Detection of Clonal Excess in Lymphoproliferative Disease by \( \kappa / \lambda \) Analysis: Correlation With Immunoglobulin Gene DNA Rearrangement

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Previous studies have suggested that analysis of the distribution of surface immunoglobulin light chain isotypes by flow cytometry provides evidence for monoclonality of B cell tumors and may detect populations of circulating tumor cells in patients with lymphoproliferative disease. We have used simultaneous flow cytometry and DNA restriction enzyme analysis on 58 samples of tissue and blood to determine whether lymphocyte populations detected by \( \kappa / \lambda \) analysis are indeed monoclonal. In \( >90\% \) of cases, abnormalities detected by flow cytometry correlated with monoclonal rearrangements of immunoglobulin genes as detected by Southern blot analysis. By analyzing tissue and blood from the same patients, we have also demonstrated that monoclonal circulating cells detected by flow cytometry reflect peripheral circulating tumor cells, since DNA from these cells shows the same immunoglobulin rearrangement as DNA from the original tumors in these patients. Although mixing studies suggested that DNA rearrangement studies were more sensitive than was flow cytometry in detecting minor populations of monoclonal lymphocytes, we found only one case in which this affected the diagnostic accuracy of the \( \kappa / \lambda \) analysis, with one notable exception, that of detection of a monoclonal proliferation of B cells that did not express surface immunoglobulin. The \( \kappa / \lambda \) test thus offers a powerful diagnostic tool in the evaluation of lymphoproliferative disease.

The evolving understanding of the ontogeny of the B lymphocyte has resulted in the development of immunologic methods that have had a great impact on the diagnosis of lymphoproliferative disease. Most malignant lymphomas are of B cell origin. Most B cells, with the exception of very early pre-B cells and mature plasma cells, express immunoglobulin on their surface. Each cell expresses only one light chain type. In normal peripheral blood and lymph nodes, there is usually a mixture of \( \kappa \)-positive and \( \lambda \)-positive cells, with two-thirds of the cells expressing \( \kappa \) and one-third expressing \( \lambda \). Because lymphoid neoplasms are usually clonal expansions of a single cell, the cells uniformly express the same light chain isotype. Consequently, B cell malignancy can frequently be suspected on the basis of the demonstration of a marked predominance of cells expressing a single light chain type.

The uniform expression of immunoglobulin light chains on monoclonal cell populations has been exploited in the development of a cytofluorometric technique to detect small populations of monoclonal B lymphocytes. This \( \kappa / \lambda \) technique is based on the direct comparison of surface immunofluorescent staining for \( \kappa \) and \( \lambda \) light chains within a cell population. Evidence of an imbalance in the normal distribution of light chain staining implies the presence of a monoclonal cell population. This technique has been used to show that a large proportion of patients with B cell lymphomas have circulating monoclonal B cells that are morphologically undetectable. \( \kappa / \lambda \) analysis has also been used to distinguish lymphoid neoplasms from benign reactive processes.

Although it has been assumed that an abnormal light chain fluorescence distribution indicates a monoclonal B cell proliferation, direct proof of this has been lacking. The availability of recombinant DNA techniques has made it possible to confirm that assumption. Immunoglobulin molecules are encoded on discontinuous genes. Early in B cell development, the immunoglobulin loci undergo gene rearrangement to form functional coding units for antibody synthesis. This rearrangement is detectable on analysis of genomic DNA and may provide evidence of monoclonality of B cells at very early stages of B cell development. This powerful tool has been used to establish the monoclonality of B cell neoplasms in which the diagnosis is hindered by confusing histology or absence of surface markers and has recently been used to establish that some lymphoid malignancies may in fact consist of more than one clone.

In this study, we have used DNA restriction enzyme analysis to assess the validity of the \( \kappa / \lambda \) test. We have confirmed that the detection of clonal excess by flow cytometry correlates well with the finding of monoclonal immunoglobulin rearrangements by DNA analysis. We have also shown that the detection by \( \kappa / \lambda \) analysis of small populations of tumor cells in the peripheral blood of patients with lymphoma reflects a population of circulating cells with the same DNA rearrangement as that seen in the primary tumors in those patients. We have identified the circumstances under which DNA restriction enzyme analysis is more informative than flow cytometry in the analysis of small populations of B cells. We conclude that the \( \kappa / \lambda \) test offers a useful and easily applied method for the diagnosis and analysis of lymphoid malignancy.

