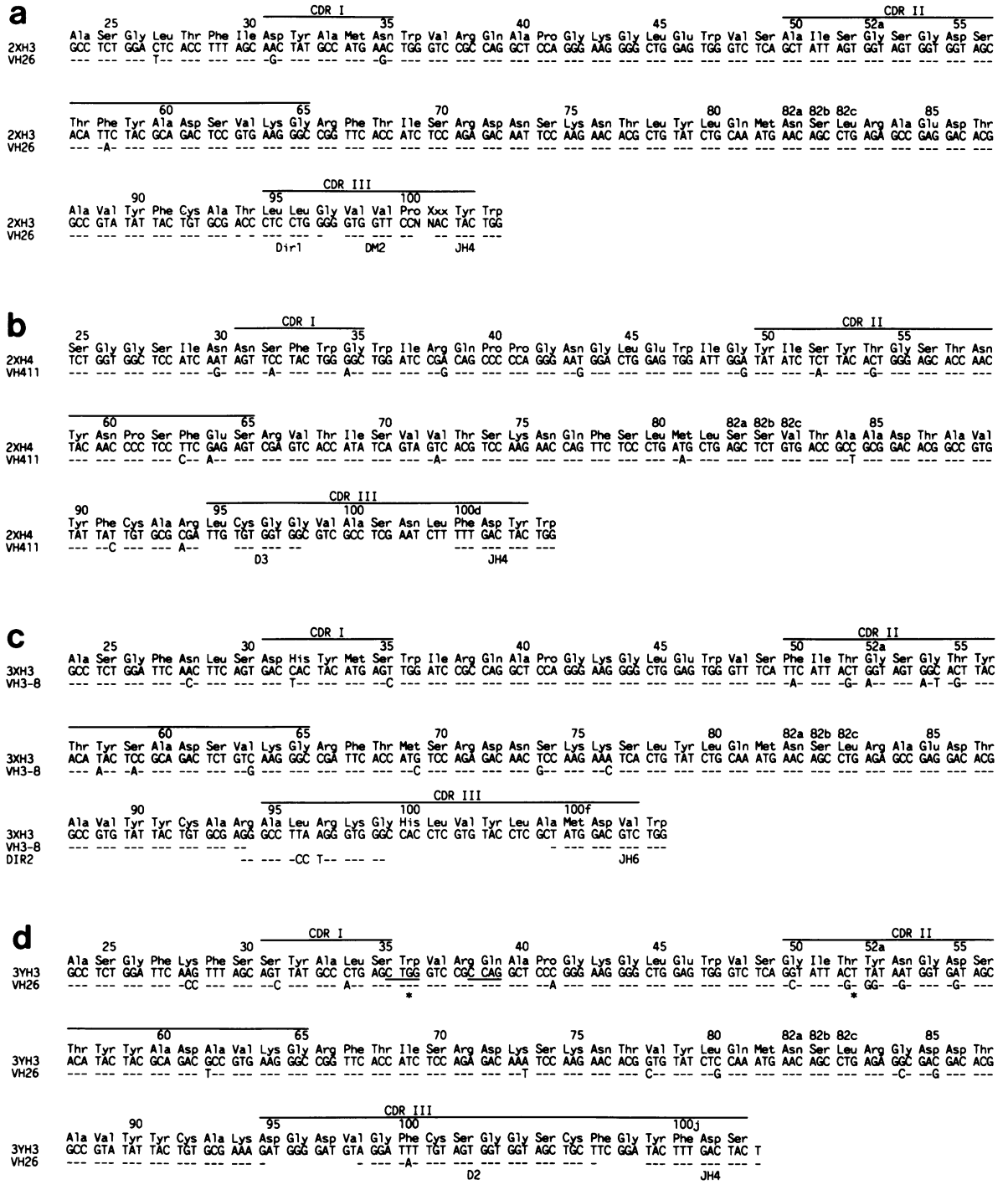

Fig. 3. Sequence analysis of clonally related V_H rearrangements. Dashes indicate sequence identity. Codons are numbered according to Kabat *et al.* (1987). Complementarity determining regions (CDRs) I–III are marked. (**a**) The sequence of the hypothetical intermediate 2XH3 of cells 2C1, 2L13 and 2L4 (Figure 4a) is compared with that of the most homologous V_H3 germline gene VH26 (Chen, 1990), the D_H gene DM2 (Ichihara *et al.*, 1988), the DIR1 gene (Ichihara *et al.*, 1988) and the J_H4 germline gene (Ravetch *et al.*, 1981). (**b**) Sequence comparison of the hypothetical intermediate in the genealogical tree of the V_H4 rearrangement of cells 2D13, 2L26 and 2L27 (2XH4, see Figure 4b). The sequence is compared with that of the V_H4 germline gene VH411 (Sanz *et al.*, 1990), the D3 D_H gene (Siebenlist *et al.*, 1981) and the J_H4 gene (Ravetch *et al.*, 1981). (**c**) Sequence analysis of the hypothetical intermediate (3XH3, see Figure 4c) in the genealogical tree of the V_H3 rearrangement of cells 3D5, 3D7, 3L13 and 3L36. The sequence is compared with that of the VH3-8 V_H3 germline gene (Winkler *et al.*, 1992), the DIR2 segment (Ichihara *et al.*, 1988) and the J_H6 germline gene (Ravetch *et al.*, 1981). (**d**) Sequence analysis of the hypothetical intermediate 3YH3 (see Figure 4d) in the genealogical tree of the V_H3 rearrangements of cells 3D1, 3D15 and 3L17. The sequence is compared with that of the VH26 V_H3 germline gene (Chen, 1990), the D2 D_H gene (Siebenlist *et al.*, 1981) and the J_H4 germline gene (Ravetch *et al.*, 1981). '*' indicates the beginning and end of the deletion in 3D15H3. A common sequence motif found near non-homologous recombination breakpoints (Chou and Morrison, 1993) is underlined. The accession numbers for the sequences reported here have been deposited in the EMBL data library under accession numbers X73160–X73213.

the dark zone no clonally related sequences were identified, but three of the 12 cells turned out to be clonally related to cells in the light zone (see below).

