A sensitive cytofluorometric technique, the "kappa-lambda test," permits detection of small numbers of monoclonal B lymphocytes (clonal excess). Such a method might represent a new diagnostic tool for diagnosis of non-Hodgkin's lymphomas, potentially providing definitive evidence of lymphomatous involvement in cases equivocal by standard immunologic methods. To determine the significance of detecting B cell clonal excess in lymphoid tissues, we applied the kappa-lambda test to cell suspensions from 60 consecutive specimens suspected of involvement by malignant lymphoma. Results were correlated with the pathologic diagnosis and with standard cell marker studies in each case. B cell clonal excess was observed in 24 of the 25 cases of non-Hodgkin's lymphoma of B cell origin, including a single case involving early detection of recurrence. None of the remaining cases, including benign reactive hyperplasia, T cell lymphoma, and Hodgkin's disease, showed evidence of B cell clonal excess. Selective examination of cell subpopulations was also achieved using this cytofluorometric method. We conclude that the detection of B cell clonal excess by the kappa-lambda test represents a new approach to the diagnosis of B cell lymphoma, which provides certain advantages over more standard methods of cell marker analysis.

THE DIAGNOSIS OF malignant lymphomas has been greatly facilitated by the use of immunologic methods to characterize lymphoid cells. Phenotypic studies of malignant lymphomas have shown that the majority of these neoplasms are of B lymphocyte origin. These tumors are thought to arise from uncontrolled expansion of a clone of B lymphocytes and that the morphologic subtype of B cell lymphoma is related to the maturation state of the abnormal B cells. Because a monoclonal population of B cells expresses exclusively either kappa or lambda immunoglobulin light chain, the demonstration of monoclonal surface or cytoplasmic light chain is supportive of a diagnosis of a B cell malignancy. Such immunologic studies are usually performed either on frozen sections of tissue or on single cell suspensions, using visual or automated (cytofluorometric) techniques. However, the presence of varying proportions of T lymphocytes, macrophages, and residual nonneoplastic B cells frequently makes the interpretation of phenotypic data difficult. It is usually not possible to detect, by standard methods, minor populations of malignant B lymphocytes in tissues having early or partial involvement by lymphoma. It would be highly desirable, therefore, to develop a single objective method of cell analysis that could circumvent these diagnostic problems.

A recently developed cytofluorometric technique allows the detection of minor populations of malignant B lymphocytes. This method, which we will refer to as the "kappa-lambda test," is based on the direct comparison of surface immunofluorescent staining for kappa and lambda light chains within a single population of cells. In normal individuals, the surface density distributions of kappa and lambda light chains of B lymphocytes are virtually identical. However, in B cell malignancies, the presence of a monoclonal population results in "clonal excess" of cells of one light chain. This clonal excess is revealed by a shift in the normal density distribution of that light chain and is detected by a measurable difference in the positions of the kappa and lambda light chain immunofluorescence curves. This difference can be quantitated by computer analysis of the light chain distribution curves. Previously, this method has been used to detect small numbers of monoclonal B cells in the blood of patients with malignant lymphoma. We now show that cytofluorometric detection of clonal excess by the kappa-lambda test is a valuable new method for diagnosing malignant lymphomas of B cell origin.

