

Antigen-driven Clonal Proliferation of B Cells within the Target Tissue of an Autoimmune Disease

The Salivary Glands of Patients with Sjögren's Syndrome

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Abstract

Structures resembling germinal centers are seen in the salivary glands of patients with Sjögren's syndrome, but it is not known whether the microenvironment of these cell clusters is sufficient for the induction of a germinal center response. Therefore, we cloned and sequenced rearranged Ig V genes expressed by B cells isolated from sections of labial salivary gland biopsies from two Sjögren's syndrome patients. Rearranged V genes from B cells within one cell cluster were polyclonal and most had few somatic mutations. Two adjacent clusters from another patient each contained one dominant B cell clone expressing hypermutated V genes. None of the rearranged V genes was found in both clusters, suggesting that cells are unable to migrate out into the surrounding tissue and seed new clusters. The ratios of replacement to silent mutations in the framework and complementarity determining regions suggest antigen selection of high-affinity mutants. These results show that an antigen-driven, germinal center-type B cell response is taking place within the salivary glands of Sjögren's syndrome patients. In view of the recent demonstration of a germinal center response within the rheumatoid synovial membrane and the existence of similar structures in the target tissues of other autoimmune diseases, we propose that germinal center-type responses can be induced in the nonlymphoid target tissues of a variety of autoimmune diseases. (*J. Clin. Invest.* 1998. 102:938–946.) Key words: autoimmunity • Sjögren's syndrome • germinal center • immunoglobulin • V gene

Introduction

Sjögren's syndrome (SS)¹ is characterized by chronic inflammatory lesions in the salivary and lachrymal glands with degeneration of the surrounding tissue and severely diminished

secretion, resulting in keratoconjunctivitis sicca and xerostomia (dry eyes and dry mouth). It can take the form of primary SS, with no other manifestations, or secondary SS in association with rheumatoid arthritis and other autoimmune connective tissue diseases such as SLE, scleroderma, and polymyositis. Approximately 25% of SS patients also have rheumatoid arthritis (1). The major autoantibodies associated with SS are rheumatoid factor, anti-Ro (SS-A), and anti-La (SS-B). Autoantibodies against salivary duct antigens are also found in the serum (2) and, recently, 95% of primary SS patients were reported to have serum antibodies against α -fodrin. The role of these autoantibodies in disease pathogenesis is unclear, but tolerization with α -fodrin prevented disease in a mouse model, suggesting an important role in disease development (3). Anti-SS-A and anti-SS-B are implicated in congenital heart block in the children of mothers with SS, due to transplacental passage of the antibodies which bind to fetal heart muscle (4–7). Infiltrating lymphoid cells are found in the salivary, lachrymal, and other exocrine glands in the respiratory tract, gastrointestinal tract, and vagina. Clusters of B and T cells resembling germinal centers, and numerous plasma cells, are found in the salivary glands of SS patients (1, 8), which produce large amounts of immunoglobulin, including rheumatoid factor, anti-Ro, and anti-La (1, 9, 10).

Ectopic germinal center-like structures can be found in nonlymphoid tissues or other abnormal sites in a variety of other autoimmune and/or inflammatory diseases, including the rheumatoid synovial membrane (11), thyroid glands of patients with Hashimoto's thyroiditis (12), the liver in chronic hepatitis after hepatitis C virus infection (13), the choroid of the eye in uveoretinitis (14), and the lung in cryptogenic fibrosing alveolitis (15).

During the immune response against a xenogeneic antigen, B cells bearing antigen-specific receptors are stimulated to proliferate and differentiate into antibody-secreting plasma cells within germinal centers. This requires the presence of follicular dendritic cells (FDCs) and activated CD4⁺ helper T cells, CD40/CD40 ligand interaction, and a cocktail of cytokines to create the microenvironment necessary for a germinal center reaction to take place. During this process a few B cells bearing the appropriate antigen receptors (~1–4 cells per germinal center) are stimulated to undergo clonal proliferation in the dark zone of the germinal center and differentiation to centroblasts, centrocytes, memory B cells, and plasma cells. Antibody-secreting plasma cells migrate out of the follicle into the surrounding tissue. Somatic hypermutation of the rearranged immunoglobulin V genes and Ig class switching are initiated within the germinal center. Centrocytes in the light zone, expressing antigen receptors which have mutated towards increased affinity for the stimulating antigen, are selected by competition for antigen on the surface of FDCs and rescue from apoptosis (16).

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1. Abbreviations used in this paper: CDR, complementarity determining region; FDC, follicular dendritic cell; RF, rheumatoid factor; R/S, replacement/silent mutation ratio; SS, Sjögren's syndrome.

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Germinal centers normally arise in primary follicles within the secondary lymphoid organs, namely the spleen, lymph nodes, and Peyer's patches, these organs providing the necessary microenvironment for the germinal center response. Recently, it was shown that B cells in germinal center-like structures in the synovial membranes of rheumatoid arthritis patients are clonally proliferating and express hypermutated Ig V genes (17, 18). This suggests that the microenvironment necessary for germinal center formation may be generated in nonlymphoid organs or tissues during an autoimmune response. Whether this is a widespread phenomenon, or is limited to rare occasions when an overactive inflammatory response generates a concentrated milieu of cytokines and inflammatory cells sufficient to bring together all the components required to generate a localized B cell response in nonlymphoid tissue, is not yet clear.