From the light zone of GC2, eight V_H and six V_x rearrangements from eight individual cells were sequenced (Table III). Five of these cells (2C1, 2C7, 2L4, 2L13 and

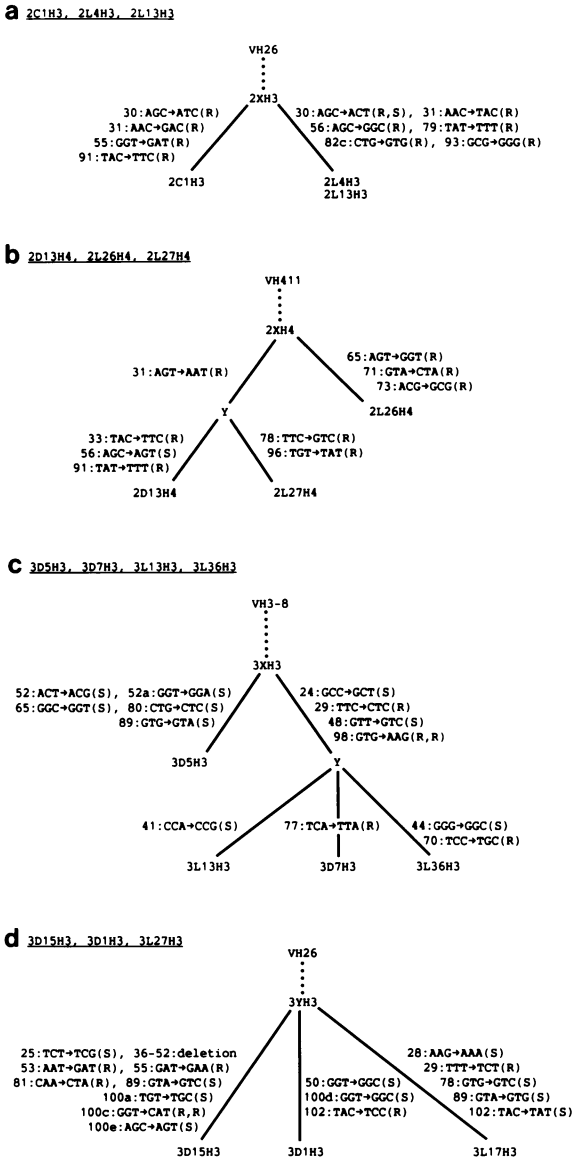

Fig. 4. Genealogical trees explaining the relationship between clonally related sequences. 2XH3, 2XH4, 3XH3, 3YH3 and Y represent hypothetical intermediates. Sequence comparisons between the intermediates 2XH3, 2XH4, 3XH3, 3YH3 and germline V_H, D_H and J_H genes are shown in Figure 3. Numbers along the branches indicate the amino acid number where the mutations are found. (**a**) Comparison of 2C1H3, 2L4H3 and 2L13H3; (**b**) comparison of 2D13H4, 2L26H4 and 2L27H4; (**c**) comparison of 3D5H3, 3D7H3, 3L13H3 and 3L36H3; (**d**) comparison of 3D15H3, 3D1H3 and 3L17H3.

2L28) and one of the cells from the dark zone (2D21) belong to one B cell clone (Table III, clone 1). This clone is characterized by an in-frame rearrangement of a V_H3 family gene and an out-of-frame V_H4 rearrangement. Two different V_χ rearrangements were obtained for this clone of cells, both of which are in-frame. Two of the three in-frame V_H3 rearrangements (2L4H3 and 2L13H3) are identical in sequence and differ from 2C1H3 by nine point mutations (Figures 3a and 4a). The sequences are most homologous to the germline gene VH26 with eight and 11 mutations for 2C1H3 and 2L4H3/2L13H3, respectively. On the assumption that VH26 is the V_H3 gene originally used in the V_H rearrangement of this clone a genealogical tree can be drawn up for the V_H3 rearrangements to explain the observed mutations in a stepwise manner (Figure 4a; in this and the other B cell clones identified the data on χ chain gene rearrangements were insufficient to design meaningful genealogical trees, however, there was no contradiction to the genealogical trees based on heavy chain V region sequences as depicted in Figure 4). The out-of-frame V_H4 rearrangements of cells 2C1 and 2C7 differ from each other by two nucleotides in the complementarity determining region (CDR) I (see Materials and methods for mutations of clonally related sequences not shown in Figures 3–5).

An in-frame $V_\chi3$ rearrangement was obtained for three cells, 2C7, 2D21 and 2L28. Two of these, 2C7K3 and 2L28K3, are identical and differ by only one nucleotide from 2D21K3 and by two nucleotides from the $V_\chi3$ germline gene L6 (not shown). A second V_χ rearrangement was amplified from 2C1, which harbours a $V_\chi2$ gene rearranged in-frame to $J_\chi2$ (not shown). The amplification of two in-frame V_χ rearrangements for this clone (2C1k2, 2C7k3; Table III), which seems to contradict the principle of allelic exclusion, could be explained by two consecutive rearrangements on one allele [first L6 to $J_\chi1$ by deletion, then A3 to $J_\chi2$ by inversion; see Lautner-Rieske *et al.* (1992) and Pargent *et al.* (1991)] as has been described earlier (Huber *et al.*, 1992). This would leave the first rearrangement in the opposite transcriptional orientation on the chromosome. Ongoing V_χ rearrangement has recently also been shown in murine B cells (Harada and Yamagishi, 1991). This process might allow autoreactive B cells to revise the specificity of their antigen receptor and thereby escape deletion (receptor editing; Tiegs *et al.*, 1993).

