In Waldenstrom’s Macroglobulinemia the Quantity of Detectable Circulating Monoclonal B Lymphocytes Correlates With Clinical Course

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Using a sensitive flow-cytometer-based method of detecting small numbers of morphologically normal monoclonal B lymphocytes, we have investigated the presence and quantity of these cells in the blood of 12 patients with Waldenstrom’s macroglobulinemia. All 12 patients, including 6 at the time of asymptomatic presentation, had such circulating mononuclear cells. By comparison, 2 of 7 patients with multiple myeloma had such cells, and prior studies have shown that 80% of patients with non-Hodgkin’s lymphoma exhibit blood involvement by these criteria. Enzymatic removal of surface immunoglobulin (Ig) with subsequent regeneration by the cells after overnight culture established that the monoclonal surface Ig being studied was of intrinsic cell membrane origin and not passively adsorbed.

Waldenstrom’s macroglobulinemia is a malignancy of B-cell origin characterized by the presence of an abnormal serum monoclonal IgM component, “plasmacytoid lymphocytes,” in the bone marrow and occasionally lymphadenopathy and hepatosplenomegaly. Previous workers measuring the number of kappa-bearing versus lambda-bearing lymphocytes in the blood of patients with macroglobulinemia have suggested that greater than 50% of affected individuals have circulating monoclonal B lymphocytes. Our laboratory and that of Ligler et al. have recently described a sensitive and rapid method by which small numbers of mono- or oligoclonal B lymphocytes can be accurately detected in blood and other fluids by taking advantage of the ability of the fluorescence-activated cell sorter (FACS) to perform accurate quantitative immunofluorescence on large numbers of cells. Such an analysis determines monoclonality by very different and more stringent criteria than that of studying the ratio of kappa/lambda-bearing lymphocytes. In addition, it provides a gross estimate of the number of mono- or oligoclonal cells present in blood. By applying this technique to the study of patients with macroglobulinemia, we have found that all of the patients studied had circulating monoclonal lymphocytes even at the time of asymptomatic presentation. Furthermore, we found a correlation between the estimate of percent abnormal cells in the blood and the clinical course of the patients with macroglobulinemia. This correlation of estimated number of abnormal cells with clinical course may also have implications for monitoring therapy in patients with other lymphoproliferative disorders.

Materials and Methods

Patients

All patients were being treated at the Division of Hematology, Brigham and Women’s Hospital, Boston, MA. This study was approved by the Human Studies Committees of the Brigham and Women’s Hospital and the Harvard Medical School.

“Kappa–Lambda” Analysis

The theory and method of analysis for detecting small numbers of mono- or oligoclonal B lymphocytes (“kappa–lambda” analysis) has been previously described. Briefly, blood mononuclear cells are isolated on a standard Ficoll-Hypaque sedimentation gradient, divided into two aliquots, and then surface labeled by indirect immunofluorescence for the presence of either kappa or lambda immunoglobulin light chains (human B cells, of course, bear either kappa or lambda light chain but not both). Each aliquot is then analyzed on a Becton-Dickinson FACS II cell sorter (Mountainview, CA). Gates are set to limit the analysis to only cells of lymphoid size, and a logarithmic amplifier (Nuclear Data, Schaumburg, IL) is used to extend the dynamic range of the measured fluorescence signal. The resulting plot of number of cells versus relative fluorescence intensity demonstrates two subpopulations in each sample, one population of nonfluorescent cells that is composed primarily of T

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cells and a second peak of immunoglobulin-labeled B cells. If one directly compares the kappa distribution with the lambda distribution in a normal individual, one finds that the two distributions are superimposable, i.e., the variation in the quantitative surface expression of light chain (the "shape" of the curves) is independent of light chain class. If the sample being analyzed contains mono- or oligo-clonal B cells, however, then the two curves are disparate. This method of analysis is somewhat analogous to immunoelectrophoresis, but examines cells rather than serum. Note that this technique does not depend on the assumption that demonstrating a larger than usual number of circulating cells with a particular surface marker constitutes a monoclonal proliferation. The number of kappa- versus lambda-bearing cells is not what is measured; rather, it is the quantitative distribution of membrane immunoglobulin on these cells. In addition, by applying a Kolmogorov-Smirnov statistical analysis comparing the curves, one can obtain a gross estimate of the percent abnormal cells in the sample causing the discrepancies in the curves. This value is known as the D value. The normal range for D values in our laboratory is 5.1 ± 3.3 (mean ± 2 SD).

Ig Re-Expression Experiments

In order to assess whether abnormalities seen in the cell sorter profiles are secondary to passively adsorbed immunoglobulin on the surface of these cells rather than to intrinsic membrane immunoglobulin, re-expression experiments were performed as follows. Isolated mononuclear cells were incubated in pronase (Sigma Chemical Co., St. Louis, MO) 1mg/ml for 1 hr at 37°C in serum-free medium. This treatment completely removed all identifiable surface immunoglobulin, as shown in Fig. 1. Cells were then allowed to regenerate surface immunoglobulin by incubation overnight at 37°C in RPMI 1640 medium (Grand Island Biological Co., Grand Island, NY) with 10% fetal calf serum (Microbiological Associates, Walkersville, MD), 2mM L-glutamine, 100 U/ml penicillin) and 50 U/ml streptomycin. Viability was greater than 90% as estimated by trypan blue staining. After this treatment, any detectable surface immunoglobulin must have been synthesized by the cells in culture rather than having been passively acquired from the patient’s serum.5

![Diagram of cell sorter profiles](image)

