SJÖGREN’S SYNDROME WITH LYMPHOMA-DEPENDENT PRODUCTION OF ANTI-RO52 AUTOANTIBODIES

By

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To all who supported me during my research
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Marginal zone B cell lymphomas from gastric tissue and salivary glands are thought to derive from antigen driven responses. However, the target antigens are mostly unknown. Here we report a patient with extranodal marginal zone lymphoma arising in the parotid gland in whom a marked and highly specific increase in anti-Ro52 antibodies was seen at time of tumor diagnosis followed by a significant decrease following tumor excision. There was no change in the levels of anti-Ro60/SS-A or anti-La/SS-B autoantibodies or rheumatoid factor over the same period. Both the tumor and the increasing/decreasing anti-Ro52 antibodies expressed κ-light chain. The patient’s lymphoma B cells used a VH3-7 heavy chain that showed evidence of somatic hypermutation and an unmutated VκIII (A27) light chain. Although this light chain is utilized by rheumatoid factors, the expressed/reassembled lymphoma heavy and light chains did not have either rheumatoid factor activity or specificity for the Ro52 antigen. Reactivity with other self-antigens also was not seen. The data suggest that the presence of the B cell lymphoma was driving the production of anti-Ro52 autoantibodies, most likely by providing a source of antigen.
CHAPTER 1
INTRODUCTION

In 1933, the Swedish ophthalmologist Henrik Sjögren described his clinical and histological findings of xerostomia and keratoconjunctivitis sicca among a female cohort of patients (1). This disease would later go on to bear his name, Sjögren’s syndrome (SS), and is a chronic autoimmune disease characterized by a progressive mononuclear infiltration of the exocrine glands that primarily affects women at a female to male ratio of 9:1. The disease can occur at any age, with a peak incidence between 40-50 years of age (2). For a diagnosis of Sjögren’s syndrome to be confirmed, clinicians use the American European Consensus Criteria (AECC) (3). The AECC classification criteria uses clinical symptoms (dry eyes and mouth), histopathology (lymphocytic infiltrations of salivary gland), and the production of autoantibodies to Ro (SS-A), La (SS-B) to confirm a diagnosis of Sjögren’s syndrome.

Clinical Aspects

Sjögren’s syndrome can exist by itself (primary SS) or in conjunction with other autoimmune diseases such as systemic lupus erythematosus or rheumatoid arthritis (secondary SS) (2). The primary clinical symptoms of SS are dry eyes (keratoconjunctivitis sicca) and dry mouth (xerostomia).

To confirm ocular dryness, tear production is measured using the Schirmer test (≤ 5 mm wetness at 5 minutes is considered diagnostic). Rose Bengal staining can also be performed to identify corneal abrasions, a common complication of keratoconjunctivitis sicca. Oral dryness is manifested by decreased salivary flow, which can also be measured and a value of ≤ 1.5 ml/15 minutes is considered abnormal. The clinical features of Sjögren’s syndrome derive not only from glandular tissue destruction via an autoimmune exocrinopathy but also by a disruption in the cascade of saliva production. Inhibition of neuronal innervation, aquaporin destruction, and
cytokine influence has all been suggested to contribute to the pathogenesis of this disease. In addition to keratoconjunctivitis sicca and xerostomia, a wide variety of extraglandular symptoms of primary SS have been reported, including Raynaud's phenomenon, chronic nonerosive polyarthritis, arthralgias, interstitial nephritis, renal tubular acidosis, peripheral neuropathy, demyelinating disease, cryoglobulinemia, dry cough, interstitial lung disease and lymphoma (2). Elevated risk of marginal zone B cell lymphoma, most commonly of the mucosa-associated lymphoid tissue (MALT) type, is a particularly important extraglandular manifestation of Sjögren’s syndrome which was first recognized in 1964 (4). The risk of developing lymphoma is 16-45-fold higher in patients with primary SS than in the general population (5).

**Pathology**

Sjögren’s syndrome is characterized pathologically by a progressive mononuclear infiltration of the lachrymal and salivary glands. The cellular population of the infiltrates is comprised mainly of T cells (75-85%), but also contains B cells (5-10%), dendritic cells and, later in the disease, plasma cells (6). In contrast, healthy salivary gland tissue is devoid of such lymphocytic infiltrations.

The T cell infiltrate in the salivary glands of SS patients are predominately of the CD4+ phenotype. The ratio of CD4+ to CD8+ T cells is usually about 3:1 (6). These mature T cells express the αβ phenotype and are CD45RO+, suggestive that they are memory/inducer cells. These infiltrating T cells also express increased levels of adhesion molecules, such as L-selectin, which may have important implications in the sustained inflammatory environment seen in the salivary gland (7).

In adults, B cells are generated in the bone marrow and migrate to the periphery where they mature at secondary lymphoid organs. Survival of immature B cells in the periphery is dependent upon specific cellular interaction and cytokine influence. There are several subsets of
B cells in humans including B-1, follicular (FO), and marginal zone (MZ). B-1 cells reside in the peritoneum and represent the main source of natural antibody production. These cells respond to antigens without the need for T cell help (thymus independent, TI) and show little to no evidence of class switching or somatic hypermutation of their immunoglobulins (8).

Follicular, also known as B-2, B cells are the traditional B cells that differentiate into memory or plasma cells upon T cell activation (thymus dependant, TD) and stimulation. Follicular B cells develop from transitional B cells and reside in the follicles of secondary lymphoid organs and participate in both T cell dependant and T cell independent immune responses. Follicular B cells are IgD$^{\text{high}}$, IgM$^{\text{lo}}$, CD23$,^+$, CD1$^{\text{lo}}$ (9). Upon activation, FO B cells enter a germinal center where they undergo affinity maturation and somatic hypermutation. Marginal zone B cells, which also develop from transitional B cells, reside in the marginal zone of the spleen, surround germinal centers and in humans, recirculate. Marginal zone B cells are able to respond to both TI and TD antigens (9). The marginal zone B cell subset is of particular importance in patients with SS due to their increased risk of marginal zone lymphomas.

Marginal zone B cells are so named because they are located extrafollicularly in secondary lymphoid organs and are IgM$^{\text{high}}$, IgD$^{\text{lo}}$, CD21$,^+$, CD1$^{\text{+}}$, CD5$,^-$, CD10$,^-$, and CD23$^-$ (10). MZ B cells can be further divided based upon the presence or absence of surface IgD. IgM$^{+}$ only MZ B cells are primarily TI responders and have unmutated $V_H$ immunoglobulins. IgM$^{\text{high}}$ IgD$^{\text{lo}}$ MZ B cells, also known as memory B cells due to the presence of CD27, have mutated $V_H$ immunoglobulins and respond to TI antigens and TD antigens (9). Evidence that MZ B cells have mutated $V_H$ genes suggests that these B cells are either post GC B cells that now reside in the marginal zone, or they have undergone an antigen independent mutation of their $V_H$ genes. While the latter is a departure from standard beliefs of B cell somatic hypermutation, it would
explain the findings of IgM⁺, CD27⁺ memory cells found in patients with X-linked hyper IgM syndrome (a condition where B cells lack CD40 and are not activated by T cells and there is no germinal center formation (8). The ability of MZ B cells to respond to both TI and TD antigens allows them to participate in germinal center reactions and could be a subset responsible for the production of autoantibodies.

The B cell population of the lymphocytic infiltrates in SS can be divided into CD20⁺ B cells and the CD38⁺/CD27⁺ plasma/memory cells (6). The infiltrating B cells of the salivary gland may home to the tissue in response to increased levels of B cell activating factor (BAFF) expressed by the infiltrating T cells or endothelial cells (11). B cell activating factor is an important B cell survival signal required for the persistence of B cells in tissue that can also help transform transitional type I B cells to transitional type II cells. These CD20⁺, CD21⁺, IgM⁺, IgD⁺ B cells may then differentiate into marginal-zone or follicular lymphocytes (12, 13). This mechanism may allow for the activation and differentiation of naïve alloreactive cells in the ectopic tissue of the salivary gland under the direction of T cell stimulation, proinflammatory cytokines and BAFF resulting in the production of autoantibodies (14). Interestingly, MZL rarely disseminate from their location of origin, one reason may be due to local BAFF expression. The stimulation of B cells by BAFF may be responsible for their persistence in extranodal locations, and lack of this signal outside of the inflammatory site could result in apoptosis.

