Abstract

Although surface immunoglobulin plays a central role in the differentiation and growth of normal B-cells, its role in the growth of human B-cell malignancies is largely a matter of conjecture. Human follicular lymphomas are attractive systems to study in part because they are clonal and sharing many similarities with germinal center B-cells which are critically dependent on antigen selection for survival. Nucleotide sequence information was determined for the immunoglobulin heavy chain variable genes expressed by two cases of follicular lymphoma. In addition, the germ line variable gene counterparts were also cloned and sequenced from biopsy material obtained from both of these patients. Numerous mutations from germ line were present in the variable genes from both of these cases, many of which accumulated during expansion and growth of these lymphomas. Moreover, the mutations that accumulated during tumor expansion were distributed in a manner that almost certainly was dependent on positive selection presumably mediated by contact with an antigen. These data indicate that antigen selection is probably important for the growth and clonal evolution of follicular lymphomas.

Introduction

The survival of normal B-cells critically depends on the expression and proper functioning of surface immunoglobulin molecules or antigen receptors. Disruption of surface IgM expression appears to arrest B-cell development at a very early stage (1). Moreover, unless engagement of surface immunoglobulin occurs, usually through binding to an appropriate antigen or antiidiotype antibody, a virgin B-cell will typically die within a few days after leaving the bone marrow. In the appropriate environment, however, antigen receptor binding can lead to B-cell activation, proliferation, and differentiation into antibody secreting plasma cells or long lived memory B-cells (2,3).

Considering the central role played by the antigen receptor in normal B-cell mitogenesis, it is reasonable to suspect that the growth of some B-cell malignancies may also depend on signals received through their surface immunoglobulin molecules. In this report, we will focus primarily on the role antigen receptor mediated stimulation and selection may play in the growth and clonal evolution of low grade human FL(4). As outlined below, these slow growing, relatively common non-Hodgkin’s lymphomas appear to have many of the characteristics of normal GC cells which require antigen receptor stimulation for survival.

Results and Discussion

Origin of Follicular Lymphomas. Based on a variety of phenotypic features, follicular lymphomas resemble normal GC cells and are often positive for CD10 (common acute lymphoblastic leukemia antigen) and negative for CD5 (4, 5). These properties allow FL to be easily distinguished on a cellular level from some of the other common low grade indolent B-cell malignancies such as CLL which is typically CD5 positive and CD10 negative. Recent evidence also suggests that the adhesion molecules expressed by FL and GC cells may be identical (6).

The growth of FL occurs in a highly organized nodular pattern that in many respects resembles the architecture of a normal germinal center typically found within secondary lymphoid follicles (7). The dendritic cells and T-cells which are invariably present in both the normal and malignant follicles may be providing important signals and/or factors necessary for lymphoma cell growth (8). Support for this notion has recently been provided by findings that cognate interactions with allospecific T-cells may induce in vitro proliferation of FL cells (9).

Another general property of FL is the ability to selectively mutate their rearranged immunoglobulin genes. This was first appreciated by examination of the mechanism whereby some of these lymphomas became resistant to treatment with monoclonal antiidiotype antibodies (10, 11). The property of immunoglobulin gene hypermutation distinguishes FL from a variety of other types of human B-cell malignancies which have been found to mutate their expressed immunoglobulin genes only infrequently if at all (12, 13). In addition, it further supports a GC cell origin for FL since selective hypermutation of immunoglobulin genes is believed normally to occur primarily if not exclusively in GC B-cells (14, 15). Based on a variety of criteria, FL are monoclonal B-cell proliferations (16-18). However, occasional multiple rearranged bands may be present on Southern blot analysis of the immunoglobulin locus. This occasional appearance of oligoclonality in FL, however, appears to be an artifact that results from point mutations that sometimes alter or introduce enzyme recognition sites in the immunoglobulin genes (19).

One important difference between FL and GC cells is the presence of a t(14;18) chromosomal translocation that can be found in almost all of these malignancies (20, 21). The t(14;18) translocation results in deregulation of the bcl-2 protooncogene which causes constitutive expression of high levels of normal bcl-2 protein (22-24). The bcl-2 protein is not acutely transforming but appears to block programmed cell death or apoptosis and also can greatly prolong the survival of various B-cell lines (25-27). Interestingly, the bcl-2 protein appears to be selectively absent from normal GC B-cells (28), most of which are destined to die by apoptosis.

