Immunophenotypic Profile of Lymphoplasmacytic Lymphoma/Waldenström Macroglobulinemia

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Abstract

We retrospectively reviewed the immunophenotypic profile of 75 cases of lymphoplasmacytic lymphoma/Waldenström macroglobulinemia (LPL/WM) analyzed by flow cytometry. All patients had monoclonal IgM (median, 2,100 mg/dL [21 g/L]) in serum and were considered clinically to have WM. The neoplastic cells, in all cases, expressed monoclonal immunoglobulin light chain (κ, 55; λ, 20) and CD19, and every case assessed was positive for CD20 (n = 68) and CD52 (n = 60). The results for other antigens assessed in decreasing frequency of positivity were as follows: surface IgM (26/28 [93%]), CD79b (11/13 [85%]), CD11c (13/16 [81%]), CD25 (5/7 [71%]), CD23 (17/28 [61%]), CD38 (24/50 [48%]), FMC7 (11/29 [38%]), CD22 (4/12 [33%]), CD5 (3/65 [5%]), and CD10 (1/38 [3%]). These results show that the immunophenotype of LPL/WM is variable and overlaps with other B-cell lymphoproliferative disorders. CD23, usually of dim intensity, and CD11c are expressed commonly in LPL/WM. Rare CD5+ and CD10+ cases of LPL/WM also exist.

In 1944, Waldenström described 3 patients, 2 of whom had fatigue, epistaxis, lymphadenopathy, worsening anemia, low serum fibrinogen levels, and high serum viscosity due to an abnormal high-molecular-weight serum protein. This protein subsequently was shown to be monoclonal IgM. Waldenström macroglobulinemia (WM), as the syndrome later was designated, initially was thought to be associated with a variety of lymphoid neoplasms that can secondarily cause hyperviscosity syndrome. Subsequently, WM was combined with lymphoplasmacytic lymphoma (LPL) and was designated lymphoplasmacytic lymphoma/Waldenström macroglobulinemia in the 2001 World Health Organization (WHO) classification. The concept of WM was defined further by a consensus group at the Second International Workshop on WM held in Athens, Greece, in 2002. In this workshop, the group defined WM as a distinct clinicopathologic entity characterized by bone marrow infiltration by LPL associated with IgM monoclonal gammopathy. It is recognized, however, that monoclonal IgM in serum is not unique to LPL/WM and that LPL/WM must be distinguished from other low-grade B-cell lymphoproliferative disorders that can be associated with IgM paraprotein, such as chronic lymphocytic leukemia/small lymphocytic lymphoma and marginal zone B-cell lymphoma. In such cases, the question arises as to whether flow cytometric immunophenotypic analysis can be helpful for differential diagnosis.

The WHO classification states that the neoplastic cells of LPL/WM usually are positive for monotypic surface immunoglobulin light chain, IgM, CD19, and CD20 and are negative for CD5, CD10, and CD23. Similarly, Owen and colleagues studied 98 cases of LPL/WM by flow cytometry and observed this immunophenotype in 90% of cases, with CD5...
and CD23 expressed in 5% and 1% of cases, respectively. By contrast, San Miguel and colleagues reported that the immunophenotype of LPL/WM is more variable, with at least 20% of cases being positive for CD5 or CD23.

We retrospectively analyzed the flow cytometric immunophenotypic findings in a large group of previously untreated LPL/WM cases at our institution. All cases had a serum IgM paraprotein and morphologic findings in bone marrow compatible with LPL/WM and clinically were considered to be WM.

Materials and Methods

After approval from the institutional review board, we searched the files of our institution for cases of LPL/WM involving bone marrow and analyzed by flow cytometric immunophenotypic analysis accessioned from January 1995 through September 2004. The original diagnoses were confirmed by review of bone marrow aspirate smears and biopsy specimens, the results of serum protein electrophoresis and immunofixation studies, and the medical records. All patients had serum monoclonal IgM protein and clinically were considered to have WM. Only untreated cases were included in the study.

