Follicular Lymphoma with Monocytoid B-Cell Proliferation: Molecular Assessment of the Clonal Relationship between the Follicular and Monocytoid B-Cell Components

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SUMMARY: Although a number of studies have recognized that follicular lymphomas may be accompanied by a prominent proliferation of monocytoid B-cells, the clonal relationship between these components has not been adequately assessed. Using laser capture microdissection, we isolated the follicular and monocytoid B-cell components from four well-characterized cases of follicular lymphoma with prominent monocytoid B-cells. DNA from each component was analyzed using polymerase chain reaction (PCR)-based methods to assess for clonal rearrangements of the immunoglobulin heavy chain gene (IgH) and for the presence of the *bcl-2* gene major breakpoint region/joining region (MBR/JH) DNA fusion products by conventional PCR and fluorescence melting curve analysis. Evidence of clonal identity was established in the follicular and monocytoid B-cell components of three cases by demonstration of IgH gene rearrangements of identical size using IgH PCR, by comparison of complementarity determining region III (CDRIII) DNA sequences, or by detection of *bcl-2* MBR/JH fusion products of identical size and/or melting temperature. Molecular analysis of the fourth case revealed a monoclonal and MBR/JH-positive follicular component accompanied by a polyclonal and MBR/JH-negative monocytoid B-cell proliferation. We conclude that the follicular and monocytoid B-cell components of this variant of follicular lymphoma are clonally identical in the majority of cases. However, in a minority of these cases, the monocytoid B-cell component is reactive. Larger studies that assess the prognostic significance of follicular lymphoma with monocytoid B-cells will benefit from molecular studies that assess the clonal relationship of both components. (*Lab Invest* 2000, 80:1593–1599).

Follicular lymphoma is one of the most common forms of non-Hodgkin's lymphoma in the Western world, accounting for approximately 22% to 34% of non-Hodgkin's lymphoma in the United States (The Non-Hodgkin's Lymphoma Pathologic Classification Project, 1982; The Non-Hodgkin's Lymphoma Classification Project, 1997; Armitage and Weisenburger, 1998). Follicular lymphoma is a clinically indolent, incurable neoplasm, and patients have a median survival time of approximately 8 to 10 years from the time of diagnosis (Horning, 1993). In recent years, a number of studies have reported cases of follicular lymphoma that also display prominent monocytoid B-cell proliferations (Fisher et al, 1995; Hernandez et al, 1995; Mollejo et al, 1994; Nathwani et al, 1992, 1997; Ngan et al, 1991; Plank et al, 1995; Schmid et al, 1996;

Sheibani et al, 1988; Slovak et al, 1993). These cases exhibit clusters of monocytoid B-cells with abundant pale cytoplasm and ovoid-to-slightly-reniform nuclei with open chromatin that surround neoplastic follicles in the marginal/interfollicular zones of lymph nodes. In a recent study, 9% of cases of follicular lymphomas had a prominent monocytoid B-cell component (greater than 5% monocytoid B-cells), and these cases also had significantly shorter failure-free survival and overall survival than pure follicular lymphomas (Nathwani et al, 1999). Nathwani et al (1992, 1997, 1998) have suggested that such cases may represent malignant transformation of a pluripotent/precursor memory B-cell that is capable of differentiating into multiple cell types. Although other investigators have attempted to infer clonal relatedness by assessment of immunoglobulin light chain restriction in both the follicular and monocytoid B-cell components of these tumors (Hernandez et al, 1995; Schmid et al, 1996), the clonal relationship between these components has not been adequately assessed at the genetic level.

In this report, we evaluate four cases of follicular lymphoma with greater than 5% monocytoid B-cells

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Figure 1.