MATERIALS AND METHODS

Kappa-Lambda Test

Single cell suspensions were prepared by teasing freshly obtained tissues with forceps in Hanks' balanced salt solution containing 5% fetal calf serum. In cases yielding poor cell viability (greater than 20% dead cells), viable cells were obtained following centrifugation over Ficoll-Hypaque. Staining for kappa and lambda light chains was performed as previously described. Briefly, aliquots of 0.5–1.0 x 10^6 cells were incubated with saturating amounts of F(ab')2 fragment of rabbit antibody specific for human kappa or lambda light chains, washed, and stained with saturating amounts of fluoresceinated goat antibody to rabbit IgG. Cells stained with the second antibody alone comprised the control. Peripheral blood lymphocytes from normal individuals served daily as cellular controls. The stained cells were fixed with 1% paraformaldehyde in phosphate-buffered
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The preparation and specificities of the antibodies used have been previously described.3 The samples were analyzed sequentially on a FACS Analyzer (Becton Dickinson Electronics, Mountain View, CA). This instrument is a flow cytometer that simultaneously measures electronic volume and single cell fluorescence excited by filtered light from a mercury-arc lamp. Typically, 1–2 x 10⁶ cells/sample were analyzed, and the data, in the form of fluorescence intensity distribution curves, were stored in a microcomputer. The fluorescence distributions for kappa and lambda light chains could be directly compared visually by superimposing the curves on the flow cytometer display screen. In normal individuals, the distribution profiles for kappa and lambda light chains are nearly identical with regard to shape and peak position. The presence of an abnormal clone (or clones) is detected by the presence of a “shift” in the fluorescence distribution curves of kappa and lambda light chains relative to one another. The basis of the kappa-lambda test is depicted graphically and explained in greater detail in Fig. 1. A microcomputer was used to calculate the difference between the two curves, as previously described,4 using the Kolmogorov-Smirnov test.4 The advantage of this method is that the resulting calculated difference, or “D” value, is independent of the relative numbers of kappa- and lambda-positive cells and provides a quantitative comparison of the two fluorescence distribution profiles.

In addition, the flow cytometer permits fluorescence analysis of cells within selected size ranges. Analysis was generally performed by choosing volume gates so as to include the majority of cells present, while excluding debris or clumped cells. In some cases, cells in a given sample were divided into “small” and “large” cells by selecting appropriate cell volume gates and were separately analyzed for light chain distribution patterns. The Coulter volume of cells was determined by previous calibration of the instrument with various sizes of polystyrene beads (Duke Scientific, Palo Alto, CA). In general, cells 6.5–8.4 μ in diameter were considered “small,” and cells 8.5–13.0 μ were considered “large.” Small cells were similar to peripheral blood lymphocytes in size distribution.

Pathology and Standard Marker Studies

Histologic sections of paraﬁlm-embedded tissue were prepared by standard methods. Immunoperoxidase studies were performed on paraﬁlm-embedded tissues using methods previously described.5 Immunoperoxidase studies on acetone-ﬁxed cryostat sections were performed using either a four-step peroxidase-antiperoxidase (PAP) method6 or a three-step avidin-biotin method.7 The PAP method was performed by serial incubation of sections with primary mouse antibody, followed by rabbit anti-mouse IgG, swine anti-rabbit IgG (Dakopatts A/S, Copenhagen, Denmark), and finally with peroxidase-rabbit-antiperoxidase reagent (Cappel Laboratories, West Chester, PA). Staining by the avidin-biotin method was performed using primary antibody followed by biotinylated horseradish peroxidase complex (Vector Laboratories, Burlingame, CA). Staining was achieved by incubation of the sections with 0.5% 3,3’-diaminobenzidine and 0.01% H₂O₂. Primary antibodies used included OKT3, OKT4, OKT8, OKT11 (Ortho Diagnostics Systems, Raritan, NJ), Leu-1, Leu-2a, Leu-3a (Becton Dickinson, Mountain View, CA), and Bl (Coulter Immunology, Hialeah, FL). Mouse monoclonal antibodies to human kappa and lambda light chains were kindly provided by Dr. Lee Nadler (Dana-Farber Cancer Institute, Boston, MA). Single cell suspensions were analyzed for T cells as determined by the binding of sheep erythrocytes.8 B cells were determined by the binding of 7S antibody-coated ox erythrocytes (Fc receptor)9 and the binding of complement-coated zymosan particles (complement receptor).14 Surface light chains were detected by fluorescence microscopy following one-step immunofluorescent staining, as previously described.15 The ﬁnal pathological diagnosis employed the Rappaport classiﬁcation system for malignant non-Hodgkin’s lymphomas16 and included information concerning the phenotype of the neoplastic cells. Cases were collected prospectively, and the data from cytofluorometric analyses15 were combined with the pathological diagnosis only upon completion of the study.