Similarly, it is not known whether the germinal center-like structures in the salivary glands of SS patients contain all the components required to generate a local, antigen-driven B cell response, or whether autoantibody-producing cells migrate independently into the salivary glands from the secondary lymphoid organs. To distinguish between these two possibilities, we cloned and sequenced rearranged Ig V genes from B cells in germinal center-like clusters, isolated by microdissection from frozen sections of labial salivary gland biopsies from SS patients. The results show that antigen-driven, clonal B cell proliferation and hypermutation accompanied by antigen selection take place within the salivary glands of these patients. Taken in conjunction with our observation of a germinal center reaction in the rheumatoid synovium and histological evidence for germinal centers in other diseases, this suggests that germinal center reactions in nonlymphoid target tissues are a general feature of autoimmune disease.

Methods

Salivary gland biopsies. Minor labial salivary glands were excised from two female patients (BW, 65 yr old with primary SS; SG, 39 yr old with secondary SS and polyarthritides). The biopsies were immediately embedded in Tissue-Tek OCT embedding medium (Miles, Giessen, Germany), frozen on cardice, and stored at -70°C . 6- or 8- μm sections were cut on a cryomicrotome (Leitz), mounted on slides coated with 2% 3-amino-propyltriethoxy silane (Sigma Chemical Co., St. Louis, MO) in acetone, dried at ambient temperature for 1 h, then 50°C for 1 min, and fixed in acetone for 10 min. The slides were stored at -70°C in the presence of a desiccant (anhydrous CaCl_2) until required.

Immunohistochemical staining of tissue sections. Frozen sections were stained with mouse monoclonal antibodies specific for B cells (anti-CD20; DAKO A/S, Glostrup, Denmark), T cells (anti-CD3; DAKO A/S), FDCs (Wue-2), and plasma cells (Wue-1) (A. Greiner, University of Würzburg, Würzburg, Germany), followed by rabbit anti-mouse Ig (DAKO A/S) and the alkaline phosphatase-antialkaline phosphatase (APAAP) complex (DAKO A/S). Immune complexes containing APAAP were detected by incubation with new fuchsin substrate (DAKO A/S) and the sections counterstained with Mayer's hematoxylin (Sigma Chemical Co.).

Microdissection of B cell clusters and preparation of DNA. B cell clusters in frozen sections of salivary gland biopsies were identified by staining with anti-CD20 as described above and excised under sterile Scott's tap water substitute (0.35% NaHCO_3 , 0.2% MgSO_4) with sterile blood lancets using an inverted microscope. The excised tissue was digested in 20 μl proteinase K (0.7 mg/ml; Boehringer Mannheim, Mannheim, Germany) at 50°C for 1 h and the enzyme was inactivated at 95°C for 10 min.

Amplification and cloning of rearranged Ig V genes. The meth-

ods used for amplification and cloning of rearranged Ig V genes have been described previously (18) and are therefore only described in outline here. To avoid contamination of reagents with amplified Ig V genes, all procedures before PCR took place in a separate laboratory from that where PCR amplification and all subsequent steps using amplified DNA were performed. The DNA in 10 μl of the digested tissue was amplified for 36 cycles with a mixture of primers specific for rearranged V_H or V_L genes. Two methods were used. In the first method, a mixture of 5' (back) primers corresponding to the first framework region of each of the V_H or V_L gene families was used in conjunction with primers for each of the J_H or J_L exons to ensure that all possible rearranged V_H and V_L genes were amplified in the first PCR (19). The product was purified by agarose gel electrophoresis and the region corresponding to 350 bp was cut out and melted in 200 μl of sterile distilled water. 2 μl of this, or a 1:500 dilution, was added to a series of tubes containing separate 5' (back) primers for each V_H , V_K , or V_λ family and subjected to a second round PCR (40 cycles).

Alternatively, nested primers were used. In the first PCR, these corresponded to the leader sequence for each V_H family and a universal J_H primer (20). 0.5 μl of the first PCR product was subjected to a second PCR without prior purification, using internal primers as described above. Duplicate negative control samples containing water instead of DNA were always amplified in parallel with the DNA from the digested tissue. These produced no detectable band after the second amplification. Amplified DNA from the second PCR was purified by agarose gel electrophoresis, extracted with silica gel (Jetsorb, Genomed, or Easy Pure; Biozym Diagnostik, Oldendorf, Germany), ligated into plasmid vector pCRII, and cloned using the TA cloning system (Invitrogen Corp., Carlsbad, CA).

Sequencing and analysis of rearranged V genes. Cloned, rearranged Ig V genes were sequenced from both ends by a modified *Taq* sequencing protocol using -40 or $+40$ digoxigenin end-labeled primers (TIB Mol Biol, Berlin, Germany), as described previously (21). Alternatively, sequences were determined by the DyeDeoxy terminator method using an automatic DNA sequencer (Applied Biosystems, Inc., Foster City, CA). Germline Ig V genes giving the best match to the cloned DNA sequences were identified by searching the human Vbase Sequence Directory (Tomlinson, I.M., S.C. Williams, O. Ignatovich, S.J. Corbett, and G. Winter, M.R.C. Centre for Protein Engineering, Cambridge, UK). Sequences were compared with the corresponding germline gene using the DNA Plot 1.4 program (W. Müller, Institut für Genetik, Köln, Germany). Family trees based on V gene sequences with identical V-D-J or V-J rearrangements were constructed by analysis of shared and unshared mutations.