**Autoantibodies and Other Immunological Abnormalities in Sjögren’s Syndrome**

Patients with Sjögren’s syndrome develop antibodies against both intracellular and plasma membrane bound proteins. Anti-Ro (SS-A) and anti-La (SS-B) antibodies, rheumatoid factor (RF) (anti-immunoglobulin antibodies), antinuclear antibodies, anti-muscarinic receptors and an overall hypergammaglobulinemia are several clinically important self-antigens to which many
patients with SS mount an immune response (2, 15, 16). Although hepatitis C and HIV infection both can cause sicca symptoms with a Sjögren’s syndrome-like pathological picture, autoantibody production (e.g. anti-Ro/SS-A and La/SS-B) is very rare in virally-induced sicca syndrome (17, 18). These autoantibodies may be present as early as 10 years before the clinical presentation of disease (19).

**Anti-Ro60 (SS-A), Anti-Ro52 and Anti-La (SS-B) Autoantibodies**

Anti-Ro60 (SS-A), anti-Ro52 and anti-La (SS-B) autoantibodies are used clinically as a diagnostic marker of Sjögren’s syndrome, although they are not specific for this condition. In addition to being associated with sicca symptoms, autoantibodies to Ro60, Ro52 and La are linked with congenital heart block in neonates (20).

Antibodies against Ro60 kDa occur in 50-90% of patients with SS (21). Ro60 (SS-A) is associated as a protein-RNA complex with Y RNA’s, designated Y1-Y5. Y RNA’s are small non-coding cytoplasmic RNAs which are transcribed by RNA polymerase III (22). The function of Y RNA in cells is currently unknown. The secondary structure of the Y RNA’s contains a long double stranded stem structure, where Ro60 binds, and a loop structure. The function of Ro60 is to bind to misfolded, defective, small RNAs which are eventually degraded (23).

Approximately 25% of SS patients have anti-La antibodies (24). The concentration of La protein is nearly 50-fold higher in the cell than Ro52 and Ro60 (25). La associates with newly synthesized RNA polymerase III transcripts, including precursor tRNA’s and 5S ribosomal RNA (26). In addition, La binds to newly transcribed Y RNA (23). A DNA-RNA unwinding activity has also been observed for the La protein and an ability to inhibit the activation of the interferon-inducible protein kinase (PKR) has also been shown (27).

The 52 kDa Ro antigen (Ro52) is recognized by autoantibodies in sera from many patients with Sjögren’s syndrome (28). Although autoantibodies from patients with SLE and SS can bind
to both Ro52 and Ro60, Ro52 is a structurally distinct protein whose physical interaction with Ro60 has not been conclusively shown (23). The Ro52 kd ribonucleoprotein has 2 zinc-finger motifs located at its N-terminus and a leucine zipper domain located in the central region of the protein (29). Zinc finger domains are important for DNA and RNA binding while leucine-zipper domains are important for protein-protein interactions and dimer formation (30). These domains could be important for Ro52’s association with IRF-8 and its ability to act as a transcription factor. In experiments, an increase expression of cellular Ro52 lead to a decrease in cell growth and induced cell death in cell culture (31). Ro52 has also been shown to be interferon inducible and associates with IRF-8 which enhances cytokine expression in macrophages (32). Several monoclonal antibodies directed against Ro52, isolated from pSS patients, have been produced by EBV/hybridoma. These antibodies showed a biased use of VH3 immunoglobulin heavy chain as well as mutations in the CDR regions (20). Interestingly, patients with Sjögren’s syndrome and SLE have an increased expression of Ro52 in PBMCs (31). This abnormal increase in Ro52 expression may account for the presence of anti-Ro52 antibodies found in these patients.

**Rheumatoid Factor**

Rheumatoid factor is one of the most common autoantibodies found in patients with primary Sjögren’s syndrome and is found in the sera and saliva of 60-80% of primary SS patients. Rheumatoid factor is directed against antigenic determinants on the Fc region of human immunoglobulin and may be of the IgM (most common), IgG, or IgA isotype (15, 33). Rheumatoid factor is also seen in other autoimmune diseases such as RA and SLE (15). Rheumatoid factor antibodies were originally discovered in patients with rheumatoid arthritis (RA) and are used clinically for diagnosis. The presence of RF is frequently encountered in hepatitis C infections, a disease that is interestingly associated with Sjögren’s-like lymphocytic infiltration of the salivary glands (18, 34). Rheumatoid arthritis is one of the conditions
associated with secondary SS. Rheumatoid arthritis is characterized by inflammation of the synovium and an infiltration of macrophages, and T and B lymphocytes that form ectopic lymphoid tissue (15, 35). This inflammatory environment and the presence of cellular infiltrates are analogous to those seen in the salivary glands of patients with pSS. Although RF is relatively common in the sera and saliva of pSS patients, it is also can be found in normal sera, especially following immunizations and is seen in patients with chronic infections such as bacterial endocarditis (36). Rheumatoid factors aid in the activation of the classical complement pathway for the removal of antigen/antibody complexes post-infection. Evidence also shows many rheumatoid factors contain a particular light chain, VκIII, in a close to germline configuration (37, 38).

In RA, rheumatoid factor has been suggested to contribute to the pathogenesis of the disease by forming immune complexes that may activate or interact with Fc receptors (via IgG), leading to an inflammatory response of the synovium. Examination of preferential gene usage among RF autoantibodies in Sjögren’s patients has revealed a restricted use of heavy and light chain genes. RF’s in SS peripheral blood as well as B cells infiltrating the salivary glands of SS have been shown to preferentially use κ-light chains, particularly VκA27 (39, 40). In circulating antibodies, use of the VH1, VH4 and VH3 heavy chain genes are predominantly expressed (15). The role of rheumatoid factor in SS is unknown.

**Anti-Muscarinic Receptor Autoantibodies**

Salivary secretion is accomplished by cholinergic stimulation of muscarinic type 3 receptors (M3R) found on salivary acinar cells. Binding of acetylcholine to M3R triggers an increase in [Ca2⁺]. The increase in [Ca2⁺] in turn activates K⁺ and Cl⁻ channels, which drive fluid secretion in acinar cells. Secretion of saliva cannot occur without the increase in [Ca2⁺]. Antibodies directed against M3R have been found in patients with primary SS and have been
shown to be pathogenic (14, 41). Infusion of primary SS IgG into Ig\textsuperscript{null} NOD mice caused reversible salivary gland hypofunction (42). It has also been shown that primary SS sera exposed to mouse and human submandibular acinar cells inhibited Ca\textsuperscript{2+} mobilization by ~50\% (41). This effect was reversible upon the removal of the sera. Anti-muscarinic antibodies have also been shown to inhibit the translocation of aquaporins to the plasma membranes (43). Aquaporins are transmembrane proteins involved in water transport through epithelial cells. There are several isoforms of aquaporins (AQP), all of which have specific locations. Mice deficient in AQP-5, located in the apical membrane of acinar cells, showed a decrease of 65\% in their salivary secretion rate compared to wild-type mice. Analysis of tears shows that AQP-5 cannot be detected from primary SS patients but is present in normal controls. This is due to acinar cell destruction by the lymphocytic infiltrates. Expression of AQP-1 has been found to be decreased by 38\% in salivary glands of SS patients (44), possibly explaining the decrease in salivary flow. The presence of anti M\textsubscript{3}R antibodies alone however, is unable to account for all of the pathogenic features of Sjögren’s syndrome.