This study was approved by the Human Subjects Committee at the Brigham and Women’s Hospital.
RESULTS

Kappa-Lambda Analysis of Lymphoid Tissues

Suspended lymphoid cells from 60 consecutive pathologic specimens were studied by cytofluorometric analysis for cell surface kappa and lambda light chains. Tissues included 45 lymph nodes, 7 spleens, 3 tonsils, and 5 masses. The pathologic diagnoses are shown in Table 1. There were 25 cases of lymphoid malignancies of B cell origin, comprising a variety of histologic subtypes. Because the purpose of the study was to determine the value of the kappa-lambda test in diagnosing B cell malignancies, all other cases were grouped together as controls (35 cases). These controls included benign and reactive processes, malignant lymphomas of T cell origin, and Hodgkin’s disease. The diagnosis of atypical hyperplasia or abnormal immune response was made when histologic features were atypical, but conventional cell surface marker studies failed to reveal diagnostic abnormalities. Cases were diagnosed as B cell lymphoma based on histologic features combined with phenotypic data obtained by standard noncytofluorometric techniques. Cases of tissue involvement by chronic lymphocytic leukemia and hairy cell leukemia were included among the B cell lymphomas, as the malignant cells in these disorders are most commonly of B cell origin, as was true in these cases.

To perform the kappa-lambda test, suspended cells were stained for kappa and lambda light chains, and the surface fluorescence distribution patterns were directly compared by cytofluorometry. In each control case, no significant difference was noted in the fluorescence distribution profiles for kappa and lambda light chains (see example in Fig. 2A). In contrast, cells from tissues involved by B cell malignancies almost always showed obvious differences in kappa and lambda light chain distribution profiles (see examples in Fig. 2). Note that B cell malignancies associated with low density of surface immunoglobulin (such as CLL, Fig. 2B) readily demonstrated differences in light chain staining profiles. Computer analysis of the light chain distributions yielded a “D” value that reflects the degree of difference of the immunofluorescence distribution curves for kappa and lambda light chains. D values for all cases are shown in Fig. 3. The mean D values (± standard deviation) for the control group and the B cell lymphoma group were 4.79 ± 1.94 and 27.3 ± 16.0, respectively. These differences were highly significant (p < 0.0001). More importantly, if D = 10 were chosen as the upper limit of normal to include all control cases, 24 of 25 cases of B cell

![Fig. 2. Kappa-lambda light chain fluorescence distributions in tissues from four patients. Each panel shows the distributions of fluorescence for kappa (bright dots) and lambda (dim dots) light chains. The horizontal axis is fluorescence intensity; the vertical axis indicates the relative number of cells. (A) Reactive (benign) lymph node; (B) chronic lymphocytic leukemia involving spleen; (C) poorly differentiated lymphocytic lymphoma, diffuse; (D) “histiocytic” lymphoma, diffuse. Malignancies were shown to be of B cell origin by other cell marker studies. D values, which are proportional to difference between pairs of kappa and lambda curves, are indicated.](https://www.bloodjournal.org/content/1082/10/1082.full)
lymphoma showed a $D$ value above the normal range. The one false-negative case was unusual, in that the malignant cells lacked detectable surface immunoglobulin by immunofluorescence staining; however, immunoperoxidase studies revealed cytoplasmic monoclonal immunoglobulin. Thus, it was possible to easily explain the failure of the kappa-lambda test to detect clonal excess in that case, as only surface light chains are detected by the cytofluorometric method. Importantly, in one case described in detail below (designated "evolving" lymphoma), early recurrence of non-Hodgkin's lymphoma was detected by an abnormal kappa-lambda test, whereas histologic and conventional marker studies were not diagnostic of lymphoma.

**Analysis of Cell Subpopulations by the Kappa-Lambda Test**

Lymphoid tissues, whether benign or malignant, usually contain varying proportions of morphologically large and small lymphoid cells. Therefore, in some cases, we examined separately the large and small cell subpopulations for the presence of clonal excess. This was done on the flow cytometer by selecting a restricted range of cell volumes to be analyzed for surface fluorescence. The size range of the small cell fraction was selected to be similar to normal peripheral blood lymphocytes (see Materials and Methods). Cells were divided into small cell and large cell fractions for analysis, regardless of whether a clear bimodal cell volume distribution was detected. The kappa-lambda test was performed, and $D$ values were calculated separately for large and small lymphoid cells. Examples of subpopulation kappa-lambda analysis of a benign lymph node and two cases of B cell lymphoma are shown in Fig. 4, along with the $D$ values of the cell subsets. The results of all the cases studied are shown in Fig. 5. In the control group, no abnormalities were detected in the light chain distributions of small and large cells. In contrast, all cases of B cell lymphoma studied in this manner showed abnormalities among either the small or large cell population, but not always both. All cases of poorly differentiated lymphocytic lymphoma and mixed lymphocytic-"histiocytic" lymphoma showed clonal excess in both the small and large cell subpopulations. Thus, in most cases, clonal excess was demonstrated in both large and small cell subpopulations regardless of their relative numbers.