The two centroblasts 2D13 and 2D18 and the two centrocytes 2L26 and 2L27 represent a second clone of B cells in GC2 (Table III, clone 2). The sequences of an in-frame VH4 rearrangement—amplified from 2D13, 2L26 and 2L27—differ from each other by 5–7 bp and from the most homologous known V_H4 germline gene (VH411) by 16–18 bp (Figures 3b and 4b). A hypothetical genealogical tree of the three V_H4 sequences is shown in Figure 4b. In addition, the clone is characterized by an in-frame $V_\chi1$ and an out-of-frame $V_\chi4$ rearrangement. The $V_\chi1$ rearrangements of 2L26 and 2L27 differ from each other by five mutations and harbour 18 and 15 bp differences, respectively, from the $V_\chi1$ germline gene L9 (not shown). An unmutated $V_\chi4$ gene, rearranged out-of-frame to $J_\chi1$, was amplified from 2D18 and 2L27 (not shown).

Taken together, the sequence analysis of V_H and V_χ rearrangements amplified from cells of GC2 shows a distinction between centroblasts in the dark zone and centrocytes in the light zone (Figure 1b). Whereas V gene sequences of most centroblasts show little or no somatic mutation and most of the cells are clonally unrelated to other B cells in the germinal centre, seven of eight light zone cells for which sequences were obtained belong to either of two B cell clones whose members appear to be intermingled in the light zone area. Ongoing somatic mutation in the course of clonal growth is evident because of sequence differences between clonally related rearrangements.

Germinal centre 3, derived from a cervical lymph node of a 23 year old adult

In the case of GC3, sequence data were obtained for seven centroblasts from the dark zone and 10 centrocytes from the light zone (Figure 2 and Table IV). Thirteen of these 17 cells could be assigned to either of three different B cell clones.

Table IV. V region sequence analysis of germinal centre cells derived from GC3

Cell	V gene family	Germline gene[a]	Base pairs difference	Clone	In-frame?	Ref.
Dark zone						
3D1	VH3	VH26	18	1	+	1
3D5	VH3	VH3-8	20	2	+	2
3D7	VH3	VH3-8	19	2	+	2
3D15	VH3	VH26	19	1	−	1
3D15A	VK3	3L20K3	10	3	+	a
3D26	VK3	3L20K3	10	3	+	a
3D33	VK3	3L20K3	11	3	+	a
Light zone						
3L9	VH3	DP51	19		−	3
3L11	VH1	?		3	−	
3L13	VH3	VH3-8	19	2	+	2
3L17	VH3	VH26	20	1	+	1
3L19	VH3	VP	0		+	4
3L20	VK3	3D15AK3	10		+	a
3L31	VH1	DP14	18	3	+	3
	VH1	?			−	
	VK3	3L20K3	10		+	a
3L33	VK4	VK4	0		−	5
3L34	VK3	3L20K3	10	3	+	a
3L36	VH3	VH3-8	20	2	+	2

The sequences are compared with those of the most homologous germline genes. Most of the cells can be assigned to one of three B cell clones. An in-frame rearrangement is indicated by '+', a non-functional (out-of-frame) one by '−'. References for germline genes: 1: Chen (1990); 2: Winkler *et al.* (1992); 3: Tomlinson *et al.* (1992); 4: Denny *et al.* (1986); 5: Klobeck *et al.* (1985).
[a]The VK3 sequences of 3D15A, 3D26, 3D33, 3L31 and 3L34 are most homologous to the rearranged VK3 gene of 3L20.

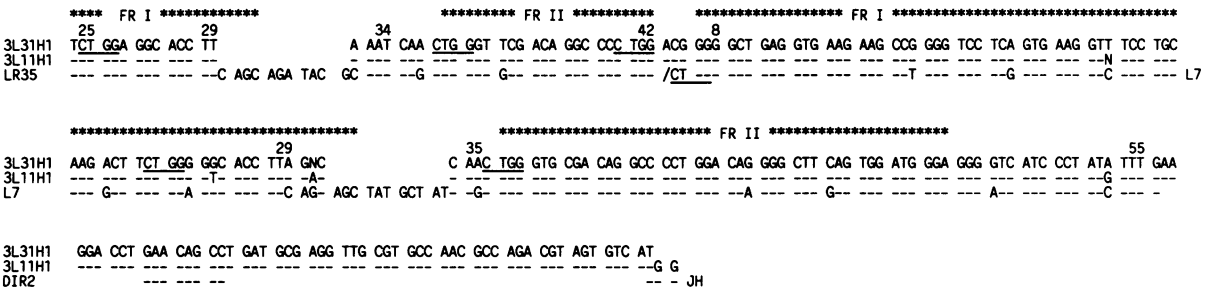

Fig. 5. Sequence analysis of the non-functional V_H1 rearrangement of cells 3L31 and 3L11. Dashes indicate sequence identity. Amino acid numbering is according to Kabat *et al.* (1987). Sequences are compared with those of the LR35 V_H1 gene (Ravetch *et al.*, 1981), the L7 V_H1 gene (Kipps and Duffy, 1991) and the DIR2 gene (Ichihara *et al.*, 1988). A common sequence motif found near non-homologous recombination breakpoints (Chou and Morrison, 1993) is underlined.