Immunophenotypic analyses were performed on RBC-lysed, whole bone marrow aspirate samples. A total of 2 × 10⁶ cells per tube were stained, lysed, and then washed before staining. For the detection of cytoplasmic antigens, fixation and permeabilization steps were taken before staining using the Fix & Perm reagent kit (Caltag Laboratories, Burlingame, CA). We performed 3- or 4-color flow cytometric immunophenotypic analysis using the FACSscan or FACSCalibur instrument (BD Biosciences, San Jose, CA) as previously described. Negative staining levels were set by comparison with isotype control samples.

Because these cases were studied during a number of years, the antibodies used varied with time. Most recently (the past 2 years), a phycoerythrin-conjugated anti-CD19 antibody and an allophycocyanin- or fluorescein isothiocyanate–conjugated anti-CD38 antibody were used simultaneously in all combinations. The antigens assessed in at least a subset of cases included immunoglobulin light chains, surface IgM, CD5, CD10, CD11c, CD19, CD20, CD22, CD23, CD25, CD38, CD45, CD52, CD79b, and CD138. All antibodies were obtained from BD Biosciences.

For this study, for an antigen to be considered positive, we used an arbitrary cutoff of 20% or more analyzed events. An immunoglobulin κ/λ ratio of less than 0.5 or more than 5 was considered evidence of monoclonality. For cases with more than 1 sample analyzed by flow cytometry over time, the immunophenotypic profiles were compared.

For cases that expressed CD5, CD10, and CD23, we also performed immunohistochemical staining using formalin-fixed, paraffin-embedded tissue sections of the bone marrow core biopsy specimens and antibodies specific for CD23 (1B12, dilution 1:15, Novocastra Laboratories, Newcastle upon Tyne, England), bcl-6 (dilution 1:10, DAKO, Carpinteria, CA), and cyclin D1 (AM29, dilution 1:15, Zymed Laboratories, South San Francisco, CA; and, more recently, SP4, dilution 1:40, Lab Vision, Fremont, CA), as described previously. Briefly, after deparaffinization and rehydration in graded alcohols and xylene, endogenous peroxidase was blocked with hydrogen peroxide. Heat-induced epitope retrieval was performed by heating slides in EDTA buffer at pH 8.0, using the Handy Steamer Plus (Black and Decker, Towson, MD); slides then were cooled for 20 minutes. Immunostaining was completed using the LSAB2 detection kit (DAKO) with appropriate positive and negative control samples.

Conventional G-band karyotype analysis was performed as described previously. Statistical comparison of various parameters was performed using the 2-tailed Student t test. The difference was considered to be significant at a P value of less than .05.

Results

A total of 77 cases of LPL/WM with flow cytometry immunophenotypic data were identified in our files. In 2 cases (3%), bone marrow aspirate samples were of suboptimal quality, although the biopsy specimen was involved by lymphoma. The aspirate specimens for these 2 cases were considered not representative and are not discussed further. The remaining 75 cases represent the study group.

Clinical and Pathologic Findings

There were 45 men and 30 women, with a median age of 60 years (range, 34-82 years). Of the 75 patients, 67 were symptomatic, and fatigue was the most common complaint; 8 patients were asymptomatic. No evidence of lymphadenopathy or splenomegaly was found in 44 patients, 11 patients had lymphadenopathy detected only by computed tomography (CT) scan, 10 patients had lymphadenopathy detected by physical examination and CT scan, 6 patients had splenomegaly detected only by CT scan, and 4 patients had clinically detectable lymphadenopathy and splenomegaly.

The median level of total serum IgM was 3,900 mg/dL (39 g/L; range, 400-12,000 mg/dL [4-120 g/L]); reference range, 29-214 mg/dL [0.29-2.14 g/L]), and the median level of serum monoclonal IgM was 2,100 mg/dL (21 g/L; range, 0.2-7.5 g/dL [2-75 g/L]). The median level of polyclonal γ immunoglobulin was 500 mg/dL (5 g/L; range, 100-1,300 mg/L [1-13 g/L]);
reference range, 500-1,400 mg/dL [5-14 g/L]) and was decreased in 36 cases (48%). In 49 cases (65%), Bence Jones protein was detected in the urine with a median level of 36 mg in 24 hours (range, 2-88 mg/24 h).