Two of the nine cases of "histiocytic" lymphoma showed abnormal $D$ values among only the large cell subpopulation. In one such case (shown in Fig. 4C), residual normal follicles were present, and suspended cells contained both kappa- and lambda-positive cells; immunoperoxidase studies demonstrated monoclonal surface light chain on the large lymphoid cells. In the
In most cases, the abnormal B cells comprised the majority of cells present, with insignificant numbers of B cells of the other light chain type. Such cases presented little diagnostic difficulty. However, in 6 of 24 cases (24%) of B cell malignancies examined, conventional suspension studies alone yielded ambiguous or nondiagnostic results that did not readily suggest a monoclonal B cell proliferation. The relevant immunologic studies on these cases are shown in Table 3. It can be seen that indeterminate conventional suspension studies were due to several factors, including (A) the presence of polyclonal staining for light chains; (B) the presence of abundant T cells, which sometimes comprised the majority of cells present; (C) faint staining for surface light chain (case 2); and (D) nonspecific cytophilic staining (case 6). The phenotype of the tumor could most often be resolved by tissue section immunoperoxidase studies, especially in cases having nodularity. Although cells staining for the other (nonmalignant) light chain type were often present in tissue section studies, their relative numbers or distribution (e.g., internodular) indicated that these cells were not part of the malignant process. However, in one case (no. 6) not even immunoperoxidase studies could provide definite criteria for a diagnosis of lymphoma due to polyclonal staining for light chains. (This case is discussed in detail later.) It is important to note that kappa-lambda analysis detected clonal excess in all cases, regardless of the relative numbers of malignant cells present. Conventional cytofluorometric analysis would also have indicated the presence of polyclonal B cells in several cases (see example in Fig. 2B); however, kappa-lambda analysis considers only the differences in shape and position of the light chain distribution profiles, not the relative numbers of positively staining cells.
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Table 3. Comparison of Conventional Marker Studies and Kappa-Lambda Analysis

<table>
<thead>
<tr>
<th>Case</th>
<th>Diagnosis</th>
<th>Cell Suspension Studies</th>
<th>Immunoperoxidase§</th>
<th>Kappa-Lambda Analysis</th>
<th>D Value</th>
<th>“Abnormal”</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Small</td>
<td>Large</td>
</tr>
<tr>
<td>1</td>
<td>Lymphocytic lymphoma with plasmacytoid features</td>
<td>55</td>
<td>40</td>
<td>30</td>
<td>8</td>
<td>Predominant staining for kappa light chain, cytoplasmic pattern</td>
</tr>
<tr>
<td>2</td>
<td>“Histiocytic” lymphoma, diffuse</td>
<td>35</td>
<td>48</td>
<td>16</td>
<td>4</td>
<td>Faint staining for kappa light chain, surface pattern, on majority of cells; scattered T cells</td>
</tr>
<tr>
<td>3</td>
<td>Mixed lymphocytic-“histiocytic” lymphoma, nodular and diffuse</td>
<td>35</td>
<td>43</td>
<td>13</td>
<td>33</td>
<td>Nodular areas show monoclonal staining for lambda light chain, surface pattern</td>
</tr>
<tr>
<td>4</td>
<td>Mixed lymphocytic-“histiocytic” lymphoma, nodular and diffuse</td>
<td>70</td>
<td>30</td>
<td>25</td>
<td>1</td>
<td>Monoclonal staining for kappa light chain within nodules; abundant T cells between nodules and scattered within nodules</td>
</tr>
<tr>
<td>5</td>
<td>Poorly differentiated lymphocytic lymphoma, nodular</td>
<td>57</td>
<td>45</td>
<td>40</td>
<td>17</td>
<td>Monoclonal staining for kappa light chain confined to nodules, surface pattern; abundant T cells between nodules and scattered within within nodules</td>
</tr>
<tr>
<td>6</td>
<td>Evolving lymphoma</td>
<td>22</td>
<td>70</td>
<td>Indeterminate</td>
<td></td>
<td>Polyclonal staining for light chains between and within follicles, lambda slightly more frequent than kappa</td>
</tr>
</tbody>
</table>