MATERIALS AND METHODS

Patient selection. Fifty-eight samples of tissue and peripheral blood from patients at the Brigham and Women's Hospital who had proven or suspected lymphoproliferative diseases were obtained for analysis. Diagnoses are outlined in Table 1. The non-Hodgkin's lymphomas included a broad range of histologic types. Because the
**Table 1. Summary of Patients Studied**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic lymphocytic leukemia (B cell)</td>
<td>1 8</td>
</tr>
<tr>
<td>Hairy cell leukemia</td>
<td>1 3</td>
</tr>
<tr>
<td>Non-Hodgkin's lymphoma</td>
<td>18*  17†</td>
</tr>
<tr>
<td>Reactive</td>
<td>6  2</td>
</tr>
<tr>
<td>Lymphosarcoma cell leukemia</td>
<td>0  1</td>
</tr>
<tr>
<td>Hodgkin's disease</td>
<td>1  0</td>
</tr>
</tbody>
</table>

* Includes 15 B cell type, 1 T cell, 1 null cell, and 1 true histiocytic cell by immunoperoxidase studies.
† All patients had non-Hodgkin's lymphomas of demonstrated B cell type.

It was intended to compare the capabilities of k/λ and Southern blot analysis in detecting clonal B cell proliferations, we were concerned mainly with the phenotypes of the various lymphomas. Each diagnosis was confirmed by routine histology, and the phenotype was established by immunoperoxidase staining of cryostat sections. All studies were performed with the approval of the BWH human subjects committee.

**k/λ analysis.** Lymphocytes were obtained by Ficoll-Hypaque centrifugation of peripheral blood or cell suspensions of tissue samples. The cells were separated into three aliquots. One was reserved for DNA analysis. The other two aliquots were labeled with rabbit heteroantisera to immunoglobulin light chains, one with k and the other with λ. The staining procedure has been described previously. Cells (1 to 2 x 10^6) from each sample were analyzed for fluorescence on a flow cytometer, and histograms were obtained by plotting relative cell number vs log fluorescence intensity. The two curves obtained in this manner were analyzed for evidence of a monoclonal population. The studies were performed on a FACS Analyzer (Becton Dickinson Immunocytometry Systems, Mountain View, Calif). This instrument measures electronic volume and single-cell fluorescence excited by filtered light from a mercury-arc lamp.

The basis for interpretation of histograms obtained by k/λ analysis is outlined in Fig 1 (similar to that previously published; see ref. 6). Detection of clonal excess depends on the empirical observation that normal k and λ histograms are identical in shape. Each curve shows a frequency distribution of fluorescence around a mean fluorescent intensity. This mean fluorescence intensity is identical for both k-staining and λ-staining cells, and is independent of the relative numbers of cells that are positive for each light chain. As noted above, k cells normally outnumber λ cells by a ratio of ~2:1. However, when the two curves are normalized for cell number, they become superimposable as shown in the upper panel (Fig 1). The presence of an abnormal clone (or clones) is detectable by the presence of a shift in the fluorescence distribution curves as shown in the lower panel (Fig 1). The proliferation of a clone of cells with a uniform fluorescence intensity of a single light chain causes a shift of that curve relative to the other unaffected light chain; as a result, the two curves are no longer superimposable. Differences are usually obvious on inspection of the curves. Curves were also analyzed by computer, using the Kolmogorov-Smirnov test to derive a "D" value to express the differences between them. This value reflects the magnitude of the deviation of the two curves and is expressed as a percentage of the total number of cells analyzed. It is independent of the number of cells staining for each light chain and provides a quantitative comparison of the two fluorescence distributions. Previous studies in this laboratory have shown that the range of D values seen in the evaluation of blood from normal individuals is 5.1 ± 3.3 (± 2 SD). A D value of 10 is thus considered to be the upper limit of normal. Occasional false-positive results have been observed in individuals with nonlymphoproliferative disorders and, rarely, in normal individuals. Consequently, studies must be correlated with appropriate clinical and histologic information.

Flow cytometry also allows fluorescence analysis of cells within selected size ranges. In some cases, cells were divided into "small" and "large" cells by selecting appropriate cell volume gates; each group was then analyzed separately for light chain distribution.

**DNA rearrangement analysis.** High mol wt DNA was prepared from Ficoll-Hypaque gradient-purified mononuclear cells obtained as noted above. Most samples contained between 5 x 10^6 and 10^7 cells. Cells were lysed with 1% Triton X-100 in 32% sucrose at 4 °C to obtain nuclei. Nuclei were incubated overnight at 37 °C in 0.5% SDS and 0.2 mg/mL of Proteinase K. This was followed by phenol-chloroform extraction and ethanol precipitation as described by Bell et al. DNA was digested with appropriate restriction enzymes, size-fractionated by agarose gel electrophoresis and transferred onto nitrocellulose paper by the method of Southern. Filters were hybridized to nick-translated, 32P-labeled probes of the immunoglobulin k and λ light chain loci and were washed at 53 °C in 0.1% SDS/0.15 mol/L of NaCl-0.00 15 mol/L of Na citrate prior to autoradiography.