Mutational Analysis of Immunoglobulin Genes: Rationale and Methods. The role of antigen selection during the clonal expansion of B-cells can be studied by analyzing the mutations that accumulate within immunoglobulin genes. The antigen binding component or variable region of a function heavy chain gene is a complex composed of a V, D, and J gene segment that are brought together during B-cell development forming a single transcriptional unit (29). Although the precise number is not known, it has been estimated that in humans there may be over

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4 The abbreviations used are: FL, follicular lymphomas; GC, germinal center; V, immunoglobulin heavy chain variable (gene segment); CDR, complementarity determining regions; FWR, framework regions; S, silent (mutation); R, replacement (mutation); CLL, chronic lymphocytic leukemia; PCR, polymerase chain reaction; J, joining (gene segment); D, diversity gene segment; HTLV-1, human leukemia T-cell leukemia virus 1.

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100 different V gene segments in the typical germ line (30) along with over 30 diversity segments that can link up with 1 of the 12 different joining gene segments (31). Mutations introduced by the immunoglobulin gene hypermutation mechanism are mostly localized to the variable regions and can occur in either the FWR or CDR (32-34). Contact with antigen is thought to be mediated primarily by amino acid residues in the CDR (35). Thus, mutations that result in amino acid R in the CDR can potentially alter antigen binding and be selected on this basis.

As a result of selection, immunoglobulin genes from B-cells that have been positively selected by antigen often harbor a disproportionate number of R mutations in their CDR (36, 37). For random mutations in a protein region not under functional constraints, the ratio of R to S mutations should be close to 2.9 (38). However, positive selection of R mutations can dramatically increase the R/S ratio to levels severalfold above this value. To better analyze the significance of mutations that occur in CDR, especially when small numbers are present, Shlomchik et al. (37) developed a binomial probability model that takes into consideration the total number of mutations that are present in both the FWR and CDR. This model assumes that mutations occur randomly and independently and therefore distribute between the FWR and CDR according to the size of these regions, roughly 75 and 25% for the FWR and CDR of typical V gene segments, respectively. Because many FWR nucleotides apparently cannot sustain mutations if immunoglobulin function is to be preserved, this model also assumes that twice as many R mutations occurred in the FWR than were actually observed. Mutational analyses of immunoglobulin genes using these techniques have been used to distinguish whether the production of various types of autoantibodies is antigen driven or the result of polyclonal activation (37, 39).

Identification of Germ Line V Genes. The germ line V genes were identified in two cases of FL so that a precise identification of the sites and types of any mutations in the lymphomas V genes could be made. For the CG lymphoma, it was determined that a mutated version of a previously reported germ line gene from the relatively small VH4 family, VH4-21, was being used. We initially suspected that the CG V gene segment was a mutated version of VH4-21 or a closely related gene after comparing it to all 10 of the known functional VH4 family germ line genes (40, 41) and noting the positions of nucleotide differences among all of these genes (42). We subsequently determined that this patient’s “VH4-21-like” germ line repertoire consisted of only an unmodified VH4-21 gene and that, therefore, all of the nucleotide differences between the CG V gene and the published VH4-21 gene were indeed mutations. This was accomplished by amplifying VH4 germ line genes from this patient by the polymerase chain reaction and sequencing all of the clones that hybridized to an oligonucleotide that was selected to identify any that were potentially related to VH4-21 or the lymphoma V segment (43).

A different approach was taken to identify the germ line element for the MT lymphoma, in part because the MT V gene belonged to the large VH3 family where many if not most of the members have yet to be identified. For this case, the germ line gene was cloned from a size selected library prepared from MT genomic DNA using for hybridization a 1-kilobase probe derived from a region approximately 1 kilobase 5' of the expressed V gene. This upstream region probe identified a single band on Southern blot analysis of germ line DNA digested with a variety of different enzymes. The V gene identified in this manner, MTGL, is identical to the V gene expressed by the MT lymphoma over a large 5' upstream region with the exception of one mutation. This gene represents a previously undescribed member of the VH3 family (44).