*Percent cells binding sheep erythrocytes.
†Percent complement receptor-positive, determined by rosetting of complement-coated zymosan particles.
‡Surface staining for kappa (K) and lambda (L) light chains determined by fluorescence microscopy.
§Performed on cryostat sections, acetone-fixed.
ID > 10.0 significant for clonal excess.
*Due to nonspecific cytophilic staining.

Detection of “Evolving Lymphoma” by Kappa-Lambda Analysis

One case in which sequential lymph node biopsies were analyzed is of particular interest. The patient had been previously treated for malignant non-Hodgkin’s lymphoma of presumed B cell origin (undifferentiated type), resulting in complete remission. Multiple lymph node biopsies from different sites were performed subsequently to diagnose recurrent adenopathy, most of which revealed reactive hyperplasia by histologic and immunologic criteria. A biopsy taken after 20 mo revealed histologic features of atypical follicular hyperplasia; immunologic studies demonstrated polyclonal B cells (Table 3, case 6). A kappa-lambda test showed an abnormal shift in the lambda light chain distribution profiles of both small and large cell subpopulations (D = 11.8 and 23.5, respectively). This biopsy was felt to represent an evolving recurrence of non-Hodgkin’s lymphoma, although definite diagnostic features of lymphoma were not present. Finally, a biopsy performed 5 mo later showed a diffuse “histiocytic” lymphoma that stained monoclonally for lambda light chain by tissue immunoperoxidase. A kappa-lambda test performed subsequently on a lymphomatous node again revealed a shift in the lambda light chain distribution, particularly among the large cells. Thus, we feel it is likely that the kappa-lambda test had detected relapsing lymphoma 5 mo prior to obvious recurrence, as (A) both the abnormal clone detected earlier by the kappa-lambda test and the subsequent lymphoma were of the same light chain type (lambda), and (B) the clonal abnormality was most readily demonstrated among the large lymphoid cells, and the subsequent lymphoma was of a large cell type.

DISCUSSION

The kappa-lambda test is a rapidly performed cytofluorometric assay capable of detecting “clonal excess” of B lymphocytes. By this method, the fluorescence distribution profiles of cells stained for kappa and lambda light chains are directly compared. The
presence of B cell clonal excess is detected by measuring a significant deviation of the light chain fluorescence distribution curves relative to one another, quantitated by a “D” value.6 The major advantage of the kappa-lambda test is that populations of clonally expanded B cells (i.e., “clonal excess”) can be detected within a heterogeneous mixture of polyclonal B cells. This assay has proven useful in detecting abnormal clones of B cells in the blood of patients with B cell lymphoma.6 Using a similar method, Ligler et al.7 demonstrated that clonal excess of B cells could be detected in tissues as well as in the blood in some cases of malignant lymphoma. We felt that the kappa-lambda test might be particularly useful in the diagnosis of B cell lymphomas because: (A) malignant lymphomas are thought to arise from an abnormal clonal expansion of lymphoid cells;8 (B) the majority of non-Hodgkin’s lymphomas are of B cell origin;7 and (C) in some cases of B cell lymphoma, the malignant lymphoid cells comprise a minority of the total cells present18 and may be intermixed with benign polyclonal B cells, making interpretation of conventional marker studies difficult.