One clone, represented by cells 3D5, 3D7, 3L13 and 3L36 (Table IV, clone 2), is characterized by an in-frame rearrangement of a V_H3 family gene with 91% homology to the V_H3 germline gene VH3-8 (Figures 3c and 4c). The four rearrangements differ from each other by 2−11 point mutations. On the assumption that VH3-8 is the germline gene originally used for this rearrangement a genealogical tree can be drawn to explain the relationship between the four sequences (Figure 4c).

A second B cell clone in GC3, defined by cells 3D1, 3D15 and 3L17 (Table IV, clone 1), uses a V_H3 family gene with highest homology to V_H26 (Figures 3d and 4d). The two sequences of 3D1H3 and 3L17H3 represent in-frame rearrangements, whereas the sequence of 3D15H3 shows a 50 bp deletion of framework region (FR) II and part of CDRII. In addition to the deletion the three sequences differ from each other by 8−13 point mutations. The corresponding genealogical tree is shown in Figure 4d.

Sequences of six further cells could be assigned to a third B cell clone in GC3 (3D15A, 3D26, 3D33, 3L11, 3L31 and 3L34; Table IV, clone 3). The non-functional V_H1 rearrangements of 3L11 and 3L31 show several abnormalities: fragments of two V_H1 family gene segments are rearranged to each other, the first one being joined at amino acid position 42 to position 8 of the second one (Figure 5). Both V gene segments show a deletion of 11 nucleotides at the end of FRI and the beginning of CDRI. The homology to V_H1 sequences ends at position 55 in CDRII of the downstream V_H segment. At the 3′ end of the sequence only a short stretch of eight nucleotides shows homology to a D_H-like element (DIR2), and three nucleotides 5′ of the J_H primer sequence are homologous

to J_H germline sequences. Therefore, despite these abnormalities, the sequences show the characteristic features of $V_HD_HJ_H$ rearrangements. The fact that the two sequences harbour at least two mutations (Figure 5) is an additional indication that these unusual sequences represent true $V_HD_HJ_H$ rearrangements as somatic hypermutation is largely restricted to rearranged V genes (reviewed by Kocks and Rajewsky, 1989). Aberrant V_H-V_H genes have been described before (Deane and Norton, 1990; Brokaw *et al.*, 1992) and, interestingly, a recent report (Chou and Morrison, 1993) indicates that two specific tetranucleotide sequences, which are also found near the recombination sites in the sequences shown here, may be important in non-homologous recombinations involving immunoglobulin sequences (Figure 5). However, it is also possible that this unusual V_H sequence did not arise somatically but represents a rearrangement of a pseudogene encoded in the germline.

An in-frame $V_\chi 3$ rearrangement was amplified for cells 3D15A, 3D26, 3D33, 3L31 and 3L34 (not shown). The sequence of 3D33K3 shows one point mutation relative to the other sequences which are identical to each other.

For the light zone of GC3 four additional sequences were obtained which could not be assigned to any B cell clone (Table IV), although three of them (the V_χ rearrangements of 3L20K3 and 3L33K4 and the out-of-frame V_H rearrangement of 3L9H3) may well belong to one or the other of the two clones for which only an in-frame V_H rearrangement was identified.

In summary, GC3 is characterized by the proliferation of three B cell clones which as in the case of GC2 grow in an intermingled fashion and which are subject to ongoing somatic hypermutation (Figure 2b). However, in contrast to GC2, all cells analysed from the dark zone of GC3 appear to express mutated V region genes and belong to the three B cell clones which dominate both light and dark zones of this germinal centre.

Discussion

Reliability and efficiency of single cell gene amplification

The main technical risk of the present approach lies in its extreme sensitivity: since the amplification products from single cells should trace back to single genes, contamination by a single target molecule would be sufficient to give rise to a false positive result. Contamination by single molecules can never be formally excluded. In addition, the amplification reaction from a single cell cannot be repeated, so that at this level the method lacks an important element of scientific experimentation, namely reproducibility. It is the reproducibility of the *pattern* of gene rearrangements on which the present approach relies. Fortunately, in the analysis of rearranged V region genes contamination with cloned DNA from previous experiments can usually be easily identified in that mostly such genes are unique in sequence for a given B cell clone. On the other hand, if the same sequence was amplified from two or more germinal centre cells we cannot formally rule out the possibility that some PCR products result from cross-contamination. This could be true, for example, for the (identical) $V_\chi 3$ sequences which we obtained from four cells of GC3 (3D15A, 3L31, 3D26 and 3L34).

Despite these problems, several lines of evidence indicate that the approach chosen in the present analysis yields a reliable picture of clonal diversification and expansion of B cells in human germinal centres. (i) In no case was the same sequence or pair of related sequences obtained for cells from two different germinal centres although the cells were amplified in parallel. (ii) A double blind control experiment in which isolated B and T cells were analysed in parallel yielded 25 amplification products from a total of 20 B cells, but only two such products from 19 T cells (see Materials and methods). (iii) The fact that a clear genealogy of somatic mutations in clonally related cells was seen within germinal centres can hardly be explained by cross-contamination of samples.

Ideally, we should be able to amplify from each B cell one (in-frame) or two $V_HD_HJ_H$ and $V_\chi J_\chi$ rearrangements, respectively. The latter would not necessarily be true for cells expressing λ chain (40% of the B cells in the human), but even those mostly retain one or two $V_\chi J_\chi$ joints in their genome (Graninger *et al.*, 1988).