**Polyclonal Hypergammaglobulinemia**

Patients with SS frequently develop polyclonal hypergammaglobulinemia, a manifestation also seen commonly in other autoimmune diseases and infections (8). Polyclonal hypergammaglobulinemia may be explained, in part, by an increased production of cytokines such as IL-6 and IL-10. IL-6 is crucial for antibody production and in B cell differentiation. Interestingly, patients with active SS have higher levels of IL-6 secreting cells in the peripheral blood than non-active SS patients (45). This increase in IL-6 could be responsible for an increase in autoantibody production which results in the observed active disease state.
Autoantibody Production

The production of autoantibodies in Sjögren’s syndrome is in response to self peptides, the same peptides that are tolerized to in normal individuals. Specific antigens are targeted in SS, e.g. Ro52, La, and interestingly they are only targeted in specific locations such as the salivary gland. The autoantibodies produced show evidence of being antigen driven. Analysis of immunoglobulins isolated from SS patients show evidence of somatic hypermutation and clonal variation (20, 46). Within the salivary gland, antigen specific B cells and local production of autoantibodies has been detected (47, 48). Interestingly, examination of immunoglobulin light chain usage shows a restricted use of κ-chain among infiltrating B cells in the salivary gland (49). The most looming question in autoimmune disease is how self peptides become immunogenic. Several possibilities ranging from apoptosis to virus involvement could be responsible for the presentation or generation of immunogenic self peptides.

Potential Role of Apoptosis in the Generation of Autoantibodies

During apoptosis, programmed cell death, cells undergo characteristic changes leading to DNA fragmentation and cellular death. Apoptosis occurs as a normal process during development, in cell turn-over, and in injured cells, i.e., damaged or infected cells. Binding of the FAS/FASL stimulates cells to undergo apoptosis. Upon binding, the cell shrinks, develops apoptotic blebs on the surface, and selected proteins are targeted for proteolytic degradation (50, 51). Several autoantigens, such as Ro52 and La, have been shown to cluster and concentrate within these surface blebs and apoptotic bodies during apoptosis (50). If these apoptotic cells are not cleared by macrophages, the altered and newly accessible self peptides may now be immunogenic, especially when presented in the context of inflammation (51). Although apoptosis is a regular occurrence, massive apoptosis could overwhelm the normal mechanisms which prevent necrosis and subsequent antigen presentation. Recent work also suggests that the
epithelial cells of the salivary gland can act as non-professional antigen presenting cells, possibly presenting self peptides (52). Stimulation of an epithelial cell line with type 1 interferon was shown to upregulate the expression of HLA-DR, ICAM-1, VCAM, and co-stimulatory molecule CD80. Incubation of IFN stimulated endothelial cells with alloreactive T cells isolated from SS patients lead to a proliferation of alloreactive T cells whereas unstimulated endothelial cells were unable to stimulate induce proliferation (52). This observation is important in terms of the cellular environment and interaction that occurs in the salivary gland of SS. The increase in inflammation observed in SS salivary glands could allow resident endothelial cells to incorrectly display self peptides and stimulate alloreactive T cells, which in turn stimulates B cells and the production of clinically observed autoantibodies.

**Altered Self Peptides and Autoantibody Production**

There are several conditions and events that can alter self peptides making them immunogenic. Viral proteins can bind to host proteins causing abnormal processing of the latter with presentation of cryptic T cell epitopes. This can lead to antibody responses to the host protein. For example, an autoimmune response can be generated against the p53 tumor suppressor protein when it complexes with SV40 large T antigen, which persists after viral clearance (53). This finding suggests that viruses may have an initial role in the development of autoantibodies which persists after viral clearance. The development of Sjögren’s-like lesions in the salivary gland of patients with hepatitis C or HIV infection is consistent with a viral etiology (18, 54). Epstein-Barr virus has been proposed, since it can establish latency in the salivary glands and in B cells as well (55). Somatic mutations also could lead to immunogenicity of self antigens. Mutations of p53 in breast cancer can lead to autoantibody formation, for example (56, 57). Chromosomal translocations can also create autoantigens with abnormal structure. The resulting fusions proteins can, in some cases, stimulate autoimmunity (58). A number of
translocations involving API2 and MALT (t(11;18)) and the bcl-2 and JH locus (t(14;18)) have been reported in salivary gland MALT lymphomas (59). However, it is not known whether any of these autoantigens are involved in translocations.

**Lymphoid Neogenesis: A Possible Site of Autoantibody Production**

Secondary lymphoid organs are genetically preprogrammed during ontogeny and are located in functionally distinct locations throughout the body. These organs are the locations of lymphocyte maturation. Within these secondary lymphoid organs are germinal centers, specialized structures that arise during an immune response and aid in affinity maturation and somatic hypermutation of B cell immunoglobulin genes (8). Tertiary lymphoid organs however, arise under environmental influences and are extranodal. Three critical events are shown to be required for the formation of ectopic lymphoid tissue; inflammatory cytokine production (especially TNF-α), lymphoid chemokine production by stromal cells, and HEV development. Like lymph nodes, ectopic lymphoid tissue can contain germinal centers and follicular dendritic cell networks. B cells within the ectopic lymphoid tissue can undergo terminal differentiation to plasma cells which have been detected in the synovium of rheumatoid arthritis and salivary glands of Sjögren’s syndrome, two sites of lymphoid neogenesis (60, 61). Ectopic lymphoid tissue formation (lymphoid neogenesis) occurs in many other autoimmune diseases as well, including the stomach of *H. pylori* infection and the liver in hepatitis C infection (62). Interestingly, there is a high incidence of B cell lymphoma in association with lymphoid neogenesis (62). This suggests that proliferating B cells in these ectopic tissues may be proliferating in the absence of normal stimulatory requirements, allowing autoreactive B cells to proliferate.

**Lymphoid Neogenesis in Sjögren’s Syndrome.** A common feature of SS is the presence of ectopic, lymphoid tissue in the salivary glands. Lymphoid follicles are more common in
primary SS (69.4%) than in secondary SS with RA 46% or SLE 23% (63). The higher incidence of ectopic lymphoid tissue may help contribute to the higher incidence of lymphoma in pSS by allowing B cells to undergo SHM which could increase the chance of an oncogenic event.

Chemokines essential for recruiting B cells, T cells, and antigen presenting cells, including BCA-1, CCL19 (ELC), and CCL21 (SLC), have been found in the salivary glands of SS patients (64, 65). Germinal-like structures within the glands contain CD21, CD35⁺ follicular dendritic cells, CD20⁺, CD10⁻, CD38⁻ B cells as well as CD20⁺, CD27⁺ memory cells (66). However, these GC like structures lack the typical presence of centroblasts and centrocytes (12). It has also been shown that these ectopic lymphoid tissues produce tissue specific, disease relevant antibodies (66) and that a correlation has been found between ectopic GC formation and autoantibody serum levels (67). Interestingly, patients with SS show a decreased population of CD27⁺ memory B cells in peripheral blood and an accumulation in the salivary gland compared to normal healthy controls and patients with SLE (68). This finding further supports the theory that B cells home to the salivary gland, possibly in response to a locally produced antigen. The homing of lymphocytes may be due in part to the increased expression of BAFF within these germinal centers (12). The formation of these GC like structures has been shown to rely on the local expression of BAFF and IFN-γ in the salivary gland and a positive correlation between high levels of BAFF and IFN-γ and germinal center formation in salivary glands of patients with SS have been shown (69). More importantly, autoreactive B cells could be escaping apoptosis and negative selection in ectopic lymphoid tissue due to the increased expression of BAFF. Consistent with this idea, low apoptosis frequency and increased levels of the anti-apoptotic protein BCL-2 have been shown in ectopic GC B cells in Sjögren’s syndrome (62).
Links Between Autoimmunity and B Cell Neoplasia

Primary SS patients are prone to develop a benign lesion in the salivary gland that is known as myoepithelial sialadenitis (MESA). Prominent features of MESA include a prominent lymphoid infiltrate with reactive germinal centers and lymphoepithelial lesions. These lymphoepithelial lesions are a combination of lymphoid cells and myoepithelial and epithelial cells. A general hyperplasia and thickening of the ducts is also observed (70). As this benign lesion progresses, the ductal lumens become occluded with the formation of epimyoepithelial islands. These epimyoepithelial islands consist of aggregates of ductal epithelium cells and B cells. The normal salivary gland tissue is steadily replaced by this tissue. An important histological transformational change from MESA to MALT lymphoma is the formation of “halos” by centrocyte-like or monocytoid B cells that surround the epimyoepithelial islands. These monocytoid B cells are nearly identical phenotypically to marginal zone B cells (71). The mechanism behind the progression from MESA to MALT is not fully understood but may be due to continued antigenic stimulation in the salivary gland. Evidence in support of this comes from the detection of a monoclonal expansion of B cells in the salivary gland but with a histologically benign phenotype (70). There is a variable period of time between the identification of MESA and lymphoma, with a reported range of 6 months to 29 years (70).