Follicular lymphoma with prominent monocytoid B-cell component. *A*, Before laser microdissection. Hematoxylin-eosin staining; original magnification, $\times 20$. *B*, Same case after laser capture microdissection of follicular and monocytoid B-cell components. Hematoxylin-eosin staining; original magnification, $\times 20$.

using laser capture microdissection to isolate the follicular and accompanying monocytoid B-cell components. Both components were subjected to molecular analysis for the presence of clonal rearrangements of the immunoglobulin heavy chain gene (IgH PCR) and for t(14;18) (q32;q21) major breakpoint region/joining region (MBR/JH) fusion products of identical size (MBR/JH PCR). We also employed a recently developed methodology that uses fluorescence melting curve analysis for the identification of the MBR/JH translocation product by its unique melting temperature (T_m) (Bohling et al, 1999). Using these methods, we provide evidence of clonal relatedness in the follicular and monocytoid B-cell components in three of four cases, with the fourth case consisting of a monoclonal t(14;18) MBR/JH-positive follicular lymphoma accompanied by a polyclonal t(14;18) MBR/JH-negative monocytoid B-cell proliferation.

Results

Using laser capture microdissection, we isolated the follicular and monocytoid B-cell components from four well-characterized cases of follicular lymphoma with greater than 5% extrafollicular monocytoid

B-cells (Fig. 1). The integrity of the DNA isolated from each microdissected sample was established by successful PCR amplification of the human β -globin gene (Fig. 2A, Table 1). In each case, DNA from each component was analyzed by PCR for clonal rearrangements of IgH (Fig. 2, B to D), and for the presence of the MBR/JH fusion product by conventional PCR (Fig. 2E) and a fluorescence PCR method (Fig. 3, A to C) using the double-stranded DNA binding dye SYBR Green and a thermal cycler integrated with a fluorometer. We have previously demonstrated that PCR amplification followed by fluorescence melting curve analysis is up to 100-fold more sensitive for the detection of the MBR/JH translocation than conventional PCR followed by detection by ultraviolet transillumination of ethidium bromide-stained agarose gels (Bohling et al, 1999).

The molecular findings from IgH PCR, MBR/JH PCR, and fluorescence melting curve analyses are summarized in Table 1. IgH PCR analysis (Fig. 2, B and C) revealed clonal rearrangements of identical band size in both the follicular and monocytoid B-cell components in Cases I, II, and III. In the fourth case, IgH PCR was monoclonal in the follicular component but polyclonal in the monocytoid B-cell component. MBR/JH fusion products (Fig. 2D) of identical band size were detected in the follicular and monocytoid B-cell components of Cases I, II, and III and only in the follicular component of Case IV, corroborating the results obtained by IgH PCR in all four cases (Table 1). Fluorescence melting curve analysis also detected the presence of the MBR/JH chromosomal translocation in both the follicular and monocytoid B-cell components of Cases I and II, and in the follicular component of Case IV, whereas the monocytoid B-cell component of Case IV was negative (Fig. 3, A to C). Because of technical difficulties, we were unable to obtain melting curve data from Case III.

We also compared DNA sequence data from IgH PCR products obtained from both the follicular and monocytoid B-cell components of selected cases. DNA sequence analysis of IgH PCR products from Case I revealed identical complementarity determining region III (CDRIII) sequences in the follicular and monocytoid B-cell components (Table 2). In Case III, CDRIII sequences in the monocytoid B-cell component differed from the sequence of the follicular component of this case by two single base substitutions (Table 2), suggesting the possibility of somatic hypermutation in the monocytoid B-cell component. DNA sequence analysis of Case II was unsuccessful despite repeated attempts, presumably because of the presence of inhibitors within the DNA specimen.

Discussion

The simultaneous occurrence of two or more distinct histologic subtypes of malignant lymphoma has been documented in the pathology literature as early as 1948 by Custer and Bernhard (1948). Although immunophenotypic analyses have refined the ability to recognize the relationship between morphologically

Figure 2.