It is apparent from our results that under the present experimental conditions successful amplifications are much less frequent than ideally expected. Even if the efficiency of amplification is expressed as the frequency of cells for which at least one V gene rearrangement could be amplified, the efficiencies range from 55% for mantle zone B cells to 25% for cells in the light zone. The former value was surprisingly constant in three experiments, namely the analysis of GC2 and 3 and the double blind control experiment. It may represent the upper limit of the sensitivity of the method whose limitations are manifold: the cells are micromanipulated from frozen sections with a thickness of about one cell diameter, so that in most cases part of the nucleus is missing. The oligonucleotide primers may not amplify all V_H and V_χ rearrangements, because of either the usage of hitherto unknown V genes or somatic mutations. Incubation with proteinase K may not in every instance lead to amplifiable DNA in the absence of further purification steps. Finally, since the sections were not stained with a B cell-specific antibody, some of the isolated cells could be non-B, e.g. T cells [~7% in the mantle zone according to Lusheng *et al.* (1983)].

That the amplification efficiencies in germinal centre cells were lower than those in mantle zone cells (Table I) could again be merely due to technical reasons. Centroblasts are larger than both mantle zone B cells and centrocytes. Therefore, the likelihood of isolating the entire cell nucleus is lower for centroblasts than for the smaller cells. In the case of the centrocytes of the light zone the presence of a considerable fraction of non-B cells has to be taken into account (~14% T cells; Lusheng *et al.*, 1983). However, the low amplification efficiency observed for cells in the light zone of GC2 and 3, and the dark zone of GC3 (Table I), could also be explained by the oligoclonality and somatic hypermutation of the B cells within these structures: the amplification efficiency must drop dramatically if the cells of an entire clone are refractory to V gene amplification, be it because of the particular V genes involved or the occurrence of shared mutations at sites complementary to the primers. In addition, as discussed further below, the hypermutation process as such may negatively affect the amplification efficiencies. These possibilities have to be taken

into account when the clonal composition of GC2 and 3 is evaluated (see below). In the worst (and unlikely) case, the true number of major clones populating these germinal centres could be about double those observed experimentally.

Mantle zone B cells express a diverse repertoire of unmutated V region genes

The origin and function of the small, resting, IgM and IgD expressing B cells in the mantle zone have been a matter of much debate (Kroese *et al.*, 1990). The mantle zone surrounds the germinal centres and corresponds to what is called a primary B cell follicle when a germinal centre is absent. Nine of the 11 rearrangements isolated from mantle zone cells involve unmutated V region genes and this may be true for the remaining two also. In addition, there was no indication of any clonal relationships between mantle zone B cells or to cells in the adjacent germinal centre. These results support the hypothesis that the mantle zone (and therefore also primary B cell follicles) originates from and represents the large pool of small recirculating B cells in the body which are excluded from the rapidly proliferating cells in the germinal centre (Liu *et al.*, 1992) and, at least at young age, largely express germline encoded V region genes, in analogy to what is seen in the mouse (Weiss and Rajewsky, 1990; Gu *et al.*, 1991).

Pattern of ongoing somatic hypermutation in proliferating germinal centre cells

The present data formally demonstrate that somatic hypermutation is ongoing in germinal centre B cells in the course of proliferation, in that V region sequences differing by mutation were isolated from different members of proliferating B cell clones *in situ*. Although the PCR products were directly sequenced we never saw the incorporation of more than one base into a given position on the sequencing gels. This argues against models of somatic hypermutation which predict that a cell undergoing hypermutation should contain multiple copies of a given rearranged V gene which differ from each other by mutations (Manser, 1990; Steele, 1991). Even if mutations were introduced in the process of DNA replication, i.e. the S phase of the cell cycle, we would expect distinct copies of the mutating V gene in the cell in G_2 phase. Our sequence data are insufficient at this point to exclude this latter model, but the model could be directly tested by isolating G_2 phase germinal centre B cells by flow cytometry and subjecting them to single cell V gene amplification. Other models of somatic hypermutation, on the other hand, predict the results obtained in the present analysis, such as the classical model of Brenner and Milstein (1966), which is based on error-prone repair of lesions specifically introduced into rearrranged V genes. Indeed, if such a process occurs at high frequency in germinal centre B cells, the efficiency of V gene amplification by PCR might be negatively affected as we have experimentally observed (see above).

That somatic hypermutation can occur at a very high rate (on the order of one or more mutations per cell division) is again born out by the present results, in accordance with earlier work in the mouse (see Kocks and Rajewsky, 1989; Jacob *et al.*, 1991a). In most cases, homologous V gene sequences isolated from different members of a B cell clone expanding in the germinal centre differed from each other by mutation.

The analysis of genomic rearranged V genes from single cells appears ideally suited to investigate which types of somatic mutation are introduced into these genes. It was not surprising that we almost exclusively observed point mutations, in agreement with earlier data (Kocks and Rajewsky, 1989). An exception was a 50 bp deletion in the in-frame VH3 rearrangement of the centroblast 3D15. Deletions of a few base pairs have been observed in the past in the analysis of somatic hypermutation (see Kocks and Rajewsky, 1989). Although we cannot formally exclude a PCR artefact in the present case, we consider it more likely that larger deletions are occasionally introduced, but were previously missed for technical reasons (e.g. lost in the course of gel purification of cloned PCR products obtained from cell populations). Like in the case of the non-functional V_H1 rearrangement of clone 3 in GC3 (Figure 5) tetranucleotide motifs often found at non-homologous recombination breakpoints are seen near the left recombination site (Figure 3d; Chou and Morrison, 1993). The 50 bp deletion in centroblast 3D15 renders the in-frame VH gene rearrangement of this cell non-functional. It is known from earlier work that somatic mutants expressing non-functional antibodies or lacking antibody expression altogether are rapidly eliminated from the cell population (Weiss *et al.*, 1992). This is also evident from the distribution of replacement and silent mutations in clones 1 and 2 from GC3 where a striking counterselection against replacement mutations is observed (Table V). Because of such counterselection the hypermutation mechanism may introduce deletions and other 'lethal' mutations such as stop codons more frequently than the available data disclose.