Results

Phenotypic characterization of lymphoid cells in the salivary glands. Clusters of B and T cells resembling germinal centers were identified in frozen sections of SS salivary gland biopsies by staining with anti-CD20 and anti-CD3. A cell cluster in biopsy BW was filled with B cells over the whole area, whereas the T cells were more loosely distributed within the cluster. Staining for FDCs revealed that they were distributed throughout the whole cell cluster, in intimate contact with the B and T cells (Fig. 1). No plasma cells were found within the cluster but a few were present in the surrounding tissue, around the ducts. The biopsy from patient SG contained two large clusters of lymphocytes separated by a narrow band of glandular tissue. Cells within the clusters stained for CD20, CD3, and FDC antigen (Fig. 2). The B cells were densely distributed throughout both clusters, whereas the T cells were confined to approximately half of the total area and FDCs were distributed in isolated patches of cells within, or on the edge of, the T cell zone (Fig. 2, E and F). As in cluster BW, very few plasma cells were

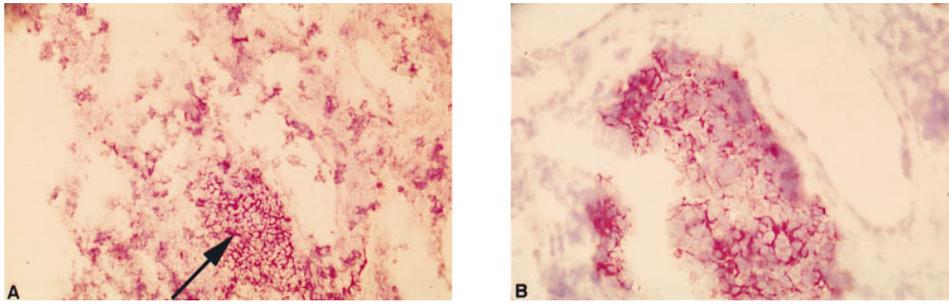


Figure 1. Immunohistochemical staining of a labial salivary gland biopsy from SS patient BW: (A) anti-CD20 ($\times 200$), (B) anti-FDC ($\times 400$). The oval cluster of B cells, T cells, and FDCs is indicated by the arrow in A.

found within the clusters, but large numbers of plasma cells were present in the surrounding tissue (Fig. 2 G). These structures resemble the germinal centers seen in secondary lymphoid organs during an immune response. The zone of T cells, which also contained B cells and FDC, in SG-L and SG-R resembles the light zone of a germinal center, whereas the area

of B cells more sparsely populated with T cells and FDC resembles the dark zone of centroblasts in a germinal center, but there was no evidence of a mantle zone. The absence of a mantle zone suggests that the proliferating B cells did not expand in a primary follicle, as occurs in germinal centers in secondary lymphoid organs. The number of B cells in the three clusters