β -globin, immunoglobulin heavy chain gene (IgH), and major breakpoint region/joining region (MBR/JH) PCR of microdissected follicular and monocytoid B-cell components from hematoxylin-eosin stained sections. **A**, β -globin PCR. Lanes 1–8, Follicular (F) and monocytoid B-cell (M) components of Cases I to IV. **B**, IgH PCR. Upper Lane 1, DNA fragment size marker (L). Upper Lane 2, Negative DNA control from reactive human tonsil (–). Upper Lane 3, Positive DNA control from the SUDHL-6 cell line (+). Upper Lanes 4–7, Follicular (F) and monocytoid B-cell (M) components of Case I, each in duplicate. Upper Lane 8, Control without template DNA (H₂O only). Lower Lane 1, DNA fragment size marker (L). Lower Lane 2, Negative DNA control from reactive human tonsil (–). Lower Lane 3, Positive DNA control from the SUDHL-6 cell line (+). Lower Lanes 4–7, Follicular (F) and monocytoid B-cell (M) components from Case II, each in duplicate. Lower Lane 8, Control without template DNA (H₂O only). **C**, IgH PCR. Lane 1, DNA fragment size marker (L). Lane 2, Negative DNA control from reactive human tonsil (–). Lane 3, Positive DNA control from the SUDHL-6 cell line (+). Lane 4, Control without template DNA (H₂O only). Lanes 5–8, Follicular (F) and monocytoid B-cell (M) components from Case III, each in duplicate. **D**, IgH PCR. Lane 1, DNA fragment size marker (L). Lanes 2–5, Follicular (F) and monocytoid B-cell (M) components from Case IV, each in duplicate. **E**, MBR/JH translocation PCR. Lane 1, DNA fragment size marker (L). Lane 2, Negative DNA control from reactive human tonsil (–). Lane 3, Positive DNA control from the SUDHL-6 cell line (+). Lanes 4–11, Follicular (F) and monocytoid B-cell (M) components of Cases I to IV.

distinct lymphoid proliferations, molecular genetic studies remain the gold standard for definitive establishment of clonal identity between synchronous or metachronous lymphoproliferative processes. The importance of establishing clonal identity at the genetic level is clearly underscored by previous reports of discordant immunoglobulin light chain restriction between low-grade and transformed lymphomas with identical immunoglobulin heavy chain rearrangements (Miyamura et al, 1990; Nakamine et al, 1992).

In this study, we examined the relationship between two components of an unusual variant of non-Hodgkin's lymphoma consisting of morphologically distinct areas of follicular lymphoma accompanied by a prominent monocytoid B-cell proliferation (Fisher et al, 1995; Hernandez et al, 1995; Mollejo et al, 1994; Nathwani et al, 1992, 1997; Ngan et al, 1991; Plank et al, 1995; Schmid et al, 1996; Sheibani et al, 1988; Slovak et al, 1993). The ability to accurately recognize this entity may be of more than academic interest, because a recent study suggests that patients with follicular lymphoma with monocytoid B-cells have a significantly shorter survival than those with pure follicular lymphoma (Nathwani et al, 1999). Thus, an assessment of the clonal relationship between the follicular lymphoma and monocytoid B-cell components might be useful for prognostic purposes.

Previous studies of cases of follicular lymphoma with monocytoid B-cells were limited to the demonstration of immunoglobulin light chain restriction as definitive evidence of clonal identity, or to the utilization of whole tissue sections for the assessment of individual components of the lymphomatous process by genetic analyses. In this study, however, we isolated follicular and monocytoid B-cell components using laser capture microdissection and subjected them to multiple genetic analyses that included IgH PCR, conventional and fluorescence melting curve MBR/JH PCR, and CDRIII DNA sequence analysis. Using IgH PCR, we demonstrated clonal immunoglobulin heavy chain gene rearrangements of identical size in both components in three of four cases. The follicular component of the fourth case yielded a reproducibly clonal band, and the accompanying monocytoid B-cell proliferation exhibited a polyclonal pattern. DNA sequences of multiple IgH PCR products from follicular and monocytoid B-cell components of Cases I and III revealed identical CDRIII sequences in both components of Case I and sequences that differed from the follicular component by only two single base pair (bp) substitutions in the monocytoid B-cell component of Case III. The intraclonal variation exhibited by Case III provides support for the hypothesis that continued somatic hypermutation occurs in these tumors. In Case IV, the follicular component was monoclonal and the monocytoid B-cell proliferation yielded a polyclonal smear pattern. MBR/JH fusion products of identical size and/or T_m were demonstrated in both the follicular and monocytoid B-cell components in Cases I, II, and III, whereas only the follicular component of Case IV had detectable MBR/JH fusion products.