**Fig. 1.** Monoclonal Ig re-expression by peripheral blood lymphocytes following pronase treatment. (A) The labeling characteristics of untreated blood mononuclear cells isolated from patient 12. The bright curve represents labeling for surface kappa light chain expression, the dim curve labeling for surface lambda light chain expression. Number of cells is plotted on the vertical axis; log relative fluorescence intensity on the horizontal axis. Note the monoclonal labeling with anti-kappa chain antibody. (B) The same analysis immediately following pronase treatment of the cells. Note the loss of surface Ig. (C) The same analysis 24 hr after pronase treatment, with the cells cultured in RPMI-1640 and fetal bovine serum. Note the return of monoclonal labeling. (The number of cells at full scale for the vertical axis in panel C is approximately half that of panel A.) The slight increase in heterogeneity of kappa fluorescence intensity after resynthesis may be due to the variable ability of the cells to completely regenerate normal amounts of surface Ig following pronase treatment. The post-treatment loss of some lambda labeling on the cells is probably due to the loss of nonspecific binding of serum Ig that had not been fully eliminated prior to pronase treatment.

Table 1 summarizes the results of the initial kappa–lambda studies on 12 patients with macroglobulinemia. Nine of the 12 were studied at time of presentation with disease, 3 after a 2–7-yr history of macroglobulinemia; 6 patients were asymptomatic and had been initially diagnosed when an abnormal laboratory study had appeared on routine screening examination. All of the patients had significant numbers of abnormal circulating monoclonal B lymphocytes as detected by kappa–lambda analysis. D values ranged from being minimally abnormal (D = 9) in 2 patients to grossly elevated (D = 90) in a patient who developed a dramatic peripheral lymphocytosis after 7 yr of classic macroglobulinemia. There was no correlation of the estimated number of abnormal circulating lymphocytes with IgM level, symptoms, total white blood count, or total lymphocyte count at presentation. In contrast, only 2 of 7 patients studied with multiple myeloma had abnormal studies at the time of active disease (data not shown).

In order to assess whether the abnormalities seen in the cell sorter profiles might be due to passively adsorbed immunoglobulin on the surface of these cells, re-expression experiments, dependent on the synthesis of immunoglobulin by the cells, were performed with the blood cells from 6 of the 12 patients. All 6 such
experiments showed an abnormal kappa–lambda analysis following re-expression of immunoglobulin, confirming that the abnormal cell sorter profiles were secondary to intrinsic membrane immunoglobulin. In 3 patients, the $D$ values following the re-expression experiment increased slightly (less than 25% change), while in 3 patients, the $D$ values decreased following re-expression.

Serial studies were performed on 7 of the 12 patients, summarized in Table 2. In all cases where a clinical response to therapy and a decrease in serum IgM level was seen, there was a corresponding decrease in the number of abnormal cells estimated by the $D$ value of the K-S test (patients 1, 4, 5, 11). In those cases in which there was neither clinical nor serum M-component evidence of response, the $D$ value likewise increased or remained constant (patients 6, 7, 9).

### DISCUSSION

Previous workers, beginning with Preud’homme and Seligmann, have shown that the majority of bone marrow plasma cells in patients with Waldenstrom’s contain immunoglobulin of only mu heavy chain type and a single light chain class. Examining the blood lymphocytes in such patients, these authors found the majority of patients to have an excessive number of IgM-bearing cells and altered ratios of kappa- versus lambda-bearing cells, even in the presence of normal total lymphoid counts. More recently, Pettersson and colleagues, using the ratio of kappa-bearing to lambda-bearing cells in the peripheral blood, concluded that they could find circulating monoclonal cells in 9/16 patients studied. Using different criteria for monoclonality, the kappa–lambda analysis, we have found circulating monoclonal cells in 12 of 12 patients with Waldenstrom’s macroglobulinemia. We have demon-
strated that the monoclonal surface immunoglobulin measured in this assay was synthesized by the cells and not passively adsorbed to the surface. These circulating abnormal lymphocytes were found even in totally asymptomatic patients at presentation, leading us to believe that this disorder is not limited to the bone marrow at presentation but is truly systemic and involves all lymphocyte-containing organs. The contrast between the frequent finding of detectable circulating malignant cells in Waldenstrom's macroglobulinemia and their relative rarity in multiple myeloma underscores the biologic differences in these disorders. Both in this aspect and its clinical course, Waldenstrom's more closely resembles the non-Hodgkin's lymphomas, in which 80% of patients have circulating monoclonal cells detectable by the kappa–lambda analysis.6

Interestingly, the estimated number of such abnormal circulating cells (the $D$ value) does not correlate with serum IgM levels at presentation. This is as expected if the rate at which IgM is synthesized and secreted by the malignant cells differs from patient to patient or if the majority of the IgM is secreted by cells other than those that circulate. On the other hand, the $D$ value does correlate with both the clinical course and serum IgM level for a given patient over time, implying that the number of circulating monoclonal cells is a rough estimate of the overall "bulk" of disease in macroglobulinemia. Such serial quantitative measurements of circulating monoclonal cells may be useful in assessing the early effect of various therapeutic regimens in Waldenstrom's. We do not as yet know whether quantitative estimates of circulating monoclonal cells in other lymphoproliferative diseases would also reflect alterations in tumor burden; however, the results in macroglobulinemia and the observation that in chronic lymphocytic leukemia increasing total lymphoid count often reflects increasing "bulk" disease would suggest that this is at least an avenue of possible fruitful investigation.

REFERENCES

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