Gene Rearrangements in Malignant Lymphomas*

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ABSTRACT

The use of molecular methods to diagnose malignant lymphoma, while firmly established in reference centers, has not been well evaluated at the community level. A group of 57 specimens from patients with non-Hodgkin's lymphomas (NHL), lymphoid leukemias (LL), and a variety of other lymphoproliferative lesions with the Southern blot methodology have been studied by us. Molecular probes to the joining regions of the heavy (JH) and light (Jk) immunoglobulin chains and the beta (Jβ1-2) chain of the T cell receptor genes were used. Gene rearrangements were detected in 90 percent of all NHL/LL with a 95 percent detection rate specifically for B-NHL/LL. In comparison, phenotypic analysis by immunoperoxidase stains favored a B phenotype in 75 percent of those cases, while flow cytometry assigned 63 percent of cases to a B cell lineage. Gene rearrangements were detected in four of six cases of T-NHL for a rate of 67 percent. The six other lymphoproliferative lesions included Hodgkin's disease, Castleman's disease, and a case of lymphoid hyperplasia. No rearrangements were detected in these specimens. The studies allowed development of increased confidence in the diagnosis of NHL/LL on ever smaller specimens. The availability of these studies has also helped establish a priority of handling all specimens so that the most appropriate studies can be performed to yield the most useful diagnostic information.

Introduction

It has now been well documented that non-Hodgkin's lymphomas (NHL) represent a monoclonal growth of neoplastic lymphoid cells.1,2,3 While the basic neoplastic process can usually be diagnosed and classified by standard histological methods, a significant minority of cases defy accurate diagnosis by such methods. Furthermore, confirmation of monoclonality and identification of the lymphocyte lineage require other methods. Monoclonality in B cell lymphomas is typically manifested by lambda or kappa light chain restriction, and evidence for this immunophenotype is often sought by the use of immunoperoxidase stains and flow cytometry. A significant portion of malig-
nant lymphomas of B cell origin do not express immunoglobulin markers; in such cases, evidence of monoclonality and cell phenotype remain undetermined. In addition, flow cytometric immunophenotyping can frequently be misleading owing to admixture of large numbers of non-neoplastic reactive lymphocyte populations. Lastly, neither immunoperoxidase staining nor flow cytometric immunophenotyping typically allows identification of a clonal marker in T cell processes.

The phenomenon of specific immune response, which is genetically programmed, allows both T and B lymphocytes to seek out and specifically destroy foreign agents such as viruses, bacteria, and tumor cells. This process is initiated by contact of an antigen with the immune system in a manner which allows selection of immunoglobulin or T cell receptor configurations which best "fit" or react with that antigen. Each of these configurations is represented by a parent lymphocyte, the deoxyribonucleic acid (DNA) of which has been rearranged, i.e., selectively customized and shortened to allow synthesis of the specific immunoglobulin or T cell receptor. In the process of the normal immune response, the rearrangement of DNA remains so diverse among the lymphocytes that no single clone of cells can be recognized; that is, the process is polyclonal in nature. Lymphoid neoplasms, on the other hand, represent large clones of identical cells having the same rearranged DNA structure. This rearranged DNA is uniquely different from all other lymphocytes as well as DNA from non-lymphocytic cells which do not undergo DNA rearrangement.

The Southern blot procedure allows identification of specific DNA sequences of monoclonal lymphocyte populations which constitute as little as 5 percent of a mixed cell population. In addition, determination of which genes have been rearranged, that is, whether heavy or light immunoglobulin chain or T cell receptor genes or some combination thereof, usually permits identification of the cell lineage.

While the Southern blot technique is regarded as a complex, labor intensive procedure perhaps only suited to a reference laboratory setting, our interest and experience with malignant lymphomas has prompted us to evaluate the use of this technique in a large community hospital laboratory. Further motivation was given by the knowledge that the initial biopsy in a local setting often represents the only opportunity to obtain diagnostic tissue unaltered by various therapeutic modalities. It is the purpose of this communication to report our experience, to compare our results with previously published reports, and to comment on the impact of this procedure in a busy clinical laboratory.

Materials and Methods

Fifty-seven specimens from 55 patients with various lymphoid lesions were evaluated. The specimens included lymph nodes, soft tissue, gastrointestinal tract, peripheral blood, effusions, and bone marrow. All cases were examined by standard histological techniques and classified according to the Working Formulation. All cases had analysis of DNA by Southern blotting, while many cases were also examined by immunoperoxidase staining and flow cytometric immunophenotyping. The immunoperoxidase stains were performed on B-5 fixed paraffin embedded tissues. All cases were studied with leukocyte common antigen, CD45R and MT1 antibodies. A smaller number of cases also had stains for lambda and kappa light chains or non-lymphoid antigens.