Overall, patients with primary SS have a 16-45-fold increased risk of developing B cell lymphomas (5) and 85% of these lymphomas are non-Hodgkin’s lymphomas (NHL) of the marginal zone subtype. Extranodal marginal-zone B-cell lymphoma of the salivary gland is an indolent neoplasm that usually presents with localized disease and does not disseminate rapidly. Mucosa-associated lymphoid tissue lymphomas usually arise in the MALT that is acquired secondary to infectious or autoimmune conditions such as Helicobacter pylori infection in the stomach, Hashimoto’s thyroiditis in the thyroid gland, or Sjögren’s syndrome-associated MESA.
in the salivary gland. The initial precipitating inflammatory event in SS is still unknown. Others have suggested that viruses could be responsible for an oncogenic transformation, similar to the mechanism seen in hepatic carcinoma or in cervical cancer (34, 72-74). To date, examination of SS MALT lymphomas have not been able to detect the presence of virus including EBV viral protein LMP, viral nucleic acid of EBV, human herpes virus 8, or human T lymphotropic virus-1 (75). However, there are several striking similarities between marginal zone lymphomas seen in hepatitis C infected patients and marginal zone lymphomas in SS patients. It has been well documented that patients with hepatitis C are at an increased risk for hepatocarcinoma as HCV is hepatotropic, but they also have a 35-fold increased risk of developing non-Hodgkins lymphoma (76). Hepatitis C is a lymphotropic virus and viral genomic DNA has been detected in peripheral B cells (34), further supporting a possible role in lymphoma. Both SS and HCV infections share several overlapping symptoms, including sicca and mixed cryoglobulinemia. Of interest is that type II MC and non-Hodgkin’s lymphoma in HCV patients share similar rearranged Ig genes (18), indicative that the MC could be a precursor to NHL. Evidence also exists to support an antigen driven event in the oncogenesis during HCV infections. Antiviral treatment in patients with HCV and lymphoma showed a decrease in viral load and tumor regression in 75% of cases (76). The decrease in viral load may be analogous to the eradication of H. pylori, both producing a loss of antigenic stimulus followed by tumor regression. In addition, patients with MC and HCV-NHL show a biased use of VH1-69 and VκA27 genes which are mutated in comparison to germline sequences (77). Similar usage of heavy chain genes between MZL in SS and HCV patients may be due to the same antigen. While the mechanism behind the progression from mixed cryoglobulinemia to lymphoma is unknown, the process mimics the antigen driven response seen in H. pylori
There is evidence that MALT lymphomas could be antigen driven in SS. The first is through the analysis of B cell immunoglobulin genes. Several groups have reported a biased use of heavy and light chain genes among pSS B cell MALT lymphoma (78-80). A biased use of VH1-69 and VH3 heavy chains indicate that these lymphomas could be responding to the same antigen in different patients. Also, examination of the immunoglobulin rearrangements shows significant mutations in the variable region of the immunoglobulins, especially CDR3 (78-80). This suggests that the B cells have undergone affinity maturation and somatic hypermutation at a germinal center, possibly at the ectopic lymphoid tissue present in the salivary gland, in response to an unknown antigen. Additional evidence suggestive of an antigen driven response is in *H. pylori* gastric MALT lymphomas where eradication of *H. pylori* results in a complete remission of the lymphoma in 80% of cases (81).

**Antigen Specificity of Extranodal B Cell Neoplasms**

It is thought that the oncogenic event that occurs in marginal zone B cell lymphoma is due to a constant presentation of antigens, foreign or self, to B cells leading to continued proliferation. Upon eradication of *H. pylori* infection, gastric MALT lymphoma regresses, supporting the theory that the lymphoma is antigen driven. Although V_H and V_L chains of tumor immunoglobulins show evidence of SHM, indicative of being antigen driven, recent evidence shows that the MALT tumor immunoglobulins are not directed against *H. pylori* surface antigens (82). In fact, an extensive array of self and *H. pylori* associated antigens were examined for antigen specificity using recombinantly produced tumor immunoglobulins but none of the examined immunoglobulins showed specificity for the tested antigens. This observation may be due to *H. pylori* specific T cells which stimulate autoreactive B cells in the gastric mucosa to proliferate through cytokine production and CD40/CD40L interaction. Marginal zone lymphomas of the gastric MALT have been shown to proliferate in the presence of T cells
specific for *H. pylori* but not in the presence of *H. pylori* and anti-CD40L (83). This reveals a contact dependency of B cells and T cells in this response. In further support of an antigen driven response in these lymphomas, patients with hepatitis C and MZL have been treated with antiviral therapy. Treatment with antiviral therapy lowered the viral load and produced a regression of tumor in a majority of cases (72). Unlike *H. pylori*, a putative antigen has been postulated to be responsible. It has been shown that recombinantly produced tumor immunoglobulin derived from a HCV-NHL showed antigenic specificity towards a hepatitis c viral protein, E2 (84). Since this finding however, no other reports of antigen specificity of lymphoma immunoglobulins in HCV patients has been reported. Unlike MZL in HCV, antigen specificity has not been determined in gastric MALT lymphomas or in SS MZL. The similarity of gene usage between MZL in pSS and MZL in HCV but not in gastric MZL support a causative role of HCV in the development of MZL (1). This evidence supports the hypothesis that constant antigen stimulation, albeit different in these examples, can lead to lymphoma. It has been shown that MALT lymphomas in SS patients exhibit gene rearrangements indicative of affinity maturation and somatic hypermutation and some MALT lymphomas have shown rheumatoid factor reactivity (85, 86).

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Table 1-1 Heavy chain gene usage among MALT lymphomas: Examination of published usage of heavy chain genes among salivary gland, gastric, and hepatitis C virus (HCV) MALT lymphoma.
CHAPTER 2
MATERIALS AND METHODS

A wide array of cellular, immunological, and molecular techniques were employed in the course of completing this thesis project. This chapter outlines the techniques utilized for the completion of the project, and provides a sufficient degree of detail to facilitate their adaptation and/or replication by other investigators. This chapter highlights general protocols developed by the laboratory for experiments completed during this thesis.

Analysis of Autoantibodies by Immunoprecipitation

Autoantibodies in the patient’s sera were evaluated by immunoprecipitation using radiolabeled K562 cell extract and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described (87). The specificities of the patient’s autoantibodies were verified by comparing with anti-Ro60 (SS-A) and anti-La (SS-B) prototype sera. Normal human serum and an irrelevant IgG1-κ-chain recombinant antibody from a chronic lymphocytic leukemia patient (CLL) were used as negative controls.