Table 1. Summary of Molecular Findings

Molecular Analysis	Case I		Case II		Case III		Case IV	
	F	M	F	M	F	M	F	M
IgH PCR	+	+	+	+	+	+	+	—
Conventional MBR/JH PCR	+	+	+	+	+	+	+	—
Fluorescence MBR/JH PCR	+	+	+	+	●	●	+	—
β -globin PCR	+	+	+	+	+	+	+	+

F, follicular component; M, monocytoid B-cell component; +, amplicon detected; —, no amplicon detected; ●, unsuccessful analysis, IgH, immunoglobulin heavy chain gene.

It has been postulated that monocytoid B-cells are related to the marginal zone cells of reactive follicles (Spencer et al, 1985), and it has also been suggested that follicle center cells might be capable of undergoing differentiation into marginal zone B-cells (Nathwani et al, 1992, 1997, 1998; Schmid et al, 1996). It is therefore conceivable that some follicular lymphomas might retain the capacity to undergo marginal zone B-cell differentiation. Support for a common origin of neoplastic monocytoid B-cells and follicle center cells has come from recent studies in which identical light chain restriction was detected in the follicular and monocytoid B-cell components of cases with coexisting monocytoid B-cell and follicular lymphoma (Hernandez et al, 1995; Schmid et al, 1996). However, other investigators have considered this entity to be a composite lymphoma with two unrelated components (Ngan et al, 1991). Our study provides additional evidence for a common clonal origin for the follicular and monocytoid B-cell components in the majority of follicular lymphomas with prominent monocytoid B-cell proliferations. In a minority of these cases, the accompanying monocytoid B-cell component probably represents marginal zone hyperplasia. Future clinicopathologic studies of follicular lymphomas with monocytoid B-cells will need to distinguish between these two groups. This study also demonstrates the utility of laser capture microdissection for the molecular assessment of complex tumors and suggests that this technology should be used more frequently to characterize lymphomas with unusual morphology and phenotype.

Materials and Methods

Clinical Samples

Well-characterized cases of follicular lymphoma with prominent monocytoid B-cell components were obtained from the files of the University of Texas M. D. Anderson Cancer Center, Houston, Texas, and the University of Utah Health Sciences Center, Salt Lake City, Utah. These cases were identified using criteria as previously described, ie, a prominent monocytoid B-cell proliferation comprised at least 5% of the entire lesion (Nathwani et al, 1999), with a minimum of 25% of neoplastic follicles surrounded by a monocytoid B-cell layer of four or more cells in thickness. Histopathologic evaluation was performed independently by two pathologists (LJM and KSJE-J), and only cases

in which both pathologists concurred were used for this study.

Immunohistochemical Studies

Immunohistochemical studies were performed on all cases using formalin-fixed paraffin-embedded tissue sections, an avidin-biotin peroxidase complex technique, and an automated immunostainer (Ventana Medical System, Tucson, Arizona). Studies were performed with the following panel of monoclonal antibodies at the indicated dilutions: CD3 (1:300), CD20 (1:600), BCL-2 (1:40), and kappa and lambda immunoglobulin light chains (1:600) (DAKO, Carpinteria, California); CD43 (1:200) (Becton-Dickinson, Mountain View, California); CD10 (1:200) (Novocastra Laboratories, Newcastle, United Kingdom); and BCL-6 (1:150) (Santa Cruz Biotechnology Inc., Santa Cruz, California). The neoplastic cells were positive for CD10, CD20, BCL-2, and BCL-6, and negative for CD3 and CD43, supporting follicle center B-cell origin (data not shown). Immunoglobulin light chain studies revealed monotypic kappa light chain restriction in both the follicular and monocytoid B-cell components of Cases I, II, and III, but were inconclusive in Case IV (data not shown).