The median extent of bone marrow involvement by tumor was 50% (range, 10%-95%). The main patterns of involvement were diffuse (n = 27) and interstitial (n = 22), but other patterns or mixtures of patterns also were observed, including nonparatrabecular and interstitial, 7; diffuse and interstitial, 6; paratrabecular and interstitial, 5; nonparatrabecular, 4; paratrabecular, 3; paratrabecular and nonparatrabecular, 1; and paratrabecular, interstitial, and focally diffuse, 1. There was no statistically significant correlation between pattern or extent of bone marrow involvement and the presence of symptoms.

Conventional cytogenetic studies were performed on bone marrow aspirate material in 41 cases. The results showed a normal diploid karyotype in 32, an abnormal karyotype in 6, and insufficient aspirate material for analysis in 3. In the 6 cases with cytogenetic abnormalities, 2 had complex karyotypes and 4 had simple abnormalities, including trisomy 8 (1 case), trisomy 18 (1 case), additional 6q chromosome (1 case), and an additional X chromosome (1 male case). The translocation t(9;14)(p13;q32) was not identified.

**Immunophenotypic Findings**

The flow cytometric immunophenotypic results are provided in Table 1. Immunoglobulin light chain restriction was detected in all cases—κ in 55 cases and λ in 20 cases. Surface IgM was detected in 26 of 28 (93%) cases analyzed. All cases tested were positive for CD19, CD20, and CD52. Most cases assessed expressed CD79b, CD11c, CD25, and CD23. Thirty to forty percent of cases were positive for CD23. Thirty to forty percent of cases were positive for CD19, CD20, and CD52. Most cases assessed expressed CD79b, CD11c, CD25, and CD23. Thirty to forty percent of cases were positive for CD23. Thirty to forty percent of cases were positive for CD3 (n = 28), CD4 (n = 27), CD8 (n = 27), and CD138 (n = 16). None of the CD11c+ cases had moderate to bright expression of CD22.

In 24 cases, more than 1 bone marrow specimen was examined at our institution. In the interval between specimens, patients received chemotherapy, most often a combination of 2-chlorodeoxyadenosine, cyclophosphamide, and rituximab. The median interval between specimens was 25 months (range, 2-79 months). Although not all markers were repeated, in 17 patients with persistent disease, the immunophenotypic profile was identical. In the other 7 cases, the subsequent bone marrow aspirate specimens did not have monotypic B cells. In 5 of these cases, there was no morphologic evidence of disease, and in 2 cases, focal involvement by lymphoma was detected only in the bone marrow biopsy specimens.

In 5 cases, concurrent fine-needle aspiration of an involved lymph node with flow cytometric immunophenotypic analysis was performed. In all 5 cases, the immunophenotype of the neoplastic cells in the lymph node was identical to that in the bone marrow.

**CD23+ Cases**

Of 28 cases, 17 (61%) were CD23+. The median level of total serum IgM for this group was 2,900 mg/dL (29 g/L; range, 700-11,400 mg/dL [7-114 g/L]), and the median level of serum monoclonal IgM was 1,800 mg/dL (18 g/L; range, 400-6,500 mg/dL [4-65 g/L]). Because CD23 expression is considered uncommon or rare in LPL/WM, as stated in the WHO classification, we focused additionally on this group. In all cases, CD23 expression was dim (first logarithmic percentile) as judged by visual examination of fluorescence intensity. The paraffin blocks were available for all 17 cases, on which we also assessed CD23 by immunohistochemical methods. In 12 cases, CD23 was negative; in 4 cases, scattered CD23+ cells were present, representing approximately 5% of cells; and in 1 case, approximately 20% of the neoplastic cells were CD23+. In no case were LPL/WM cells uniformly or strongly positive.

When the 17 CD23+ cases were compared with the 11 CD23– cases, there was no statistically significant difference in patient age (P = .182), serum monoclonal IgM level (P = .635), total serum IgM level (P = .510), and extent of bone marrow involvement (P = .908). The residual polyclonal serum γ immunoglobulin level was higher in cases of CD23+ LPL/WM than in cases of CD23– LPL/WM (P = .018).