Expression and Purification of Recombinant Autoantigens

Recombinant Ro52, Ro60/SS-A, La/SS-B, were produced as described (28). Briefly, each gene was amplified from cDNA obtained from human peripheral blood mononuclear cells and cloned into the PET28a expression vector with an N-terminal 6-histidine tag. *E. coli* BL21-DE3 was transformed with the plasmids and induced to produce fusion protein with 2 mM IPTG. After four hours, the bacteria were centrifuged and lysed. The recombinant proteins were purified using a Ni-NTA resin column (Sigma-Aldrich, St. Louis, MO) and eluted in 6 M urea.
ELISA

Anti-Ro60, Ro52, and La ELISAs

The wells of microtiter plates (Immobilizer Amino; Nunc, Naperville, IL) were coated with 1 µg/ml purified recombinant antigen in borate buffered saline overnight at 4°C. Plates were washed with TBS-Tween 20 after each step and blocked with 0.5% bovine serum albumin (BSA)-150 mM NaCl, 2 mM EDTA, 20 mM Tris pH 7.5, 0.3% Nonidet P-40 (NET/NP40) for 1 hour at room temperature as described (88). Human sera were diluted 1:4000 for the Ro52 and La ELISAs, and 1:500 for the Ro60 ELISA. Antibody binding was detected with goat anti-human immunoglobulin antibodies (γ, κ, or λ chain specific, 1:1000 dilution from Southern Biotechnology, Birmingham, AL). The assay was developed using diethanolamine/phosphatase buffer (Sigma-Aldrich) and absorbance was determined 405 nm using a VERSA\textsubscript{max} ELISA plate reader (Molecular Devices). Using serial 1:5 dilutions of high-titer anti-Ro52 or La antibody-positive serum, the absorbance was converted to units using a standard curve. ROC analysis was used to determine cut-off values for positive sera. Optimum sensitivity and specificity were 90% and 80% respectively and the corresponding cut-off values for the anti-Ro52 and La ELISAs were 2.21 and 1.5 units respectively. Samples were considered positive for anti-Ro60 if the absorbance was higher than the mean of 10 healthy controls + 3 S.D.

Rheumatoid Factor ELISA

Rabbit IgG (Sigma-Aldrich) was coated on microtiter plates (Nunc) at a concentration of 1 µg/ml overnight at 4°C. Plates were washed with TBS-Tween 20 after each step and blocked as above. Patient sera and recombinant antibodies were diluted 1:200 in blocking buffer and incubated for 1.5 hours. Goat anti-human IgG and IgM were diluted at 1:1000 and incubated for 1.5 hours. Samples were developed as above. Samples were considered positive for rheumatoid factor if the absorbance was higher than the mean of 10 healthy controls + 3 SD.
Anti-dsDNA ELISA

S1 nuclease treated calf thymus DNA (Sigma-Aldrich) was coated overnight at 4°C on microtiter plates (Nunc) at a concentration of 3 µg/ml in Reacti-Bind DNA coating solution (Pierce, Rockford, IL). Patient sera or recombinant antibody were tested at a 1:500 dilution and incubated for 2 hours at room temperature. Binding was detected with goat anti-human IgG antibody (1:1000) and the assay was developed as above. High-titer anti-dsDNA antibody-positive serum was used to generate a standard curve and the optical readout was converted to units using SoftMax Pro (Molecular Devices, Sunnyvale, CA).

Purification of Genomic DNA

Genomic DNA from biopsy tissue was purified using the Pinpoint Slide DNA Isolation System (Zymo Research, Orange CA) according to the manufacturer’s protocol. Briefly, 5 µm sections of paraffin-embedded tissue were washed twice in xylenes at room temperature for 30 min. The sample was then re-hydrated for two minutes per step in graded ethanol-water solutions (100%, 70%, 50%, and 0%). The sample was then air dried and digested with proteinase K for 4 hours at 55°C. The digested product was heated at 96°C for 10 minutes to inactivate proteinase K. The isolated DNA was analyzed on a 1% agarose gel in Tris-borate-EDTA buffer.

Immunoglobulin V-D-J Sequence Analysis. To determine the VDJ segment usage, 1 µl of genomic DNA from the tumor was amplified by PCR. For the heavy chain, VH1-7 forward primers were used individually along with a JH consensus reverse primer (89). For light chain, VkI/II and VkIII forward primers were used along with Jkf, Jk2E, Jk2I, Jk5E, or Jk5I reverse primers (89). Each primer contained a restriction site that permitted cloning of the DNA fragment. The reaction was carried out in a 20 µl volume containing a total concentration of 1.25 nM pooled VHF/VLF primers and 2.5 nM JH primers, 1X PCR buffer, 1.5 mM MgCl₂, 2.5
mM dNTPs, and 0.05 units of Taq DNA polymerase (Invitrogen, San Diego, CA). The reaction was carried out with a PTC-100 Programmable Thermal Controller (MJ Research, Inc., Waltham, MA) as follows: initial denaturation at 95°C for 12 min, denaturation at 94°C for 40 sec, annealing at 60°C for 45 sec, and extension at 72°C for 45 sec. After 35 cycles, extension was continued at 72°C for an additional 10 min. The \( V_H \) and \( V_L \) PCR products, which contained \( V_H\text{-}D\text{-}J_H \) or \( V_L\text{-}J_L \), were cloned into a TA vector (Invitrogen) and sequenced using an Applied Biosystems Model 373 Stretch DNA Sequencer. Sequences were compared against germ line sequences using the Ig-Blast Software (NCBI).

Heavy and light chain DNA were inserted in front of the human \( \gamma 1 \) or \( \kappa \) constant regions of vectors described previously by Wardemann, et al. (90). Briefly, the amplified DNA fragment and vector were digested with \textit{PmaII} and \textit{SalI} for \( IGHV\text{-}D\text{-}J \) and with \textit{AgeI} and \textit{BsiWI} for \( IGKV\text{-}J \) and ligated using T4 DNA ligase. Plasmids were transformed into \textit{E. coli} BL21 DE3 and purified using the Qiagen miniprep plasmid purification kit according to the manufacturer’s protocol (Qiagen, Valencia, CA).

**Expression of Recombinant Antibody**

293T human embryonic kidney fibroblasts were cultured in DMEM (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum, 4 mM L-glutamine and penicillin/streptomycin. Log phase cells were co-transfected with 30 \( \mu \)g of heavy chain and 30 \( \mu \)g light chain encoding plasmid DNA in 120 \( \mu \)l Lipofectamine 2000 (Invitrogen) (91). Cells were grown at 37°C for six days before harvesting the supernatant. Recombinant antibodies were purified using ImmunoPure Immobilized Protein G (Pierce). Immunoglobulin in the cell culture supernatant was quantified using an anti-human Ig\( \kappa \) ELISA (Southern Biotechnology) with purified IgG\( \kappa \) from human serum as standard. The purified recombinant antibody was adjusted to a concentration of 0.1 mg/ml. Assembly of the H and L chains was verified by purifying the
antibodies using protein G-Sepharose followed by elution and analysis by SDS-PAGE (Coomassie blue staining and western blot analysis using anti-κ specific detection antibodies).

**Flow Cytometry**

A single cell suspension was generated from the salivary gland biopsy specimen by digesting with collagenase and analyzed by flow cytometry as described (92, 93). The isolated cells were stained with the following labeled antibodies: anti-κ light chain-FITC, anti-λ light chain-FITC, anti-CD19-PE, CD5-FITC, anti-CD23-PE, anti-CD10-FITC, anti-CD19-PerCP-Cy5.5, and anti-CD20-PE (anti-light chain antibodies were from Dako North America, Carpinteria, CA; all others were from BD Biosciences, San Jose, CA). Isotype controls were used to evaluate background fluorescence. Isolated tumor cells were washed in staining buffer (PBS supplemented with 0.1% NaN₃ and 1% bovine serum albumin). Antibodies were added at the manufacturer’s suggested concentrations. Cell suspensions were washed twice in PBS before acquiring data on a FACSCalibur flow cytometer (BD Biosciences) and analyzing with FCS Express Version 2 (DeNovo Software, Thornhill, ON, Canada). At least 20,000 events per sample were analyzed using size gating to exclude dead cells.
CHAPTER 3
CASE REPORT

A 58-year-old woman was seen in the Autoimmune Disease Clinic for primary Sjögren’s syndrome and right parotid swelling and tenderness. She noted the onset of fatigue, dry eyes, and dry mouth in 1980 and had recurrent bilateral parotid swelling in 1982-3. In 1997 she underwent rheumatological evaluation for parotid swelling, oral, ocular and vaginal dryness, and arthralgias. She was found to have a positive ANA (1:1280 titer, speckled pattern), and positive anti-Ro60 (SS-A) and La (SS-B) autoantibodies and rheumatoid factor. Her γ-globulin level was 4.4 g/dL and she had a white blood cell count of 3600 (25% lymphocytes). A Schirmer test was abnormal. In 2001 she was referred to the University of Florida with persistent sicca symptoms and intermittent right parotid swelling and tenderness. The presence of anti-Ro60 and anti-La (SS-B) autoantibodies was confirmed by immunoprecipitation (Fig. 3-1A) and high titer anti-Ro52 autoantibodies were detected by ELISA (Fig. 3-1B). The ESR was 46 and γ-globulin level was 4.6 g. Serum protein electrophoresis did not show an M-spike. The parotid swelling initially responded well to hydroxychloroquine. However, in August 2002, she had recurrent right parotid swelling every 1-2 weeks and in November 2002, the swelling became persistent. A computer guided tomography (CT) scan of the right parotid revealed a 1 cm mass (Fig. 3-2A) and a needle biopsy obtained for flow cytometry showed a predominance of B-cells bearing κ light chain but was non-diagnostic of lymphoma. An excisional biopsy was performed revealing an encapsulated mass of the right parotid gland (Fig. 3-2B). There was no apparent extension of the tumor outside of the solitary mass. Hematoxylin & eosin staining revealed lymphoepithelial lesions characteristic of an extranodal MALToma (Fig. 3-2C, D).