Laser Capture Microdissection and DNA Extraction

Laser capture microdissection of follicular lymphoma and associated monocytoid B-cell components was performed on 5- μ m-thick hematoxylin and eosin stained sections from formalin-fixed paraffin-embedded tissue as previously described (Fig. 1) (Fend et al, 1999). DNA extraction from microdissected follicular and monocytoid B-cell components was also performed as previously described (Fend et al, 1999). The integrity of the microdissected DNA samples was established by successful PCR amplification of the human β -globin gene from each sample (Fig. 2A).

IgH PCR Analysis

IgH PCR analysis was performed using a previously described protocol and primers (Lombardo et al, 1996). Briefly, amplification of rearranged immunoglobulin genes was accomplished using a Perkin-Elmer 2400 thermocycler (Perkin-Elmer, Norwalk, Connecticut), a "hot start" technique using TaqStart

Figure 3.

MBR/JH PCR fluorescence melting peak analysis. A, Characteristic melting peaks of the positive (SUDHL-6 cell line), negative (reactive human tonsil), and no template (H_2O) controls, along with fluorescence melting peaks for the follicular and monocytoid B-cell components from Case I. The melting temperatures (T_m) for both components are identical in duplicate reactions ($T_m = 87.5^\circ C$). B, Characteristic melting peaks of the positive (SUDHL-6 cell line), negative (reactive human tonsil), and no template (H_2O) controls, along with fluorescence melting peaks for the follicular and monocytoid B-cell components from Case II. The melting temperatures (T_m) for both components are identical in duplicate reactions ($T_m = 88.5^\circ C$). C, Characteristic melting peaks of the positive (SUDHL-6 cell line), negative (reactive human tonsil), and no template (H_2O) controls, along with a fluorescence melting peak for the follicular component from Case IV. The melting temperature (T_m) for the follicular component is identical in duplicate reactions ($T_m = 88.25^\circ C$). Note that no melting peak was obtained for the monocytoid B-cell component.

Antibody (Clontech, Palo Alto, California), and immunoglobulin heavy chain variable region (VH-FRIII; 5'-ACA CGG C[C/T]G T[G/A]T ATT ACT GT-3') and joining region (JH; 5'-ACC TGA GGA GAC GGT GAC

C-3') consensus primers. Crude extract (1 or 2 μl) from microdissected tissue and 1 μl of 50 ng/ μl positive and negative controls were used as the template for 35 cycles of amplification consisting of denaturation at $96^\circ C$ for 1 minute, annealing at $56^\circ C$ for 1 minute, and elongation at $74^\circ C$ for 1 minute. PCR products were analyzed on ethidium bromide-stained 2% Metaphor agarose gels (FMC Bioproducts, Rockland, Maine) and detected by ultraviolet transillumination. PCR analysis was performed in quadruplicate with consistent results for each microdissected sample.

Conventional MBR/JH and Fluorescence-Based PCR Analysis

Conventional PCR and fluorescence melting curve analyses for detection of the MBR/JH translocation were performed as previously described by Bohling et al (1999). Briefly, conventional PCR analysis was performed with paired oligonucleotide primers specific for the major breakpoint region (MBR) of the *bcl-2* gene (5'-GAG TTG CTT TAC GTG GCC TG-3') and the immunoglobulin heavy chain joining region (JH) (5'-ACC TGA GGA GAC GGT GAC C-3') (Segal et al, 1994; Stetler-Stevenson et al, 1988). PCR products were analyzed on ethidium bromide-stained 1.5% SeaKem LE agarose gels (FMC Bioproducts, Rockland, Maine) and detected by ultraviolet transillumination. Amplification reactions containing a discrete band in the expected electrophoretic migration range of 80 to 300 bp were considered to be positive for the MBR/JH translocation.