Analysis of Lymphoma V Genes. The immunoglobulin V genes expressed by two different cases of FL were analyzed in an attempt to evaluate the potential role antigen selection may have played during the clonal evolution of these tumors. Both of these lymphomas had clinical, phenotypic, and histological characteristics typical of FL and had (14;18) translocations. Additional information about these cases can be found elsewhere (44, 45).

For the CG lymphoma, DNA isolated from four different tumor biopsies taken over a 2-year time period was analyzed. Consensus sequences for the tumor V genes could be determined for each biopsy in a straightforward manner using an asymmetrical PCR sequencing technique. In addition, nucleotide sequence information was also obtained from multiple VH4 genes cloned following PCR amplification from the DNA isolated from each biopsy. In this way, it was concluded that a single clone containing 23 total V gene substitutions relative to the germ line VH4-21 gene dominated the tumor cell population in the first three biopsies (Fig. 1). The maximum number of substitutions observed in any of the V gene sequences was also 23 with the exception of a few clones that also contained one additional mutation that was not found in any of the other clones or sequences and could, therefore, have been an artifact of the amplification procedure (46). The consensus sequence for the fourth biopsy contained only 17 mutations and was missing 8 substitutions that were present in the other V consensus sequences. However, because this biopsy was obtained after treatment of the patient with an antiidiotype antibody and the lymphoma cells in this biopsy failed to stain with the treatment antibody, the antiidiotype therapy may have played a role in the expansion of this clonotype.

In evaluating lymphoma V gene sequences for evidence of antigen selection, analysis should include only those mutations that accumulated in the tumor cells. Based on the available information, it is possible that some if not all of the mutations that were shared among all of the V sequences occurred in a precursor B-cell prior to transformation. It is clear, however, that mutations that are not shared by all of the clonally related V gene sequences must have accumulated after lymphomagenesis in a tumor cell. For the clone that dominated the first three CG biopsies, the location (FWR or CDR) as well as type (R or S).

Fig. 1. V gene sequence of dominant CG lymphoma clone. Three of the four biologies of the CG lymphoma all gave identical V gene consensus sequences. This sequence is shown here relative to the germ line VH4-21 gene; dashes, identity. Boldface capitals, nucleotide differences that result in amino acid replacements. Lower case, substitutions that do not produce changes in amino acid residues. The functional domains defined by Kabat et al. (61) are indicated above the sequences as FWR or CDR and the appropriate number.
Strong evidence for positive selection of CDR R mutations was also obtained following a similar analysis of the V genes expressed by the MT lymphoma. In this case, nucleotide sequence information was obtained from multiple V gene clones generated by both PCR and molecular cloning from a single FL tumor biopsy. Two dominant FL clonotypes were identified within this biopsy that were subsequently shown by hybridization studies using clonotype specific oligonucleotides to comprise 70 and 30% of the tumor cell population, respectively. Starting from FWR1, the V segment of the major clonotype contained a total of 41 substitutions relative to germ line while the minor clonotype contained only 13 substitutions. Because only 4 mutations were shared among all of the V gene sequences, it can be inferred that most of the substitutions in both the major and minor clone accumulated following lymphomagenesis during clonal expansion of the tumor. As shown in Table 1, an unexpectedly high number of R mutations were present in the CDR of both the major and minor clones, 15 and 5, respectively, which is the signature of positive selection as discussed above. The probability that either of these distributions arose by chance is only \( P < 0.025 \) and \( P < 0.042 \), for the major and minor clones, respectively.