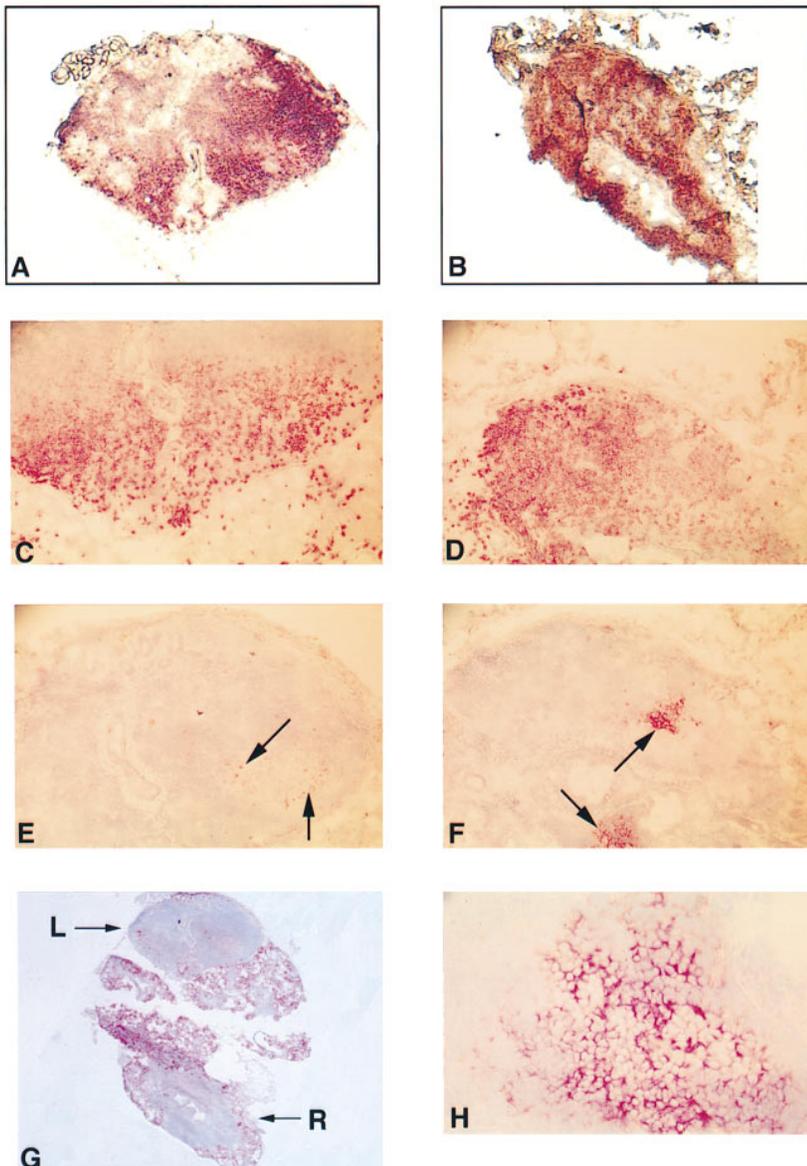


Figure 2. Two adjacent germinal center-like cell clusters in a labial salivary gland biopsy from patient SG. (A) SG-L and (B) SG-R stained with anti-CD20 ($\times 100$). (C and D) The same clusters stained with anti-CD3 showing large numbers of T cells in the lower half of SG-L (C) and the upper half of SG-R (D) ($\times 100$). (E and F) Clusters SG-L and SG-R stained for FDCs with monoclonal antibody Wue-2 ($\times 100$). Areas containing FDCs are indicated by arrows. (G) Section containing clusters SG-L and SG-R stained for plasma cells with monoclonal antibody Wue-1. The glandular tissue surrounding the cell clusters is packed with plasma cells. Very few are inside the clusters ($\times 20$). (H) Higher power magnification of the upper area of FDCs indicated by an arrow in F ($\times 400$).

was estimated by counting the number of B cells across the long and short axes in an anti-CD20-stained section from the mid-region of the cluster and the number of sections in which part of the cluster could still be seen. Assuming that the clusters approximate to the shape of a prolate ellipsoid, cluster BW contained $\sim 6,000$ – $7,000$ B cells, whereas SG-L and SG-R were much larger, containing ~ 7 – 9×10^5 B cells. Multiple clusters of B cells, T cells, and FDC were also seen in minor salivary gland biopsies from two other patients with primary SS.

Cluster BW represents an early stage of germinal center reaction. The cellular components necessary for a germinal center response are present in cluster BW, namely B cells, T cells, and FDCs capable of presenting antigen to the B cells. Of 27 rearranged V genes isolated from the cluster, five independent V_H -D-J and two V_L -J rearrangements were shared by two or more cloned genes (Table I). The single base difference in one out of four DP-10 V_H genes is below the level of the *Taq* polymerase error and is therefore probably an artifact of the PCR. The number of base differences between the remaining V genes is above the *Taq* polymerase error rate of 1.7×10^{-3} for V_H or 1.2×10^{-3} for V_K , i.e., approximately one error per two V_H genes or per three V_K genes (22). However, only one of these groups of rearranged V genes included sequences which could be ascribed to parental and daughter cells on the basis of shared mutations [DP-79(a)]. The other groups [3D279d, DP-79(b) and DP-38] each contained only two members which shared identical rearrangements. The DP-38 sequences had only one base difference between them, which could be ascribed to PCR-induced error. The 3D279d and DP-79(b) pairs of V_H genes differed from their partners by six and three bases respectively, suggesting that they are the result of cell division and mutation.

Table I. Rearranged V Genes Isolated from Cluster BW

Section	V gene family	Germline V gene	No. of sequences isolated	Av. No. of mutations/V region	Mean R/S	
187	VH3	3D279d	2	19.5	2.9	
		DP49	1	17		
	VH4	DP79* (a)	4	2.3	0.5	
189	V_{K1}	DP79* (b)	2	19.5	2.9	
		DPK9 [‡]	1			
		DPK30	1	4	3.0	
190	VH1	DP10	4	0.3	> 1	
		DP10 [‡]	1	1		
	VH3	DP38	2	6.5	2.3	
	V_{K1}	DPK9 [‡]	1			
	V_{K5}	EV15	1	5	> 5	
		EV15 [‡]	2			
	$V_{\lambda 1}$	Iv2046	1	9	> 9	
	$V_{\lambda 2}$	DPL13	1	6	5.0	
	$V_{\lambda 3}$	DPL16* (a)		1	0	—
			DPL16* (b)	2	10	3.0

Rearranged Ig V genes isolated from a cell cluster in a labial salivary gland biopsy of patient BW were identified with the best matching germline V gene in the human Vbase Sequence Directory using a fasta search. Sequences with independent V-D-J rearrangements are listed separately as a and b. *V genes with different rearrangements; [‡]Nonfunctional gene.

12 light chain V genes were cloned and sequenced from the same cluster, 6 V_K and 6 V_λ (Table I). Four of the six rearranged V_K genes contained stop codons and were therefore nonfunctional. These consisted of two pairs of genes using identical rearrangements of the germline genes DPK9 and EV15, respectively. The two functional V_K genes were unrelated. One pair of V_λ genes [DPL16(b)] shared the same V-J rearrangement and one of these genes was heavily mutated, suggesting that they are members of the same clone. No single B cell clone dominates this cluster.

Cluster SG-L and SG-R each contain a dominant expanded clone of B cells. Most rearranged V_H genes isolated from two cell clusters (SG-L and SG-R) in the salivary gland biopsy of patient SG were members of only two B cell clones, one from each cluster. 5 out of 8 of the V_H genes from SG-L used germline V gene VH328 with the D exon DXP'1 and J_H 5b. 21 out of 29 V_H genes from SG-R used germline V gene DP10 with D exon DA-1 and J_H 4b, respectively (Fig. 3). All genes using the same V_H /D/J combination had identical junctional sequences and were therefore members of the same B cell clone. All of the eight remaining V_H genes isolated from cluster SG-R used the germline gene DP65 and were nonfunctional rearrangements containing stop codons but had different mutation patterns. Since all functional V_H genes from this cluster were derived from the same clone of B cells, it seems likely that the nonfunctional DP-65 genes were also derived from this clone and had undergone somatic mutation during proliferation. Similarly, all 11 V_λ genes isolated from cluster SG-R shared the same rearrangement of germline gene DPL16. Only four functional V_K genes were isolated from this cluster. Two of these shared the same germline gene (B3) and they contained few mutations (Table II). Thus, it would appear that the majority of cells in this cluster are members of the same clone.