The probes used for Southern analysis are shown in Fig 2. The appropriate restriction sites for each probe are shown. V-J recombination in the light chain loci gives rise to an altered 5' restriction
enzyme site and alters the size of the germline restriction fragment. Detection of one or two bands of altered size is indicative of monoclonal rearrangement of the light chain genes. Polyclonal proliferations yield small clones of varied size and do not appear as distinct bands on Southern blot hybridization. All DNAs were also probed with a probe derived from the heavy chain J region (not shown). Because heavy chain rearrangement occurs before light chain rearrangement, all DNAs which displayed light chain rearrangement had also undergone alterations in their heavy chain locus. No DNAs analyzed had a heavy chain rearrangement in the absence of light chain rearrangement.

The number of λ constant region genes has been shown to vary from individual to individual. Consequently, the 8-kilobase (kb) fragment diagrammed in Fig 2 may be 13, 16, or 18 kb. Because this locus has a polymorphic nature, granulocytes from patients with confusing λ gene rearrangements were run as controls. Such polymorphism does not exist in the κ locus; therefore, similar comparisons were unnecessary.

RESULTS

Detection of lymphoma cells by κ/λ and DNA restriction analysis. The ability to detect clonal excess by flow cytometry, as reflected in the D value, is correlated with the findings on DNA analysis in Fig 3. A κ/λ shift (indicated by a D value >10) was detected in 32 of 35 (92%) of cases in which monoclonal immunoglobulin gene rearrangements were seen on DNA analysis. The three false-negative κ/λ studies, as well as the single case in which a positive κ/λ study was not corroborated by gene rearrangement studies, will be discussed in detail below.

Characteristic results of flow cytometry and DNA rearrangement studies are compared in Fig 4. Panel A shows results obtained by each method in analysis of a lymph node involved by a κ-expressing lymphoma. There is a clearly detectable κ shift by flow cytometry, which correlates with an obvious rearrangement of the κ light chain locus seen on Southern blot analysis. The abnormal result in Fig 4A is compared with the analysis of a benign hyperplastic lymph node, in which the κ/λ curves are superimposable (D < 10), and there is no evidence of a clonal gene rearrangement.

Lymphoid tissue usually contains cells of variable size. As noted above, flow cytometry allows the separate analysis of cell subpopulations of different size within a total cell population. Previous studies have shown that analyzing cells in this way allows detection of a clonal excess which may be seen only in one such subpopulation. Figure 4B illustrates such a case. By flow cytometry, a clear λ shift is seen only in the large cell population, which constituted a minority of the suspended cells. This correlated with a λ locus rearrangement which was easily detected on DNA restriction fragment analysis of unsorted cells.

Correlation of blood abnormalities with tissue involvement. Tissue and blood from the same patient were obtained in ten cases as outlined in Table 2. Although only one of the 10 showed morphological evidence of peripheral blood involvement with lymphoma, κ/λ and DNA analyses were positive for lymphoma cells in four cases. All four showed identical DNA rearrangement studies in tissue and blood, confirming that shifts seen by flow cytometry correlated with the presence of circulating tumor cells.

Results of blood and tissue studies performed on one such patient are shown in Fig 4C. Flow cytometry reveals a clonal excess of λ-bearing cells in both tissue and blood. DNA analysis confirms that the clone detected in peripheral blood contains a rearrangement of the λ locus identical to that seen in the original tissue specimen.