Flow cytometry of cells isolated from the mass again revealed a predominance of CD19+ B-cells bearing κ light chain (Fig. 3-3A). On further analysis, the CD19+ cells were found to be
CD5+, CD10+, CD23+, and CD20+ (Fig. 3-3B), consistent with a marginal zone B-cell phenotype, confirming the diagnosis of an extranodal marginal zone lymphoma.

A CT scan of the chest and pelvis and a bone marrow biopsy did not reveal any additional nodal involvement. Further therapy was not felt to be necessary and the patient has remained free of lymphoma for five years.
Figure 3-1. Patient’s autoantibody profile. A, Immunoprecipitation of radiolabeled K562 cells using patient sera, anti-La and anti-Ro prototype sera, and serum from a normal healthy control (N). B, Anti-Ro52 ELISA using recombinant human Ro52 as antigen. Normal human sera (NHS, n = 20) and anti-Ro52+ sera (n = 20) were tested at a 1:50 dilution. Arrow indicates the result using serum from the patient reported here.
Figure 3-2. Imaging and pathology of right parotid mass. A, Computed topography of patient’s right parotid gland showing a 1.5 cm mass (arrow). B, Low power hematoxylin & eosin stain of the mass removed from the patient’s right parotid gland. C, High power hematoxylin & eosin staining showing lymphocytic infiltration and lymphoepithelial lesions characteristic of MALT type B-cell lymphoma (arrows).
Figure 3-3. Flow cytometry of cells isolated cells from the parotid mass. A, Staining for κ (left) and λ (right) positive CD19+ B cells. Arrow indicates the malignant B cell population, which was κ+ and λ-. B, Staining of CD19+ B cells for CD5 (left) and CD23 (middle) and staining of B cells for CD20 and CD10 (right). Arrows indicate malignant cell population.
CHAPTER 4
RESULTS

Selective Reduction in IgGκ Anti-Ro52 After Excision of Lymphoma

Analysis of serial serum samples revealed an increase in anti-Ro52 antibodies from 275 units before the diagnosis of lymphoma to 750 units when lymphoma was diagnosed four months later (Arrows, Fig. 4-1A). Following excision, anti-Ro52 antibodies fell to 300 units over the next six months (Fig. 4-1A).

Analysis of anti-Ro52 light chains showed a striking preferential usage of κ-light chains. The κ/λ ratio increased from 17 to 40 with the onset of lymphoma and decreased back to 17.6 after excision. In striking contrast, the κ/λ ratio in the total serum immunoglobulin ranged from 0.8-1.6 over the same time period and was never higher than 1.7 (Fig 4-1B). Further analysis of the anti-Ro52 antibodies indicated that the anti-Ro52 κ antibodies increased with the onset of the tumor and decreased after excision (Fig. 4-1C) whereas anti-Ro52 λ antibodies exhibited little change or even increased slightly after the tumor was removed (Fig. 4-1D). These results raised the possibility that the lymphoma was either driving the immune response to Ro52 or was secreting anti-Ro52 autoantibodies.

To further assess the specificity of the response, rheumatoid factor, anti-La and anti-Ro60 levels were also examined longitudinally (Fig. 4-2). Although low level rheumatoid factor was detected by latex fixation, on ELISA using rabbit γ-globulin as an antigen, the patient’s RF levels fell within the normal range (Fig. 4-2A). The binding to rabbit γ-globulin remained essentially unchanged over the period of observation (2001-2004), including the period between 4/02 and 11/02 when the lymphoma developed and was excised (Fig. 4-2A). Similarly, there was little change in anti-Ro60 (SS-A) antibody levels over this time (Fig. 4-2B). Her low level
anti-La (SS-B) antibodies increased from 14 to 26 units from 4/02 until 8/02, but then increased slightly more to 28 anti-La units after surgery (Fig. 4-2C).

Together, the data suggest that this patient’s κ-chain bearing anti-Ro52 antibodies specifically increased with the onset of her B-cell lymphoma and decreased after it was removed. Consequently, we examined the possibility that the marginal zone lymphoma was producing anti-Ro52 antibodies.

**Identification of Lymphoma Heavy and Light Chains**

Using purified genomic DNA from the lymphoma, we amplified the immunoglobulin heavy and light chain genes from the malignant B cells. A strong band at ~350 bp was obtained using the VH3 forward primer but not with VH 1, 2, 4, 5, 6 primers (Fig. 4-3A). TLR3 and β-actin primers amplified the expected 490 bp and 297 bp fragments. Further analysis using primers specific for VH3A and VH3B showed that the tumor H-chain was VH3A (Fig. 4-3B). The light chain was amplified using VkIII (forward) and Jκ2I (reverse) primers, but not with other combinations (Fig. 4-3C). Thus, the tumor expressed a VH3 H-chain and a VkIII L-chain.

**Lymphoma Bears a Mutated V3-7 H Chain**

The heavy-chain aligned with the germline V3-7 sequence (Fig. 4-4, top left). Sequence analysis showed that it had a total of 12 mutations, 9 within the CDR regions. Two of the 9 CDR mutations were replacement mutations vs. 2 of 3 in the framework regions (Fig. 4-4, top right). The tumor L-chain sequence closely matched the germline A27 sequence (Fig. 4-4, bottom) a member of the VkIII gene family (94). There were two mutations, both in the framework region 3. The CDR 1, 2, and 3 sequences were unmutated (Fig. 4-4, bottom).

**Expression of Recombinant Lymphoma B-cell Antigen Receptor**

To determine if the lymphoma B-cells had antigen specificity, the immunoglobulin heavy and light chains were inserted into expression vectors that individually express the cloned
fragment as an IgG1 heavy-chain and a κ-light chain (90). Human embryonic kidney cells were transiently co-transfected with the heavy and light chain constructs and the recombinant IgG1 antibody was purified from culture supernatant (Fig. 4-5). The recombinant antibody showed no reactivity with human Ro52 in ELISA. (Fig. 4-6A). To exclude the possibility that low pH used to elute the recombinant antibody inactivated it, we also tested recombinant antibody directly from the supernatant (without further purification) and again saw no binding (not shown). We then tested for reactivity with other autoantigens. The antibody showed no reactivity with Ro60 (SS-A) (Fig. 4-6B) or with rabbit IgG (RF, Fig. 4-6C). There also was no reactivity with La (SS-B) and the U1-A antigen (not shown). The recombinant antibody showed no reactivity with any of the cellular proteins produced by K562 (erythroleukemia) cells by immunoprecipitation (Fig. 4-6D). Similar negative results were obtained using cell extract from a human salivary gland ductal epithelium cell line (not shown).