In the two B cell clones expanding in GC2 the hypermutation mechanism appears to operate with different efficiency on different V gene rearrangements in the cells of either clone: in both cases significantly fewer mutations were seen in the rearranged V_x than in the V_H genes (Table III and Figure 4a and b), a phenomenon which has also been observed in the analysis of clonally related hybridomas in the mouse (Clarke *et al.*, 1990; Rickert and Clarke, 1993). Perhaps V genes differ in hypothetical, *cis*-acting elements which target the hypermutation mechanism to the appropriate location. However, the absence of somatic hypermutation in a rearranged V_x gene could also result from a secondary V_x gene rearrangement which, if it involves inversion, could place the initial V_x joint far away from the C_x locus (Weichold *et al.*, 1990; Huber *et al.*, 1992). Work by Sharpe *et al.* (1991) has shown that an enhancer element downstream of C_x is essential for full activation of the somatic hypermutation mechanism.

Clonal and intraclonal diversity, compartmentalization and dynamics of germinal centres

A large fraction of the cells in GC2 and 3 for which sequence data were obtained could be assigned to two or three major B cell clones (Figures 1b and 2b). As pointed out above, the true number of such major clones could be somewhat higher, up to a factor of 2. This is in very good agreement with earlier work in rodents, in which germinal centres were estimated to originate from one to six B cell precursors whose progeny was identified by genetic markers (Kroese *et al.*, 1987; Liu *et al.*, 1991a). As the present analysis shows, these clones grow in an intermingled fashion and presumably can reach very large sizes: we estimate that GC2 and 3 contain

Table V. Mutant selection in B cell clones expanding in GC2 and GC3: replacement versus silent mutations

GC	Clone	Length	No. of mutations		R/S ratio			
		FR/CDR	FR	CDR	Observed		Random	
					FR	CDR	FR	CDR
2	1	1.8:1	7	4	6:1	4:0	3.0:1	3.2:1
	2	1.6:1	4	5	4:0	4:1	2.7:1	3.5:1
				sum	10:1	8:1		
3	1	1.5:1	9	5	3:6	2:3	3.3:1	3.6:1
	2	1.3:1	8	9	3:5	4:5	3.3:1	3.2:1
				sum	6:11	6:8		

The designation of clones is as in Tables III and IV. R/S: ratio of replacement (R) to silent (S) mutations. Only nucleotide differences between clonally related sequences are considered. Two mutations in a codon are counted as two separate events. The random R/S ratio was calculated for the common precursors in the genealogical trees (2XH3, 2XH4, 3XH3 and 3YH3) taking the codon composition into account.

$\sim 1 \times 10^4$ B cells each (see Materials and methods). If the majority of these cells represent four to six clones of approximately equal size, then each clone has a size of ~ 2000 cells and has thus gone through more than 10 generations. Within a clone, most cells apparently express distinct antibody V regions, so that each germinal centre generates many thousands of B cells with different antibody binding sites which are all derived from the few binding sites expressed by the clonal precursors and presumably mostly selected for binding to just a few epitopes of some immunizing antigen(s).

We were surprised to find in the dark zone of GC2 a large population of dividing cells most of which expressed germline-encoded V region genes unrelated to those expressed by the clones dominating the light zone of GC2 and which appeared clonally unrelated among each other. Since we identified five independent in-frame V_H rearrangements in these cells, they must originate from a minimum of five precursor cells. This, together with the presence of cells belonging to the two major clones of GC2, raises the number of B cell precursors for the dark zone of this germinal centre to a minimum of seven. However, the true number of precursor cells is likely to be substantially larger: considering the five independent V_H rearrangements, the probability of picking five clonally unrelated cells from a population of cells from five clones of equal size, is 0.04. This probability rises to 0.15 assuming seven and to 0.3 assuming 10 such clones.

Although other interpretations are possible, we take these data to suggest that initially, germinal centres are populated by a polyclonal set of antigen-activated B cells which proliferate in the dark zone and largely express unmutated V region genes. Through an unknown signal somatic hypermutation is turned on in these cells and rare somatic mutants expressing high affinity antibodies are selected for further expansion in the light zone of the germinal centre, as postulated by MacLennan and colleagues (MacLennan *et al.*, 1991). Unselected cells are rapidly eliminated, whereas high affinity mutants may go through multiple rounds of proliferation and mutation in the dark zone. At the end of this process, the germinal centre is exclusively populated by members of the few clones which have won in the competition. GC3 (Figure 2b) would be representative of this stage of germinal centre development. Significantly, in the clones which have survived in this germinal centre we see strong counterselection against replacement mutations as one might expect for a late stage of somatic evolution of antibody affinity (Table V). In contrast, at an earlier stage, when the system is still in search of an optimal selection, replacement mutations may predominate (GC2; Table V).

In this picture, the clonal complexity of germinal centres estimated in earlier work to be in the order 1 to 6 does not reflect the number of progenitor cells originally populating the germinal centre, but that of the surviving clones.