We applied the kappa-lambda test to 60 consecutive biopsy specimens suspected of being involved by malignant lymphoma. An advantage of this study was that, in each case, the kappa-lambda analysis was correlated with tissue diagnosis and with conventional marker studies performed by several methods on suspended cells and on tissue sections. Control cases (cases other than B cell malignancy) all failed to show abnormal light chain distributions by the kappa-lambda test, with D values of less than 10. Importantly, these controls included cases of Hodgkin’s disease and T cell lymphoma, demonstrating the specificity of this test for B cell malignancies. In contrast, all but one case of B cell lymphoma showed abnormal light chain distributions. The one false-negative case consisted of a “histiocytic” lymphoma of B cell origin that lacked detectable surface immunoglobulin. In that case, only immunoperoxidase studies on paraffin-embedded tissue revealed the B cell nature of the neoplasm. Such a case illustrates the importance of relating findings from various types of studies in order to resolve discrepancies and to arrive at the correct phenotype of the tumor.

Another advantage of cytofluorometric analysis is the ability to selectively analyze subpopulations of cells. Tissues involved by lymphoma frequently contain a range of cell sizes from small to large, their relative proportions contributing to the histologic classification of the tumor.16 Our observation of clonal abnormality of both large and small cells in most lymphomas suggests that, in most cases, the morphological heterogeneity is due to varying maturation states among the malignant cells. The process of intratumor differentiation could be further explored by detailed phenotypic studies of tumor cell subpopulations.

In two cases of “histiocytic” lymphoma, the malignant clone could be detected only among the large lymphoid cells. These cases emphasize the practical importance of subpopulation analysis in diagnosis. The small lymphoid cell component frequently includes numerous T lymphocytes and residual B cells, which can hinder the detection of malignant cells. Also, large lymphoid cells may be lost in greater proportion in preparation of cell suspensions18 and might be mistakenly excluded from analysis. Study of the large lymphoid cells revealed abnormalities in every case of B cell lymphoma studied, regardless of the relative numbers of large cells present.

In general, there was excellent correlation between the “abnormal” light chain detected by the kappa-lambda test and the light chain type of the lymphoma as determined by combined conventional suspension and immunoperoxidase studies. Although many immunologic markers were studied, we have chosen to emphasize the light chain findings in these cases, as light chain monoclonality is as yet the most reliable marker for B cell lymphoma.19 Of particular interest was the 24% of our cases in which conventional cell suspension studies alone were indeterminate for B cell lymphoma. Other investigators have also noted that a significant proportion of lymphoma cases studied in cell suspension lack definite monoclonal light chain staining.1,19 Although the reasons for such indeterminate studies varied among our cases, the presence of small numbers of malignant B cells, abundant T cells, and polyclonal B cells were common factors. In such cases, simple enumeration of cell surface markers is obviously of limited usefulness, whether performed by microscopy or by cytofluorometry. Kappa-lambda analysis provides a unique advantage in suspension studies because the detection of abnormal (malignant) clones is independent of their numbers, within the limits of sensitivity of the assay. Kappa-lambda analysis would provide an advantage, especially in situations where only suspended cells are available (e.g., malignant effusions) or in institutions lacking facilities for immunoperoxidase studies.

Our sequential study of a case of relapsing lymphoma illustrates the potential usefulness of kappa-lambda analysis in the early detection of B cell lymphoma. Because patients treated for lymphoma require close follow-up and are frequently rebiopsied upon developing new adenopathy, kappa-lambda analysis might detect recurrent disease much earlier than standard methods, allowing treatment before the development of more advanced
disease. In addition, it may be possible to detect the emergence of a malignant B cell clone in patients with autoimmune or immunodeficiency-related B cell proliferations, some of whom eventually develop B cell lymphoma.2

The analysis of surface immunoglobulin light chains using the kappa-lambda test provides unique advantages over more standard methods of cell surface analysis. Compared to cell marker studies performed by visual methods, cytofluorometric analysis is objective and rapid, permitting the study of large numbers of cells. The surface density of a particular marker may be determined by cytofluorometry, and the phenotypes of cell subpopulations may be selectively studied. The kappa-lambda test provides the unique advantage of detecting abnormal clones of B lymphocytes independent of their relative numbers. As shown in the present study, the presence of B cell clonal excess appears to be highly specific for B cell lymphomas. Most important, it may be possible to detect clonal abnormalities at a time when morphology and conventional marker studies are nondiagnostic. This feature may be important in allowing early diagnosis of new or recurrent B cell lymphoma.

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Cytofluorometric detection of B cell clonal excess: a new approach to the diagnosis of B cell lymphoma

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