That the isolation of rearranged V genes from a single clone is not an artifact due to selection bias during the PCR amplification is shown by the identification of multiple, independently rearranged V genes from cluster BW, peripheral blood lymphocytes (data not shown), and from a pharyngeal tonsil germinal center (18) using the same protocol. Approximately 10,000 cells were present in each section of SG-L and SG-R removed for PCR amplifications, so, if other B cell clones were present in significant numbers we would expect to have detected them among the 8 V_H genes cloned from SG-L and the 30 V_H genes cloned from SG-R. Of course, we cannot rule out the possibility that another clone(s) might be present in sections that were not analyzed.

None of the cells isolated from SG-L or SG-R contained the V_H -D-J rearrangement present in the cells of the neighboring cluster, showing that B cells had not migrated between the two clusters.

Clonal B cell proliferation within the salivary gland is accompanied by hypermutation of immunoglobulin V genes in SS. Five rearranged V_H genes and one rearranged V_K gene from cluster BW were hypermutated (17–20 point mutations per V gene). The remainder had relatively low levels of base differences (0–9) when compared with the germline gene giving the best match. The average number of base changes in the latter V genes, which are all members of groups with identical rearrangements, is 3.7 ± 2.1 (SD). Although low, this is higher than the *Taq* polymerase error rate and therefore is likely to be the result of somatic mutation. All members of the two clones from patient SG were hypermutated, SG-R having from

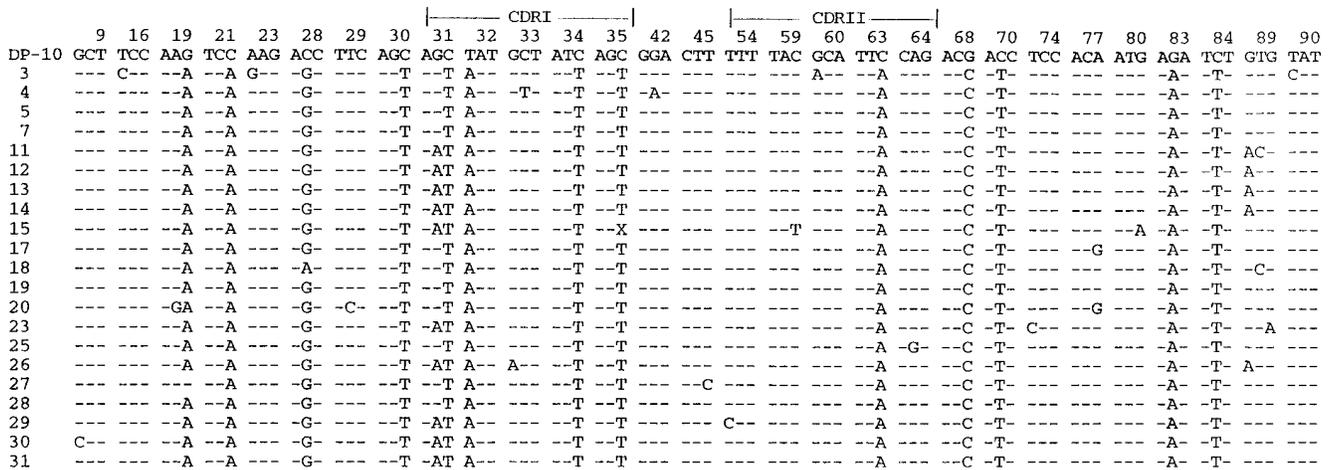


Figure 3. Alignment of sequences of V_H genes isolated from cluster SG-R with germline gene DP-10. Only codons containing base changes compared with the germline gene are shown. All genes had an identical sequence at the junction with DA-1 and J_H4b and are therefore members of the same B cell clone.

13 to 20 mutations per V_H gene, with a mean of 14.8±1.7 (SD) and 8.9±1.2 per V_λ gene. The clone in cluster SG-L contained from 24 to 27 mutations per V_H gene with a mean of 26.0±1.1 (Table II).

Three of the five groups of V_H genes from cluster BW whose members share identical V-D-J rearrangements have sufficient differences to suggest that they are daughters of a parental cell that divided within the salivary gland but only one of these groups [DP79 (a)] included an identifiable parental cell (Table I and Fig. 4). In clusters SG-L and SG-R, we see a stepwise accumulation of mutations in V_H and V_λ genes enabling us to draw genealogical trees arising from the original naive B cell expressing the corresponding germline gene (Fig.

4). This clearly demonstrates that the two clones developed within the salivary gland by proliferation of a common precursor accompanied by V gene diversification through somatic hypermutation.

Is B cell proliferation within the salivary gland driven by antigen selection? Hypermutation within a germinal center is closely linked with antigen-induced B cell proliferation, so it is reasonable to deduce from the above data that the response within the salivary gland is also driven by antigen. The second phase of this process, during an immune response against a xenoantigen, is selection of B cells expressing high-affinity antigen receptors resulting from rare mutations. It is generally believed that this process manifests itself as an increase in the ratio of replacement (R) to silent (S) mutations, particularly in the complementarity determining regions (CDRs) (23). In contrast, the R/S ratios of heavy and light chains from all three clusters were significantly lower than would be expected for random mutations (Tables I and II). The mean R/S ratio in cluster BW was 1.8±1.2 for V_H genes and 2.9±1.4 for V_L genes. All of the 21 members of the DP-10 V_H clone which dominates cluster SG-R had consistently low R/S ratios in both the framework regions (mean = 1.2±0.23) and CDRs 1 and 2 (mean = 0.91±0.2), suggesting selection against replacement mutations. Mean R/S ratios for the clone expressing V_H328 in SG-L were 1.3±0.1 in the framework region and 2.3±0.4 in CDRs 1 and 2, also indicating negative selection in the framework region. Only the CDR regions of the V_λ genes in SG-R had high R/S ratios (Table II).