Fluorescence melting curve analysis was accomplished using the same MBR and JH primers used in conventional analysis and performing rapid cycle PCR reactions in glass capillary tubes in a microvolume fluorometer (LightCycler; Idaho Technology, Idaho Falls, Idaho) (Wittwer et al, 1997). SYBR Green I (Molecular Probes, Eugene, Oregon), which interacts with double-stranded DNA, was included in each reaction at a 1:30,000 dilution. Sequential monitoring of fluorescence signals was performed at the end of the extension phase of each amplification cycle. All reactions included a negative DNA control from reactive human tonsil, a positive DNA control from the Stanford University diffuse histiocytic lymphoma (SUDHL)-6 cell line, and a control without template DNA (H_2O only). After PCR amplification, the PCR products were cooled to $45^\circ C$ and then slowly heated to $100^\circ C$ at a rate of $0.2^\circ C$ per second. Since the T_m of a particular segment of DNA is related to its specific DNA sequence, GC content, and length, deductions about similarities between DNA sequences can be made based on their T_m s. Fluorescence melting peaks were derived from initial fluorescence melting curves (fluorescence [F] versus temperature [T]) by plotting the negative derivative of fluorescence over temperature versus temperature ($-dF/dT$ versus T). Samples with characteristic fluorescence melting curve profiles were considered to be positive for the MBR/JH translocation (Bohling et al, 1999). Assays were performed

Table 2. IgH PCR Product Sequencing: Comparison of CDRIII DNA Sequences^a

CASE	3' END OF V _H	N	D _H	N	J _H
<div style="display: flex; justify-content: space-around;"> D_HQ52 J_H4 </div>					
GERMLINE	ACTGT		CTGAGCTGAGAA		ACTTTTGACTACTGGGGCCAAGGAACCCCTGGTC
IF		GAG	T---A-G-----	GGA	-----G-----
IM		---	-----	---	-----
<div style="display: flex; justify-content: space-around;"> DA1 J_H1 </div>					
GERMLINE	TGT		TGACTACAGTAA		CAGGGCACCCCTGGTCACCGTC
IIIF		GCAAAA	--G-CC-----G-	GTCACC	-----TT-----C-C-A----
IIIM		-----	----T*--A*--	-----	-----

CDRIII, complementarity determining region III; M, monocytoid B-cell component; F, follicular component; V, variable region; N, non-templated nucleotides; D, diversity region; J, joining region; T*, A*, single base substitutions in the monocytoid B-cell component of Case III.

^aCDRIII DNA sequences from follicular and monocytoid B-cell components of Cases I and III. Germline sequences and diversity and joining region designations are included with each case. Sequences from follicular and monocytoid B-cell components are aligned with their respective germline D_H and J_H segments. Dashes (---) represent identity with the germline sequence.

in quadruplicate with consistent results for each microdissected sample.

DNA Sequencing

Direct DNA sequencing of IgH PCR products was performed following purification and concentration of amplification products using the QIAquick PCR Purification Kit (QIAGEN, Valencia, California) and Microcon-50 microconcentrator columns (Amicon, Beverly, Massachusetts). Purified PCR products were bidirectionally sequenced using IgH PCR primers and analyzed on the ABI Prism 377 with dye terminators (PE Applied Biosystems, Foster City, California). Cloned IgH PCR products were also bidirectionally sequenced after ligation into the pCR2.1 vector according to manufacturer's instructions (Invitrogen, San Diego, California) using M13 reverse and T7 promoter primers. In each case, at least four clones corresponding to the follicular and monocytoid B-cell IgH products of identical size were selected for DNA sequencing.

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