Discrepancies between flow cytometry and DNA analysis. Previous studies have shown that 10% mononclonal B cells can be routinely detected by flow cytometry. 1 The relative sensitivities of the flow cytometric method and Southern blot analysis were directly compared in mixing studies. Varying numbers of cells from the DAUDI cell line...
Fig 4. Characteristic DNA and \( \kappa / \lambda \) analyses. Representative examples of tissue and blood analyses are shown. (A) Typical rearrangement of the kappa light chain locus in lymphocytes from a lymph node involved with lymphoma, with the corresponding abnormal study by flow cytometry (\( \kappa \), bright dots; \( \lambda \), dim dots; \( D = 53.7 \)). This study is compared with that of a benign reactive lymph node which shows no light chain abnormality by \( \kappa / \lambda \) analysis (\( D < 10 \)) and germline \( \kappa \) chain genes with no clonal rearrangement. (B) Analysis of a lymph node with a smaller population of abnormal cells; \( \kappa / \lambda \) analysis was performed by selective analysis of cells by size. An abnormal clone was detected only in the large cell population (\( D = 25 \)); the corresponding \( \lambda \) light chain rearrangement is seen on restriction enzyme analysis of DNA. Note the presence of the 5-kilobase (kb) fragment which, as noted above, represents hybridization to a known pseudogene. (C) Comparison of blood and tissue specimens from a patient with B cell non-Hodgkin’s lymphoma (case 9, Table 2). DNA analysis shows two rearranged bands in the \( \kappa \) locus which are seen on analysis of both blood and tissue. The 8-kb band is very faint consistent with rearrangement of \( \text{CX}2 \) involving nearly every cell. The germline band is easily seen in the blood analysis, because only a portion of the cells are tumor cells and contain the rearrangement. The DNA studies are compared with the results by flow cytometry. Again, the cells from the tumor show a single population of nearly uniform fluorescence intensity (\( D = 52.4 \)), whereas this is seen as only a part of the cell population in peripheral blood (\( D = 46.1 \)).

(a monoclonal B cell line that exhibits bright surface \( \kappa \)-staining) were mixed with normal spleen cells, and aliquots were subjected to both \( \kappa / \lambda \) and Southern blot analysis. The lowest proportion of monoclonal B cells detected by \( \kappa / \lambda \) analysis (ie, \( D > 10 \% \)) was 5%, whereas a distinct band could still be detected on Southern blots with 1% malignant cells. This level of sensitivity of Southern blot analysis is similar to results reported by others.\(^{11}\) The level of sensitivity of the \( \kappa / \lambda \) study is in fact somewhat better than previously reported; this is probably owing to the fact that the DAUDI cells exhibit particularly bright staining. Therefore, Southern blot analysis is approximately five to ten times more sensitive than \( \kappa / \lambda \) analysis in detecting monoclonal B cells. Despite this increase in sensitivity, DNA studies detected no lymphoma cells in peripheral blood samples that were not detected by the \( \kappa / \lambda \) test. The overall incidence of involvement of peripheral blood with lymphoma cells was 50% by both methods.

In only one of the three false-negative \( \kappa / \lambda \) tests on tissue samples was the sensitivity of the DNA analysis the probable reason for detecting the clonal cell population. The other two cases involved lymphoma cells with no detectable surface immunoglobulin; in both cases, the monoclonal population was well in excess of 10% of the total cells. The one false-positive case occurred in a patient diagnosed as having true histiocytic lymphoma. This diagnosis was based on immunoperoxidase and histocytochemical studies which showed that the large malignant cells lacked T cell-related or B cell-related antigens or immunoglobulin light chains, but were positive for monocyte/macrophage-related antigen (MO-2), intracellular lysozyme, and \( \alpha \)-naphthyl acetate esterase activity.
The misassignment of light chain type to a small clone of abnormal cells is a potential error of the \( \kappa/\lambda \) test. A given clone is detectable because it has a uniform fluorescence. This fluorescence may be either brighter or dimmer than the mean fluorescence of a population of normal cells. Consequently, when the distortion of the curve is small, it is often difficult to ascertain whether the clone is of one light chain or another, although there may clearly be a clone present. This is, of course, not a problem with DNA analysis. In this study, only one sample was assigned an inappropriate light chain type by flow cytometry.

**DISCUSSION**

By the use of DNA restriction enzyme analysis, we have confirmed that the light chain imbalance detected by the \( \kappa/\lambda \) test is reflective of a monoclonal cell population in patients with non-Hodgkin’s lymphoma. Further, we have documented that clonal excess detected in peripheral blood by flow cytometry correlates with the presence of circulating lymphoma cells identical to those present in tumor tissue.

The \( \kappa/\lambda \) test appears to be a very sensitive means of assessing cell populations for the presence of abnormal clones of B cells. In an analytic cytology lab equipped with a flow cytometer, it offers significant advantages over DNA analysis. It is less time-consuming and does not require radiolabeled probes. The \( \kappa/\lambda \) test may also be done on as few as 10⁶ cells, whereas DNA studies usually require 5 × 10⁶ cells.