**CD5+ and CD10+ Cases of LPL/WM**

The clinicopathologic features of the 3 CD5+ cases and the CD10+ case of LPL/WM are discussed in detail.

One CD5+ case was a 60-year-old woman who complained of short-lived episodes of lightheadedness. She had no lymphadenopathy or hepatosplenomegaly detected by physical examination or imaging studies. The CBC count was normal.
The serum monoclonal IgM was 2,900 mg/dL (29 g/L). Bence Jones protein was 8.8 mg/24 h. A bone marrow biopsy specimen revealed hypercellular (70%-80%) bone marrow with multiple lymphoid aggregates in a paratrabecular and non-paratrabecular pattern. Immunostaining for cyclin D1 was negative. Bone marrow aspirate smears showed a population of lymphoplasmacytic cells. Flow cytometric immunophenotyping of the aspirate specimen revealed that neoplastic cells were positive for monotypic surface immunoglobulin κ, CD5, CD19, CD20, and CD52 and negative for CD10 and CD38. CD23 was negative by immunohistochemical analysis.

A second CD5+ case was a 52-year-old man who had chest pain. He had no lymphadenopathy or hepatosplenomegaly detected by physical examination. Imaging studies revealed no lymphadenopathy or hepatomegaly and “slight enlargement of the spleen” (no measurement provided). The CBC count was normal except for slight thrombocytopenia (platelet count, 132 × 10^3/µL [132 × 10^9/L]; reference range, 140-440 × 10^3/µL [140-440 × 10^9/L]). The serum monoclonal IgM was 5,900 mg/dL (59 g/L). Bence Jones protein was 24 mg/24 h. A bone marrow biopsy specimen revealed hypercellular (95%) bone marrow infiltrated by small lymphocytes, approximately 50% of all cells. Bone marrow aspirate smears showed lymphoplasmacytic cells. Flow cytometric immunophenotyping of the aspirate specimen revealed that neoplastic cells were positive for monotypic surface immunoglobulin λ, IgM, CD5, CD19, CD20, and CD52 and negative for CD23 and CD38.

A third CD5+ case was a 61-year-old man who was asymptomatic, but whose CBC count was abnormal at the time of a routine checkup. He had no lymphadenopathy or hepatosplenomegaly detected by physical examination and imaging studies. The CBC count showed mild leukocytosis (WBC count, 12,500/µL [12.5 × 10^9/L]; reference range, 4,000-11,000/µL [4.0-11.0 × 10^9/L]) and absolute lymphocytosis (lymphocyte count, 6,500/L [6.5 × 10^9/L]; reference range, 1,000-4,800 [1.0-4.8 × 10^9/L]; hemoglobin and platelet values were normal. The serum monoclonal IgM was 400 mg/dL (4 g/L). A bone marrow biopsy specimen revealed hypercellularity with a lymphoid infiltrate in an interstitial pattern. Immunostaining for cyclin D1 was negative. Bone marrow aspirate smears showed scattered small mature lymphoid cells. Flow cytometric immunophenotyping of the aspirate specimen revealed that neoplastic B cells were positive for surface and cytoplasmic immunoglobulin κ, IgM, CD5, CD11c, CD19, CD20, CD22 (dim), CD25, CD52, and CD79b and negative for CD3, CD4, CD10, CD23, and CD138.

The CD10+ case was a 60-year-old man who was asymptomatic, but an elevated serum level was detected at the time of a routine checkup. He had no lymphadenopathy or hepatosplenomegaly detected by physical examination and imaging studies. The CBC count was normal. The serum monoclonal IgM was 4.5 g/dL [45 g/L], and Bence Jones protein was 83 mg/24 h. A bone marrow biopsy specimen revealed hypercellular (70%) bone marrow with increased interstitial lymphocytes representing approximately 20% to 30% of all cells; there were no paratrabecular infiltrates. Immunostains of the bone marrow biopsy specimen showed that the neoplastic B cells were positive for CD20 and the plasma cells were positive for...
CD138. The neoplastic cells were negative for bcl-6. Bone marrow aspirate smears showed a slightly increased number of lymphocytes with a plasmacytoid differentiation. Flow cytometric immunophenotyping revealed that the neoplastic B cells were positive for surface and cytoplasmic immunoglobulin λ, CD10, CD19, CD20, and CD52 and negative for CD5.