It is clear that the verification of this model will require a kinetic analysis which can be more easily performed in experimental animals. Such an analysis being under way, we would like to point out that the present approach of analysing cells picked from their histological microenvironment in molecular terms through gene amplification, should be useful in the context of many other physiological and pathological processes. The range of possible applications could be considerably broadened by modifying the technique such that it also allows the analysis of gene expression in single cells isolated from histological sections.

Materials and methods

Tissues

Two human lymph nodes which had been taken out for diagnostic reasons were analysed. GC2 is derived from an inguinal lymph node of a 6 year old child, and GC3 from a cervical lymph node of a 23 year old adult who presented with tonsillitis.

Staining of frozen sections

For immunostaining 10 μm thick frozen sections were put on glass slides, air dried and incubated either with the OKT3 antibody (Ortho Diagnostics) or with the antibody Ki-67 (gift of Dr H.Lemke, Kiel) for 30 min at room temperature. After short washes with Tris-buffered saline, the slides were incubated with biotinylated Fab fragments of a rabbit anti-mouse monoclonal antibody (E413, Dako Diagnostics, Hamburg, 1:400) for 30 min. Following another washing step the slides were incubated with streptavidin–biotin labelled with alkaline phosphatase (K391, Dako Diagnostics, Hamburg) for 30 min. After washing, bound alkaline phosphatase was visualized by staining with new fuchsin. The slides were counterstained with haematoxylin.

Micromanipulation of single cells

The stained sections were incubated with 5 mg/ml collagenase H (Boehringer, Mannheim) in PBS. Single cells were mobilized under the microscope (Olympus) with the help of a hydraulic micromanipulator (Narishige) using 600× magnification. The cells were then aspirated using a micropipette fixed to a second micromanipulator. After the isolation of a cell a photograph was taken to allow the exact localization of that cell in the histological microenvironment. Isolated cells were put into 20 μl PCR buffer (50 mM

Table VI. Sequences of oligonucleotides used as primers for the amplification of rearranged V_H and V_x genes

V_H1	5'-ACTAGTCGACCTCAGTGAAGGT<CT>TCCTGCAAGGC-3'
V_H2	5'-ACTAGTCGACGTCCTGCGCTGGTGAAA<GC>CCACAC-3'
V_H3	5'-ACTAGTCGACGGGTCCCTGAGACTCTCCTGTGCAG-3'
V_H4	5'-ACTAGTCGACCCTGTCCCTCACCTGC<AG>CTGTC-3'
V_H5	5'-ACTAGTCGACAAAAAGCCCGGGGAGTCTCTGA<AG>GA-3'
V_H6	5'-ACTAGTCGACCTGTGCCATCTCCGGGGACAGTG-3'
$3'J_H1,2,4,4$	5'-ACCTGAGGAGACGGTGACCAGGGT-3'
$3'J_H3$	5'-TACCTGAAGAGACGGTGACCATTGT-3'
$3'J_H6$	5'-ACCTGAGGAGACGGTGACCGTGGT-3'
$5'J_H1,3,4,5$	5'-ACTAGTCGACGGTGACCAGGGT<GCT>CC<CT>GGCC-3'
$5'J_H2$	5'-ACTAGTCGACAGTGACCAGGGTGCCACGGCC-3'
$5'J_H6$	5'-ACTAGTCGACGGTGACCGTGGTCCCTTGCC-3'
V_x1	5'-TGATGTCGACATCC<AG>G<TA>TGACCCAGTCTCC<AT>TC-3'
V_x2	5'-TGATGTCGACAG<TA>CTCCACTCTCCCTG<CT>CCGTCA-3'
V_x3	5'-TGATGTCGACTCCAG<GC>CACCCTGTCT<GT>TGTCTC-3'
V_x4	5'-TGATGTCGACTCCCTGGCTGTGTCTCTGGGC-3'
V_x5	5'-TGATGTCGACAGTCTCCAGCATTCATGTCAGCGA-3'
V_x6	5'-TGATGTCGACTT<CT>CTCTCTGTGACTCCA<GA><GA>GGAG-3'
$3'J_x1,2,4$	5'-ACTCACGTTTGAT<TC>TCCA<GC>CTTGGTCC-3'
$3'J_x3$	5'-GTACTTACGTTTGATATCCACTTTGGTCC-3'
$3'J_x5$	5'-GCTTACGTTTAATCTCCAGTCGTGTCC-3'
$5'J_x1,2$	5'-TGATGTCGACTTGAT<CT>TCCA<GC>CTTGGTCCC<CT>TGGC-3'
$5'J_x3$	5'-TGATGTCGACTGATATCCACTTTGGTCCCAGGGC-3'
$5'J_x4$	5'-TGATGTCGACTGATCTCCACCTTGGTCCCTCCGC-3'
$5'J_x5$	5'-TGATGTCGACTAATCTCCAGTCGTGTCCCTTGGC-3'

The V_H and V_x primers hybridize to the FRI of the respective family. '< >' denotes a nucleotide mix at this position. The $5'J_H$ and $5'J_x$ primers were used at equimolar concentrations. The V_H1 primer also hybridizes to members of the newly defined V_H7 family (Mortari *et al.*, 1992).

KCl, 10 mM Tris−HCl pH 8.4, 0.01% gelatin, 1.5 mM $MgCl_2$) containing 1 ng/μl 5S rRNA and stored at −20°C.