Because the MT tumor underwent progression to a diffuse large cell lymphoma, we also had the opportunity in this case to explore the role of antigen selection in this process. Histological progression of low grade FL to diffuse large cell lymphoma, a more aggressive disease, occurs in more than one-half of cases within 10 years of diagnosis (47, 48). For the MT diffuse tumor, multiple V gene clones obtained by PCR and molecular cloning were evaluated from two biopsy sites. The consensus V gene from within this biopsy that were subsequently shown by hybridization studies using clonotype specific oligonucleotides to comprise 70 and 30% of the tumor cell population, respectively. Starting from FWR1, the V segment of the major clonotype contained a total of 41 substitutions relative to germ line while the minor clonotype contained only 13 substitutions. Because only 4 mutations were shared among all of the V gene sequences, it can be inferred that most of the substitutions in both the major and minor clone accumulated following lymphomagenesis during clonal expansion of the tumor. As shown in Table 1, an unexpectedly high number of R mutations were present in the CDR of both the major and minor clones, 15 and 5, respectively, which is the signature of positive selection as discussed above. The probability that either of these distributions arose by chance is only \( P < 0.025 \) and \( P < 0.042 \), for the major and minor clones, respectively.

Selection for Surface Immunoglobulin Expression. In contrast to the CDR, fewer R mutations were observed in all of the V gene FWR from both of these cases than would be expected by chance. In the absence of selection, R mutations should occur approximately 3 times as frequently as S mutations (38). As can be appreciated from Table 1, however, approximately equal numbers of R and S mutations were observed in the FWR of the dominant clonotypes from both cases. This was also true for all of the V gene clones that were analyzed from either case including those from the MT diffuse tumor. These data suggest that the immunoglobulin function is being actively maintained on the lymphoma cells even after progression by selection against R mutations in the FWR similar to what has been reported for normal B-cells. It has been proposed that approximately one-half of the potential FWR R mutations are destructive of immunoglobulin function and are therefore not seen if immunoglobulin must be maintained (37).

Finding diminished numbers of R mutations in FWR of the V genes in the CG and MT tumors is consistent with the notion that surface immunoglobulin expression is maintained by most FL. This has been suggested by past studies because, despite ongoing mutation in the immunoglobulin genes that presumably occurs in most of these tumors (11), the vast majority of FL express surface immunoglobulin even after treatment with anti-idiotypic antibodies (5, 16, 45). Based on these data, it appears that surface immunoglobulin is important for the survival of FL cells. Although it is tempting to speculate that immunoglobulin expression is maintained in order to transmit mitogenic signals generated by antigen binding, other explanations could also be proposed.

Remaining Questions about Selection. The nature of the selective force, presumably antigen, that appears to be controlling the clonal expansion of the CG or MT lymphomas was not examined. In general, very little is known about the reactivity of FL with given antigens. However, the possibility that self-antigens may play a role in FL growth has become more attractive in light of a recent report showing that of 31 FL cases examined, 8 were found to express immunoglobulin with autobody activity (49). It is particularly interesting in this regard that the CG lymphoma expresses a somatically mutated version of VH4–21 since this particular V gene seems to be frequently associated with autobody activity (39, 50, 51).

In addition, the issue of how long antigen selection occurred during clonal expansion of these tumors is not known, nor is whether continual antigen binding may be required for tumor cell growth. Because histological progression is a late occurring event in the evolution of many FL, finding suggestive evidence in the MT case for positive selection at the more aggressive diffuse large cell stage may suggest that an antigen receptor growth dependency can occur for an extended period of time. This conclusion would follow even if many of the mutations in the diffuse tumor had actually accumulated in a precursor FL clone prior to the event(s) that caused the change in histological appearance. Cytogenetic abnormalities such as translocations,

Table 1 V gene mutations accumulated after lymphomagenesis in dominant clonotypes of CG and MT follicular lymphomas

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<td>CDR1.2</td>
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Table 2 V gene mutations in MT diffuse clone not shared with MT follicular major clone

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which typically accompany progression in this disease (18), have been shown in some cases to activate additional oncogenes and at the same time eliminate immunoglobulin expression (52, 53). Even with continued immunoglobulin expression, however, the combined action of oncogene activation or the removal of tumor suppressor genes with progression may be sufficient in some cases to render FL growth independent of immunoglobulin signaling. Because of these considerations, it will be particularly interesting to analyze additional cases of FL both before and after progression to see if antigen selection may play a general role in this process.