Discussion

As defined by a consensus group at the Second International Workshop on WM in Athens in 2002, LPL/WM is a distinct B-cell lymphoproliferative disorder involving bone marrow and associated with serum IgM paraprotein. Because a variety of other types of B-cell lymphoma can be associated with serum monoclonal IgM protein, however, the diagnosis of LPL/WM also requires exclusion of other types of B-cell lymphoma, and classification often hinges on immunophenotypic data. According to the WHO classification, cases of LPL/WM usually are surface immunoglobulin–positive and CD19+CD20+CD5–CD10–CD23–. However, in our recent experience we have encountered cases that did not have this typical immunophenotype and, thus, prompted this study.

The WHO classification states that CD23 usually is absent, and the 2002 WM consensus group stated that CD23 expression is “rarely encountered” in LPL/WM. In the present

**Image 2** CD5+ lymphoplasmacytic lymphoma/Waldenström macroglobulinemia. A, Flow cytometric histogram shows a distinct population of CD5+ (y-axis) and CD19+ (x-axis) B cells. B, The bone marrow aspirate smears shows that the neoplastic cells are small, mature forms (Wright-Giemsa, ×1,000). APC, allophycocyanin; PE, phycoerythrin.

**Image 3** CD10+ lymphoplasmacytic lymphoma/Waldenström macroglobulinemia. A, Flow cytometric histogram shows a distinct population of CD10+ (y-axis) and CD19+ (x-axis) B cells. CD10 expression is relatively dim. B, The bone marrow aspirate smear shows a population of lymphoplasmacytic cells (Wright-Giemsa, ×1,000). APC, allophycocyanin; FITC, fluorescein isothiocyanate.
study, using an anti-CD23 antibody conjugated with phycoerythrin, 61% of LPL/WM cases (17/28) were CD23+. In all cases, CD23 expression was dim, and, therefore, in many cases, immunostaining for CD23 was negative or stained only a small subset of cells. Although this relatively high frequency initially was surprising to us, in fact, others also have reported CD23 expression in an appreciable subset of LPL/WM cases. For example, Matutes and colleagues\textsuperscript{13} reported that 24% of LPL/WM cases were CD23+. Similarly, San Miguel and colleagues\textsuperscript{9} found that 20% of LPL/WM cases were CD23+. More recently, Chang and colleagues\textsuperscript{14} detected CD23 in 40% of 22 LPL/WM cases, and Hunter and colleagues,\textsuperscript{15} as part of the 2004 consensus conference on WM in Paris, France, reported the detection of CD23 in 35% of cases. The explanation for the relatively high frequency of CD23 expression in this study and other recent studies compared with earlier studies is uncertain. We used a phycoerythrin-conjugated anti-CD23 antibody. Because phycoerythrin-conjugated antibodies are more sensitive than fluorescein-conjugated antibodies, this may explain the discrepancy with older studies. Owen and colleagues,\textsuperscript{8} who detected CD23 in only 1% of LPL/WM cases, also used a phycoerythrin-conjugated anti-CD23 antibody. However, this group considered antigens as positive if they were detected on at least 50% of the B cells analyzed,\textsuperscript{8} which might explain the difference in results.

Of 65 cases of LPL/WM in this study, 3 (5%) were CD5+, a percentage similar to that reported at the Athens and Paris WM consensus conferences. The consensus group considered CD5 expression in LPL/WM to be a rare event with no apparent clinical significance.\textsuperscript{7} The low peripheral lymphocyte count, absence of peripheral lymphadenopathy, lymphoplasmacytic cytologic features in bone marrow aspirate smears, and the absence of CD23 expression by flow cytometry for the relatively high frequency of CD23 expression in this study and other recent studies compared with earlier studies is uncertain. We used a phycoerythrin-conjugated anti-CD23 antibody. Because phycoerythrin-conjugated antibodies are more sensitive than fluorescein-conjugated antibodies, this may explain the discrepancy with older studies. Owen and colleagues,\textsuperscript{8} who detected CD23 in only 1% of LPL/WM cases, also used a phycoerythrin-conjugated anti-CD23 antibody. However, this group considered antigens as positive if they were detected on at least 50% of the B cells analyzed,\textsuperscript{8} which might explain the difference in results.