PCR amplification

A set of oligonucleotides was chosen as primers for PCR amplification of rearranged V_H and V_x genes. Twelve V gene family specific primers were used for the six human V_H and six V_x families together with J_H and J_x specific oligonucleotides (see Table VI; the V_H1 primer also amplifies members of the newly defined V_H7 family (Mortari *et al.*, 1992)). A semi-nested PCR approach was chosen. In the first round of amplification the 12 V gene primers and the outer (3') J_H and J_x primer mixes were used simultaneously in one tube. Taking into account the degeneracy of several of the primers a total of 45 different oligonucleotides is present in the first round. For the second round of amplification aliquots of the first round were reamplified using the same V_H and V_x primers but with nested J_H and J_x primer mixes in seperate reactions for each V gene family.

Single cells in 20 μl PCR buffer were incubated with 0.25 mg/ml proteinase K for 1 h at 50°C. The enzyme was inactivated by heating to 95°C for 10 min. The first round of amplification was carried out in the same reaction tube in a 50 μl volume containing 50 mM KCl, 10 mM Tris−HCl pH 8.4, 0.01% gelatin, 2.5 mM $MgCl_2$, 200 μM each dATP, dGTP, dCTP and dTTP, 2.8 nM each V_H, V_x, $3'J_H$ and $3'J_x$ primer (see Table VI) and 2.5 U of Taq DNA polymerase (Gibco BRL). Enzyme was added after the first denaturation step. The cycle program consisted of one cycle at 95°C for 2 min, 59°C for 4 min, 72°C for 80 s, followed by 34 cycles of 95°C for 90 s, 59°C for 30 s, 72°C for 80 s, followed by 5 min incubation at 72°C.

The second round of amplification was carried out in separate reactions for each of the six V_H and six V_x family specific primers using 1.5 μl of the first round reaction mixture in a 50 μl volume with 50 mM KCl, 10 mM Tris−HCl pH 8.4, 0.01% gelatin, 1.5 or 2.5 mM $MgCl_2$ (1.5 mM for the V_H primers, 2.5 mM for the V_x primers), 200 μM each dATP, dGTP, dCTP and dTTP, 0.125 μM of one of the V_H or V_x primers and 0.125 μM of the $5'J_H$ or $5'J_x$ primer mixes (see Table V). 2.5 U of Taq DNA polymerase were added after the first denaturation step. The cycle program consisted of one cycle at 95°C for 2 min, 61°C or 65°C for 4 min, 72°C for 80 s, followed by 44 cycles of 95°C for 90 s, 61°C or 65°C for 30 s (65°C for the V_H3 and V_H4 primers), 72°C for 80 s, followed by 5 min incubation at 72°C. All amplifications were carried out in a Trio-Thermoblock (Biometra). A 10 μl aliquot of the reaction was analysed on a 2% agarose gel.

Extreme care was taken throughout the procedure to avoid contamination by DNA: gloves were changed frequently, separate equipment and working space were used for pre- and post-PCR manipulations, and aerosol resistant pipette tips were used.

Sequence analysis

PCR products were purified by gel electrophoresis through 2.5% NuSieve GTG agarose (Biozym). An aliquot of the isolated DNA was sequenced using the ds cycle sequencing system (Gibco BRL) as recommended by the supplier. Both strands of the PCR product were sequenced with the primers used in the second round of amplification. The V gene sequences were analysed using DNASIS software (Pharmacia) and the GenBank data library (release 73).

Double blind control experiment

To test the method for efficiency and reliability of the PCR amplification from single micromanipulated cells a control experiment was carried out. For this experiment T cells were micromanipulated from the T cell zone of a lymph node section stained with an anti-CD3 antibody (OKT3) and mantle zone cells as a source of B cells were isolated from another section of the same lymph node stained with the Ki-67 antibody (Gerdes *et al.*, 1984), which allows the identification of the mantle zone of GC. Isolated cells were coded and the analysis was carried out as a double blind experiment. Twenty cells were B cells and 19 were T cells. Ten of the 20 B cells gave at least one PCR product—defined as a visible band of the expected length on an ethidium bromide stained gel—upon V_H and V_x amplification with 25 PCR products altogether. For the 19 T cells, four PCR bands were obtained (sequences not shown). Sequence analysis of these PCR products revealed that only two of them represent V gene rearrangements (an in-frame V_H and an out-of-frame V_x rearrangement), the others being due to non-specific priming in the PCR (e.g. part of the unrearranged J_H locus in one case). Such false positive sequences were never obtained for PCR products derived from B cells.

Estimation of the number of cells in the germinal centres

The number of cells seen on the sections was counted (750 and 760 cells for GC2 and GC3, respectively) and the ratio between length and width of the germinal centre sections was determined. Assuming that the volume of the germinal centres can be approximated as a spheroid and that the germinal centres are cut near the largest area, GC2 and GC3 harbour at least 12 000 and 14 000 cells, respectively. Taking into account that the dark zone of GCs mainly contains B cells whereas in the light zone >10% non-B cells are present (Lusheng *et al.*, 1993) the number of B cells in the two germinal centres may be $\sim 1 \times 10^4$.

Mutations of clonally related sequences not shown in figures

Sequences 2C1H4–2C7H4 had position 32 changed from ACT to CCT [replacement mutation (R)] and position 35a from GAC to AAC (R); 2L26K1–2L27K1 had position 32 changed from TAT to TCT (R), position 48 from ATG to ATC (R), position 52 from ACG to ACT [silent mutation (S)], position 63 from AGA to AGC (R) and position 83 from TCT to TTT (R); 2C7K3 and 2LK28K3–2D21K3 had position 29 changed from GTT to ATT (R); 3D15AK3, 3D26K3, 3D33K3 and 3L31K3–3L34K3 had position 33 changed from TTA to TTG (S).

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