Another unresolved issue is whether antigen driven proliferation is indirectly responsible for any of the secondary events that presumably cooperate with the t(14;18) in generating these malignancies. From molecular analyses of breakpoints, it has been suggested that the t(14;18) occurs as a mistake during rearrangement of the immunoglobulin gene D and J gene segments prior to V gene segment rearrangement or rearrangement of light chain gene segments (54). Because D-J joining normally occurs very early in B-cell development it has also been proposed that the t(14;18) also occurs at the same developmental stage. If this interpretation is correct, then the additional genetic events that presumably contribute to transformation must occur after the t(14;18) because FL express monoclonal heavy and light chain immunoglobulin genes that successfully completed the rearrangement process as mentioned above. It may be relevant that for both the CG and MT tumors, mutations were present that were common to all of the respective V sequences analyzed from each case. This is consistent with the possibility that these substitutions occurred prior to transformation and would suggest that both of these tumors originated in a mature B-cell after the somatic hypermutation mechanism had been activated. Placing the final transformation event after activation of the immunoglobulin gene somatic hypermutation mechanism suggests that antigen stimulation preceded transformation (2, 15) and is certainly consistent with the notion that antigen driven proliferation can also indirectly contribute to lymphomagenesis.

Selection in Other Systems. Antigen stimulation may play an important role in the evolution or development of human B-cell malignancies besides FL. Recently, Friedman et al. (55) cloned and sequenced the V genes of a lymphoma that expressed an immunoglobulin with autoantibody activity from three serial biopsies. The V gene consensus sequence for each biopsy contained mutations relative to the proposed germ line gene in a pattern that suggested antigen was involved in the selection of the identified clonotypes. Unlike the CG and MT lymphomas described above, however, fewer R mutations were found in the CDR regions than would be expected by chance alone suggesting that selection was operating in this case to presumably maintain binding to the autoantigen. Although initially this tumor was classified as a well differentiated low grade lymphoma, the last biopsy showed a predominance of poorly differentiated large cells, a more aggressive histological appearance. However, similar to the MT FL case described above after progression, it appeared that antigen selection was still operative at this late stage and, as would be expected, that the larger lymphoma cells could still bind the identified autoantigen.

It has also been suggested that antigen selection may also be important in the development of CLL, a low grade malignancy of CD5+ B-cells. Support for this notion largely rests on finding that CLL exhibits a restricted or biased use of V gene segments manifested primarily as an abundance of cases expressing genes from the small VH5 and VH6 families (56, 57). The selection hypothesis applied to CLL would contend that these malignancies develop in response to a very limited number of antigens and that antigen binding requires use of the one of the three functional V genes from the VH6 of VH5 families. It is also possible that antigen stimulation is responsible for generating biased use of V genes in normal human CD5 positive B-cell populations as has been described for certain strains of mice as a result of selection by self-antigens (58). Unfortunately, because these tumors typically do not mutate their immunoglobulin genes, similar studies to those described above for FL to help assess whether antigen selection of tumor cells occurs in individual cases will generally not be possible. In addition to the data regarding V gene use, several cases of CLL have been identified that appear to be malignant proliferations of B-cells that are responding to HTLV-1 infections (59). In these cases, interaction of surface immunoglobulin with HTLV-1 protein may play an indirect role in transformation by providing a chronic proliferation signal in an environment with HTLV-1 generated abnormalities in T-cell functioning.

Concluding Remarks. An analysis of the mutations in V genes expressed by two cases of FL suggests that clonal selection of FL cells may be akin to the selection of high affinity antibody clones that occurs in normal germinal centers. Although the mechanism of antigen driven selection in normal CG is not well understood, recent evidence indicates that induction of bcl-2 expression brought about by cross-linking surface immunoglobulin may be important (15, 60). Induction of bcl-2 expression in a GC cell may allow it to escape apoptosis or programmed cell death and further expand and differentiate into antibody secreting plasma cells or memory B-cells. Because FL express bcl-2 constitutively, these cells should be able to avoid apoptosis without interacting with antigen. Our data suggest, however, that clonal expansion of FL is dependent on interaction with antigen and that, therefore, the antigen receptor complex is providing a signal(s) in addition to bcl-2 that is required for selection.

References
Antigen Selection in Human Lymphomagenesis


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