Flow cytometric immunophenotyping was performed on suspensions of fresh tumor cells using the Becton Dickinson
FACScan 340* and the method of Boyum with modifications.\textsuperscript{7,8} The typical antibody panel included: CD45/CD14, CD4/CD8, CD10/CD19, CD5/CD20, CD22/CD3, CD7/CD33, HLA-DR, CD13, CD15/CD2, and lambda/kappa light chains. Results were expressed as supportive of lymphoid neoplasia of B or T cell type or of a reactive or normal lymphoid population. In the case of B cell neoplasia, evidence of lambda or kappa light chain restriction was sought.

**Analysis of Gene Rearrangements**\textsuperscript{†}

The procedure used was that specified for the B/T Blue Gene Rearrangement Test System.\textsuperscript{9} Deoxyribonucleic acid was extracted from tissue using a non-organic/salting out method and subsequently quantified by spectrophotometry. Partial hydrolysis of DNA was performed with each of three enzymes, EcoRI, BamHI and HindIII. Restriction fragments were separated by size using agarose-gel electrophoresis. Each gel included lanes for fragment size markers, germline controls, and sensitivity control. Periodically, a normal tissue control was included. Fragment size markers were a mixture of biotinylated and non-biotinylated fragments of DNA obtained by hydrolysis of lambda phage DNA with HindIII. Germline controls were human placental DNA (unrearranged DNA) which had been hydrolyzed with either EcoRI, BamHI or HindIII restriction enzymes. The sensitivity control was a mixture of human placental DNA restricted with each of the three aforementioned enzymes.

After electrophoresis, fragments of DNA were depurinated, denatured, and transferred to nylon membranes. Hybridization with biotinylated DNA probes followed. The probes used were J\textsubscript{H}, which hybridized to the joining region of the gene for the heavy chain of the human immunoglobulin molecule, J\textsubscript{\beta\textsubscript{1}-2}, which hybridized to the joining regions of the gene for the beta chain of the T cell receptor, and J\textsubscript{\kappa}, a probe for the joining region of the gene for the kappa light chain of the immunoglobulin molecule. After hybridization, membranes were washed and bound probes were detected colorimetrically.

The fragment size markers served to identify visually the positions of the bands. To assure that a clonal population of cells was detectable at 5 percent of the total cell population, the sensitivity control had to be detected. If rearrangement was detected for the patient's sample but the sensitivity control was not detected, the result was accepted. However, if no evidence of rearrangement was found and the sensitivity control was not detected, the test had to be repeated. Nongermline bands not due to rearrangement were excluded by established criteria.\textsuperscript{9,10}

The presence of a single rearrangement band in each of two enzyme hydrolysates or two such bands for a single enzyme was the minimal evidence for clonality with one probe. The B cell lineage was suggested if rearrangement was detected with the J\textsubscript{H} probe and not J\textsubscript{\beta\textsubscript{1}-2}. The T cell lineage was suggested if rearrangement was detected with the J\textsubscript{\beta\textsubscript{1}-2} probe but not J\textsubscript{H}. If rearrangement was detected with J\textsubscript{H} and J\textsubscript{\beta\textsubscript{1}-2}, J\textsubscript{\kappa} was used to differentiate. Under these circumstances, rearrangement detected with J\textsubscript{\kappa} suggested B cell lineage, whereas absence of rearrangement with this probe yielded inconclusive lineage results.\textsuperscript{9,10}

**Results**

A total of 57 specimens from 55 patients had both histological and Southern blotting procedures completed. Many of these cases also had immunoper-
oxidase staining and/or flow cytometric immunophenotyping performed as well. The final diagnosis of each case was assigned by correlation of all the available data in each case (table I). Fifty-one cases were classified as NHL or LL, and rearranged bands indicative of monoclonality were detected in 46 cases (90 percent). Forty-four of the cases were B-NHL, and rearranged bands were detected in 42 cases (95 percent), while B cell of origin was assigned in 41 cases (93 percent). By contrast, immunoperoxidase staining was completed on 28 of these cases with 21 of them (78 percent), assigning a B phenotype, while flow cytometric immunophenotyping was performed on 24 cases with 15 (63 percent) felt to be diagnostic of the B cell origin. There were two cases of B cell NHL which did not exhibit rearranged bands. The first of these was a large non-cleft cell lymphoma of the stomach. This was a small lesion, and, retrospectively, it appears that the specimen submitted for DNA analysis was probably inadequate. The second specimen was a diffuse large cell lymphoma in a lymph node which was germline when probed for heavy chain and T cell receptor rearrangements. Possible light chain rearrangements were not probed in this case. Therefore, while this case was assigned as an instance where gene rearrangements were not demonstrated, our current methodology would require probing with $J_K$ prior to reaching such a conclusion.