The possibility that Epstein-Barr virus (EBV) is involved in the pathogenesis of SS has been discussed (95, 96). To eliminate EBV as a possible target of the lymphoma B-cell receptor (BCR), we performed an immunoprecipitation with radiolabeled B95-8 cells, a marmoset cell line that sheds EBV. The recombinant antibody did not react with either cellular extracts or supernatant from B95-8 cells (not shown).
Figure 4-1. Temporal relationship of anti-Ro52 autoantibodies to the presence of B cell lymphoma. A, Anti-Ro52 IgG levels as a function of the date of serum collection (recombinant anti-Ro52 ELISA). B, κ/λ ratio of anti-Ro52 antibodies (ELISA) vs. total serum IgG (ELISA) over time. C, Levels of κ chain bearing anti-Ro52 antibodies (ELISA); D, levels of λ chain bearing anti-Ro52 antibodies (ELISA). Note the different Y-axis scales in panels C and D. In all panels, arrows indicate the samples immediately before lymphoma was diagnosed, at the time of diagnosis, and after surgical resection of the parotid mass.
Figure 4-2. Levels of other serum autoantibodies over time. A, Rheumatoid factor ELISA (rabbit IgG antigen); B, anti-Ro60/SS-A ELISA (recombinant human Ro60 antigen); C, anti-La/SS-B ELISA (recombinant human La antigen). Arrows indicate the samples immediately before lymphoma was diagnosed, at the time of diagnosis, and after surgical resection of the parotid mass, as in 4-1.
Figure 4-3. Identification of the heavy and light chains expressed by the lymphoma (PCR). A, Heavy chain analysis. Tumor DNA was amplified using VH1, 2, 3, 4, 5, 6, or 7 forward primers along with a JH consensus reverse primer. A 350 bp fragment was amplified by the VH3 primers (single arrow, left). TLR3 and β-actin (control) primers amplified fragments of ~490 and 297 bp, respectively (arrows on right). B, Tumor DNA was amplified using forward primers specific for VH3A and VH3B, respectively, along with JH consensus reverse primer. A specific band was amplified by the VH3A primers but not the VH3B primers (arrow). C, Light chain analysis. Tumor DNA was amplified using VkI/II and VkIII forward primers and Jkf, Jk2E, Jk2I, Jk5E, or Jk5I reverse primers. A DNA fragment of the predicted size (~400 bp) was amplified using VkIII and Jk2I primers (arrow).
Figure 4-4. Nucleotide and amino acid sequences of lymphoma heavy and light chains. A. Alignment of the tumor heavy chain with germline VH3-7 nucleotide (left) and amino acid (right) sequences. Positions of the CDR1, CDR2, and CDR3 regions are indicated by boxes. Bottom, Alignment of the tumor light chain nucleotide (left) and amino acid (right) sequences with the germline A27 sequence. Positions of the CDR1, CDR2, and CDR3 regions are indicated by boxes.
B

Light chain

A27
Tumor

CDR1

A27
Tumor

CDR2

A27
Tumor

CDR3

A27
Tumor
Figure 4-5. SDS-PAGE of recombinant antibody. SDS-PAGE of purified recombinant antibody shows heavy and light chain pairing with correct size of 25 kD and 50 kD.
Tumor immunoglobulin lacks specificity for common autoantigens. Recombinant antibody was expressed in 293 cells, purified from the culture supernatant, and tested for reactivity with various antigens. A, Anti-Ro52 ELISA. Recombinant antibody (rAb) and a control CLL IgG1 κ were tested for reactivity with human Ro52 at a concentration of 0.1 mg/ml. Reactivity of human prototype anti-Ro52 sera (positive control, n = 10) and normal human sera (NHS, negative control, n = 10) are shown on the left. B, Anti-Ro60 ELISA. Recombinant antibody (rAb) and a control CLL IgG1 κ were tested for reactivity with human Ro60 at a concentration of 0.1 mg/ml. Reactivity of human prototype anti-Ro60 sera (positive control, n = 10) and normal human sera (NHS, negative control, n = 10) are shown on the left. C, Rheumatoid factor ELISA. Recombinant antibody (rAb) and a control CLL IgG1 κ were tested for reactivity with rabbit IgG. Reactivity of human prototype rheumatoid factor positive sera (positive control, n = 10) and normal human sera (NHS, negative control, n = 10) are shown on the left. D, Immunoprecipitation using the recombinant antibody. [35S]-labeled K562 cell extract was immunoprecipitated with the recombinant antibody (rAb), the CLL IgGκ antibody, serum from the patient (Pt), Ro60 and La positive reference serum (Ref), or normal human serum (NHS).
Extranodal marginal zone lymphomas are associated with chronic inflammation due to immune or autoimmune responses in the stomach, thyroid, salivary gland, and other locations (97). Antigen stimulation is thought to play a role in the pathogenesis of these tumors, which generally bear B cell receptors that have undergone somatic hypermutation (79, 98-100). Gastric lymphomas frequently regress after eradication of *H. pylori* infection, but specificity of the lymphoma cells for *H. pylori* antigens has not been established (81, 82, 97, 101). It has been suggested that lymphoma cell growth is stimulated by *H. pylori* activated T cells via CD40-CD40L interactions (83, 97). However, tumors producing antibodies that recognize *H. pylori* antigens have not been reported, although marginal zone lymphomas producing rheumatoid factors have been identified (86, 102). In most instances, the antigen/autoantigen specificity of these B cell neoplasms and the precise nature of their link with autoimmunity remain to be elucidated.

We report a patient with an IgGκ marginal zone lymphoma that developed in association with a dramatic and highly selective increase in IgGκ anti-Ro52 antibodies. When the tumor was surgically resected, the anti-Ro52 level decreased. At the same time, there was no significant change in the levels of other autoantibodies (anti-Ro60/SS-A, anti-La/SS-B, RF) or of λ L-chain bearing anti-Ro52 antibodies. Although suggestive of the possibility that the tumor might produce anti-Ro52 antibodies, this could not be confirmed using recombinant IgG from the lymphoma.

**H-chain from the Lymphoma Shows Evidence of Antigen Selection**

Analysis of the heavy chain (V3-7) and light chain (VκIII/A27) sequences obtained from this patient’s B cell lymphoma revealed similarities with the sequences obtained from other
extranodal marginal zone lymphomas as well as evidence of antigenic stimulation. The V3-7 H-chain utilized by this patient’s lymphoma cells also has been reported in monoclonal antibodies specific for Ro52 (16, 66). The H-chain sequence shows a lesser degree of somatic mutation, especially in the CDR3 region, than reported in other marginal zone lymphomas (46, 78-80, 86). However, mutations were concentrated primarily in the CDRs, consistent with the possibility that the malignant B cells were antigen-selected. The VH family utilized most frequently by peripheral blood lymphocytes is VH3 (103). In contrast, MALT lymphomas of both nodal and extranodal sites most frequently utilize VH4 followed by VH3 and VH1 (104). VH1 and VH3 are used preferentially in MALT lymphomas originating in the salivary gland (78, 80) and MALT lymphomas expressing V3-7 have been reported previously (86). VH1 and VH4 are commonly utilized by rheumatoid factors from patients with Sjögren’s syndrome whereas VH3 is more common in rheumatoid arthritis (37, 105, 106).

In contrast to the H-chain, the L-chain from this tumor was in a near-germline configuration (Fig. 4-4). Biased use of V\kappaA27 L-chain by marginal zone lymphomas arising in the salivary glands has been reported in primary Sjögren’s syndrome (79) and both A27 and other V\kappaIII family members are frequently utilized in antibodies with rheumatoid factor activity, especially when paired with H-chains containing V1-69 and JH4 (78, 86).

**The Patient’s Malignant B cells Lack Autoantigen Specificity.** Sjögren’s syndrome is associated with a spectrum of autoantibodies, including those specific for the ribonucleoproteins Ro60 (SS-A, consisting of a 60 kDa protein bound to the Y1-4 small RNAs) and La (SS-B, a 45 kDa protein bound to RNA polymerase III precursor products) as well as autoantibodies against the 52 kDa Ro52 antigen, an interferon inducible RING finger protein that interacts with and ubiquitinates the transcription factor interferon regulatory factor 8 (IRF-8) (32). The production
of rheumatoid factor also is highly characteristic of Sjögren’s syndrome (15). The patient reported here produced high levels of anti-Ro60/SS-A, anti-La/SS-B, and anti-Ro52 autoantibodies and low levels of rheumatoid factor.