In this study, we have tried to establish the circumstances under which DNA analysis offers significant advantages over \( \kappa/\lambda \) studies. There are two major reasons for false negative-results in flow cytometry analysis: technical and biologic. The studies may be inaccurate for technical reasons on several grounds: (1) the size of the clonal population may be below the limits of detectability of the \( \kappa/\lambda \) test; (2) the curves may be difficult to interpret and may lead to assignment of the incorrect light chain type; or (3) the test may be technically inadequate. In this study, few errors in diagnosis were made as a result of technique. In only one sample of the total 58 was the abnormal population detectable by DNA analysis so small as to be undetectable by flow cytometry; in one case, the light chain type was incorrectly assigned. Hence, although DNA restriction analysis is more sensitive than \( \kappa/\lambda \) analysis, it had little effect on the diagnostic accuracy of the test.

False-negative results can also arise for biologic reasons. The \( \kappa/\lambda \) test cannot detect abnormal lymphoid populations if the cells lack surface immunoglobulin, as was true in two cases studied here. In such cases, DNA analysis obviously offers significant advantages in detecting abnormal cell populations, as has been shown by others.⁹

We observed one positive \( \kappa/\lambda \) study that was not correlated with detectable DNA rearrangements. The reason for this apparent false-positive study is unclear, but there are several possibilities. Either study may have been technically at fault, although this would be surprising with a D value of 16, which suggests a significant clonal population that should be unmistakable by \( \kappa/\lambda \) analysis and easily detectable by DNA studies. Alternatively, it may reflect the biologic nature of the tumor involved. This case was diagnosed as a true histiocytic lymphoma, based on generally accepted histologic, immunologic, and histocytochemical criteria.¹⁵ The malignant cells in this disorder phenotypically resemble normal monocytes or macrophages, and may be associated with Fc receptors with high avidity for circulating immunoglobulin.¹⁶,¹⁷ As in this case, one would anticipate a greater nonspecific binding of \( \kappa \) chains than \( \lambda \) chains, since \( \kappa \) immunoglobulin is present at approximately twice the concentration of \( \lambda \) immunoglobulin in peripheral blood. Although cells are routinely incubated at 37 °C before staining for \( \kappa/\lambda \) analysis in order to shed cytophilic antibody, this may not have been sufficient in the presence of an avid Fc receptor. This is a potential problem with the \( \kappa/\lambda \) test, and one that has been observed in patients with M components as is seen in multiple myeloma. It is not usually seen in the presence of normal levels of immunoglobulin, presumably because the nonspecific affinity of lymphocytes for antibody is low; however, it may explain false-positive results seen in patients with high levels of circulating immunoglobulin.

In the current study, we found that 50% of the patients overall with non-Hodgkin’s lymphoma of B cell type had evidence of peripheral blood involvement. This figure is somewhat less than the 80% incidence detected by \( \kappa/\lambda \) analysis recently reported.¹⁸ There are several possible explanations for the lower rate of involvement seen in the current study. First, fewer patients were included in the present study, and inclusion of more patients may have significantly altered the proportion of positive studies. Second, the prior study included many patients with long-standing or relapsed lymphoma, whereas the present study primarily included patients at or near the time of first diagnosis. The fact that circulating lymphoma cells are present in most patients with relapsed lymphoma⁹ could explain the greater incidence of positive peripheral blood findings in the earlier study.

In addition to confirming the diagnostic usefulness of the \( \kappa/\lambda \) test, our results suggest an unexpected aspect of the natural history of lymphoproliferative disease. One might have expected that use of a more sensitive test would have
revealed additional cases of peripheral blood involvement undetected by \(\kappa/\lambda\) analysis. This was not what we observed, however. There were no false-negative \(\kappa/\lambda\) tests on peripheral blood as compared with DNA rearrangement studies, suggesting that the ability to invade the peripheral blood may be an intrinsic property of certain lymphomas but not of others, despite their apparent similarity by histologic examination.

We did not select patients on the basis of the duration of their disease or on their exposure to chemotherapy, both of which may be crucial to the observation of peripheral blood involvement. Whether those patients showing no peripheral blood involvement later develop evidence of circulating clonal cells and whether it becomes detectable by DNA rearrangements before it is detectable by \(\kappa/\lambda\) analysis must be studied. Appropriate studies of patients with lymphoproliferative disease followed in a longitudinal fashion should aid in elucidating this further.

ACKNOWLEDGMENT

We would like to thank Dr Geraldine Pinkus for performing immunoperoxidase studies and Dr Brian Smith for his comments and assistance in preparing this manuscript.

REFERENCES

Detection of clonal excess in lymphoproliferative disease by kappa/lambda analysis: correlation with immunoglobulin gene DNA rearrangement

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