Of the six cases that were believed to be of T cell origin, four cases (67 percent) showed rearranged bands when probed with $J_{B-1,2}$, thus establishing monoclonality and probable T cell of origin. The two cases failing to show rearranged bands were believed to be of T cell origin based upon (1) convincing T cell predominance by immunoperoxidase staining in a case of immunoblastic lymphoma that also had equivocal flow cytometric data and (2) convincing flow cytometric data in a case of mixed large and small cell lymphoma with otherwise equivocal immunoperoxidase stain findings. Finally, there was one case of a diffuse large cell lymphoma for which neither immunoperoxidase staining nor flow cytometry was helpful in assigning monoclonality or cell of origin. This case was also negative when probed for heavy chain and T cell receptor rearrangements, but the possibility of kappa light chain rearrangement was not investigated in this case and remains unknown.

Thus far six additional specimens of lymphoproliferative lesions other than

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**TABLE I**

<table>
<thead>
<tr>
<th>Non-Hodgkin's Lymphoma/ Lymphoid Leukemias$^a$</th>
<th>Number of Cases</th>
<th>Rearrangements Detected (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td></td>
</tr>
<tr>
<td><strong>B-NHL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Small cell</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Small cleaved cell</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Mixed large and small cell</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Large cell (including immunoblastic)</td>
<td>19</td>
<td>17</td>
</tr>
<tr>
<td>Small non-cleaved cell</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Acute lymphoblastic leukemia</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>44</td>
<td>42 (95%)</td>
</tr>
<tr>
<td><strong>T-NHL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphoblastic</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Ki 1 positive large cell</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Mixed large and small cell</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Immunoblastic</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Acute lymphoblastic leukemia</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>6</td>
<td>4 (67%)</td>
</tr>
<tr>
<td><strong>Null-NHL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large cell</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$Final pathological diagnosis is based upon correlation of all histologic, phenotypic, and genotypic data in each case.
NHL/LL have been studied. These cases include Hodgkin's disease (two patients), Castleman's disease (three specimens from two patients), and a hemophagocytic syndrome with lymphoid hyperplasia (one patient). No gene rearrangements were found in this group of cases.

Discussion

The evolving understanding of the genetic basis of malignant lymphomas as monoclonal proliferations is the subject of a number of recent excellent reviews.1,2,5,11 Concurrently, there have evolved a variety of diagnostic tests designed to allow clinical laboratories to recognize molecular alterations of DNA, identify monoclonal cell populations, and assign cell lineage.3 However, because the tests continue to be complex, labor intensive, and expensive, their clinical application has heretofore been largely relegated to reference laboratory centers. Because of the relatively large number of patients with NHL and LL who undergo primary diagnostic procedures at our institution, the diagnostic challenges posed by such a group of patients are well known to us. In particular, smaller and smaller biopsy specimens are increasingly presented which are often obtained by endoscopic methods with full expectations of establishing a firm pathological diagnosis. Clearly, one cannot rely solely upon histological and cytological methods and must be prepared to preserve specimens and/or actually perform procedures such as immunophenotyping by immunoperoxidase staining and flow cytometry. To this list of procedures, the capability of molecular studies by the Southern blot method has been added in order to evaluate its role in a primary diagnostic center.12,13,14

Using the Southern blot method, monoclonality was established in 90 percent of all cases of NHL/LL (95 percent of B cell lineage tumors and 67 percent for T cell neoplasms), which is comparable to other published studies.10 Furthermore, in the case of B-NHL where there were significant numbers of cases, the ability to predict genotype by Southern blotting (93 percent) also exceeded the predicted phenotype by immunoperoxidase stains (78 percent) and flow cytometry (65 percent).