Table II. Rearranged V Genes Isolated from Clusters L and R from Patient SG

Section and cluster	V gene family	Germline V gene	No. of sequences isolated	Av. No. of mutations/V region	Mean R/S±SD	
					Framework region	CDRs
82 L	VH3	V3-49	1	9	4.0	> 4
	VH4	DP-65	2	22	2.2	> 6
		VH328	5	26±1.1	1.3±0.13	2.3±0.38
81 R	VH1	DP-10	21	14.7±1.2	1.2±0.22	0.9±0.19
	VH4	DP-65*	8			
	Vκ1	L5	2	9	3.0	4.0
	Vκ1	08/018*	1			
	Vκ4	B3	2	1.5±0.5	> 1	> 0.5
		B3*	1			
		Vκ5	B2*	1		
	Vλ3	DPL16	10	8.6±1.1	1.2±0.24	> 4±0.6
		DPL16*	1	10	1.0	> 4

Rearranged Ig V genes isolated from two cell clusters (L and R) in a labial salivary gland biopsy of patient SG were identified with the best matching germline V gene in the human Vbase Sequence Directory using a fasta search. *Nonfunctional; †Different rearrangement from DP-65 in cluster SG-L.

Discussion

The characteristic lesions in the major and minor salivary glands of patients suffering from SS contain focal infiltrates of T and B lymphocytes and plasma cells which first appear as small clusters around the ducts, later enlarging to form structures resembling germinal centers containing B and T lymphocytes with the plasma cells scattered in the surrounding glandular tissue (24). These clusters of lymphocytes have been reported frequently as monotypic in a significant number of SS patients by immunochemical staining for κ and λ light chains, a