The patient’s selective increase in serum anti-Ro52 activity coincided with the onset of the patient’s B cell lymphoma and declined following tumor resection, consistent with the possibility that the malignant B cells were specific for Ro52. However, recombinant tumor antibody showed no reactivity with human Ro52 (Fig. 4-5), whereas the patient’s serum was highly reactive (Fig. 3-1 and data not shown). As the epitopes recognized by antibodies tend to be conformational, it is possible that the recombinant antibody was reactive with an epitope that did not fold correctly when expressed in bacteria. However, this explanation is unlikely if one assumes that the increasing and decreasing levels of anti-Ro52 exhibited by the patient’s serum were due to production of anti-Ro52 by the tumor (Fig. 4-1). Despite containing a VκIII family L-chain (A27), we also were unable to demonstrate any rheumatoid factor activity (Fig. 4-5C). Reactivity with the native Ro60/SS-A and La (SS-B) proteins was not seen either (Fig. 4-5B, D). Thus, although the H-chain isolated from this patient’s marginal zone lymphoma had characteristics suggestive of prior antigen exposure, we were unable to show specificity for any of the autoantigens recognized by the patient’s serum autoantibodies.

**Possible Role of the Tumor in Stimulating Autoantibody Production**

Analysis of H and L chain sequences suggest that B cells undergo antigen-driven clonal expansion within the salivary glands of patients with Sjögren’s syndrome (100). B cells reactive with self-antigens such as Ro60/SS-A and La/SS-B have been found within the ectopic lymphoid tissue developing in the salivary glands of patients with Sjögren’s syndrome (48, 107). Similarly, rheumatoid factor B cells have been detected in rheumatoid synovium (35). Also, anti-U1-A specific B cells have been localized to ectopic lymphoid tissue in mice with
tetramethylpentadecane-induced lupus (D Nacionales, et al. Submitted). Along with the antigen-specific B cells, antigen-specific T cells home to ectopic lymphoid tissue in close proximity to B cells reactive with the same antigen (J Weinstein, et al. Submitted).

In view of the presence of autoreactive B and T cells within ectopic lymphoid tissue, it is intriguing that the formation of lymphoid structures in various locations (such as salivary gland, synovium, thyroid, liver, or stomach) is associated with the development of a unique and tissue-specific spectrum of autoantibodies (108), suggesting that the antigens/autoantigens driving the response could be produced locally within the affected tissues. Thus, the production of immunogenic Ro52 antigen by the patient’s lymphoma leading to the activation of antigen-specific but non-malignant T and B lymphocytes is potentially an alternative explanation for the data shown in Figures 4-1 and 4-2.

In fact, autoimmunity is not unusual in neoplastic diseases such as hepatocellular carcinoma (109, 110), breast cancer (56, 57), lung cancer (111), and acute myeloid leukemia (58), to name a few examples. In some cases, altered structure of the autoantigen through mutation or translocation is strongly implicated in the pathogenesis of the immune response (56-58). Although we do not know whether Ro52 expression was elevated in the lymphoma B cells, there is increased Ro52 expression in peripheral blood mononuclear cells from patients with autoimmune disease (31). Increased Ro52 expression increases ubiquitination of IRF-8, and could enhance the production of IL-12 and Type I interferon, potentially causing an excessive inflammatory response (32), altered B cell differentiation (112) and enhanced maturation of immunostimulatory dendritic cells, which could promote autoimmunity (113).

In addition to IL-12 and Type I interferon, other cytokines may have been produced by the lymphoma. Of particular interest is IL-6, a pleiotropic cytokine that plays an important role in
the maturation of antibody secreting plasma cells (114). IL-6 is produced by atrial myxomas (115) and also by some marginal zone lymphomas (116). If this patient’s lymphoma was secreting IL-6, it might have stimulated the differentiation of autoantibody secreting cells, but it is not clear why this would have been selective for anti-Ro52 unless the patient’s parotid gland selectively accumulated Ro52-specific (and not Ro60 or La-specific) B cells.

Finally, mutations in the Fas gene have been associated with both autoimmunity and non-Hodgkin lymphoma (117, 118). In one series of 150 patients, Fas mutations were found in 11%, including three extranodal MALT-type lymphomas (60%) (117). It is conceivable that the patient’s tumor contained a Fas mutation resulting in abnormal apoptosis and necrotic death of the lymphoma cells (119). The inflammation associated with necrotic tumor death might contribute to the induction of autoimmunity, but again it is unclear why the response should be selective for Ro52.

In conclusion, the temporal relationship between increased production of Ro52 autoantibodies and the presence of an extranodal marginal zone lymphoma as well as the highly selective production of anti-Ro52, and not other co-existing autoantibodies, suggests that either this patient’s lymphoma produced anti-Ro52 autoantibodies or that its presence somehow stimulated the production of these autoantibodies by non-malignant B cells. Our data are most consistent with the latter possibility, but further studies are needed to define the mechanism by which the lymphoma stimulated anti-Ro52 autoantibody production.
CHAPTER 6
CONCLUSION AND FUTURE AIMS

Unfortunately, antigen specificity of a marginal zone B cell in our patient with Sjögren’s syndrome was unable to be determined. The mechanism behind the observed increase in anti-Ro52 κ-chain specific antibodies was not elucidated through this work. Altered presentation of Ro52, in the environment of inflammation and pro B cell cytokines, by the lymphoma is the most likely candidate for the observations seen in this patient. While an exhaustive search for foreign and self antigens was conducted, there are still several causative agents which have yet to be excluded. Due to similar immunoglobulin gene rearrangements between marginal zone lymphomas in hepatitis C infected patients and marginal zone lymphomas in SS, it will be interesting to look for possible antigen specificity against hepatitis C virus, especially since the discovery of a MZL in a patient with HCV which showed reactivity towards the hepatitis C viral protein E2. In addition, certain translocations have been detected in MZL, particularly of the gastric MALT, and may play a role in their development and pathogenesis. Due to genetic abnormalities found in gastric marginal zone lymphomas, examination of our tumor for t(11:18) or t(14:18) would be a worthwhile endeavor. In addition, recent work has elucidated the function of Ro52 as an interferon inducible gene which associates with IRF-8. Due to this association, it would be interesting to look for anti-IRF8 antibodies in this patient.

The only tissue that is available from the tumor is paraffin embedded. This process does not preserve RNA and previous work to obtain RNA of high quality was unsuccessful. However, more recent techniques could allow for the isolation of intact RNA from the paraffin embedded tissue. Examination of Ro52 and IL-6 expression levels of the tumor may help illustrate a more complete picture of lymphoma in our patient.
While the reappearance of MZL’s is a rare event, it has been documented. Being vigilant in monitoring for the tumor reemergence in this patient may allow for a more thorough for antigen specificity in marginal zone lymphomas in patients with Sjögren’s syndrome.
REFERENCES


BIOGRAPHICAL SKETCH

Robert Capel Lyons was born in Albany, NY on October 29, 1982. He lived and attended school in Albany during his youth, until he moved to Port St. Lucie, FL. He attended and graduated from Port St. Lucie High School in 2000. After high school, Robert attended the University of Florida in Gainesville, where he obtained a Bachelor of Science in microbiology and cell science with a minor in chemistry. During this time, he worked as a student researcher in the Department of Medicine, Division of Rheumatology and Clinical Immunology, under Dr. Westley H. Reeves. While a senior at UF, Robert earned an EMT certification from Sante Fe Community College, graduating at the top of his EMT class. Upon graduation, Robert became shift leader for Alachua County Fire Rescue.

Robert was then admitted to and entered the master’s program at the University of Florida. He continued the research that he started as an undergraduate under the direction of Dr. Westley H. Reeves. Here Robert investigated the role autoantibody production in a Sjögren’s syndrome patient with lymphoma. After completing his M.S., Robert will enter medical school at the University of Florida. Upon graduation, Robert will apply for and complete a residency for board certification. His ultimate professional goals include becoming an academic physician with an emphasis on teaching.