Failure to detect rearrangements in all cases is explained in a number of ways. First, the enzymatically digested restriction fragments resulting from rearrangement may be of a size similar to the typical germline restriction fragments. In such cases, electrophoretic separation of the similar sized fragments will not be possible. In other instances, the restriction fragments may be of large size which do not separate well in the agarose electrophoretic medium. Conversely, the restriction fragments may be so small that they electrophoretically move beyond the point of optimal examination in the agarose. Last, rearranged bands will not be detected unless the appropriate gene probe is utilized. Our current studies involve the use of probes for the heavy (H) and light chain (L) immunoglobulin genes as well as the joining sections of the beta chain (Jβ1-2) of the T cell receptor gene. Clearly, other genes can undergo rearrangement, and, in the current context, they would remain undetected. This would appear to apply particularly to T cell receptor genes where alpha, gamma, and delta gene probes were not utilized. Others have similarly reported a lower incidence of detected rearrangements in T cell malignant lymphomas.15

In addition to determination of monoclonality, B and T cell gene rearrangements frequently serve to identify probable cell lineage. The cell lineage is described as probable because of reports indicating that gene rearrangements may cross lineage lines; that is, immuno-
globulin heavy chain genes may occasionally be arranged in T lymphocytes, and T cell receptor beta chain genes may occasionally be rearranged in B lymphocytes. As many as 25 percent of precursor B cell acute lymphoblastic leukemias have been reported to contain rearrangements of both immunoglobulin heavy chain genes as well as T cell receptor beta chain genes. Conversely, immunoglobulin heavy chain gene rearrangements have been found in some T lymphocyte neoplasms, and they have also occasionally been observed in acute myeloid leukemia. However, rearrangement of both the immunoglobulin heavy and light chain genes appears to be specific for the B lymphocyte lineage since the co-existence of heavy and light gene rearrangements has not been clearly identified in T lineage cells.  

While the procedure is truly long, labor intensive, and complex, the methodologies involve standard laboratory techniques. The pre-prepared reagents and Probe Tech 2 apparatus, which incorporates both the separation and transfer of DNA fragments into a single unit, are readily adaptable to any laboratory with experience in electrophoretic methods. Furthermore, the use of a biotinylated colorimetric system is easier and quicker than the reference method which uses radionuclides for detection of DNA bands.

The ability to make confident diagnoses of malignant lymphomas on specimens which are of limited size is improving. Whether or not the specimens represent endoscopically guided biopsies from various body organs, bone marrow, effusions or peripheral blood, they can all be obtained with minimal patient discomfort and morbidity. It is preferable to obtain 250 mg of tissue for DNA analysis, which allows for repeat testing if necessary, but successful results have been achieved with as little as 70 mg. A firm diagnosis based upon a combination of morphologic, immunophenotypic and genotypic findings of small specimens can then allow the patient to proceed to definitive therapy without the delays, cost, and infirmity that sometime follow the more traditional open biopsy procedures.

In order to approach these small specimens with confidence, the laboratory must maintain a vital interest in developing these newer techniques and fine tuning the interpretation of these studies. Our experience with a wide variety of typical lesions has allowed us to develop increasing security in our methods and interpretations. Clearly, analysis of DNA is not necessary on all lymphoid lesions, but only with such a diverse experience can one develop a sense of confidence. Only then can one apply the methods of DNA analysis to resolve those difficult cases which have eluded diagnosis by standard morphological and immunophenotypical methods.

As experience is gained, it is our intention to apply more selectively those methods which seem most appropriate on a case by case basis. Therefore, initial handling of clinical specimens at the time of biopsy has become the most critical phase in this process. Only by appropriately preserving portions of specimens for additional studies can the full capability of the diagnostic armamentarium be utilized. This point was recently emphasized with a case which was clinically highly suspicious for malignant lymphoma involving the mediastinum. A frozen section was performed and regarded as essentially diagnostic of malignant lymphoma. However, the eventual permanent microscopic sections, while highly suspicious of malignant lymphoma, were felt to be less than fully diagnostic of that lesion. Fortunately, a small portion of tissue had been saved for gene rearrangements, and when rearrangement bands were clearly detected, a firm diagnosis of malignant
lymphoma was warranted. Our institution has become increasingly restrictive in the use of frozen sections so as not to “waste” tissue that may be more appropriately used for other studies.

In addition to increasing our ability to serve better the needs of patients who are undergoing primary diagnostic procedures, the authors believe that this project has been an educational stimulus to our laboratory staff. It has, moreover, helped us to embark upon a course of molecular diagnostic studies which will increasingly find their way into our clinical laboratories of the near future. Currently, we are in the process of evaluating the molecular basis of chromosome translocations in malignant lymphomas/leukemias by use of probes to detect c-myc, bcl-2, and bcr-abl. Beyond the field of hematopathology, the emerging molecular diagnostic applications in microbiologic and genetic diseases will be more feasible largely because of the stimulus provided by this experience.

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References