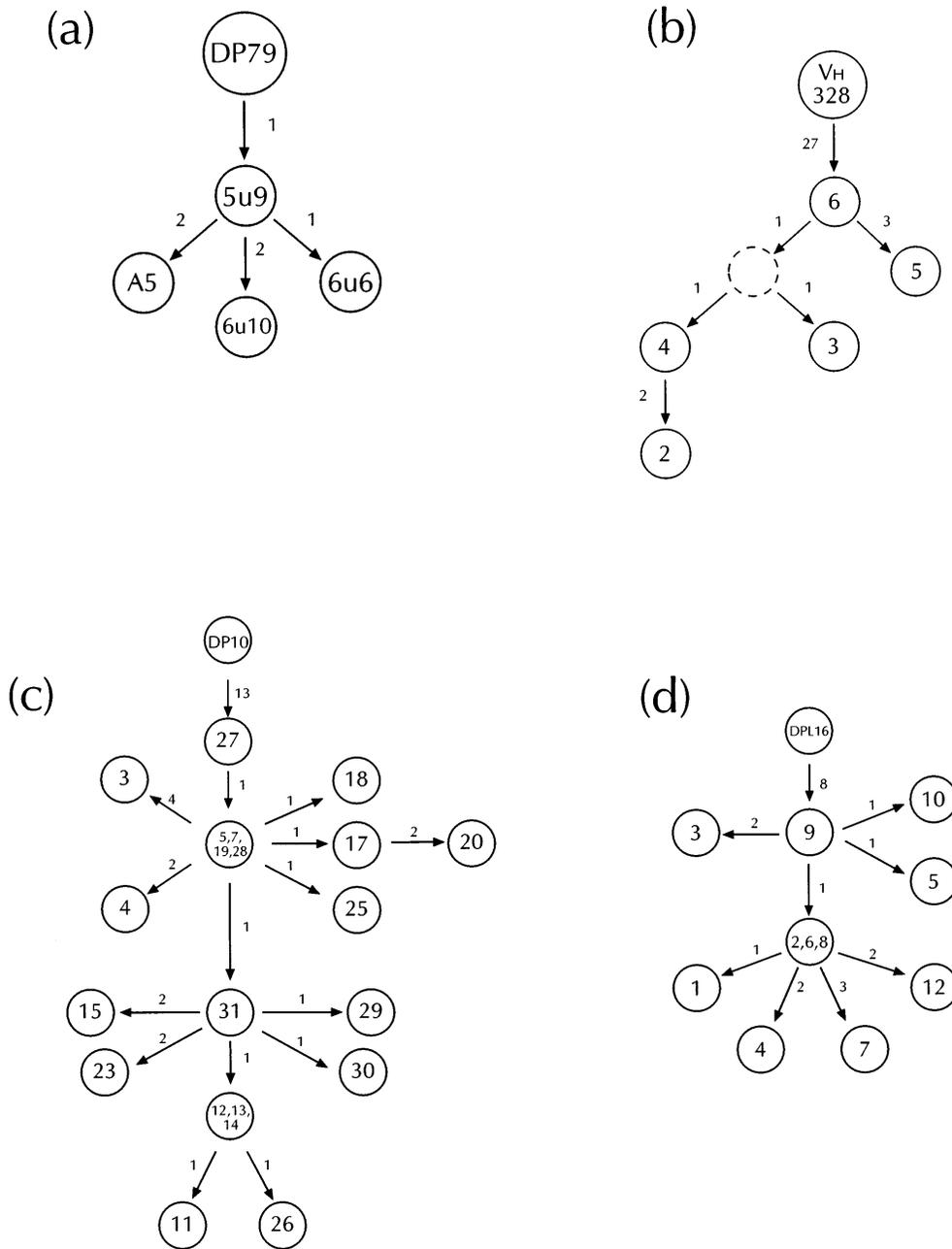


Figure 4. Genealogical trees of B cell clones identified by their recombined V genes isolated from clusters BW, SG-L, and SG-R. The members of each clone share identical sequences at the V-D-J or V-J junction. (a) Clonal relationship between V_H genes using germline gene DP79 isolated from cluster BW. (b) B cell clone using germline gene V_H 328 from cluster SG-L. (c and d) B cell clones using germline genes V_H DP-10 and V_L DPL16 from cluster SG-R. The top circle in each set represents the naive B cell expressing the germline gene. Numbers inside other circles are arbitrary numbers given to cloned genes. A circle containing more than one number indicates two or more cloned genes with identical sequences. Numbers at the sides of the arrows indicate the minimum number of point mutations required at each step. Dashed circles represent hypothetical intermediates.

pattern which is generally regarded as evidence for monoclonality. The proportion of patients reported to exhibit this pattern of staining varies from 18 to 36% (24). A monotypic staining pattern does not necessarily demonstrate monoclonality, however, as it could equally be the result of restricted expression of light chain isotype, e.g., if a response to an antigen or epitope favors one isotype or V_L gene, as has been demonstrated in the immune response to some haptens (25–29). We have shown previously that serum autoantibodies are also often clonally restricted or even monoclonal in autoimmune diseases including Hashimoto's thyroiditis, autoimmune uveoretinitis, and SLE (30–32). Southern blotting and size analysis of PCR products on polyacrylamide gel have also been used to demonstrate clonal restriction of B cells in lip biopsies of SS patients (33, 34).

The frequency of non-Hodgkin's B cell lymphoma is abnormally high in SS patients, up to 44 times the general population (35). The lymphomas are usually found in mucosal tissue, including the major salivary glands, stomach, and lungs, as well as lymph nodes, although they are relatively uncommon in the minor salivary glands, possibly reflecting the smaller mass of this tissue. It has been proposed that lymphoma may initially develop in a salivary gland from one of the clusters of proliferating B cells, possibly as a result of chronic stimulation resulting in an increased probability of neoplastic transformation. However, in only one instance is there evidence that a lymphoma arose in a salivary gland, when a gastric lymphoma was shown to have the same V-D-J rearrangement as B cells in a lip biopsy removed 15 mo previously (36). Recently, Bahler et al. (37) reported ongoing somatic hypermutation in V_H and

V_L genes of salivary gland mucosa-associated lymphomas and suggested that mucosa-associated lymphoid tissue lymphomas therefore originate from a germinal center B cell. Clonal diversification has also been reported in a B cell lymphoma secreting a monoclonal IgM cold agglutinin from a patient with autoimmune hemolytic anemia. However, the mutation rate in the V_H gene expressed by the lymphoma was much lower than the hypermutation rate in a secondary immune response (38).

To determine whether an antigen-driven germinal center reaction is taking place in the B and T cell clusters in the salivary glands and to determine the number of B cell clones present in the clusters, it is necessary to examine the sequences of the expressed, rearranged Ig V genes in individual clusters. To this end, we removed individual clusters from one-cell-thick frozen sections of labial salivary glands by microdissection, and cloned and sequenced their rearranged V genes.

All of the clusters we examined contained B cells, T cells, and FDCs with a morphology similar to germinal centers, although a mantle zone was not apparent. So far as we are aware, this is the first time FDCs have been identified within the cell clusters in SS salivary glands, showing that the cellular components necessary for an antigen-driven B cell response are all present. However, there was a marked difference in size between the cluster isolated from patient BW and the two from patient SG, the former being estimated to contain in the region of 6,000–7,000 B cells, whereas clusters SG-L and SG-R were much larger, containing in the region of $7\text{--}9 \times 10^5$ B cells, i.e., ~ 100 times larger. This is also ~ 50 times larger than two human tonsil germinal centers studied by Küppers et al. (39) using similar techniques. We do not know whether the same signals and growth controls, including cytokines, are operating within these clusters as in germinal centers but, if the cells are dividing at the same rate as the centroblasts in a germinal center, namely a generation time of 6–7 h, clusters of the size of SG-L and SG-R could be generated within 6 d. Since SS is a chronic autoimmune disease and the majority of patients do not progress to lymphoma, there is presumably some feedback mechanism limiting the growth of these clusters which may be defective in those patients who do develop B cell lymphoma.

The small cell cluster BW was polyclonal with many of the rearranged V genes containing few or no mutations. Those genes which were hypermutated were not present in large numbers (not more than two representatives of each identical rearrangement) and may therefore be expressed by memory cells rather than expanding clones, although two pairs of hypermutated genes [3D274d and DP-79 (b)], which differed by six and three point mutations respectively, appear to be derived from cells that divided within the cluster. One group of four V_H genes all had identical rearrangements but only low numbers of base differences from the germline gene, albeit above the PCR error rate, suggesting that they were also derived from B cells that had divided within cluster BW. A V_K gene (*Humkv325*), which is commonly associated with rheumatoid factor antibodies, has also been reported to be somatically mutated in B cells from a submandibular salivary gland biopsy of a patient with SS (40).