Of 38 cases of LPL/WM tested in this study, 1 (3%) expressed CD10. The patient was asymptomatic and did not have peripheral lymphadenopathy, and the morphologic features in the bone marrow were not typical for follicular lymphoma. In particular, the neoplasm in the bone marrow was not present in a paratrabecular pattern, and bcl-6 was negative. This result is in accord with other studies. Owen and colleagues\textsuperscript{8} found a similar percentage (4.5%) of CD10+ LPL/WM cases. Two other recent reports described a slightly higher percentage (10%) of CD10+ cases.\textsuperscript{14,15} By contrast, 1 group reported that all LPL/WM cases were CD10+.\textsuperscript{9} The WM consensus group indirectly acknowledges the possibility of CD10+ cases of LPL/WM stating that “the majority of cases do not express CD10.”\textsuperscript{13}

Of 16 cases in the present study, 13 (81%) expressed CD11c. This result differs from that of San Miguel and colleagues,\textsuperscript{9} who found that only 15% of LPL/WM cases were positive for CD11c, with CD11c being dim in only a subset of neoplastic cells. In that study, San Miguel and colleagues\textsuperscript{9} also suggested that CD11c expression correlated with the presence of clinical symptoms. They used a fluorescein-conjugated anti-CD11c antibody. By contrast, we used a phycoerythrin-conjugated anti-CD11c antibody. Among the 13 CD11c+ cases, 11 patients were symptomatic and 2 were asymptomatic. Among the 3 CD11c− cases, 2 patients were symptomatic and 1 was asymptomatic. Although this is a small number of CD11c+ cases, our experience does not show a correlation between CD11c expression and the presence of symptoms in patients with LPL/WM.

All 60 cases of LPL/WM tested for CD52 expressed this antigen. To the best of our knowledge, this study is the first to assess CD52 expression in a large number of LPL/WM cases. Similar results were recently reported by Owen and colleagues,\textsuperscript{16} who also found all 20 cases tested positive for CD52.\textsuperscript{16} This report also indicates a clinical benefit of monoclonal anti-CD52 antibody (alemtuzumab) in a substantial proportion of patients. We believe our observation that all cases of LPL/WM are CD52+ could be important clinically because of the availability of alemtuzumab for therapy.

Of the 77 cases of LPL/WM assessed by flow cytometry in our hospital, 2 (3%) had involvement of the bone marrow biopsy specimen, and yet bone marrow aspirate smears and flow cytometric immunophenotypic findings performed on bone marrow aspirate material showed no evidence of disease. The pattern of involvement in the bone marrow biopsy specimen was diffuse in one case and interstitial in the other; the quality of bone marrow aspirate smears was poor with no particles present. Although those 2 cases were excluded from the study group, knowledge that involvement of bone marrow by LPL/WM can be restricted to the biopsy specimen will prevent misdiagnosis and supports the need for bone marrow biopsy for staging.

Our results suggest that there is more immunophenotypic variation in LPL/WM cases than has been emphasized in the literature. In this study, CD11c (13/16 [81%]) and CD23 (17/28 [61%]) were commonly positive in LPL/WM cases. In agreement with others, CD5 (3/65 [5%]) and CD10 (1/38 [3%]) usually were negative in LPL/WM. Knowledge of this immunophenotypic variation is needed to correctly establish the diagnosis of LPL/WM because the immunophenotypic overlap with other B-cell lymphoproliferative disorders can complicate the differential diagnosis. Interpretation of cases with an “atypical immunophenotype” can be challenging. An accurate diagnosis of LPL/WM should be based on integrating morphologic, immunophenotypic, and clinical data and excluding alternative diagnoses. The constant expression of CD52 in LPL/WM suggests that alemtuzumab (anti-CD52) might have a potential therapeutic role in patients with LPL/WM, if clinically indicated.
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