The two large cell clusters from patient SG resemble fully developed germinal centers since all except one of the functionally rearranged V_H genes were derived from two clones of B cells, one in each cell cluster. The observation of large numbers of plasma cells surrounding these clusters also supports this hypothesis, compared with the less developed cluster BW

which was surrounded by much smaller numbers of plasma cells. Whether these plasma cells have migrated out of the clusters has yet to be demonstrated. Both clones are hypermutated compared with the germline genes which match them most closely. Each of the two clones must have proliferated within the clusters from common precursor cells since a genealogical tree can be drawn for each on the basis of shared and unshared mutations (Fig. 4). It could be argued that either or both of these clones may be neoplastic but it is unlikely, however, that two neoplastic clones would arise independently within the same minor labial salivary gland.

Immune responses to xenogeneic antigens under conditions of limiting antigen concentration usually result in selection for replacement mutations in the CDRs which increase antibody affinity, while replacement mutations in the framework regions that disrupt the secondary and tertiary structure of the V domain are selected against. R/S ratios in the CDRs that are significantly lower than the ratio expected to be generated by random mutations (2.9) are also regarded as evidence of antigen selection (23, 41). Despite the high frequency of mutation, indicative of a memory response, the low R/S ratios of mutations in the framework regions and CDRs of V_H328 and DP-10 indicate that selection against replacement mutations has occurred. The clonally expressed λ chain (DPL16) in SG-R also had a low R/S ratio in the framework region but a high R/S in the CDR. Low R/S ratios were also found in the CDRs of hypermutated rheumatoid factors from healthy donors (42), some anti-DNA antibodies from SLE patients (43), and some of the antibodies produced by germinal center B cells in synovial membranes from rheumatoid arthritis patients (18). These results imply that antigen selection is driving the B cell response within the salivary gland and rheumatoid synovium.

A possible explanation for lower than random R/S ratios in the CDRs during an autoimmune response is that the autoantigen will be present at high concentration within the target tissue where B cells are proliferating. Consequently, mutant B cells expressing high-affinity antigen receptors would have no selective advantage over B cells expressing receptors with lower affinity. However, negative selection would still be effective since mutations which disrupt the V domain structure would render the antigen receptor unable to bind antigen and/or unable to signal to the B cell. Therefore the cell would cease to divide. This is consistent with a scenario in which a pathogenic microorganism triggers a small number of B cells with receptors for a self-antigen (e.g., Ro or La). The response would be maintained by high concentrations of self-antigen presented on FDCs in the salivary glands. Intra- and intermolecular epitope spreading would then lead to production of other autoantibodies, e.g., from Ro to La and rheumatoid factor. There is some, albeit limited, evidence that a virus such as Epstein Barr virus, cytomegalovirus, herpes virus, or human T cell leukemia virus-1 may be the initiating etiological agent (44).

There are three possible alternative origins for the multiple B cell clusters in the salivary glands of SS patients. One is that they arise by aggregation of infiltrating polyclonal B cells, which we have ruled out by demonstrating clonal B cell proliferation within the clusters. The second is that a single B cell clone proliferates to form a germinal center in the salivary gland; B cells then migrate out into the glandular tissue to seed other clusters. The third hypothesis is that individual infiltrating B cells are stimulated independently by antigen-presenting cells at different locations to proliferate and produce separate

clusters. Since none of the cells in either of the large clusters SG-L or SG-R expressed the same V-D-J combination as cells in the neighboring cluster, even though the two clusters were separated by only a thin strip of glandular tissue, it would appear that B cell clones proliferate and mutate independently within each cluster, in agreement with the third hypothesis. This is also consistent with the observation that B cells responding to a hapten (4-hydroxy-3-nitrophenyl acetyl) proliferate and mutate independently in germinal centers and do not migrate between them (45).

The origin of the parental B cells from which the clones arose is not known but the low number of mutations in the DP79 clone found in cluster BW suggests that this clone arose from a naive B cell, whereas the clones in SG-L and SG-R, which were more heavily mutated, probably arose from memory B cells. This is consistent with a chronic autoimmune response with continual restimulation by self-antigen. The memory B cells may be generated either in a secondary lymphoid organ and subsequently migrate to the salivary gland, or they may develop in the salivary gland itself, where they become activated and induced to proliferate. Our data show that a microenvironment is built up within the salivary gland that allows a germinal center reaction to take place.

The minor salivary glands of SS patients also contain many smaller foci of B and T cells than those studied here. Whether these foci are also sites of antigen presentation by FDCs, glandular cells, or B cells, stimulating clonal B cell proliferation and diversification, is not yet known.

We have demonstrated that germinal center reactions occur in the nonlymphoid target tissues of two autoimmune diseases, namely the salivary glands of SS patients and in synovial membranes from the rheumatoid joint (18). Germinal center-like structures have also been described in other autoimmune diseases, on the basis of their immunochemical staining (12–14, 46, 47), although evidence of a germinal center reaction, as described here, has not yet been presented in these cases. The combination of histological and molecular evidence suggests that germinal center reactions in the target tissue may be a common feature of autoimmune diseases. A disadvantage of this approach is that we do not know the antigen specificity of the B cells from which the rearranged V genes were obtained. Currently, we are seeking to remedy this by constructing single chain Fv mini-libraries in expression vectors, using V_H and V_L genes cloned from cell clusters.

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